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# Plant Biotic and Abiotic Stresses Volume I

Edited by
Hakim Manghwar and Wajid Zaman

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This is a reprint of articles from the Special Issue published online in the open access journal *Life* (ISSN 2075-1729) (available at: https://www.mdpi.com/journal/life/special_issues/_plant_stresses).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

**Volume I**
**ISBN 978-3-0365-8536-9 (Hbk)**
**ISBN 978-3-0365-8537-6 (PDF)**
**doi.org/10.3390/books978-3-0365-8537-6**

**Set**
**ISBN 978-3-0365-8534-5 (Hbk)**
**ISBN 978-3-0365-8535-2 (PDF)**

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*Review*

# Genome Editing and Improvement of Abiotic Stress Tolerance in Crop Plants

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**Abstract:** Genome editing aims to revolutionise plant breeding and could assist in safeguarding the global food supply. The inclusion of a 12–40 bp recognition site makes mega nucleases the first tools utilized for genome editing and first generation gene-editing tools. Zinc finger nucleases (ZFNs) are the second gene-editing technique, and because they create double-stranded breaks, they are more dependable and effective. ZFNs were the original designed nuclease-based approach of genome editing. The Cys2-His2 zinc finger domain's discovery made this technique possible. Clustered regularly interspaced short palindromic repeats (CRISPR) are utilized to improve genetics, boost biomass production, increase nutrient usage efficiency, and develop disease resistance. Plant genomes can be effectively modified using genome-editing technologies to enhance characteristics without introducing foreign DNA into the genome. Next-generation plant breeding will soon be defined by these exact breeding methods. There is abroad promise that genome-edited crops will be essential in the years to come for improving the sustainability and climate-change resilience of food systems. This method also has great potential for enhancing crops' resistance to various abiotic stressors. In this review paper, we summarize the most recent findings about the mechanism of abiotic stress response in crop plants and the use of the CRISPR/Cas mediated gene-editing systems to improve tolerance to stresses including drought, salinity, cold, heat, and heavy metals.

**Keywords:** abiotic and biotic stress; CRISPR; mega nucleases; TALEN; ZFN

**Citation:** Yadav, R.K.; Tripathi, M.K.; Tiwari, S.; Tripathi, N.; Asati, R.; Chauhan, S.; Tiwari, P.N.; Payasi, D.K. Genome Editing and Improvement of Abiotic Stress Tolerance in Crop Plants. *Life* **2023**, *13*, 1456. https://doi.org/10.3390/life13071456

Academic Editors: Balazs Barna, Hakim Manghwar and Wajid Zaman

Received: 30 May 2023
Revised: 25 June 2023
Accepted: 26 June 2023
Published: 27 June 2023



## 1. Introduction

By the end of the year 2050, the world population is anticipated to reach up to 10 billion [1]. In this situation, increasing food crop production by 60% over the coming decades is necessary to ensure global food security [1,2]. To sustainably increased food production, additional integration of all developed relevant techniques, such as genomics, genome editing (GE), artificial intelligence, and deep learning, will be necessary [3,4]. Crop modification methods have a long history and have been used ever since the first agricultural plants were domesticated. Since then, other new methods have been created and are being developed to boost crop production and economic value even more. Traditional crop breeding techniques in the 20th century either relied on naturally occurring mutations or on mutagenesis that was created artificially [5]. Genetic research has traditionally focused on the identification and assessment of spontaneous mutations. Scientists were reliant on each other and showed that radiation or chemical treatment could increase the rate of mutagenesis [6,7]. Later approaches, suchas radiation and chemical mutagenesis, altered the genome at random sites by inserting transposon motifs that may be induced in some

animals. However, a fundamental disadvantage of conventional breeding methods is the length of time needed to breed new varieties of any crops with the required agronomic characteristics. The duration of the growing season and the maturity level of the plants (particularly long-period growers, such as trees), as well as various stages of crossing, selection, and testing during the breeding process, all have an impact on this [8]. The plant genome cannot be targeted using conventional techniques for chemical and physical mutagenesis or natural mutations. Using genetic engineering, better plants and animals may be developed more quickly [5].

The first genetically modified (GM) crops were released for sale in 1996 [9]. Generations of GM crops up to now have relied on the genome's random insertion of new DNA sequences. The possibility that the inserted gene may affect or impede the activity of other crucial nearby genes has been raised as a concern regarding this approach. In addition, public anxiety regarding GM crops is increased when talking about the introduction of 'alien' genes from distantly related organisms, which is thought to be 'unnatural' despite mounting evidence to the contrary [10,11].

The creation and use of DNA-based markers at the turn of the twenty-first century has made it possible to reduce significantly the time needed to generate new lines and varieties of agricultural crops [10–13]. All these factors have greatly helped the development of focused GE methods [14–17]. In yeast and mice, the first targeted genetic alterations were created in the 1970s and 1980s [6,8]. This gene targeting was based on the homologous recombination process, which was extremely accurate.

RNA interference (RNAi) was one of the first GE technologies [5,18,19]. Even though this technology has been successfully used in functional genomics and plant breeding [20–22], it has several drawbacks, including the unlimited insertion site of an RNAi construction into the genome and partial gene function suppression [5].

This is a marvelous time for genetics, due to advances in genetic analysis and genetic manipulation. Genome editing, the most recent crop-enhancement method, allows precise changes of the plant genome by deleting undesired genes or enabling genes to acquire new functions [23]. Numerous crops' genomes have been sequenced, and improvements in genome-editing techniques have made it possible to breed for desired features. To sustainably increase food production, additional integration of all developed relevant techniques, such as genomics, genome editing (GE), artificial intelligence, and deep learning, is necessary [24].

Advanced biotechnological methods are made possible by genome-editing tools, allowing for precise and effective targeted modification of an organism's genome. Several novel tools for genome or gene editing are available to enable researchers to modify genomic sequences precisely [25]. These techniques facilitate novel insights into the functional genomics of an organism and enable us to alter the regulation of gene expression patterns in a pre-determined region. Because of accurate DNA manipulation, genome-editing technologies, for instance, CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated systems), TALENs (transcription activator-like effector nucleases), CRISPR/Cas12a (Cpf1, CRISPR from *Prevotella* and *Francisella*1), and Cas9-derived DNA base editors, provide unprecedented advancements in genome engineering. As a result, this technology is a powerful tool that can be employed to secure the global food supply [26].

Genome editing was first proposed by Capecchi [27] in the 1980s. This method allows for the removal, modification, or addition of genetic material at specified genomic locations. Even though current GE technologies are substantially more accurate than traditional mutagenesis [28,29], the biggest barrier here is still the legitimacy of GE crops. Assessing the biosafety of such crops is a unique difficulty because it is impossible to predict the effects of single base alterations following the application of ODM and BEs [30,31].

The primary elements that affect plant growth and reduce agricultural productivity are biotic stressors [32,33] such as disease and insect pests, along with abiotic stressors [13] including cold, drought, and saline–alkali stress (Figure 1). Many crop plants that can

withstand abiotic stress have previously been created via traditional marker-assisted breeding. However, due to extensive screening [34,35] and backcrossing procedures, it takes this tactic about a decade to generate abiotic stress-resilient crops effectively [36]. Although genetically modified, stress-tolerant plants have disclosed encouraging results, several barriers still stand in the way of their widespread commercialization. In many ways, crops with genome editing differ from genetically engineered species [37]. Considering this, genome editing seems to be a sophisticated strategy to create crops that are resistant to different abiotic stress in the future, because it allows precise manipulation of different gene loci in comparably less time, which lowers the cost of crop-improvement programmes [38]. Gene-editing technology based on CRISPR/Cas might successfully target complex quantitative genes linked either directly or indirectly to abiotic stressors. The use of CRISPR-Castechnology has been linked in recent years to the establishment of disease resistance in plants by modifying gene regulation [39–42]. Currently, CRISPR/Cas-based genome editing has been efficaciously utilized to investigate tolerance against multiple abiotic stresses, including heat, drought, salt, and nutritional values in several critical agricultural plants [43,44]. In this review article, we summarize the most likely uses of the CRISPR/Cas9-mediated genome editing technique in crop plants for dealing with diverse abiotic stresses such as heat, drought, salinity, cold, herbicide etc., and we predict the tools for future advancements in the creation of crop varieties that can withstand stresses.

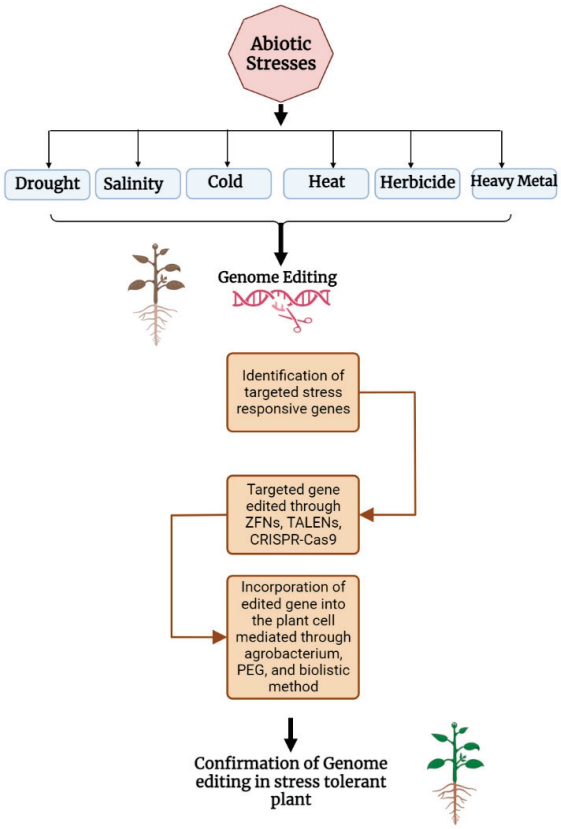


**Figure 1.** Applications of genome editing in crop improvement against abiotic stresses.

## 2. Genome-Editing Strategy

Genome editing is one of the most promising approaches to understand the genome and to improve crop plants. The fundamental mechanisms involved in genetic modification by programmable nucleases (NHEJ) are the recognition of target genomic loci and binding of effector DNA-binding domain (DBD), double-stranded breaks (DSBs) in target DNA caused by restriction endonucleases (FokI and Cas), and repair of DSBs through homology-directed recombination (HDR) or non-homologous end joining [45]. While the well-organized and error-prone NHEJ results in the deletion or insertion of nucleotides, the

less efficient and more accurate HDR results in the replacement of nucleotides. Genome-editing methods such as ZFN, TALEN, and CRISPR/Cas are being utilized to add the desired trait(s) and remove the undesirable ones. Numerous techniques are available for genome editing using either a site-specific recombinase (SSR) system or a site-specific nuclease (SSN) system. Both systems must be able to find a known sequence. The SSN system causes single or double strand DNA breaks and activates endogenous DNA repair systems. Depending on how the sites (loxP, FLP, etc.) are oriented, SSR technology, such as Cre/loxP- and Flp/FRT-mediated systems, can knockdown or knock in genes in the eukaryotic genome around the area of the target [46].

Plant genome-editing techniques have been classified into four major types based on onsite-specific endonucleases (Table 1). Those are ZFNs, meganucleases, TALENs, and CRISPR-Cas9 along with DSB-free genome editing, base editing, prime editing, and mobile CRISPR. These techniques are all discussed in detail below.

### *2.1. Zinc-Finger Nucleases*

ZFNs are assemblages of DNA recognition modules based on zinc fingers and the DNA cleavage domain of the FokI restriction enzyme. With their use, the target genome can be altered to introduce a variety of genetic changes, such as deletions, insertions, inversions, translocations, and point mutations [47]. They have two domains, the first of which is a nuclease domain and the second of which is a DNA-binding domain. The DNA-binding domain's 3- to 6-zinc finger repeats may recognize nucleotide sequences that are 9 to 18 bases long. The second domain is made up of the restriction enzyme Flavobacterium okeanokoites I (FokI), which is necessary for DNA cleavage [48].This method involves three artificial restriction enzymes, specifically ZFN-1, ZFN-2, and ZFN-3 [49]. ZFN-1: At this point, ZFN is transferred to the plant genome devoid of taking a repair template. Once it arrives at the plant genome, it makes double-stranded breaks (DSB) to the host DNA leading to non-homologous end joining (NHEJ) of DNA [50], which either produces site-specific arbitrary mutations or a small deletion or insertion. ZFN-2: Distinct from ZFN-I, a homology-directed repair (HDR) alongside a short repair template is delivered to the crop genome next to the ZFN enzyme [51]. The template DNA is homologous to the target DNA, which attaches to a specific sequence causing a double-stranded rupture. The template commences repairing with an endogenous repair mechanism which is directed to site-specific point mutations throughout homologous recombination (HR). ZFN-3: As soon as the ZFN transcribing gene is transferred to the plant genome next to the large repair template, it is called ZFN3 [51,52].

ZFN has been effectively implemented in *Arabidopsis*, tobacco, soybean, and maize [53–56]. In one example of the use of ZFNs in crop breeding, the insertion of PAT gene cassettes disrupted the endogenous ZmIPK1 gene in maize, which altered the inositol phosphate profile of growing maize seeds and improved herbicide resistance [53].ZFNs can be created utilizing various protein-engineering techniques to target essentially any unique DNA stretch [57]. ZFNs with enhanced specificity and activity have been developed to produce knockouts, which disable the gene's function, as well as gain-of-function alterations [58].

### *2.2. Meganucleases*

Longer DNA sequences (more than 12 bp) can be selectively detected and cut by meganucleases, which are endonucleases. This approach has been discovered in a wide variety of organisms, including archaebacteria, bacteria, algae, fungi, yeast, and many plant species. Meganucleases at the target region can sustain mild polymorphisms [59]. Meganucleases have been divided into five groups based on their sequence and structural features. These consist of His-Cys box, GIY-YIG, LAGLIDADG, PD-(D/E) XK, and HNH [60,61].Genome editing has mostly used members of the LAGLIDADG meganuclease (LMN) family. According to Silvaet al. [60], the name of this protein family is taken from the sequence of the main motif found in its structure. LMNs are typically expressed in the chloroplast and mitochondria of unicellular eukaryotes. The bulk of these endonucleases

are dimeric proteins that have two separate functions: they splice their own introns as RNA maturases and cleave exon sequences as specialized endonucleases [62]. I-SceI and I-CreI's genomes can be edited employing the rRNA gene of the mitochondrial DNA of *Saccharomyces cerevisiae*. The 21S contains the I-SceI gene's location. The chloroplast of *Chlamydomonas reinhardtii*, a unicellular alga, was found to contain I-CreI, which is found in the 23S rRNA gene. However, due to the difficulties in reengineering meganucleases to target specific DNA areas, their utility in genome editing is limited [63].

### *2.3. Transcription Activator-like Effector Nucleases (TALENs)*

Restriction enzymes called TALENs, or transcription activator-like effector nucleases, are designed to cleave specific DNA sequences. TALENs are made up of a nuclease that can cleave DNA in cells and a TALE domain that is intended to mimic the natural transcription activator-like effector proteins. Currently, a huge number of researchers are studying transcription activator-like effector nucleases (TALENs), which are composed of a free designable DNA-binding domain and a nuclease [64], in a variety of organisms. TALENs have recently emerged as a cutting-edge method for genome editing in a variety of species and cell types. It was discovered that TALENs may alter the genome in a variety of plants, including *Arabidopsis*, Nicotiana, Brachypodium, barley, potatoes, tomatoes, sugarcane, flax, rapeseed, soybean, rice, maize, and wheat [65,66]. According to a report, rice was the first crop in which TALENs technology was employed for enhancement. According to Li et al. [67], the main pathogen of blight disease (*Xanthomonas oryzae*) significantly reduces global rice production each year. By disrupting the genes for fatty acid desaturase (FAD), soybeans with high oleic acid and low linoleic acid levels were produced, improving the shelf life and heat stability of soybean oil [68,69]. TALENs are naturally occurring type III effector proteins created by *Xanthomonas species* that change the host plant's gene expression. The TALENs proteins comprise a nuclear localization signal, a transcriptional activation domain, and a core DNA-binding domain [70]. The nuclear localization signal helps TALENs enter the nucleus, whilst the activation domain activates the transcriptional machinery to start expressing genes [71].

### *2.4. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-Associated Protein 9 (Cas9)*

Clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) are short, repetitive genetic variations that are present in most bacterial and archaeal species. CRISPR/Cas9 and its associated proteins produce a very strong defensive system that works as a safeguard for plants against foreign agents including bacteria, viruses, and other elements. The first application of CRISPR/Cas9 in an adaptive immune system was documented in a 2007 experiment [72]. The CRISPR/Cas9 gene-editing system has revolutionized research in animal and plant biology since its usage in genome editing was first demonstrated in mammalian cells in 2012 [73]. According to Rathore et al. [23] first-generation CRISPR/Cas9 genome editing involves simple manipulationand cloning techniques that can be applied to a variety of guide RNAs to edit different locations in the targeted organism's genome (Figure 2). With the use of CRISPR/Cas, crop species can be precisely edited, opening the door to the generation of favorable germplasm and new, more sustainable agricultural systems. The genetic modification of crops can now be targeted and precise due to recent developments in CRISPR/Cas9 technology, hastening the advancement of agriculture [42]. To date, only a few species have been studied using this methodology [74].The yield, quality, disease resistance, and climatic adaptability of monocots and dicots have all been improved by the CRISPR/Cas9 system [75]. The genomes of cereal crops including wheat, maize, rice, and cotton as well as fruits and vegetables such as tomatoes and potatoes have all been altered using the CRISPR/Cas9 technique [76,77].

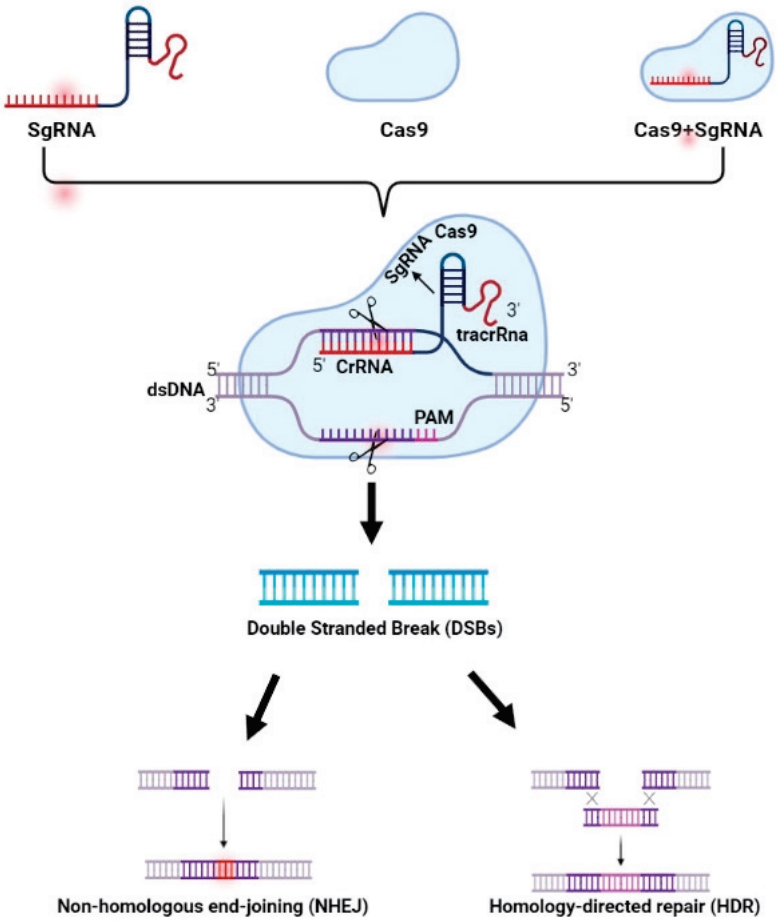


**Figure 2.** Mechanism of genome editing using CRISPR/Cas9.

According to Makarova et al. [78], the CRISPR/Cas system can be divided into three types: type I, type II, and type III. Bacteria and archaea both have type I CRISPR/Cas mechanisms based on the exact signature of the Cas protein. The Cas3 protein's endonuclease activity is used to connect to the DNA sequence [78]. In bacteria, the type II CRISPR/Cas system has been developed. The four protein pairs Cas1, Cas2, Cas4/Csn2 proteins, coupled with Cas9, make up the simplest system. The type III CRISPR/Cas system hunts for DNA and RNA in archaea, as well as infrequently in bacteria. Cas6, Cas10, and repeat associated mysterious proteins (RAMP) are markers for its presence. Cas10 protein's processing of crRNA ultimately aims to cleave DNA [78]. The *Streptococcus pyogenes* (SpCas9)-derived type II CRISPR system mostly targets the negatively regulating genes [79].

The CRISPR/Cas technique is straightforward, stable, and enables effective change compared withthe first two generations of genome-editing systems. These traits allowed CRISPR/Cas to quickly replace the traditional genome-editing methods ZFN and TALEN. The techniquewas adapted from the bacterial defense mechanism. The CRISPR/Cas mechanism is used by a variety of bacterial and archaeal species to protect themselves against invading viruses [80]. Many studies are now being conducted to improve the CRISPR/Cas system and increase the tool's ability to target the genome. For instance, non-canonical NGA and NG PAM sites in plants may be found using xCas9, SpCas9-VRQR, and Cas9-NG variants [81,82]. SpCas9 orthologues have been recognized from *Streptococcus thermophiles* (St1Cas9), *Staphylococcus aureus* (SaCas9), *Streptococcus canis* (ScCas9), and *Brevibacillus laterosporus* (BlatCas9).They have been demonstrated to amend plant genomic loci with PAM sequences of NNGRRT, NNG, NNAG AAW, and NNNCND, respectively [83,84]. Additionally, the type V Cas12a and Cas12b extracted from different bacterialsystems have been demonstrated with AT-rich PAM specifications and employed in genome editing of selected plants [85,86].

The CRISPR/Cas9 gene-editing approach has so far been used on more than 20 crop species to increase yields and reduce biotic and abiotic stress [87]. Genome-editing techniques based on CRISPR/Cas9 have been utilized to enhance agricultural disease resistance and tolerance to severe abiotic environments including salinity and drought. Three rice genes involved in regulating responses to various abiotic stress stimuli, including phytoene desaturase (OsPDS), betaine aldehyde dehydrogenase (OsBADH2), and mitogen-activated protein kinase (OsMPK2), have undergone sequence-specific CRISPR/Cas9-mediated genomic modification. CRISPR/Cas9 technology was successfully used by Shan et al. [88] to insert the TaMLO gene (mildew resistance locus O) into wheat protoplasts. It was also discovered that *Blumeria graminis* f. sp. Tritici, the agent of powdery mildew illness, is resistant to the CRISPR TaMLO knockdown (Btg). Wheat ethylene responsive factor3 (TaERF3) and wheat dehydration response element binding protein 2 (TaDREB2) are two abiotic stress-related genes that were targeted by the CRISPR/Cas9 genome-editing technology in wheat protoplasts, according to Kim et al. [89]. The CRISPR/Cas9 technology can be used in conjunction with current and upcoming breeding techniques such as speed breeding and omics-assisted breeding to boost agricultural production and ensure food security (Table 2).

**Table 1.** Comparison of different types of plant genome-editing techniques.

| Feature | ZFNs | Meganucleases | TALENs | CRISPR/Cas | References |
|---|---|---|---|---|---|
| Length of target sequence (bp) | 18–36 bp | 12–40 bp | 28–40 bp | 20–22 bp | [90,91] |
| Nuclease protein | FokI | I-SceI | FokI | Cas9 proteins | [91–93] |
| Dimerization | Required | Not required | Not required | Not required | [90–92] |
| Mode of action | Double-stranded break in target DNA | Direct conversions in targeted regions | Double-stranded break in target DNA | Double-stranded breaks or single-stranded nicks in target DNA | [94–96] |
| Repair events | NHEJ | HDR | HDR | NHEJ | [92,93,97] |
| Mutagenesis | High | Middle | Middle | Lower | [94] |
| Cloning | Necessary | Not necessary | Necessary | Not necessary | [91,98,99] |
| Creation of libraries and multiplexing | Challenging | Challenging | Challenging | Possible | [91,96,99] |
| Cost | Higher | Higher | Higher | Low | [100] |
| Types | One | One | One | Many | [101] |
| Specificity | Moderate | High | High | Low | [90,91] |
| Crop improvement | Low | Low | Low | High | [100] |
| Future use | Medium | Medium | Medium | High | [100] |

**Table 2.** List of reported targeted gene(s) via ZFNs, TALEN, and MNs gene-editing tool technologies in different plant species to develop resistant/tolerant genotypes.

| Crop | Gene | Trait | Technique | References |
|---|---|---|---|---|
| Rice | *OsQQR* | Detection of safe harbor loci herbicide | ZFNs | [102] |
| | *OsBADH2*, *OsDEP1*, *OsSD1*, *OsCKX2* | Fragrance | TALEN | [103] |
| | *Os11N3* | Bacterial blight resistance | TALEN | [67] |
| | *OsCSA* | Photoperiod sensitive male sterility | TALEN | [104] |
| | *OsDERF1* | Drought tolerance | TALEN | [104] |

**Table 2.** *Cont.*

| Crop | Gene | Trait | Technique | References |
|---|---|---|---|---|
| Wheat | *TaMLO-A1*, *TaMLO-B1*, *TaMLO-D1* | Resistance to powdery mildew | TALEN | [105] |
| Maize | *PAT* | Herbicide resistance | ZFNs | [106] |
| | *ZmIPK1* | Herbicide tolerant and phytate reduced maize | ZFNs | [53] |
| | *ZmTLP* | Trait stacking | ZFNs | [107] |
| | *ZmPDS*, *ZmIPK1A*, *ZmIPK*, *ZmMRP4* | Biosynthesis of phytic acid | TALEN | [108] |
| | *MS26* | Independent lines of male sterile plants | MNs | [109] |
| Barley | *HvPAPhy* | Phytase reduction and seed development | TALEN | [110] |
| Soybean | *DCL* | Herbicide transmission | ZFNs | [111] |
| | *FAD2-1A*, *FAD2-1B* | Low polyunsaturated fats | TALEN | [68,69] |
| Tobacco | *GUS: NPTII* | Chromosome breaks | ZFNs | [112] |
| | Endochitinase-50 gene (*CHN50*) | Emergence of resistance to herbicides | ZFNs | [113] |
| Tomato | *L1L4/NF-YB6* | Reduced contents of the anti-nutrient's oxalic acid | ZFNs | [114] |
| Cotton | *EPSPS* | Herbicide tolerance | MNs | [115] |
| | *Hppd* | Herbicide tolerance | MNs | [115] |
| Potato | *VInv* | Sugar metabolism | TALEN | [116] |

### *2.5. DSB-Free Genome Editing*

A sole histidine residue at site 840 of the HNH domain of SpCas9 cuts the PAM strand, while the aspartate at site 10 in the RuvC domain cuts the opposite strand3. Mutating both amino acids to alanines (D10A and H840A) resulted in nuclease-dead Cas9 (dCas9). dCas9 still identifies its target site and frees up the DNA in an R-loop without including DSBs. The binding of dCas9 to its solitary target site can work as a repressor of transcription and is called CRISPR interference (CRISPRi). Alternately, dCas9 can be utilized as a tool for localization of DNA effector proteins to the genome. Examples of this approach are CRISPR–DNMT3 fusion proteins and CRISPR activators (CRISPRa) for targeted methylation. DNA-alteration enzymes are combined with dCas9 to induce genetic variants for overcoming the limitations linked with DSB initiation in genome engineering [117].

### *2.6. Base Editing*

The first base editor combines dCas9 to the cytidine deaminase apolipoprotein B mRNA editing catalytic polypeptide-like (rAPOBEC1), which catalyzes the alteration from cytidine to uracil. The cell mends this uracil into thymidine, resultingin an assembly (BE1) replacing a C•G by a T•A base pair, entitled a cytosine base editor (CBE) [118]. First-generation CBEs were suppressed by uracil glycosylation. So, second-generation base editors (BE2) were invented by combining an uracil glycosylase inhibitor (UGI) with the dCas9–rAPOBEC1 combination [119].For increasing editing efficiency, dCas9 can be changed into a nickase SpCas9-D10A (BE3). The strand not altered by rAPOBEC1 is cleaved. The cell identifies this nick and starts DNA repair to solve the damage. The strand withthe base modification is used as a template for repairing the nick to yield stable integration. The BE3 architecture was furthermore ameliorated by combining an additional UGI in fusion with linker optimization to result in a fourth-generation cytosine base editor (BE4). BE4s have improved editing efficiency by approximately50%, with two-fold decline of unintended byproduct formation such as point mutations and indels [118]. Subsequent ancestral reconstitution and codon optimization led to a CBE architecture that enables the

most powerful base editing in organoids, 2D cell lines, and in vivo by improving nuclear localization and expression of the proteins [120].

### *2.7. Prime Editing*

The logic behind prime editing is to escort exogenous DNA with the modification of interest close to the Cas9 binding site. Areverse transcription (RT) domain obtained from the Moloney murine leukaemia virus was combinedwith nickase SpCas9- H840Atodevelopthe first generation of prime editors (PE1). The RT domain changes RNA into DNA tofind its template in the 3′ extension of the specially designed sgRNA, entitledthe primeediting guide RNA (pegRNA).Itguides the Cas9 in PE1 to the target site. After targetrecogination, the PAM-consistingstrand is nicked by the active HNH domain of Cas9-H840A. Then, the pegRNA extension combineswiththe nicked strand of the primer-binding site (PBS).Then, the RT domain of PE1 uses the restpegRNA(RT template) to synthesize a 3′-DNA flap containingthe edit of interest. This DNAflap is solved by cellular DNA repair procedure combining the edit of interest [121]. Theprime editing requires optimizing PE3guides andpegRNA, limiting its implementationin organoids. Threemodifications have been made forovercoming this issue. First, the utilizationof two pegRNAs in trans alongwith overlayingRT domains enhancesprime-editing competencein plants [121]. Second, engineered pegRNAs can have tmpknot or evopreqdomains combinedatthe 3′ end. These domains enhancethe stability of the pegRNA [122]. Finally, including the N394Kand R221K amino acid alterationincreases the nuclease workof SpCas9, resulting in a more efficient PE2Max [123].

### *2.8. Mobile CRISPR*

A breakthrough in the CRISPR tool, "genetic scissors" was announced by scientists of the Max Planck Institute of Molecular Plant Physiology to edit plant genomes. The discovery could speed up and simplify development of novel and genetically stable crop varieties by fusing grafting with a 'mobile' CRISPR tool. The drawing of the CRISPR/Cas9 gene scissors is transferred as RNA from the rootstock of a genetically modified plant to the grafted shoot of a normal plant. The gene scissors protein is made with the aid of the RNA. This gene scissor protein edits specific genes in flowers. Plants carry the desired gene modification in the next generation. A normal shoot is grafted onto roots containing a mobile CRISPR/Cas9, which allows the genetic scissor to move from the root into the shoot. It edits the plant DNA without leaving a trace of itself in the subsequent generations of plants. This ground-breaking turn can save cost and time and evade current limitations of plant breeding.

## 3. Genome Editing Related to Abiotic Stresses

Abiotic stresses that impact plant growth and development, such as salt, drought, extremely high temperatures, cold, and heavy metals, can reduce agricultural production by approximately 50% [124].Numerous biochemical, morphological, and physiological factors important for plant development are influenced by stress. Stresses from the environment can modify how plants behave as they develop. Most changes in plant growth and development caused by different abiotic stresses are associated with poorer yields [13]. By 2050, the rapid growth in the human population is predicted to reach 9.7 billion. The global temperature is also set to increase significantly. As plant scientists, it is hard for us to manage the food requirements of the increasing population. However, we own the capability to develop climate-flexible crop varieties that can flourish under such challenging circumstances. These varieties must be maintained in ruthless climatic conditions such as heat, drought, heavy metals, cold, or flood stresses. This requires a continuous search for newer and diverse germplasm [125,126], which was traditionally performed either entirely through development of natural variations [127,128] or by selective breeding [129,130]. Another possibility is the construction of mutant populations that are evaluated to hunt for new resources among variations that might be novel valuable mutations that in turn are included in breeding programmes. Modern genome-editing system tools such as CRISPR

facilitate the user to commence desirable genomic modifications accurately, illustrating great promise as a tool for producing novel climate-resistant plants [131]. In over 20 agronomically important crops, CRISPR/Cas mediated gene editing is widely utilized and accepted for crop improvement against different abiotic stresses [79].

Ordinarily, plants are equipped with numerous defense schemes against abiotic stresses. Among numerous defense mechanisms of abiotic stresses, the five broad-spectrum protections are regulated utilized in a complicated managing network consisting of numerous mediators and gene regulatory constituents in response to abiotic stresses [132]. During the procedure, stress hormones, particularly nitrogen oxides (NO), abscisic acid (ABA), polyamines (PAs), calcium ions ($Ca^{2+}$), hydrogen sulfide ($H_2S$), reactive oxygen species (ROS), and phytochrome B (PHYB), interact with others, either synergistically or antagonistically. The transcription factors (TFs) could alter the expression of genes and enzyme activity in a regulatory way, triggering a suitable reaction. The regulatory constituents open a lot of potential for developing multiple stress tolerance/resistance. Five main plant defenses to abiotic stresses are ROS scavengers, molecular chaperones, cuticle as the outer shield, oxylipin precursors, and osmoprotectants, along with unsaturated fatty acids, and compatible solutes [132].

### *3.1. Drought Stress*

Drought is becoming a challenge to sustainable agriculture due to the consequences of climate change, including erratic rainfall patterns and rising temperatures in many regions of the world. The greatest danger to global food security is drought stress, which is the primary factor in the catastrophic loss of agricultural production and productivity [133]. Drought alone can reduce yield by 50–70% in different crops [134]. For example, 40% yield losses due to drought stress have been reported in maize [35,135], 50% in rice [136], 21% in wheat [126,135], 27–40% in chickpea [125,137], 68% in cowpea [138] and 42% in soybean [34,139]. After the discovery of genome editing, efforts are being planned to alter the genes involved in pathways enabling drought tolerance, in order to increase farmers' acceptance of crops using these technologies. In recent years, in-depth research has helped to adapt and overcome drought stress using CRISPR-Cas9 technology (Table 3).

In many crop plants, $H_2O_2$ and abscisic acid (ABA) are frequently produced in situations of salinity or drought stress. The discovery was reported of ABA-induced transcription repressors (AITRs) as a novel transcription factor family that plays a significant role as feedback regulators of ABA signaling. Alternation in the expression of AITR genes resulted in abiotic stress tolerance, including drought and salinity in *Arabidopsis* [140,141]. A CRISPR/Cas9-induced mutation in the *Arabidopsis* OST2 structural gene exhibited drought resistance [142]. Another study found that knockout of Arabidopsis plants' genemiR169athrough CRISPR/Cas9 led to significantly improved drought tolerance [143]. Similarly, Arabidopsis' drought tolerance increased after the vacuolar H+-pyrophosphate (AVP1) regulating gene was expressed using CRISPR/Cas9 [144]. Similar results were shown when the abscisic acid-responsive element binding gene (AREB1) was activated in Arabidopsis through CRISPR/Cas9a [145]. Recently, drought tolerance in *Arabidopsis thaliana* was demonstrated via the CRISPR/Cas9 gene silencing of the trehalose (TRE1) gene [146].

Numerous studies have documented how CRISPR confers drought resistance in many plants. For instance, it has been demonstrated that increasing rice's ability to withstand drought can be attained by reducing the expression of the regulatory genes DERF1, PMS3, MSH1, MYB5, and SPP [147]. In rice plants, drought stress tolerance increased after OsERA1 was modified using CRISPR/Cas9 [148]. CRISPR/Cas9 has been employed to improve drought resistance in rice by knocking out the SRL1, SRL2, and ERA1 genes [148,149]. A CRISPR/Cas9-created ospyl9 mutant might increase rice yield and drought tolerance [150]. Indica mega rice cultivar MTU1010 with broader leaves, a decreased stomatal density, and improved leaf water retention under drought stress was developed using CRISPR/Cas9 to modify the *OsDST* gene [151]. The *OsOREB1*, *OsRab21*, *OsRab16b*, *OsLEA3*, *OsbZIP23*,

*OsSLAC1*, and *OsSLAC7* genes, which act downstream of SAPK2, were modulated in expression in the loss-of-function sapk2 mutant of rice plants developed using CRISPR/Cas, increasing their tolerance to drought stress [131].

Two genes, *RVE7* and *4CL*, have been found to be associated with drought tolerance in chickpeas. The first report of CRISPR/Cas9-mediatedediting of the chickpea protoplast was made by Badhan et al. [152]. They described knockouts of the genes *4CL* and *RVE7*, which are linked to pathways for drought tolerance. That study established a framework for potential future chickpea-genome-editing approaches [153]. Another gene, namely *ARGOS8*, responding to drought stress has been altered through genome editing. The expression of the *ARGOS8* gene increased as a result of negative regulators of ethylene signaling pathways, providing drought tolerance [154,155]. To increase the production of maize under drought stress under field conditions, the GOS2 promoter region was replaced with an *ARGOS8* promoter sequence using the CRISPR/Cas system [156].

CRISPR/Cas9 altered the *GID1* gene in tomato plants, which exhibit high leaf water content under drought conditions [157]. Additionally, *SlLBD40* gene mutation caused by CRISPR/Cas9 significantly improved drought tolerance in tomato [158]. Furthermore, use of the CRISPR/Cas technique to alter mitogen-activated protein kinases (MAPKs) revealed SlMAPK3 to be a drought stress modulator [159]. Knockout of the *SlNPR1* gene resulted in increased drought tolerance and down-regulation of drought-related genes [160].

Drought resistance of wheat was improved by CRISPR/Cas editing of wheat *TaDREB2* and *TaERF3* [89]. In wheat, a multiplex CRISPR/Cas9 assay was used to alter the *SAL1* gene, a negative regulator of drought tolerance, to increase drought tolerance at the seedling stage [161]. CRISPR/Cas genome editing of the HB12 gene can increase cotton's resistance to drought [162]. CRISPR/Cas9 was used to modify the *BnaA6.RGA* gene in oil seed crops, which significantly improved rapeseed's ability to withstand drought [163].

### *3.2. Heat/Temperature Stress*

Plants have a preferred temperature, any rise or fall in that temperature can significantly impede their development and productivity. The third most important abiotic factor is heating stress, which may decrease crop production considerably. For instance, every 1 °C augmentation in atmospheric temperature diminishes wheat yield by 6%, rice yield by 10–20%, and corn yield by 21–31% [164–166]. Significant yield losses were caused by high heat stress, which is now recognized as a severe problem that will simply become worse in the future. All phases of plant growth, from germination to harvest, are severely harmed by heat stress [167,168]. Heat stress not only increases plant mortality rates but also reduces plant quality [169,170].

In severe cases, a bad alteration in temperature results in plant mortality because plants are more susceptible to temperature changes. The ideal temperature would normally be better for crop growth and development; conditions below and above the optimum temperature have a harmful effect on productivity. For every 10 °C rise, followed by 20 °C and 30 °C, mostbiochemical and enzymatic procedures double in speed [171]. Abiotic stressors, predominantly high and low heat, have a harmful effect on the premature stage of the male gametophyte in a range of agricultural crops, including maize, rice, barley, wheat, sorghum, and chickpea [172]. Due to temperature stress, the functions of tapetal cells are diminishedduring the reproductive growth period, and the anther is dysplastic. Pollen discharge is insufficient and indehiscence happens as a result of increased heat preventing pollen grains from escalating. Plants have developed precise physiological and chemical reactions to manage temperature stress [173].

The presence of genes that are responsive to heat stress, signal transduction, and the synthesis of metabolites are only a few of the complex molecular systems that plants activate in response to heat stress. Different temperature-stress-related genes have been identified and characterized to improve plants' ability to withstand heat as a result of developments in structural and functional genomics technologies in plants. The heat stress reaction, which is connected to the accumulation of ROS, is mediated by the heat shock transcription factors

(HSFs) and the heat shock proteins (HSPs) [174].Therefore, by enhancing plants' ability to resist ROS components, temperature stress tolerance can be improved [175]. This indicated that higher tolerance might increase the antioxidant properties of crops. Plant temperature tolerance was significantly increased via metabolite production and temperature-induced gene expression. To explore the molecular processes associated with temperature stress and improve plant heat tolerance, CRISPR-Cas9 is a cutting-edge technology among all genome-editing techniques [176] (Table 3).

A cultivable HS-inducible rice mutant was created using CRISPR/Cas9 technology [177]. The orthologs of mitogen-activated protein kinase 3 and agamous-like 6 were modified using CRISPR to increase tomato sensitivity to heat stress, whereas ADP-ribosylation factor 4 enhanced tomato sensitivity to salinity shocks. According to Bouzroud et al. [178], these CRISPR-edited mutant plants had improved agronomic characteristics and were resilient to abiotic stresses. As a component for heat tolerance, BRZ1 positively regulates the formation of ROS in the tomato apoplastic area. This was confirmed by the CRISPR-Cas9-based bzr1 mutants, which showed reduced temperature tolerance and respiratory burst oxidase homolog 1 (RBOH1) with diminished hydrogen peroxide generation in the apoplast [179]. In comparison to wild-type crops, the development of CRISPR/Cas-mediated heat-stress-sensitive albino 1 (HSA1) mutants of tomato showed greater sensitivity to temperature stress [180].

The thermosensitive genic male sterile gene was altered by CRISPR in maize to promote thermo susceptible male-sterile plants [181]. In lettuce, knockouts of NCED4, a crucial regulating enzyme in abscisic acid production, allowed the seeds to germinate at a higher temperature. As a result, LsNCED4 mutants may have commercial significance in manufacturing environments with high temperatures [182]. In order to make a plant more resistant to heat, the hsps gene, which increases osmolyte levels and prevents cell protein damage, can be overexpressed [183]. The protein kinase SAPK6 and the transcription factor OsbZIP46CA1 in rice also increase the capacity for responding to heat stress [184].

### *3.3. Cold Stress*

Cold stress, which includes chilling (20 °C) and freezing (0 °C) temperatures, hinders plant growth and development and severely limits plant geographic expansion and agricultural productivity [185]. Plants are directly inhibited from responding metabolically to low temperatures, which results in osmotic stress, oxidative stress, and other types of stress. Due to mechanical damage and metabolic dysfunction caused by extreme cold temperatures, plant growth and development are halted [186]. The physiological, biochemical, and molecular behavior of plants during their growth and expansion is adversely affected by cold stressors. The photosynthetic capacity and crop anatomy are brutally impacted by cold exposure, especially throughout the winter [187,188].Cold stress during the seedling stage may cause impaired germination and emergence. Long-term exposure impairs source–sink relationships, growth, nutrient localization, and leaf chlorosis [189]. Membrane formation, which amplifies other cold-stress-related downstream processes, is the main consequence of cold stress on crops [190]. In-generic or inter-specific hybridization has been successful in boosting the cold tolerance of significant crops using conventional breeding methods. For creating non-transgenic genome-edited crops to combat climate change and ensure future food security, CRISPR/Cas9 is a clever and practical approach [191,192] (Table 4).

To increase the plant's resistance to cold, genome editing is employed to target a few of the depressant regulator transcription factors in rice. A transcription factor called OsMYB30 attaches to the amylase gene promoter and negatively affects cold tolerance. According to Lv et al. [193], under conditions of cold stress, OsMYB30 forms a compound with OsJAZ9 and slows down the expression of the amylase gene, which may contribute to increasing cold sensitivity by causing maltose buildup and starch breakdown. In order to determine the specific function of the TIFY1a, TIFY1b, and Ann3 genes in rice's ability to withstand cold stress, CRISPR/Cas9 technology has also been applied to these genes. The mutant outperformed the natural variation in terms of yield, temperature tolerance,

and amount of germination prior to harvest [194]. Using CRISPR base editing, suppression of photosynthetic genes in rice plants under cold stress has been shown to cause the white-striped leaves phenotype in the white stripe leaf 5 (wsl5) mutant line [195,196].

PRPs are proline-rich proteins that not only aid in dealing with low temperatures but also reduce nutrient loss, boost antioxidant activity, and aid in the production of chlorophyll. Rice capacity for cold tolerance was improved by the CRISPR/Cas9 deletion of OsPRP1, which encodes a proline-rich protein [197]. In a recent work using CRISPR/Cas9, three rice genes, viz., OsPIN5b, GS3, and OsMYB30were altered to increase spike length, grain size, and resilience to cold stress [198]. The CRISPR/Cas9 technology altered the G-complex-related genes i.e., OsRGA1, OsGS3, OsDEP1, and OsPXLG4 to make rice more resistant to chilling stress [199].Because tomato plants are prone to chilling stress, their fruits are more vulnerable to damage from the cold. C-repeat binding factor 1 (CBF1) was shown using CRISPR-Cas9-based cbf1 mutants to protect the tomato plant next to it from cold/chilling damage and decrease electrolyte leakage [200]. These plants also demonstrated excellent addition of hydrogen peroxide and indole acetic acid, resulting in tomato plants tolerant of chilling stress.

**Table 3.** List of reported targeted gene(s) via CRISPR/Cas9 technology in different plant species for development of tolerant genotypes against drought and heat stresses.

| Crops | Gene | Trait | Technique | References |
|---|---|---|---|---|
| Rice | *OsDERF1* | Drought | CRISPR/Cas9 | [147] |
| Rice | *SRL1*, *SRL2* | Drought | CRISPR/Cas9 | [149] |
| Rice | *OsAAA-1*, *OsAAA-2* | Drought | CRISPR/Cas9 | [201] |
| Rice | *OsNAC006* (transcription factor) | Drought and heat sensitivity | CRISPR/Cas9 | [202] |
| Rice | *OsAOX1a* | Drought resistance | CRISPR/Cas9 | [147] |
| Rice | *OsDST* | Drought and salinity | CRISPR/Cas9 | [151] |
| Rice | *OsERA1*, *OsPYL9* | Drought | CRISPR/Cas9 | [148,150] |
| Rice | *SAPK2* | Tolerance to salinity and drought | CRISPR/Cas9 | [131] |
| Rice | *OsPMS3* | Photoperiod-sensitive male-sterile | CRISPR/Cas9 | [147] |
| Rice | *Csa* | Photosensitive-genic male-sterile | CRISPR/Cas9 | [203,204] |
| Rice | *TMS5* | Thermo-sensitive genic male-sterile | CRISPR/Cas9 | [205] |
| Rice | *OsNAC14* | Drought tolerance | CRISPR/Cas9 | [206] |
| Rice | *OsPUB67* | Drought tolerance | CRISPR/Cas9 | [207] |
| Wheat | *TaDREB2*, *TaERF3* | Tolerance to drought | CRISPR/Cas9 | [89] |
| Maize | *ZmARGOS8* | Drought | CRISPR/Cas9 | [156] |
| Maize | *ZmTMS5* | Creation of thermosensitive maize lines | CRISPR/Cas9 | [181] |
| Mustard | *BnaA6.RGA* | Drought tolerance | CRISPR/Cas9 | [163] |
| Soybean | *Drb2a*, *Drb2b* | Tolerance to drought and salinity stress | CRISPR/Cas9 | [208] |
| Soybean | *GmMYB118* | Drought tolerance | CRISPR/Cas9 | [209] |
| Chickpea | *4CL*, *RVE7* | Drought tolerance | CRISPR/Cas9 | [152] |
| Tomato | *SlMAPK3* and *SlNPR1* | Drought | CRISPR/Cas9 | [159,160] |
| Tomato | *SlARF4* | Drought | CRISPR/Cas9 | [140] |
| Tomato | *SlAGL6* | Heat stress | CRISPR/Cas9 | [210] |

**Table 4.** List of reported targeted gene(s) via CRISPR/Cas9 technology in different plant species for development of tolerant genotypes against cold stresses.

| Crops | Gene | Trait | Technique | References |
|---|---|---|---|---|
| Rice | *OsMYB30* | Cold tolerance | CRISPR/Cas9 | [198] |
| Rice | *OsAnn3* | Cold tolerance | CRISPR/Cas9 | [211] |
| Rice | *OsAnn5* | Cold tolerance | CRISPR/Cas9 | [211] |
| Rice | *OsPRP1* | Cold tolerance | CRISPR/Cas9 | [212] |
| Tomato | *SlCBF1* | Cold tolerance | CRISPR/Cas9 | [200] |
| *Arabidopsis thaliana* | *AtCBF1*, *AtCBF2* | Cold tolerance | CRISPR/Cas9 | [213] |

### *3.4. Salinity Stress*

Owing to the negative consequences of climate change, salinity stress has recently become much worse [214]. Salinity stress is the second most severe abiotic danger that affects fertile lands as well as crop productivity [215]. According to Morton et al. [216] and Van Zelm et al. [217], severe salts have an impact on about one-fifth of the irrigated agricultural area. Lack of good irrigation water, a changing climate, and excessive use of chemicals such as fertilizers and pesticides prolong the process of adding more land to the salinity stress zone. According to estimates made by Jamil et al. [218], 50% of cultivable lands will be saline by 2050 due to the overuse of chemicals including fertilizers and pesticides. One of the most important and harmful factors that has a negative impact on soil quality and agricultural output is salt stress. When too many soluble salts accumulate in the crop root zone, it causes salinization of the soil because roots are unable to absorb water. Thus, osmotic stress and nutritional imbalance in plants have a negative impact on their morphology, biochemistry, and biomass, which ultimately causes irreparable plant damage [219–221].

Reactive oxygen species (ROS) are intensified by salt stress, which has a detrimental effect on crops' cellular and metabolic processes [222,223]. Lipid peroxidation, which causes membrane deterioration as well as protein and DNA damage, is a harmful effect of ROS [224]. By diminishing chlorophyll content and stomatal conductance, salt stress hinders the development of the photosystem II and the transpiratory apparatus [225]. Additionally, it decreases the water potential of the soil and leaves, which lowers plant turgor pressure by affecting water relations and causing osmotic stress [226]. Plants suffer from decreased leaf area, lower photosynthetic rate, poor seed germination, decreased biomass production, and crop yield as a result [227–229]. Salinity tolerance is the ability of a plant to maintain the equilibrium of biomass and/or output under conditions of salt stress. In order to tolerate salt, plants have several molecular and physiological mechanisms [230].

Genome editing has the capacity to improve crops; there are yet few studies on its effective application in breeding plants that can withstand saline stress (Table 5). In one such work, rice was modified to impart salt stress tolerance by editing the *OsRR22* gene, which encodes for a transcription factor (TF) involved in the control of signaling and the metabolism of cytokinins in plants [231,232]. Using CRISPR/Cas9 technology, the *OsRR22* gene was altered, and two homologous $T_2$ generations revealed improved salt tolerance with no discernible difference between the modified and wild-type lines [232]. Using CRISPR/Cas9 technology, the paraquat tolerance-3 mutations (*OsPQT3*) gave rice a high level of salt tolerance [233]. The function of *OsmiR535* in salt stress tolerance was investigated using genome-editing techniques, and it was proposed that *OsmiR535* might be knocked out using CRISPR/Cas9 to enhance salinity tolerance in rice. Additionally, a homozygous 5bp deletion in the *OsmiR535* coding region might be a valid target for raising rice's salt tolerance [234]. Furthermore, some other genes increase the ability of rice to tolerate salt, using CRISPR/Cas9 technology by eliminating the *OsbHLH024* gene and increasing the expression of the ion transporter genes including *OsHKT1*;3, *OsHAK7*, and

*OsSOS1* [235]. When the rice *OsRAV2* gene was altered using CRISPR-Cas, the rice plants were able to survive under high salt conditions [236].

**Table 5.** List of reported targeted gene(s) via CRISPR/Cas9 technology in different plant species for developing salinity tolerance.

| Crops | Gene | Trait | Technique | References |
|---|---|---|---|---|
| Rice | *OsbHLH024* | Salinity | CRISPR/Cas9 | [235] |
| Rice | *OsRR22* | Salinity | CRISPR/Cas9 | [232,237] |
| Rice | *OsRAV2*, *OsNAC041*, *OsmiR535* | Salinity | CRISPR/Cas9 | [234,236,238] |
| Rice | *OsRR9*, *OsRR10* | Salinity | CRISPR/Cas9 | [239] |
| Rice | *OsNAC041* | Salinity | CRISPR/Cas9 | [240] |
| Rice | *OsOTS1* | Salinity | CRISPR/Cas9 | [241,242] |
| Rice | *OsDST* | Drought and salinity | CRISPR/Cas9 | [151] |
| Rice | *SAPK2* | Tolerance to salinity | CRISPR/Cas9 | [131] |
| Wheat | *TaHAG1* | Salt tolerance | CRISPR/Cas9 | [243] |
| Maize | *ZmHKTI* | Tolerance to salinity | CRISPR/Cas9 | [244] |
| Soybean | *GmAITR* | Salt tolerance | CRISPR/Cas9 | [245] |
| Soybean | *Drb2a*, *Drb2b* | Tolerance to droughtand salinity stress | CRISPR/Cas9 | [208] |
| Barley | *HvITPK1* | salinity | CRISPR/Cas9 | [246] |
| Tomato | *SlHyPRP1*, *SlARF4* | salinity | CRISPR/Cas9 | [247,248] |

Improvements in salt stress tolerance were seen in tomatoes after changes were made to the 8CM and PRD domains of the hybrid proline-rich protein1 (HyPRP1) [247]. Additionally, the capability of crops to tolerate salt stress may be significantly increased by employing CRISPR/Cas9 technology to eliminate the *OsDST* genes for rice [151], *OsNAC041* [238], and HvITPK1 [246] for barley.

### *3.5. Heavy Metals Stress*

An important issue for sustainable agricultural development is heavy metals, which seriously impair plant growth and productivity [249]. Heavy metals (HMs) including Mn, Cu, Ni, Co, Cd, Fe, Zn, and Hg, among others, have accumulated in soils as a result of various human activities such the application of fertilizer, incorrect disposal of industrial waste, and unauthorized sewage disposal [250,251], or the hasty disposal of vehicle waste. They are either collected on the soil surface or leached from the soil into the groundwater [252,253]. Additionally, heavy metals cause oxidative stress by promoting the generation of hydroxyl radicals (OH), superoxide radicals, and hydrogen peroxide ($H_2O_2$) [250,254]. Plant physio-morphological activities are hampered by the accumulation of HMs, especially in the roots where they are blocked by Casparian strips or trapped by root cell walls, which eventually reduces crop output [255]. When consumed, heavy metals accumulated in plants canseriously impair human health [256].

To combat heavy metal stress in plants, CRISPR-Cas9-induced plant mutants may prove useful (Table 6). In contrast to WT Co10 plants, the oxp1/CRISPR mutant of Arabidopsis plants exhibits resistance to Cd, indicating an increased capacity for heavy metal detoxification in mutant crops [257]. Accordingly, study showed how indel mutations using gene-editing techniques could provide tolerance to heavy metals and xenobiotics in plants [257]. Increased plant tolerance to heavy metals is influenced by a variety of genes [258]. Several transporter genes in rice, including OsLCT1 and OsNramp5, are implicated in Cd absorption by the roots [259]. The amount of Cd in rice has been reduced

by CRISPR/Cas9-enabled gene-expression manipulation. Rice grains with OsNRAMP1 knocked out by CRISPR/Cas9 have decreased levels of Cd and lead (Pb) [260,261]. Eliminating an R2R3 MYB transcription factor called OsARM1 using CRISPR/Cas9 prevents rice from absorbing and transporting arsenic [262].Cesium (Cs+) absorption and translocation in rice are regulated by the *OsHAK1* gene. Using the CRISPR-Cas9 technique, the cesium permeable potassium transporter *OsHAK1* was turned inactive [263].

### *3.6. Herbicide Stress*

In order to increase crop productivity, there is a need to manage weed growth with application of herbicides. Herbicides destroy non-target plants while also causing stress to the target plants and weed plants by interfering with or changing their metabolic processes. They also leave soil residues that are hazardous to the environment [264,265].The morphological, physiological, and biochemical traits of agricultural plants have been negatively impacted by the inappropriate application of herbicides. Herbicide toxicity reduces photosynthetic activity, which has a detrimental impact on the ability of crop plants to produce yield. One of the main goals for raising agricultural productivity is the development of herbicide tolerance in crop plants. To improve herbicide resistance in plants, genome editing including ZFNs, TALENs, and CRISPR/Cas technologies is an excellent tool (Table 6).

Leucine, isoleucine, and valine are branched amino acids whose biosynthesis is catalyzed by the enzyme acetolactate synthase, which is encoded by the *ACETOLACTATE SYNTHASE* (*ALS*) gene [266,267]. It is a potential target of many herbicide improvement programmes. The recombination of acetolactate synthase using CRISPR/Cas9 produces herbicide resistance in rice [268] and in watermelons [269]. Additionally, using the same strategy and emphasizing the *ALS1* and *ALS2* genes, herbicide-resistant maize plants were produced [270]. CRISPR-based editing in the *OsALS1* gene has been used to introduce herbicide tolerance characteristics into rice [271,272]. Glyphosate is one of the most imperative and quickly adopted herbicides for function in resistant crops such as soybean, maize, sugar beet, and chili pepper. The advancement of glyphosate-resistant plants requires changes in the machinery of some genes [203]. 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme is implicated in the formation of aromatic compounds in crops with the transfer of phosphoenolpyruvate (PEP) enzyme for activating the reaction [203,273]. Glyphosate hinders the act of the EPSPS enzyme by inhibiting the add-on of glyphosate to the PEP enzyme binding sites, eventually blocking the formation of aromatic products and causing crop death [203]. The endogenous EPSPS gene of rice was targeted with CRISPR/Cas9 to produce site-specific gene incorporation and substitution, which were fully transferred to the next generation with crops 100% resistant to the glyphosate [203]. CRISPR/Cas9 was also utilized toproduce a mutation in the promoter of the EPSPS gene of chili to state this gene beneath the action of glyphosate [274]. The resulting crops were reasonably resistant to glyphosate, and additional studies advised that selecting a diverse promoter may assist in the development of entirely resistant chili [274].The modified genotypes of rice and flax now have enhanced tolerance to glyphosate as a result of the CRISPR/Cas9 change of two nucleic acid residues in the binding site of glyphosate–EPSPS [91,203]. Recently, herbicide resistance was developed in tomato plants by CRISPR-Cas9-based targeted mutations in EPSPS, PDS (phytoene desaturase), and ALS [92].

**Table 6.** List of reported targeted gene(s) via CRISPR/Cas9 technology in different plant species for tailoring herbicide and metal stress tolerance.

| Crops | Gene | Trait | Technique | References |
|---|---|---|---|---|
| Rice | *C287T* | Herbicide resistance | CRISPR/Cas9 | [274] |
| Rice | *BEL* | Herbicide resistance | CRISPR/Cas9 | [71] |
| Rice | *OsALS1* | Herbicide tolerance | CRISPR/Cas9 | [271] |

**Table 6.** *Cont.*

| Crops | Gene | Trait | Technique | References |
|---|---|---|---|---|
| Rice | *EPSPS* | Herbicide resistance | CRISPR/Cas9 | [203] |
| Rice | *SF3B1* | Herbicide resistance | CRISPR/Cas9 | [72] |
| Wheat | *ALS* | Herbicide resistance | CRISPR/Cas9 | [275,276] |
| Maize | *ALS1 and ALS2* | Herbicide resistance | CRISPR/Cas9 | [270] |
| Maize | *MS26* | Herbicide resistance | CRISPR/Cas9 | [270] |
| Soybean | *ALS1* | Resistant to Chlorsulfuron | CRISPR/Cas9 | [277] |
| Tomato | *ALS* | Resistant to Chlorsulfuron | CRISPR/Cas9 | [278] |
| Tomato | *SlEPSPS* | Herbicide resistance | CRISPR/Cas9 | [92] |
| Tomato | *SlALS1*, *SlALS2* | Herbicide resistance | CRISPR/Cas9 | [92] |
| Tomato | *Slpds1* | Herbicide resistance | CRISPR/Cas9 | [92] |
| Rice | *OsTubA2* | Base editing | CRISPR/Cas9 | [279] |
| Rice | *OsHAK1* | Low cesium accumulation | CRISPR/Cas9 | [263] |
| Rice | *OsPRX2* | Potassium deficiency tolerance | CRISPR/Cas9 | [280] |
| Rice | *OsARM1* | Increase tolerance to higharsenic | CRISPR/Cas9 | [260] |
| Rice | *OsLCT1* | Less cadmium accumulation | CRISPR/Cas9 | [259] |

## 4. Conclusions and Prospects

Plants serve as sources of food, fiber, medicine, biofuels, and other goods. Farmers need new, superior cultivars in order to increase crop output and feed both the nation and the world. Plant breeders need a variety of tools for this purpose, including genomics and marker-assisted molecular breeding. Scientists can now implant desired traits more precisely and faster than in the past. Meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR) system are genome-editing tools that have been used with greater accuracy and efficiency than conventional breeding to enhance the quality of staple, oilseed, and horticultural crops. Today, there are several successful cases of "genome editing." In order to edit genes accurately in the genomes of model and crop plants as well as a range of other organisms, genome editing employs designed nucleases as potent tools that target certain DNA sequences. A study of the literature on transcriptomics, biotechnology, genomics, and phonemics has shown that this novel approach to crop development is effective. CRISPR/Cas9-based genome editing is a genuinely innovative strategy. With genome editing, crops can effectively incorporate a variety of genetic traits. When these precise and powerful methods are applied to expedite plant breeding, they create certain outcomes. In order to accomplish a second Green Revolution and meet the escalating food demands of a quickly growing global population under constantly changing climatic conditions, plant breeding will advance with the help of this multidisciplinary approach. By overcoming the limitations of current transgenic techniques, genome-editing technology ushers in a new era of improved plant genetics. This information may be proved useful to plant breeders and researchers in their thorough evaluation of the use of various gene-editing tools to improve crops by focusing on the targeted gene. We believe that CRISPR/Cas9 technology islikely to bridge the GMO and societal divide in upcoming days.

**Author Contributions:** Writing—original draft preparation, R.K.Y., S.T., N.T., R.A., S.C., P.N.T. and D.K.P.; writing—review and editing, M.K.T. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Screening and Selection of Drought-Tolerant High-Yielding Chickpea Genotypes Based on Physio-Biochemical Selection Indices and Yield Trials

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**Abstract:** Chickpea production is seriously hampered by drought stress, which could be a great threat in the future for food security in developing countries. The present investigation aimed to screen the drought-tolerant response of forty desi chickpea genotypes against drought stress through various physio-biochemical selection indices and yield-attributing traits. Principle component-based biplot analysis recognized PG205, JG2016-44, JG63, and JG24 as tolerant genotypes based on physiological selection indices. These genotypes retained higher relative water content, stomatal conductance, internal $CO_2$ concentration, and photosynthetic rate. ICC4958, JG11, JAKI9218, JG16, JG63, and PG205 were selected as tolerant genotypes based on biochemical selection indices. These genotypes sustained higher chlorophyll, sugar and proline content with enhanced antioxidant enzyme activities. With respect to yield trials, JAKI9218, JG11, JG16, and ICC4958 had higher seed yield per plant, numbers of pods, and biological yield per plant. Finally, JG11, JAKI9218, ICC4958, JG16, JG63, and PG205 were selected as tolerant genotypes based on cumulative physio-biochemical selection indices and yield response. These identified drought-tolerant genotypes may be further employed in climate-smart chickpea breeding programs for sustainable production under a changing climate scenario.

**Keywords:** chickpea; drought stress; selection indices; drought tolerant genotypes

**Citation:** Tiwari, P.N.; Tiwari, S.; Sapre, S.; Babbar, A.; Tripathi, N.; Tiwari, S.; Tripathi, M.K. Screening and Selection of Drought-Tolerant High-Yielding Chickpea Genotypes Based on Physio-Biochemical Selection Indices and Yield Trials. *Life* **2023**, *13*, 1405. https://doi.org/10.3390/life13061405

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 24 April 2023
Revised: 14 June 2023
Accepted: 15 June 2023
Published: 17 June 2023



## 1. Introduction

Legumes play a significant role in human diet because they not only complement the nutrients in a cereal diet but also improve the taste and texture of staple dishes [1,2]. Chickpea is a nutrition-rich grain legume and serves as an inexpensive source of high-quality daily protein as compared to animal protein, so is vital for nutritional security in developing countries, especially the vegetarian people of India [3,4]. It also serves as an enhancer of soil fertility through biological nitrogen fixation and fits in various crop rotation systems for the improvement of soil fertility [5,6]. It is also known as Bengal Gram or Garbanzo, and originated from Turkey [7]. It is ranked third after dry beans and peas worldwide [8,9]. Globally, chickpea occupies 14.8 Mha area, spanning over 59 countries, with an annual production of 15.1 million tons [10]. The major global production of chickpea comes from Asian countries; India shares 70% of the global chickpea area and 67% global chickpea production as the largest chickpea-producing country, followed by Pakistan, Turkey, Australia and Myanmar [11]. Based on seed morphological traits, chickpea is separated into two groups, i.e., desi type with microsperma and Kabuli type with macrosperma [12,13].

Desi type is more important than Kabuli type, as it covers approximately 80–85% of global chickpea production [14]. Desi chickpea is a potential source of nutritional components, i.e., high-quality proteins composed of albumins and globulins in large quantities, amino acids, essential fatty acids, trace elements and minerals [15].

Chickpea is frequently grown as rainfed crop in arid and semiarid regions, where water requirement is mainly received with either seasonal rainfall or stored moisture under soil [16,17]. In the last few years, unpredicted climatic changes resulting in high temperature (heat stress) and unusual rainfall (floods) and drought stresses are becoming major threats for crop production [18–22]. Among climatic changes, low moisture and high temperature stresses are the most important yield-limiting stresses in chickpea [23]. Chickpea is most sensitive to water stress at pre-flowering and early pod filling stages [1,4]. It is estimated that terminal drought alone can cause up to 50% of yield losses in chickpea [4,24].

Genetic improvement could be a less expensive and more long-lasting solution for better drought adaptation in chickpea than agronomic options. However, an understanding of yield maintenance under low water supplies becomes increasingly difficult because of several mechanisms employed by plants for maintaining growth and development [25]. To experience better stability of grain yield under drought, trait-based breeding strategies are being increasingly emphasized above yield-based breeding because grain yield is greatly affected by genotype × environment interactions and depicts low heritability [26]. Trait-based breeding also enhances the probability of crosses, which result in additive gene action under drought conditions.

For chickpea breeders, the breeding of drought-tolerant cultivars has been a tough task because of the unavailability of good selection indices. The lack of genetic divergence and a good source of resistance/tolerance to different abiotic stresses has been a major obstacle in the development of high-yielding drought-tolerant chickpea cultivars [27]. The screening and selection of chickpea germplasm line (s) based on diverse morpho-physiological and biochemical traits becomes a pre-requisite for crop improvement under drought stress [26]. Although similar efforts have been made with a major focus on morpho-physiological and biochemical traits contributing to drought tolerance in chickpea [4,16,26,28], limited detail about the terminal stage drought tolerance of the same genetic material are available. Thus, to fill this gap, the present investigation was conducted to assess the effect of terminal drought stress in chickpea genotypes by evaluating key drought-tolerant indicator traits and to select high-yielding drought-tolerant chickpea genotypes, especially those cultivated in India.

## 2. Materials and Methods

The experiment was performed in a randomized completely block design (RCBD) with three replications during the post-rainy seasons of 2020–2021 and 2021–2022 under a rainout shelter at Biotechnology Centre, Jawaharlal Nehru Krishi Vishwa Vidyalaya (JNKVV), Jabalpur (23°10′ N 79°59′ E). To study the effect of normal irrigated and terminal drought-stressed conditions on the morpho-phenology, physiology, biochemistry, yield and other traits of desi chickpea at reproductive stage, forty chickpea genotypes, including drought-resistant types, released varieties and advanced breeding lines, were obtained from Lead Centre, All India Coordinated Research Project (AICRP) and the Department of Plant Breeding Genetics, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur, Madhaya Pradesh, India (Supplementary Material Table S1). The field was prepared with 1 m wide bed flanked by 0.45 m furrows and fertilized with di-ammonium phosphate (DAP) containing nitrogen (18.0 kg/ha) and phosphorus (20.0 kg/ha). Seeds were treated with Bavistin (2.0 g per kg seed weight), Chlorpyriphos 20EC (10.0 mL per kg seed) and Rhizobium (5.0 g per kg seed). Seeds were sown at a depth of 2–3 cm manually, maintaining a row-to-row distance of 45 cm. For the uniform emergence of seedlings, 20 mm irrigation was applied immediately after sowing. Thinning was performed after two weeks of seed germination to maintain a plant-to-plant distance of 10 cm within rows. Subsequently, drought stress was imposed by

withholding the water supply to the stressed set of plots before the onset of pod initiation up to the harvesting [29].

### *2.1. Physiological Traits*

Relative water content (RWC) and canopy temperature depression (CTD) were estimated according to Gontia-Mishra et al. [30] and Purushothaman et al. [26], respectively. The leaf gas exchange parameters, viz., photosynthesis rate (Pn), stomatal conductance ($g_s$), transpiration rate (Tr) and internal $CO_2$ concentration (Ci), were recorded using a portable infra-red gas analyzer (IRGA) LiCor-6400 (LiCor Instruments, Lincol, NE, USA).

### *2.2. Biochemical Traits*

Chlorophyll content was estimated according to Gontia-Mishra et al. [30], while protein content was determined using an extraction buffer, as mentioned in the Bradford assay [31]. To determine the oxidative stress of a cell, hydrogen peroxide ($H_2O_2$) content, lipid peroxidation content (malondialdehyde; MDA) and electrolyte leakage (EL) were measured as described by Velikova et al. [32], Naservafaeito et al. [33] and Sachdeva et al. [28], respectively. To estimate the osmolytes accumulation of a cell, free proline content of leaf using ninhydrin [30] and total soluble sugar content using an anthrone reagent methodology [34] were determined.

To determine the enhanced activity of antioxidant enzymes, crude enzyme was extracted using an enzyme extraction buffer. Superoxide dismutase (SOD) activity was determined according to Sharma et al. [35], and one unit of enzyme activity was defined as the amount of enzyme that decreased the absorbance by 50%. The estimation of peroxidase (POD) activity was performed following Rao et al. [36], and enzyme activity was calculated as per extinction coefficient of tetra-guaiacol $\in$ = 26.6 $mM^{-1}$ $cm^{-1}$. Catalase (CAT) activity was estimated according to Aebi et al. [37], and enzyme activity was calculated as the amount of $H_2O_2$ decomposed per min. Ascorbate peroxidase (APX) activity was determined as described by Nakano et al. [38], and enzyme activity was calculated as per extinction coefficient of ascorbate $\in$ = 2.8 $mM^{-1}cm^{-1}$.

### *2.3. Morpho-Phenological Traits, Yield and Yield Attributing Traits*

Plant height was recorded from the ground level to the shoot tip. The date when half of the plants in a replication had at least one flower opened and the date when more than 75% of the pods of a plant turned brownish yellow from the days after sowing (DAS) were recorded as days to 50% flowering (DTF) and days to maturity (DTM), respectively. At the time of harvesting, all the seed-filled pods of a plant were counted as numbers of pods (NOP), and the weight of the plant including the pods was recorded as biological yield per plant (BYPP). The harvested seeds of a plant were weighed to obtain seed yield per plant (SYPP). Harvest index (%) was calculated as the ratio between seed yield per plant and biological yield per plant multiplied by 100.

### *2.4. Statistical Analysis*

From each treatment, three plants were randomly selected to record the various drought-related morpho-phenological, physio-biochemical, and yield traits in two successive Rabi seasons (2020–2021 and 2021–2022). The data of both seasons were pooled for all 40 chickpea genotypes under both water conditions. The significance was established by analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) at $p < 0.05$ using STAR V2.0.1 and SPSS V20 software, respectively. Principle component analysis (PCA) and PCA-based biplots were constructed to select reliable chickpea genotype (s) under drought-stressed conditions using XLSTAT software. Cluster analysis was also constructed, employing algometric hierarchical clustering for all chickpea genotypes under drought stress by applying STAR V2.0.1.

## 3. Results

### *3.1. Effect of Terminal Drought Stress on Physiological Traits*

Under terminal drought-stressed condition, all studied physiological traits were significantly decreased in comparison to normal irrigated conditions in all chickpea genotypes (Supplementary Materials Tables S2 and S3). A higher RWC was maintained by genotype JG63 (77.66%), whereas lower RWC was noted in the genotype JG2016-36 (57.03%) (Figure 1). Higher CTD was obtained by genotype ICCV19616 (2.18 °C), whereas the lowest CTD was reported in genotype JG6 (1.08 °C). In terms of *C*i, the highest value was achieved in genotype PG205 (195.9 µmol $CO_2$ $m^{-2}s^{-1}$), whereas the lowest *C*i was recorded in genotype JG2022-75 (123.78 µmol $CO_2$ $m^{-2}s^{-1}$) (Figure 2). Figures 2 and 3 show that genotype JG2016-44 exhibited the maximum *P*n (18.31 µmol $CO_2$ $m^{-2}s^{-1}$) and gs (0.31 mol $H_2O$ $m^{-2}s^{-1}$), while the minimum *P*n (10.31 µmol $CO_2$ $m^{-2}s^{-1}$) and gs (0.17 mol $H_2O$ $m^{-2}s^{-1}$) were found in genotype JG2022-75. Higher *T*r was maintained in genotype JG2016-44 (15.4 mmol $H_2O$ $m^{-2}s^{-1}$),and the lowest was seen in genotype JG2022-75 (8.62 mmol $H_2O$ $m^{-2}s^{-1}$).

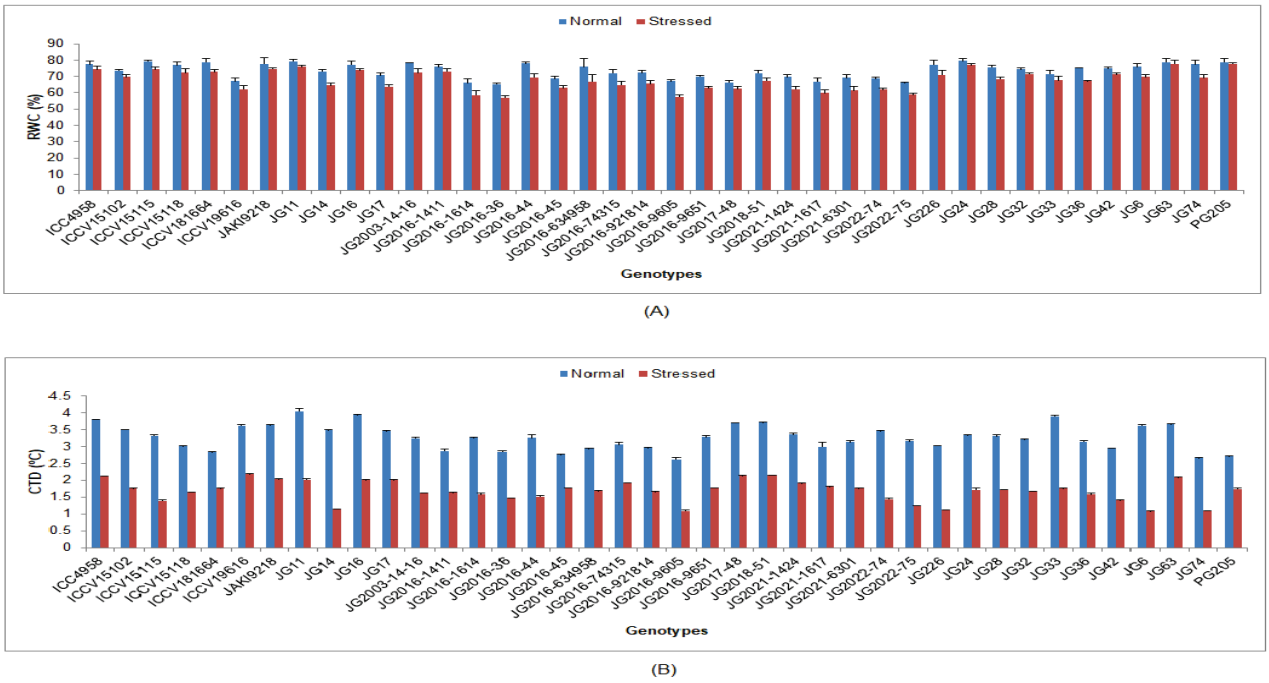



**Figure 1.** Effect of terminal drought stress on (**A**) RWC and (**B**) CTD of studied chickpea genotypes, where RWC and CTD indicate relative water content and canopy temperature depression, respectively.

### *3.2. Effect of Terminal Drought Stress on Biochemical Traits*

Under terminal drought-stressed conditions, chlorophyll a, b and protein content were significantly reduced, whilst $H_2O_2$ content, EL, lipid peroxidation (MDA) and antioxidant enzyme activities were significantly enhanced as compared to normal irrigated condition in all investigated chickpea genotypes (Supplementary Tables S4 and S5). Higher Chl 'a' was maintained by genotype JG16 (0.41 mg/g FW) (Figure 4), while higher Chl 'b' by genotype ICC4958 (0.31 mg/g FW). Higher protein content was upheld by genotype JG2021-6301 (0.47 mg/g FW), whilst the minimum was documented in genotype JG74 (0.34 mg/g FW) (Figure 5). Minimum $H_2O_2$ content was recorded in genotype JG6 (3.39 mmol/g FW), while maximum enrichment in $H_2O_2$ was found in genotype JG2021-6301 (42.93%). Higher EL was observed in genotype JG2016-634958 (45.08%), whilst the minimum was found in genotype JG11 (34.49%) (Figure 6). Minimum MDA content was noticed in genotype JG2016-1411 (2.05 nmol/g), whereas the maximum was documented in genotype ICC4958 (16.29%). Higher TSS content was maintained by genotype ICC4958 (2.07 mg/g FW),

whereas the lowest was recorded in genotype JG2016-9605 (1.60 mg/g FW) (Figure 7). Higher proline content was detected in genotype JG11 (89.18 µg/g FW), while the lowest was noticed in genotype JG2022-75 (55.83 µg/g FW).

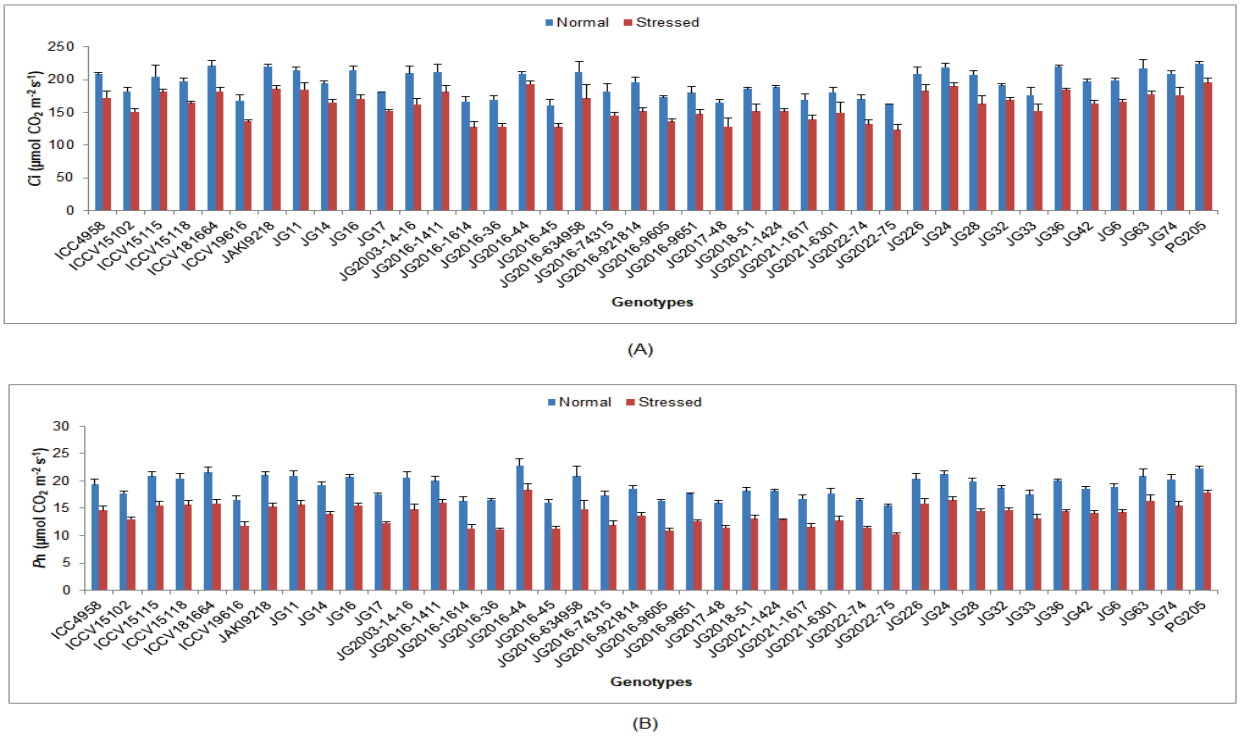



**Figure 2.** Effect of terminal drought stress on (**A**) Ci (**B**) *P*n of studied chickpea genotypes, where *C*i and *P*n indicate internal $CO_2$ concentration and photosynthesis rate, respectively.

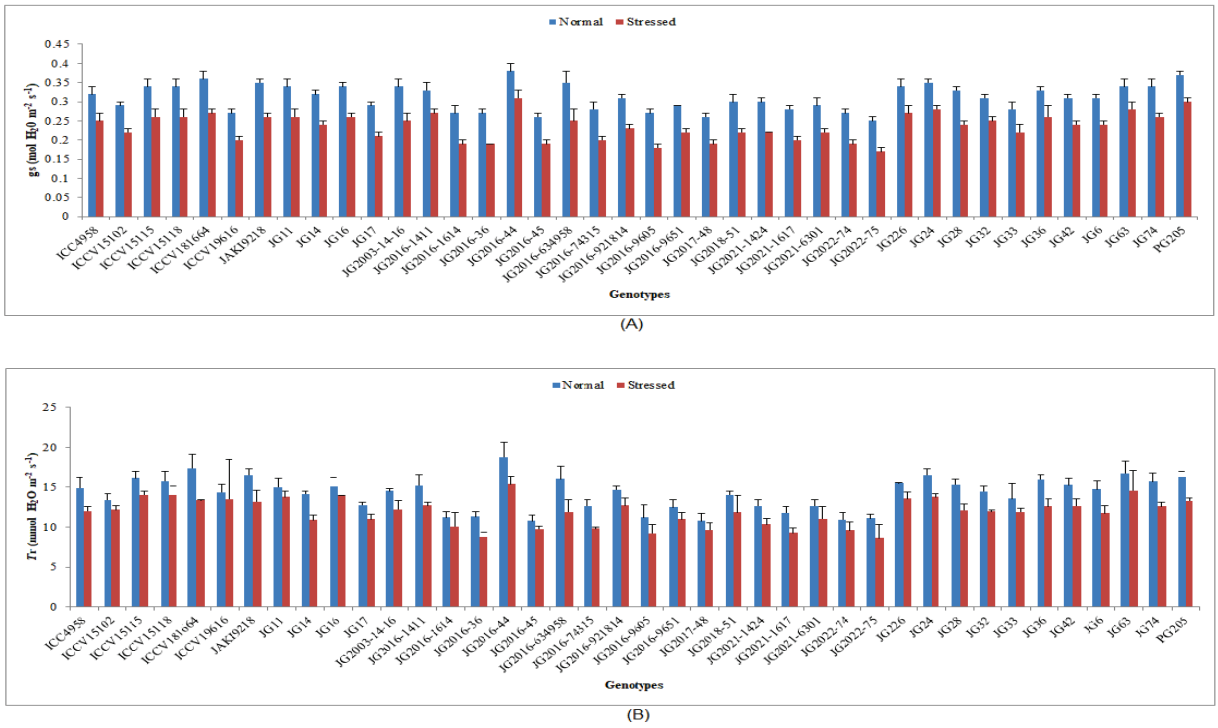
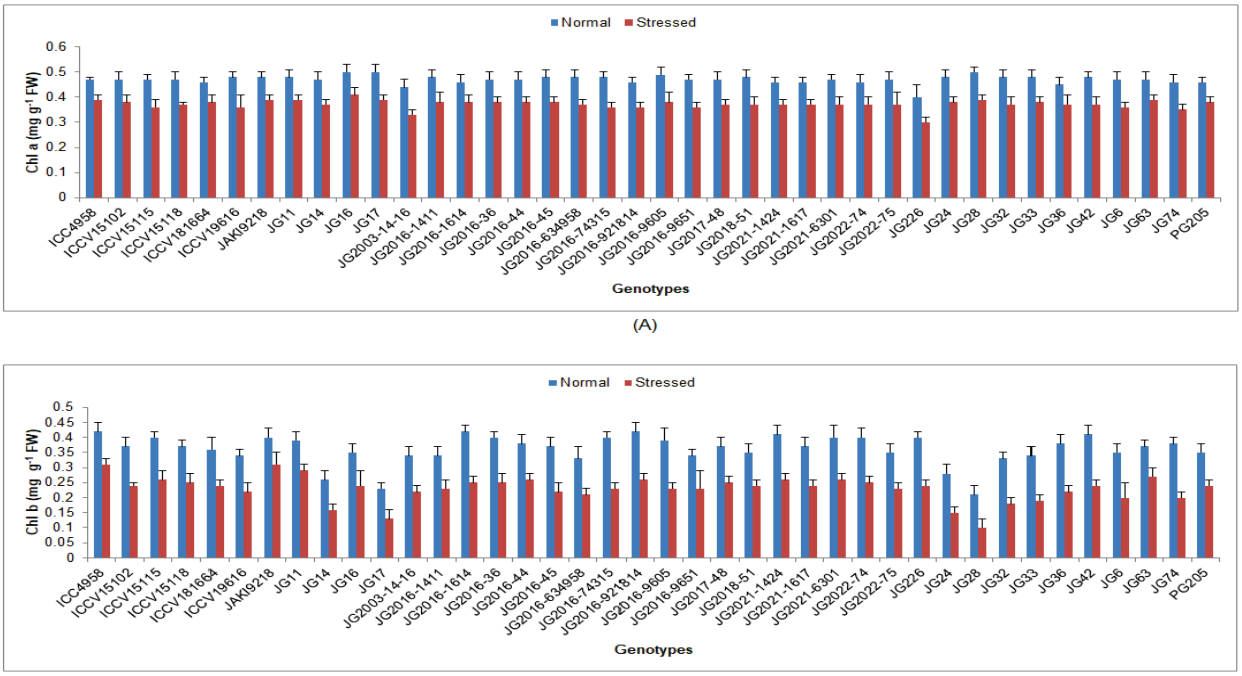



**Figure 3.** Effect of terminal drought stress on (**A**) gs and (**B**) *T*r of studied chickpea genotypes, where gs and *T*r indicate stomatal conductance and transpiration rate, respectively.

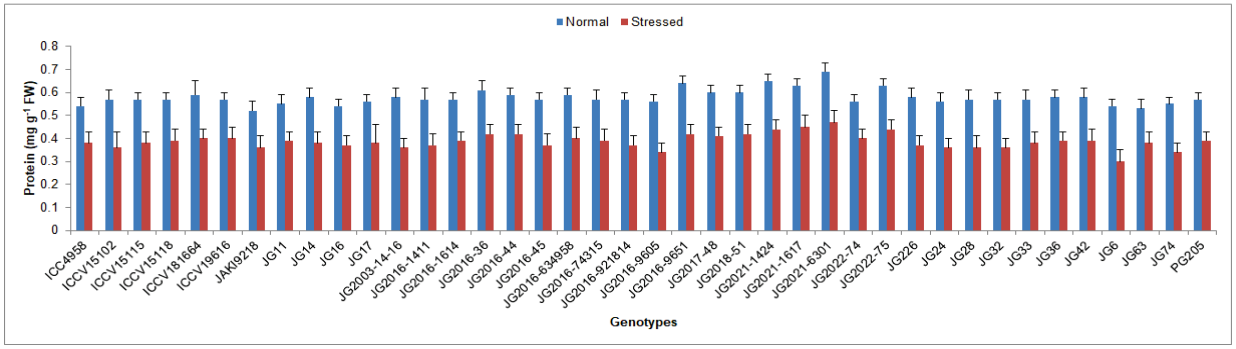



(A)

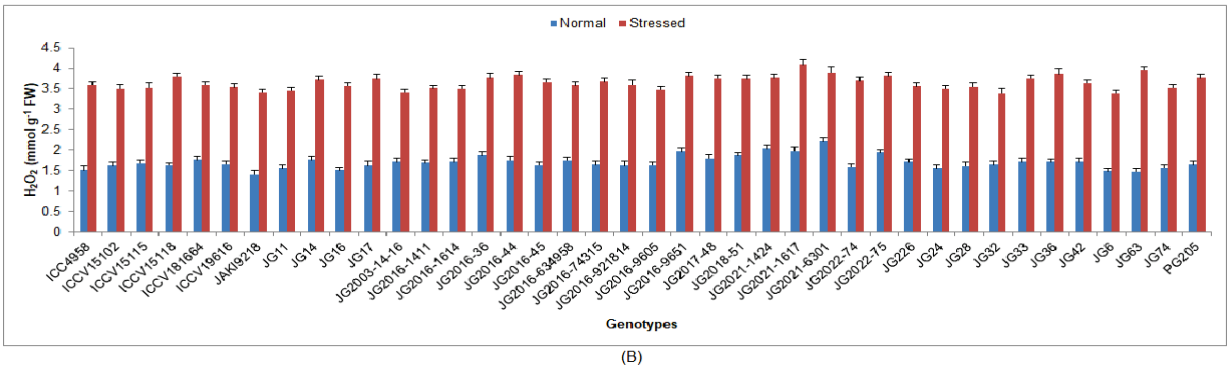



(B)

**Figure 4.** Effect of terminal drought stress on (**A**) Chl a and **(B**) Chl b content of studied chickpea genotypes, where Chl a and Chl b indicate chlorophyll a and chlorophyll b, respectively.

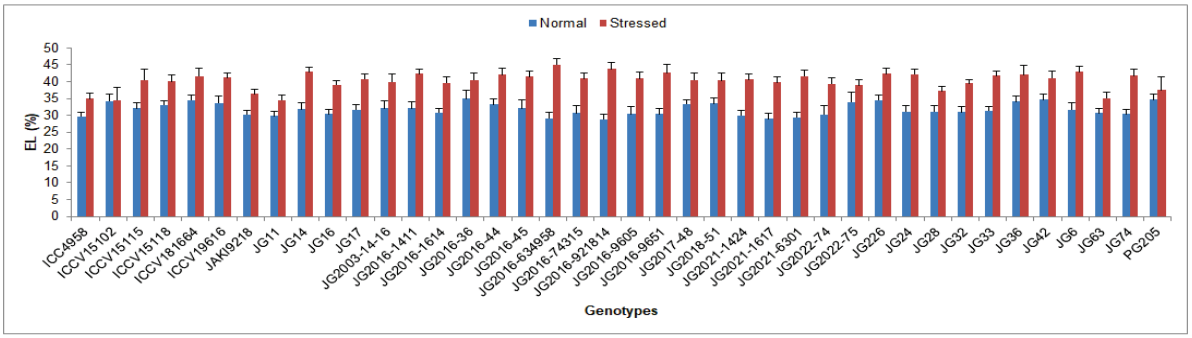



(A)

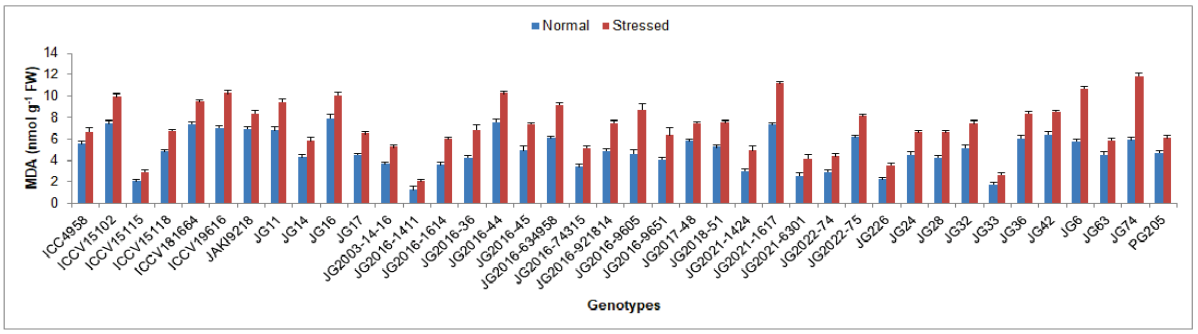



(B)

**Figure 5.** Effect of terminal drought stress on (**A**) protein content and (**B**) $H_2O_2$ content of studied chickpea genotypes, where $H_2O_2$ indicates hydrogen peroxide.

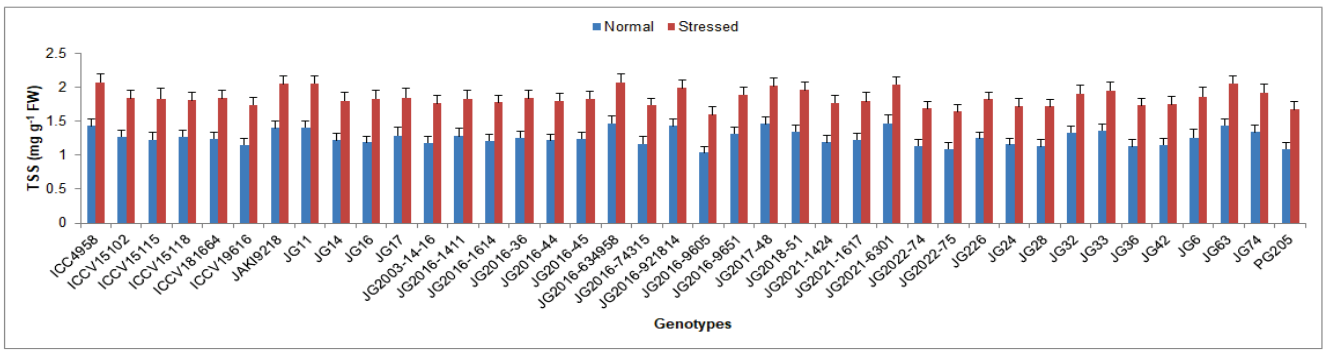



(A)

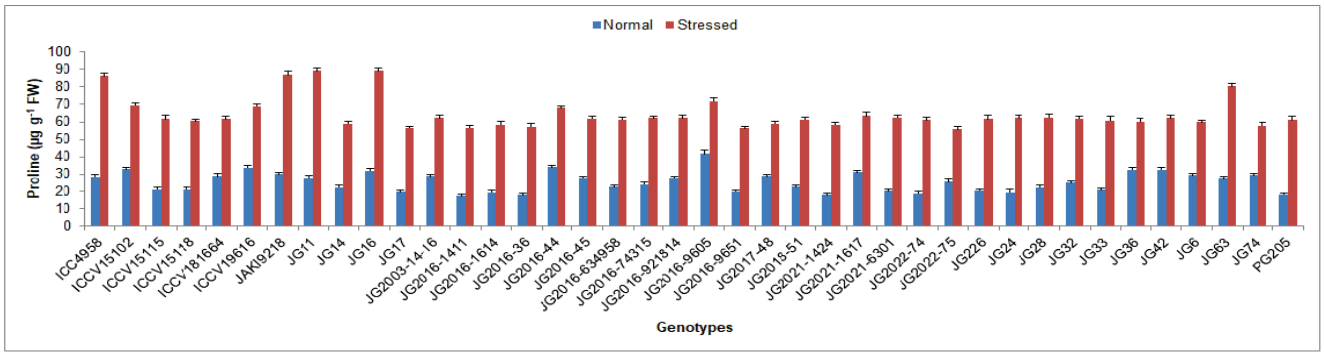



(B)

**Figure 6.** Effect of terminal drought stress on (**A**) EL (%) and (**B**) MDA content of studied chickpea genotypes, where EL and MDA indicate electrolyte leakage and malondialdehyde, respectively.



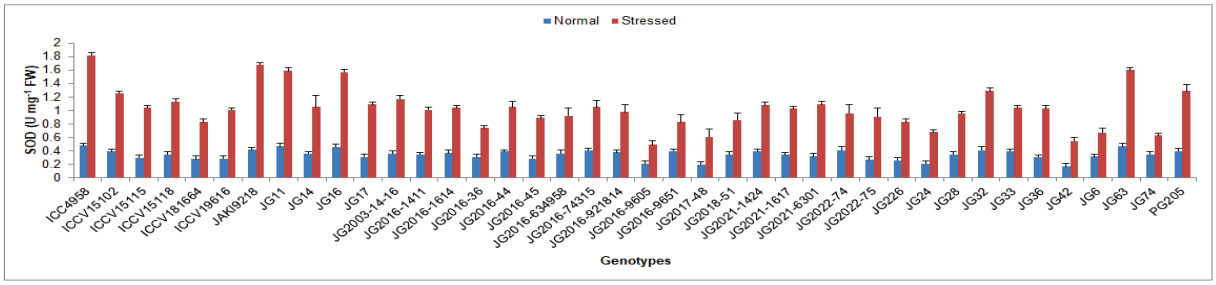

(A)



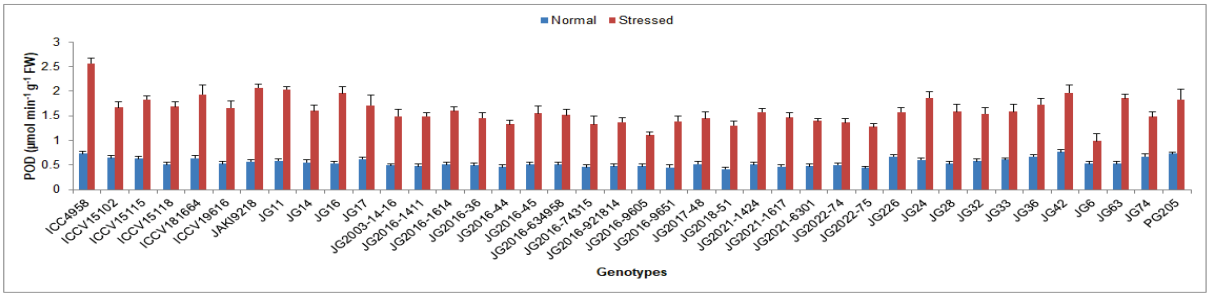

(B)

**Figure 7.** Effect of terminal drought stress on (**A**) TSS content and (**B**) proline content of studied chickpea genotypes, where TSS indicates total soluble sugar.

Higher SOD was maintained by genotype ICC4958 (1.82 U/mg FW), while the minimum was recorded for the genotype JG206-9605 (0.49 U/mg FW) (Figure 8). Higher POD was sustained in genotype ICC4958 (2.57 µmol/min/g FW), whilst the minimum was evidenced in genotype JG6 (0.99 µmol/min/g FW). Higher CAT was maintained by genotype ICC4958 (4.52 µmol/min/g FW), whereas the minimum was perceived in genotype (JG6 2.77 µmol/min/g FW) (Figure 9). Higher APX was exhibited by the genotypePG205 (16.54 µmol/min/g FW), whilst the lowest was found in genotype JG6 (8.43 µmol/min/g FW).

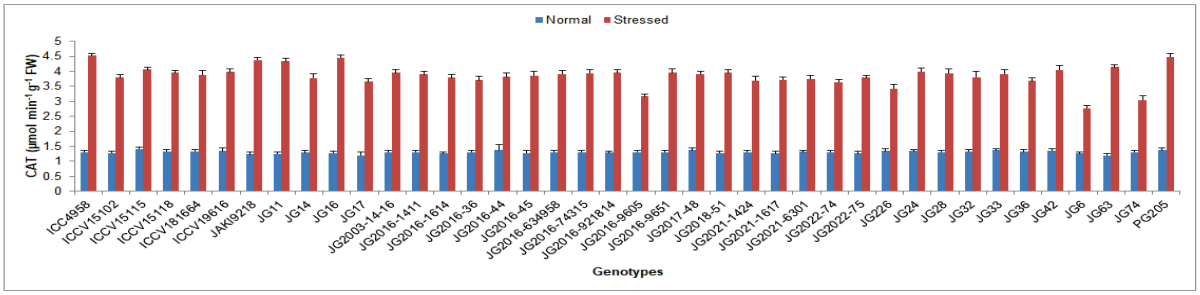



(A)

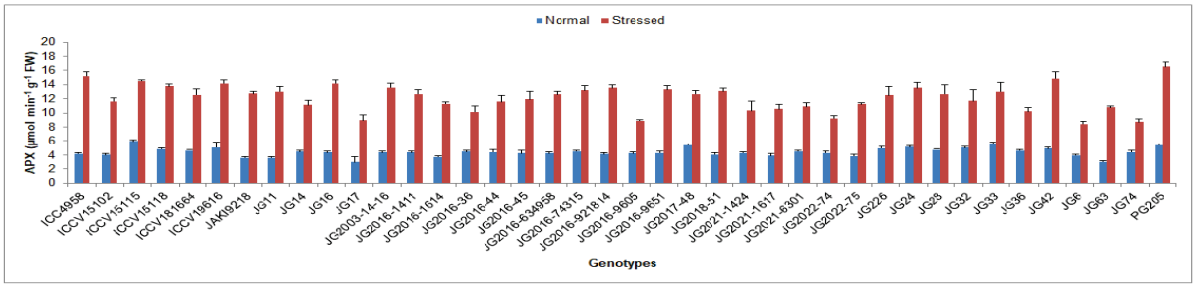



(B)

**Figure 8.** Effect of terminal drought stress on (**A**) SOD and (**B**) POD activity of studied chickpea genotypes, where SOD and POD indicate superoxide dismutase and peroxidise.




(A)




(B)

**Figure 9.** Effect of terminal drought stress on (**A**) CAT and (**B**) APX activity of studied chickpea genotypes, where CAT and APX indicate catalase and ascorbate peroxidise, respectively.

### *3.3. Effect of Terminal Drought Stress on Yield and Its Attributing Traits*

Under the drought-stressed condition, yield and its accrediting characters were significantly reduced in all the studied chickpea genotypes compared to the normal irrigated condition (Supplementary Tables S6 and S7). In terms of genotypic response, the lowest DTF was documented in genotype JG11 (54.3 DAS), while the maximum was in genotype JG32 (74.91DAS) (Figure 10). Lower DTM was documented in genotype ICC4958 (98.13 DAS), whilst maximum DTM was investigated in genotype JG74 (119.42 DAS). Higher NOP was maintained by genotype JG16 (65.25), whereas the minimum was observed in genotype JG14 (30.25) (Figure 11). Higher SYPP was upheld by genotype JG11 (11.42 g), the while minimum was observed in genotype JG74 (6.14 g). Higher BYPP was sustained in genotype PG205 (34.77 g), and the minimum was shown in genotype JG74 (19.33 g) (Figure 12). Higher HI was exhibited by the genotype JAKI9218 (43.29%), whereas the minimum was found in genotype JG36 (28.18%).

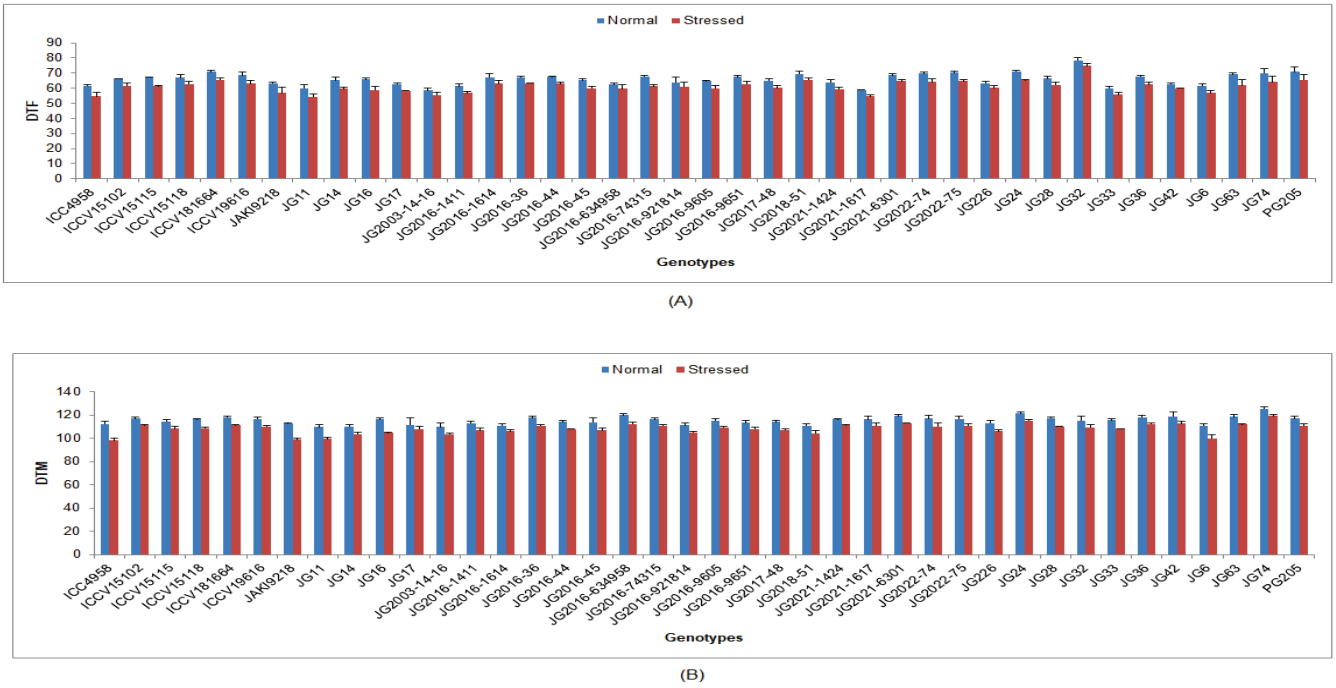



**Figure 10.** Effect of terminal drought stress on (**A**) DTF and (**B**) DTM of studied chickpea genotypes, where DTF and DTM indicate days to 50% flowering and days to maturity, respectively.

### *3.4. Principle Component Biplot Analysis*

For a more reliable identification of genotypes with a maximum value for one or more traits, genotype by trait biplots were constructed for PC-I and PC-II for all genotypes and all traits under all treatments (Figure 13, Supplementary Table S8). Biplot analysis clearly distinguished the drought-associated traits into positively correlated traits (<90°), independent traits (=90°), and negatively correlated traits (>90°). The RWC, CTD, *P*n, *g*s, and *C*i were identified as positively correlated traits among the studied physiological traits; chl a, chl b, TSS and proline contents, including antioxidant enzymes activities, viz., SOD, POD, CAT, and APX, were proved to be positively correlated traits among the studied biochemical traits. Similarly, SYPP, NOP, and BYPP were also considered positively correlated traits among the studied yield and its attributes. These cumulative positively correlated physio-biochemical traits, yield, and its attributing traits contributed more towards the drought tolerance of chickpea genotypes, and so can be treated as markers for terminal drought tolerance in chickpea.

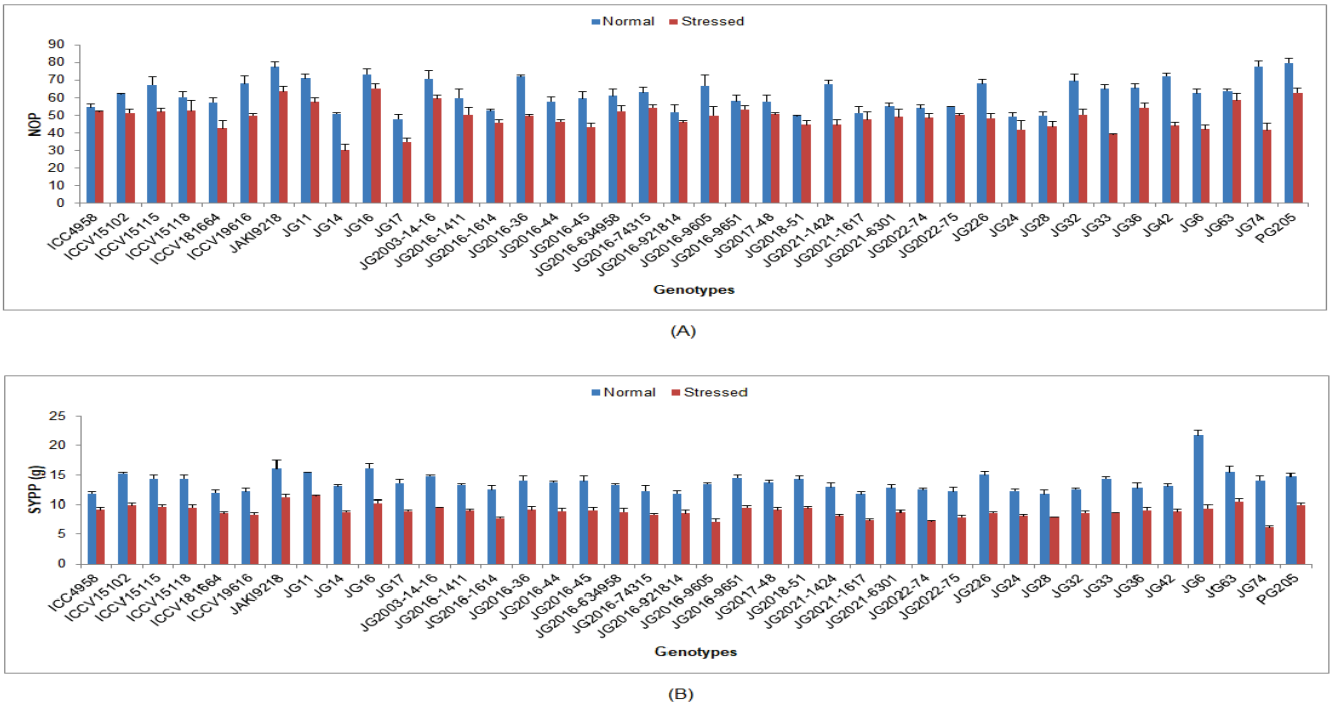



**Figure 11.** Effect of terminal drought stress on (**A**) NOP and (**B**) SYPP of studied chickpea genotypes, where NOP and SYPP indicate number of pods and seed yield per plant, respectively.

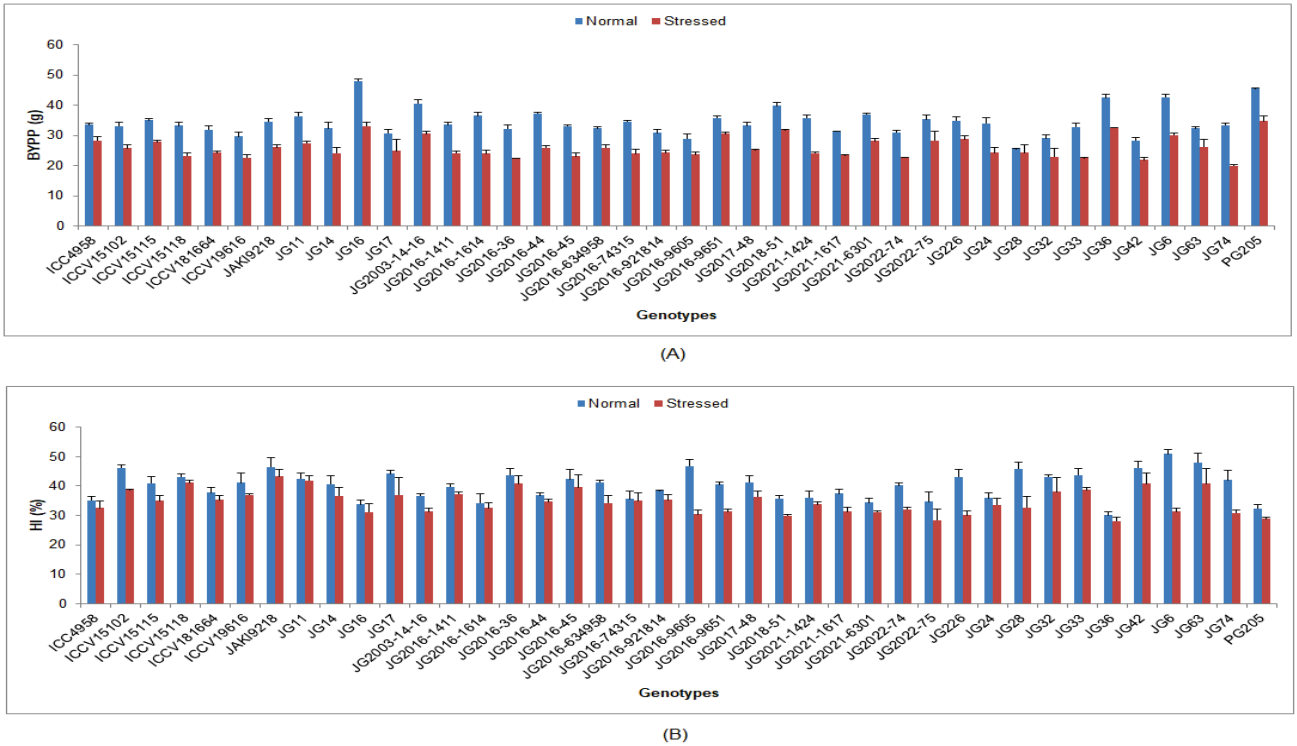



**Figure 12.** Effect of terminal drought stress on (**A**) BYPP and (**B**) HI (%) of studied chickpea genotypes, where BYPP and HI indicate biological yield per plant and harvest index, respectively.

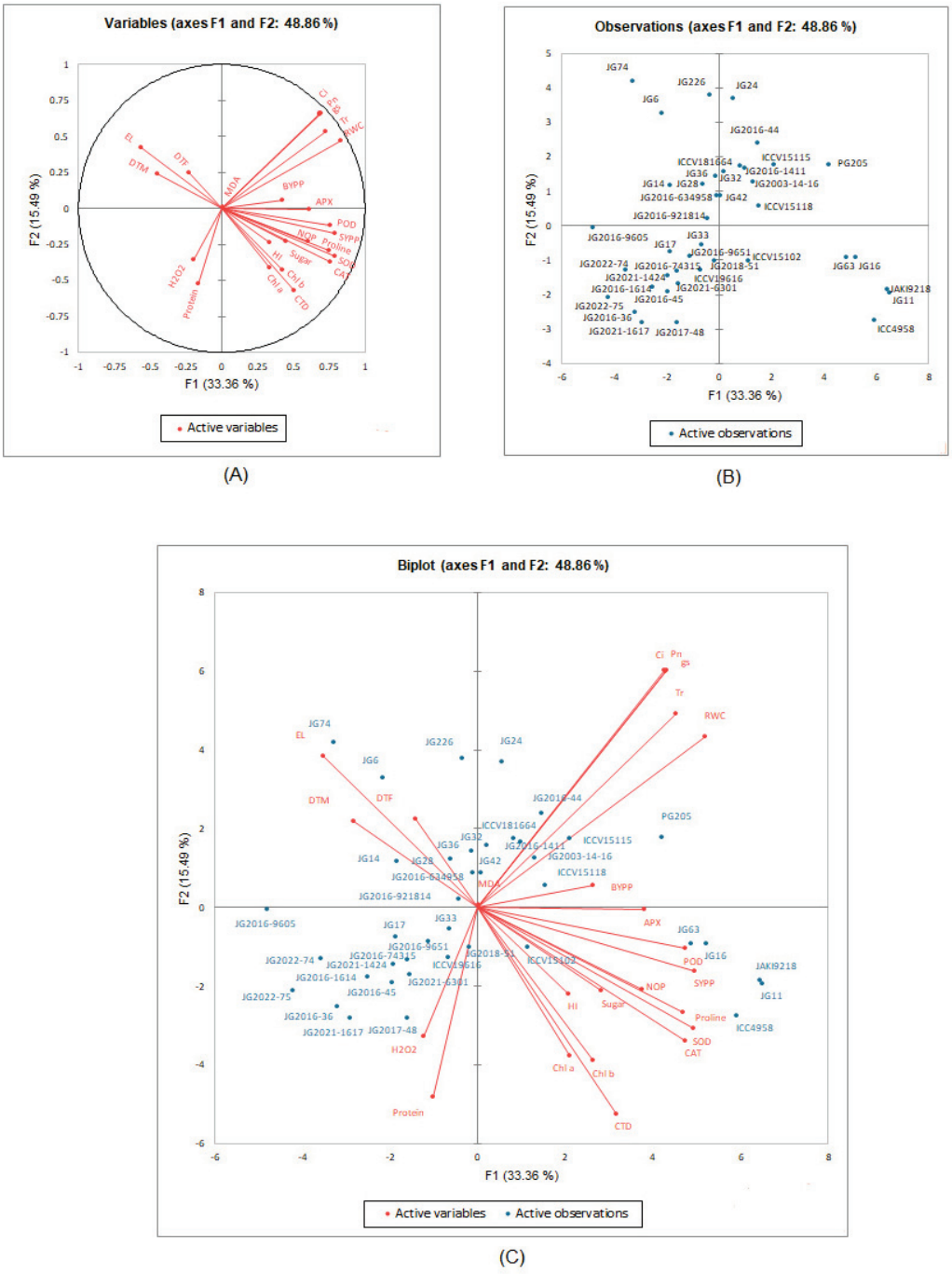



**Figure 13.** PCA biplots depicting (**A**) relationships between the traits measured, (**B**) performance of chickpea genotypes, and (**C**) combined (**A** + **B**) under terminal drought stressed condition. In the active variables, RWC, CTD, Ci, Pn, gs, Tr, Chla, Chlb, EL, MDA, $H_2O_2$, SOD, POD, APX, DTF, DTM, NOP, SY, BY, and HI indicate the relative water content, canopy temperature depression, internal $CO_2$ concentration, photosynthesis rate, stomatal conductance, transpiration rate, chlorophyll a, chlorophyll b, electrolyte leakage, malondialdehyde, hydrogen peroxide, superoxide dismutase, peroxidase, ascorbate peroxidase, days to 50% flowering, days to maturity, number of pods, seed yield, biological yield and harvest index, respectively.

In biplots, the genotypic performance can be estimated by the distance of the genotype from the origin of the biplot. The distant genotypes could have the greatest values for one or more traits. The PCA biplot distinguishes the ICC4958, JG11, JAKI9218, JG16, and JG63 genotypes as distant genotypes with strong positive correlation with CAT, SOD, proline,

TSS, POD, and APX selection indices. These genotypes could have the greatest values for these selection indices. The bilpot also distinguishes JG6 and JG74 genotypes as distant genotypes with strong negative correlation with these selection indices. These genotypes could have minimum values for these selection indices. The rest of the genotypes could have medium values for these selection indices. Further, cluster analysis was performed using morpho-physiological and biochemical data under the stress condition. The agglomerative clustering categorized forty genotypes into two major clusters (Figure 14). Major cluster I consisted of six genotypes, viz., JG16, ICC4958, JAKI9218, JG11, JG63, and PG205. Major cluster II consisted of two subclusters. Sub-cluster I also contained six genotypes, viz., JG74, JG2016-9605, JG6, JG226, and JG-2003-14-16Sub-cluster II contained the rest of the genotypes.

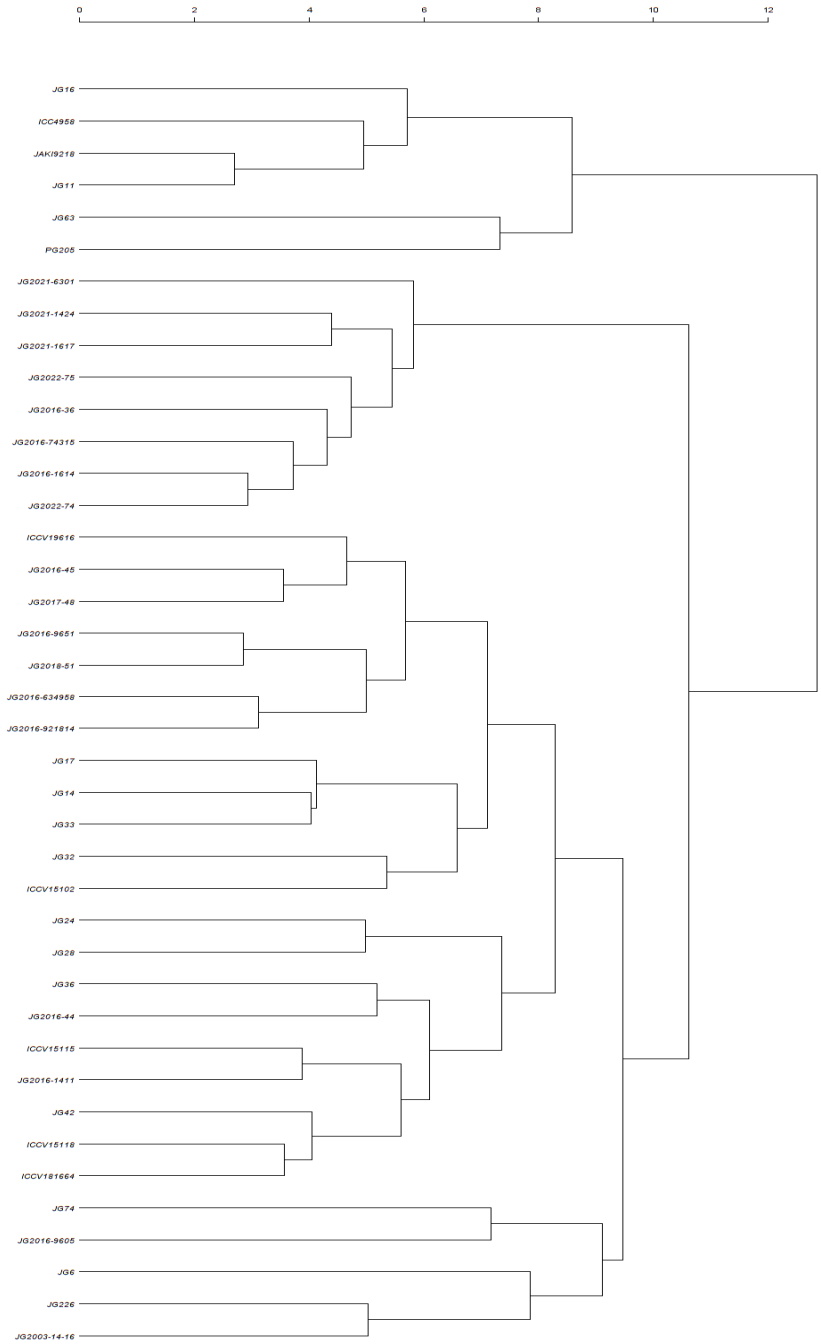



**Figure 14.** Agglomerative clustering of studied chickpea genotypes under terminal drought-stressed condition.

## 4. Discussion

Abiotic stresses are almost interlinked, causing morpho-physiological, biochemical, and molecular alteration that negatively affects crop growth and development, crop efficiency, and ultimately yield [4,20–22]. The prevalence of inconsistent rainfall and extreme temperature (drought and heat) is proposed to increase soon owing to climate change [39]. Low moisture and heat affect chickpea growth and may be observed in early morphological stages that ultimately affect seed yield indirectly. The present findings revealed that under normal sown conditions, there was a substantial increase in plant growth and development compared to under drought-stressed conditions.

Plant water status is the primary factor that affects crop yield and quality. The present investigation reveals that drought stress caused a significantly reduced RWC content in the leaves of genotypes. RWC decreased in lesser magnitude in drought-tolerant genotypes; this may be because of their more extended root systems, which could complement water lost by transpiration. Under drought-stressed conditions, the ability of a plant to maintain the turgor pressure and related physiological processes has great significance, and it is related to drought resistance in terms of osmoregulatory activities. Drought stress leads to the dehydration of plants and a decline in RLWC, which can result in stomatal closure [40–43]. CTD was also decreased under the stressed conditions compared to normal conditions. The drought-tolerant chickpea genotypes demonstrated higher CTD under drought-stressed conditions than other genotypes, showing their extraordinary ability to maintain a canopy cooler than the rest. CTD has already been utilized as a selection indicator for tolerance to drought and high-temperature stress in early-generation selections [44,45]. A positive correlation of CTD with yield was also observed in chickpea under heat-stressed and drought-stressed conditions [26]. Various other studies have also described a comparable pattern of decreases in CTD under heat- and water-stressed conditions in chickpea genotypes [26,39].

Under terminal drought-stressed condition, the gas-exchange parameters were also decreased in all studied chickpea genotypes. The most negligible reduction was evidenced in tolerant genotypes compared to other genotypes. The decrease in internal $CO_2$ concentration and leaf photosynthetic rate under drought-stressed conditions appears to be mediated by stomatal closure, as demonstrated by the reduced stomatal conductance and transpiration rate [46,47]. In this investigation, pigment and protein content were also reduced under stress conditions, and less reduction was documented in tolerant genotypes compared to other genotypes. Chl 'a', Chl 'b' and total chlorophyll content in chickpea leaves was shown to be degraded with increasing days of irrigation intervals compared with unstressed plants. The water deficit condition decreased chickpea growth, chlorophyll content and photosynthetic efficiency when plants were exposed to irrigation levels of 100, 60, 40 and 20% of the field capacity [48]. Protein molecules play a crucial role in the proper functioning of the cell. In this study, protein content decreased in all genotypes under drought stress, and the most negligible reduction was detected in tolerant genotypes compared to other genotypes [49]. Reduced photosynthesis under drought stress reduces or even stops protein synthesis. Abiotic stresses caused a reduction in protein production, possibly due to various factors involved [50].

Water stress enhances the production of ROS such as alkoxy radicals, singlet oxygen, $O_2^{\bullet -}$, $OH^{\bullet}$, $H_2O_2$, etc. Increased $H_2O_2$ content induces oxidative stress with several adverse effects, including electrolyte leakage, associated membrane damage, and lipid peroxidation. In this research, tolerant genotypes showed a lesser increase in $H_2O_2$, EL and MDA contents than other genotypes. Under drought stress, similar findings of increased leaf $H_2O_2$ [44], EL and MDA content [39,41] were also reported in chickpea. Under terminal drought-stressed conditions, the chickpea genotypes accumulated osmolytes. Drought-tolerant genotypes accumulated higher osmolyte levels, suggesting that osmolytes might be proved an appropriate indicator for evaluating drought tolerance in chickpea. In the shoots of the chickpea plants, proline content was significantly increased under moderate and severe drought-stressed conditions compared with untreated plants [50]. Although

water stress induced a significant increase in leaf proline content of the sensitive cultivar (Azad), leaf proline content in the tolerant cultivar (Arman) strongly increased [48]. Owing to unpredicted changes in climate, pulses become more sensitive to oxidative damage by the overproduction of ROS such as $H_2O_2$, hydroxyl, and superoxide radicals. Specialized enzymatic antioxidants, i.e., POD, SOD, APX and CAT, are activated and act as the first line of defense for detoxification of the effects of ROS [51]. In the present study, increased activity of SOD, POD, CAT, and APX was investigated in all genotypes under drought-stressed conditions over the normal condition. A higher activity level was evidenced in tolerant genotypes compared to other genotypes. Several earlier researchers also reported a similar increased level of antioxidant enzyme activities under water stress conditions in chickpea. SOD, POX and catalase activities were significantly enhanced in moderate (50% FC) and severe (25% FC) conditions under drought stress [47]. CAT, SOD, POX, APX and GR activities were markedly increased in chickpea shoots under moderate and severe drought-stressed conditions [46]. CAT, SOD, POX, APX and GR activities were markedly enhanced in chickpea plants under drought stress [4,48,50] circumstances as well.

Under the normal irrigated condition, the maximum grain yield per plant was documented by genotype JG6, tracked by the genotypes JG16, JAKI9218, and JG11. The maximum yield per plant was yielded by genotype JG11, tailed by genotypes JAKI9218, JG16, and ICC4958 under terminal drought stress. In this investigation, the tolerance of genotypes JG11, JAKI9218, JG16, and ICC4958 against drought stress was perhaps due to the higher number of pods per plant, the better accumulation of osmolytes, i.e., sugar and proline, and the greater activities of antioxidant enzymes, viz., SOD, POD, CAT, and APX. Similarly reduced yield attributes including the numbers of pods and numbers of seeds per plant, and hundred-seed weight under moderate and severe drought-stressed conditions have also been observed in chickpea, allowing us to conclude that the synthesis of enzymatic and non-enzymatic antioxidants and proline content in stressed plants helped in the protection of plants under drought-stressed conditions [50]. A significant difference was investigated among the genotypes based on different biochemical, morphological, and physiological parameters. The chickpea genotypes, viz., GGP-1260, PGP-1426, and PB-1, were considered drought-tolerant genotypes based on their higher plant biomass production, pod yield, harvest index, and having the highest activities of POD, CAT, and SOD. Under drought stress, the drought-tolerant genotypes retained higher plant yield with lower reductions in CI, RWC, MSI, numbers of secondary branches, and biomass [28]. An integrated approach involving physio-biochemical traits and multi-environmental yield trials was performed for screening and selecting drought-tolerant chickpea genotypes and allowed us to conclude that higher RWC, CMS, glycine betaine, and proline content conferred a more significant capability for drought stress tolerance in chickpea [16]. In another investigation, the reduction in growth and yield of the tolerant cultivar was less compared to the susceptible cultivar DUSHT, probably due to the accumulation of higher antioxidant enzyme activities, suggesting the protective role of enhanced antioxidant enzyme activities of plants under water-stressed conditions [51].

PCA biplot is the most effective multivariate analysis for evaluating the genotypic performance and traits interaction. It is being extensively utilized to dissect the traits correlation in different crop plants by several researchers [16]. PCA biplots provided a new understanding of drought-tolerance mechanisms and plant responses under drought-stress conditions [28]. Under the stressed condition, biplot analysis based on principal component and correlation analysis established a strong positive association of SYPP with POD, NOP, proline, SOD, CAT, APX, and sugar content, signifying their greater utilization in selecting high-yielding drought-tolerant genotypes. Genotypes ICC4958, JAKI9218, JG11, JG16, and JG63 performed better under the stressed condition, with a smaller reduction in NOP and BYPP, including a higher accumulation of osmolytes (proline and sugar) and enhanced antioxidant enzyme (POD, SOD, APX, and CAT) activity. Further, the agglomerative clustering also supported the result obtained from biplot analysis and grouped tolerant and susceptible genotypes in separate clusters. Major cluster I contained tolerant genotypes,

while sub-cluster I consisted of susceptible genotypes. Our findings follow the results of Sachdeva et al. [28], who also observed a strong positive association with RWC, chlorophyll index (CI), membrane stability index (MSI), numbers of secondary branches (SB) and yield traits and negative associations with drought-susceptibility index (DSI), 100-SW and days to maturity under drought-stressed conditions through principal component analysis based on biplot and correlation analysis. Genotypes ICC4958, Pusa1103, BGD72, CSG8962, ICCV97309, ICCV10, ICCV03311, ICCV05308, ICCV3403, and ICCV10313 were identified as the most drought-tolerant genotypes, with higher values of lower DSI and DTM and high RWC and MSI values under drought-stressed conditions at both vegetative and reproductive stages based on PCA-biplot analysis. Similarly, Shah et al. [16] also utilized biplot analysis to select superior chickpea genotypes under drought stress and concluded that genotypes D0091–10, D0085–10, K010–10, K005–10, 08AG016, D0078–10, 08AG004, 09AG002, D0080–10, K002–10 and D0099–10 proved superior in yield as well as physio-biochemical performances under drought-stressed multiple environmental conditions. Furthermore, genotype by-trait (GT) biplots were constructed for a more reliable identification of genotype with maximum value for multiple traits in chickpea for all genotype under stress conditions [4].

## 5. Conclusions

The identification of new genetic resources that are tolerant to drought-stressed conditions is crucial. However, simultaneously, attention has been given to identifying suitable physiological and biochemical markers that can be employed to distinguish the tolerant and susceptible genotypes. The PCA biplots revealed that POD, NOP, proline, SOD, APX, CAT and sugar content showing strong positive association with SYPP could be used as selection indices to distinguish between tolerant and sensitive genotypes. ICC4958, JAKI9218, JG11, JG16, JG63, and PG205 performed better in the terminal drought-stressed condition with higher accumulation of proline and sugar, enhanced activity of POD, SOD, APX, and CAT enzyme activities and smaller reduction in NOP. Due to the unavailability of quantitative real-time polymerase chain reaction (qRT PCR), expression analysis of drought-associated genes could not be performed. So, further analysis of gene expression and the nutritional profiling of drought-tolerant chickpea genotypes may be performed to further explore the genetic traits of the selected drought-tolerant genotypes.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/life13061405/s1. Table S1. Details of the 40 chickpea genotypes used for present study. Table S2. Pooled physiological responses of various chickpea genotypes under normal irrigated condition. Table S3. Physiological responses of various chickpea genotypes under terminal drought stressed condition. Table S4. Pooled biochemical responses of various chickpea genotypes under normal irrigated condition. Table S5. Biochemical responses of various chickpea genotypes under terminal drought stressed condition. Table S6. Yield and yield attributing trait responses of various chickpea genotypes under normal irrigated condition. Table S7. Yield and yield attributing trait responses of various chickpea genotypes under terminal drought stressed condition. Table S8. PC Scores of studied chickpea genotypes under terminal drought stressed condition.

**Author Contributions:** Conceptualization: S.T. (Sharad Tiwari); Methodology: P.N.T. and S.S.; Software: P.N.T. and S.T. (Sharad Tiwari); Resources: S.T. (Sharad Tiwari); and A.B.; Writing original draft: N.T. and S.T. (Sushma Tiwari); Review and editing: M.K.T. and S.T. (Sharad Tiwari). All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding for research.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The first author acknowledges the Indian Council of Agricultural Research (ICAR) for the award of the ICAR Junior/Senior Research Fellowship (F. No. EDN/1/25/2015-Exam Cell) for PhD degree program.

**Conflicts of Interest:** The authors declare no conflict of interest.

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*Brief Report*

# Arbuscular Mycorrhizal Fungi Response on Soil Phosphorus Utilization and Enzymes Activities in Aerobic Rice under Phosphorus-Deficient Conditions

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**Abstract:** The prominence of arbuscular mycorrhizal fungi (AMF) in sustainable rice production has long been recognized. However, there is little information about AMF response in aerobic rice cultivation under phosphorus (P)-deficient conditions. The aim of this experiment was to compare and determine the preeminent AMF effects on rice mycorrhizal colonization, responsiveness, P utilization, and different growth-promoting traits under P-deficient conditions. Different AMF genera *viz*. (*Funneliformis* sp., *Rhizophagus* sp., *Glomus* sp., *Acaulospora* sp., and *Claroideoglomus* sp.) in four different aerobic rice varieties developed by ICAR-NRRI, India (CR Dhan 201, CR Dhan 204, CR Dhan 205, and CR Dhan 207) were investigated using the check P-susceptible variety (IR 36) and the P-tolerant variety (Kasalath IC459373). Data analyzed through linear modeling approaches and bivariate associations found that AMF colonization was highly correlated with soil enzymes, particularly fluorescein diacetate (FDA) and plant P uptake. The microbial biomass carbon (MBC) and FDA content were significantly changed among rice varieties treated with AMF compared to uninoculated control. Out of four different rice varieties, CR Dhan 207 inoculated with AMF showed higher plant P uptake compared to other varieties. In all the rice varieties, AMF colonization had higher correlation coefficients with soil enzymes (FDA), MBC, and plant P uptake than uninoculated control. The present study indicates that AMF intervention in aerobic rice cultivation under P-deficient conditions significantly increased plant P uptake, soil enzymes activities and plant growth promotion. Thus, the information gathered from this study will help us to develop a viable AMF package for sustainable aerobic rice cultivation.

**Keywords:** arbuscular mycorrhizal fungi; aerobic rice; soil enzymes; phosphorus utilization; P-deficient; plant growth promotion

**Citation:** Mitra, D.; Panneerselvam, P.; Senapati, A.; Chidambaranathan, P.; Nayak, A.K.; Mohapatra, P.K.D. Arbuscular Mycorrhizal Fungi Response on Soil Phosphorus Utilization and Enzymes Activities in Aerobic Rice under Phosphorus-Deficient Conditions. *Life* **2023**, *13*, 1118. https://doi.org/10.3390/life13051118

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 3 April 2023
Revised: 28 April 2023
Accepted: 29 April 2023
Published: 30 April 2023



## 1. Introduction

Rice (*Oryza sativa* L.) is a major agricultural crop and staple food that feeds more than half of the world's population, is grown in >100 countries with 90% of the total global production coming from Asia [1]. In India, rice is cultivated in an area of 45 million hectares and contributes to a great extent to national food security. Additionally, Asia alone consumes 90% of the freshwater diverted to agriculture in the entire world [2,3]. This will soon be a burden on the ecological balance in many areas, leading to water scarcity. In this case, aerobic rice cultivation is a modern practice for cultivating rice crops with durable water soil and suited, high-yielding varieties that are sown directly dry [4]. This approach saves water significantly; in China, the aerobic rice system of cultivation used 55–56% less water as compared to the traditional transplanted system of cultivation with water productivity that is 1.6–1.9 times higher [5]. To keep pace with the changing scenario, an estimated 22 varieties and 2 hybrids have been released for aerobic conditions in India [4]. According to Ghasal et al. [6], dry and aerobic soil can reduce the natural

supply of phosphorus (P), making the application of P fertilizer more crucial for rice grown aerobically. P is necessary for all living organisms, and is a crucial nutrient for the expansion and development of the plants [7–9]. Phosphorus makes up about 0.2% of a plant's dry biomass and is mostly present in tissue components such as phospholipids, nucleic acids, and adenosine triphosphate (ATP) [10]. P is the second most limiting nutrient after nitrogen (N) [11]. It may decrease agricultural productivity and slow down plant growth and development. P exists in three different forms in the soil: organic P, soluble inorganic P, and insoluble inorganic P [7]. The amount of total soil phosphorus varies between 30 and 65% in organic forms, which are unavailable to plants, and 35 to 70% in inorganic forms [12]. Organic P can be found in soil microorganisms and dead plants and animals. P becomes unavailable in the soil because of fixation and immobilization, and 70–90% of phosphate fertilizers become fixed in the ground [13,14]. Soil microorganisms, mainly arbuscular mycorrhizal fungi (AMF), play a key role in mobilizing phosphorus from the soil into plant-available forms [15–18]. In the root cortical cells of their host plants, AMF create highly branching fungal structures called arbuscules, with which they exchange inorganic minerals, particularly phosphorus, and carbon molecules. AMF are one of the most prevalent organisms in the mycorrhizosphere [19,20] and have interactions and colonization with more than 200,000 different species of host plants with more than 240 different AMF morphotypes [21]. The exploration of mycorrhizal symbiosis is one of the most promising methods for creating resource-efficient and resilient agricultural systems [20,22]. Several studies have reported AMF diversities in rice [23–25], but very limited information is available on their performance in aerobics under P-deficient conditions [26]. Additionally, some studies indicated that AMF have a host preference [27] and their performance will vary depending on different agroecosystems [28]. In aerobic rice cultivation, soil P fixation is one of the major problems which causes P deficiency in the soil resulting in yield reduction. The main idea of this study is whether the intervention of suitable AMF will resolve the issue of soil P deficiency in aerobic rice cultivation. Hence, the present study was conducted to evaluate the effect of AMF on P uptake and growth promotion in popular aerobic rice varieties under P-deficient conditions.

## 2. Materials and Methods

### *2.1. Low P Soil Sampling, Site Description, and AMF Inoculum and Propagation of AMF*

Low-phosphorus (P) soil was collected from Krishi Vigyan Kendra (KVK), Santhpur, ICAR—NRRI, Cuttack, Odisha (20°27′45.08″ N; 85°52′58.76″ E) for the experiment and analysis. The initial properties of the experimental soil were analyzed (Table 1). The sterilized soil was used for a pot experiment. The soil-based single AMF inoculum *viz*. *Funneliformis* sp., *Rhizophagus* sp., *Glomus* sp., *Acaulospora* sp. and *Claroideoglomus* sp. received from Microbiology, ICAR—the National Rice Research Institute (ICAR-NRRI), India, were used in this experiment together with inoculum containing 115–120 AMF spores/g of soil, which was multiplied using finger millet (*Eleusine coracana*) as the host plant in sterile soil using a trap culturing method (Figure 1) [27,29].

**Table 1.** Initial soil properties of the experimental soil sample.

| pH (1:2.5, Soil: Water Suspension) | Electrical Conductivity (dS/m) | Available Phosphorus (kg/ha) | Available Nitrogen (kg/ha) | Available Potassium (kg/ha) |
|---|---|---|---|---|
| 6.53 ± 0.06 | 0.48 ± 0.03 | 6.003 ± 0.59 | 236.75 ± 3.65 | 136.86 ± 3.97 |

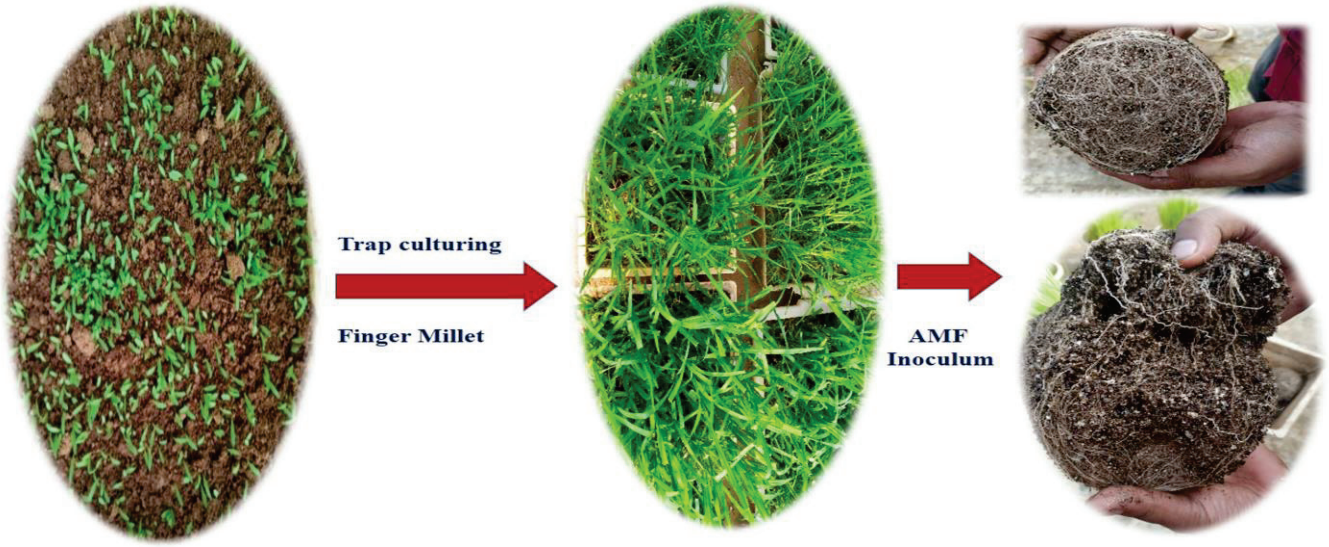


**Figure 1.** Monospecific AMF spore propagation using trap cultures and finger millet as host plant.

### *2.2. Experimental Site and Pot Experiment*

The experiment was conducted during the 2020–2021 *Rabi* season (the Indian cropping season starting from the onset of winter from October-November until spring in March–April) in a controlled net house condition at Microbiology, the ICAR-National Rice Research Institute (NRRI), Cuttack, Odisha (latitude—20°25′ N, longitude—85°55′ E with an altitude of 24 m above mean sea level). The pot (5 kg) experiment was conducted with five different species of AMF and six rice varieties with three replications. The treatment details are as follows, T0: Control, T1: *Funneliformis* sp., T2: *Rhizophagus* sp., T3: *Glomus* sp., T4: *Acaulospora* sp. and T5: *Claroideoglomus* sp. In this experiment, four aerobic rice varieties *viz*. V1: CR Dhan 201, V2: CR Dhan 204, V3: CR Dhan 205, V4: CR Dhan 207 (CR Dhan 201, 204, 205, and 207 developed by ICAR-NRRI, Cuttack), and two check varieties *viz*. V5: IR 36 (P-susceptible) and V6: Kasalath IC459373 (P-tolerant) were used, and were collected from the Crop Improvement Division, ICAR-NRRI, Cuttack, India. After germination, three plants per pot were maintained. Soil (completely homogenized and transported to the laboratory in a cool pack) and all the plant samples from each pot were collected from all treatment after 60 days in order to estimate the AMF colonization, growth parameters (root length, shoot length, leaf area, chlorophyll, fresh and dry biomass), P uptake, soil chemical, microbial and enzymatic activities analysis [30].

### *2.3. Assessment of AMF Colonization and Spore Count*

The method developed by Phillip and Hayman [31] was used to evaluate the rice root colonization of AMF [32]. Freshly collected root samples were gently washed to remove soil that was attached to the root surfaces, submerged in 10% potassium hydroxide (KOH) solution, and autoclaved for 15 min at 121 °C. The KOH solution was decanted, and the treated roots were rinsed with tap water three times until no brown colour appeared in the rinsed water. The treated root samples were further immersed in 2% hydrochloric acid (HCl) solution for 5 min. Without being rinsed with water, HCl was decanted, and the root samples were stained with 0.05% trypan blue (HiMedia, Maharashtra, India) in lacto-glycerol (400 mL lactic acid + 400 mL glycerol + 100 mL water) and autoclaved for 15 min at 121 °C. After autoclaving, the stained solution was decanted, and the roots were de-stained with lacto-glycerol solution to remove the excess stains and used for microscopic observations. The slide was prepared by keeping 10 segments of the stained root on a clean glass slide and observed under a compound microscope (Zeiss Stemi 508, Oberkochen, Germany). The method described by McGonigle et al. [33] was used to calculate the percentage of root colonization.

AMF root colonization was calculated using the formula:

$$\% \text{ of colonization} = \text{no. of root segments colonized} \div \text{total no. of root segments} \times 100$$

### *2.4. Phosphorus Estimation in Plant Sample*

Collected plant samples were dried in a hot air oven maintained at 60 °C for up to 5 days in order to attain a constant weight. The determination of P concentration in the plant sample was carried out using the vanadomolybdophosphoric acid method with a spectrophotometer [30,34]. A quantity of 1 gm of the dried plant sample and 10 mL of the concentrate $HNO_3$ were added and kept overnight, following which 10 mL of tri-acid ($HNO_3$, $H_2SO_4$, $HCLO_4$ in a ratio of 9:4:1), was added and mixed properly. The mixture was kept in a hot plate at 100 °C for 1 h under a temperature rise up to 200 °C until the content reduced to 2–3 mL and turned colourless. The content was cooled and 10 mL of diluted HCL was added and filtered through Whatman No. 42. The filtrate volume was made up to 100 mL with distilled water. A quantity of 5 mL of the digested sample was taken and 10 mL of vanadomolybdate reagent was added (Merck, Darmstadt, Germany) and kept for 30 min. The absorption of the sample was measured at 420 nm with a spectrophotometer (Analytikjena specord-200, Jena, Germany). A standard curve was prepared with a phosphate solution (0.2195 gm of $KH_2PO_4$ in 500 mL of distilled water + 25 mL of 7N $H_2SO_4$ and made up to 1 L) and the P content of the plant sample was calculated from the standard curve.

### *2.5. Estimation of Soil Chemical, Enzymatic and Microbial Properties*

The activity of the acid (AcP) and alkaline (AkP) phosphatase of soil samples was estimated by the method of Tabatabai and Bremner [35], using p-nitrophenyl as a substrate and expressed in l g of p-nitrophenyl phosphate (*p*NP) released per gram of soil per hour. Soil fluorescein diacetate activity (FDA) measurement was carried out by using Scherer and Ross [36] as modified by Adam and Duncan [37]. The concentration of fluorescein released during the assay was calculated using the calibrating graph produced from the 0–5 μg fluorescein $mL^{-1}$ standard and expressed as μg fluorescein $h^{-1}g^{-1}$ soil [27]. Dehydrogenase activity (DHA) was estimated by the method of Casida et al. [38]), using triphenyltetrazolium chloride (TTC) as a substrate. Microbial biomass carbon (MBC) was determined using the chloroform fumigation extraction (CFE) method [39].

### *2.6. Statistical Analysis*

The R version 4.2.2 [40] was used for statistical computing. For the identification of important variables related to AMF colonization in plants, a stepwise regression model was constructed using the "stepAIC" function available in the MASS package [41]. The Pearson correlation was constructed using the "ggpairs" function available in the GGally package [42].

## 3. Results and Discussion

Rice crops are very sensitive to water stress and reduction in water inputs with a consequent decline in yield [43]. Approximately 75% of the rice is produced by a conventional flooding method, and 3000–5000 L of water is needed to produce 1 kg of grains [4,44]. Researchers have developed several technologies to reduce water inputs in rice such as alternate wetting and drying, raised bed rice cultivation, saturated soil culture, a system of rice intensification, ground cover systems, and raised bed systems [45]. Some of the modern technologies additionally require puddling and ponded water during crop growth. In rice cultivation, the aerobic rice has been introduced to minimize the use of water, which is one of the promising water-saving technologies in rice production [46,47]. Aerobic rice reduces water use by 27–51% by limiting water loss due to seepage, percolation, and evaporation and increases water productivity by 32–88% [48]. It has been well documented that microorganisms enhance plant growth under abiotic stress [49].

### *3.1. Seed Germination of Rice Varieties*

The seed germination percentages of four different aerobic rice varieties (CR Dhan 201, CR Dhan 204, CR Dhan 205, and CR Dhan 207), as well as another P-susceptible variety (IR 36) and P-tolerant variety (Kasalath IC459373) are given in Figure 2. CR Dhan 204 and 207 rice varieties showed the highest germination percentages. However, all the rice types had germination rates of >90%.

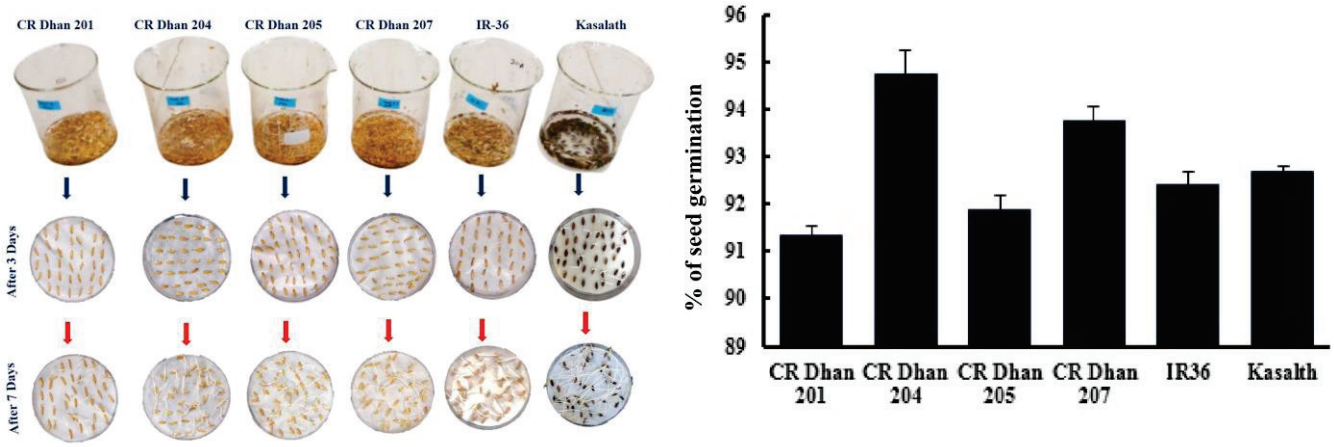


**Figure 2.** Percentage of seed germination of six rice varieties.

### *3.2. AMF Root Colonization in Different Aerobic Rice Varieties*

AMF symbiosis increases nutrient and water uptake in plants by external hyphae, regulation of stomatal conductance and the increased activity of antioxidant enzymes. Under aerobic conditions, rice plants readily form mycorrhizal associations as compared to submerged conditions where the anoxic environment limits the mycorrhizal infection process [50,51]. Rice can also be grown with alternate irrigation to reduce the water input and to create aerobic conditions for better AMF fungi colonization in rice roots. Therefore, an investigation was undertaken to understand the benefits of AMF association for rice plant growth and development under aerobic conditions. Narwal et al. [44] found a 20% increase in the plant biomass and 58% higher colonization of *Glomus intraradices* and *G. mosseae* (currently *Funneliformis mosseae*) in upland rice varieties (Pyari, Satyabhama, CR Dhan 205 and CR Dhan 202) compared to lowland rice varieties (Pusa Basmati (PB) 1509, PB 1121, Pusa Sugandha 5 and PB 1612) in pot experiments with sterile soil. The AM plants enhanced the activities of glutamine synthetase and nitrate reductase; the rice genotypes with higher nitrate reductase and glutamine synthetase (Pyari and Satyabhama) also exhibited more (20%) biomass production and plant N content by 36% [44]. In our study, the results of the different AMF-inoculated rice varieties and its root colonization, presented in Figure 3, indicated that *Funneliformis* sp., *Rhizophagus* sp. and *Glomus* sp. showed higher colonization in CR Dhan 207 (91.75, 91.72 and 87.97%, respectively) and CR Dhan 204 (85.43, 83.19, and 75.37%, respectively), while the other genera of AMF recorded a root colonization in the range of 54.38–74.98%.

### *3.3. Effect of AMF Inoculation on Physiological and Agronomic Properties*

Inoculation of AMF played an important role in the improvement of the biomass chlorophyll contents and physiological and agronomic parameters of the plant. It is widely believed that the inoculation of AMF provides the highest efficiency to host plants for plant growth. As shown in Figure 4, our results demonstrated that AMF inoculation in different rice varieties significantly increased the agronomic parameters, including root length (cm), shoot length (cm), leaf area ($m^2$), chlorophyll (SPAD), fresh biomass (gm), and dry biomass (gm) compared to the control. The highest shoot and root lengths were found in IR36 (53.40 cm) and CR Dhan 207 (23.973 cm) with the treatment of *Rhizophagus* sp. (Figure 4a,b). In the rice variety CR Dhan 207 (34.127 $m^2$), treatment with *Glomus* sp.

showed the best improvements for the leaf area (Figure 4c). Chlorophyll (SPAD) levels were highest in CR Dhan 204 (32.73) with *Rhizophagus* sp.; CR Dhan 204 (32.53) with *Claroideoglomus* sp.; Kasalath IC459373 (32.43) and CR Dhan 207 (32.40) with *Acaulospora* sp. treatment (Figure 4d). The *Funneliformis* sp. treated with CR Dhan 207 (4.32 gm) and *Rhizophagus* sp. treated with Kasalath IC459373 (2.466 gm) had the maximum performance in terms of plant fresh biomass and dried biomass, respectively (Figure 4e,f). However, the plant growth parameters viz. root length, leaf area, chlorophyll and plant biomass showed themselves to be significantly higher in CR Dhan 207 and CR Dhan 204 inoculated with *Rhizophagus* sp., *Glomus* sp., *Funneliformis* sp., and *Acaulospora* sp.

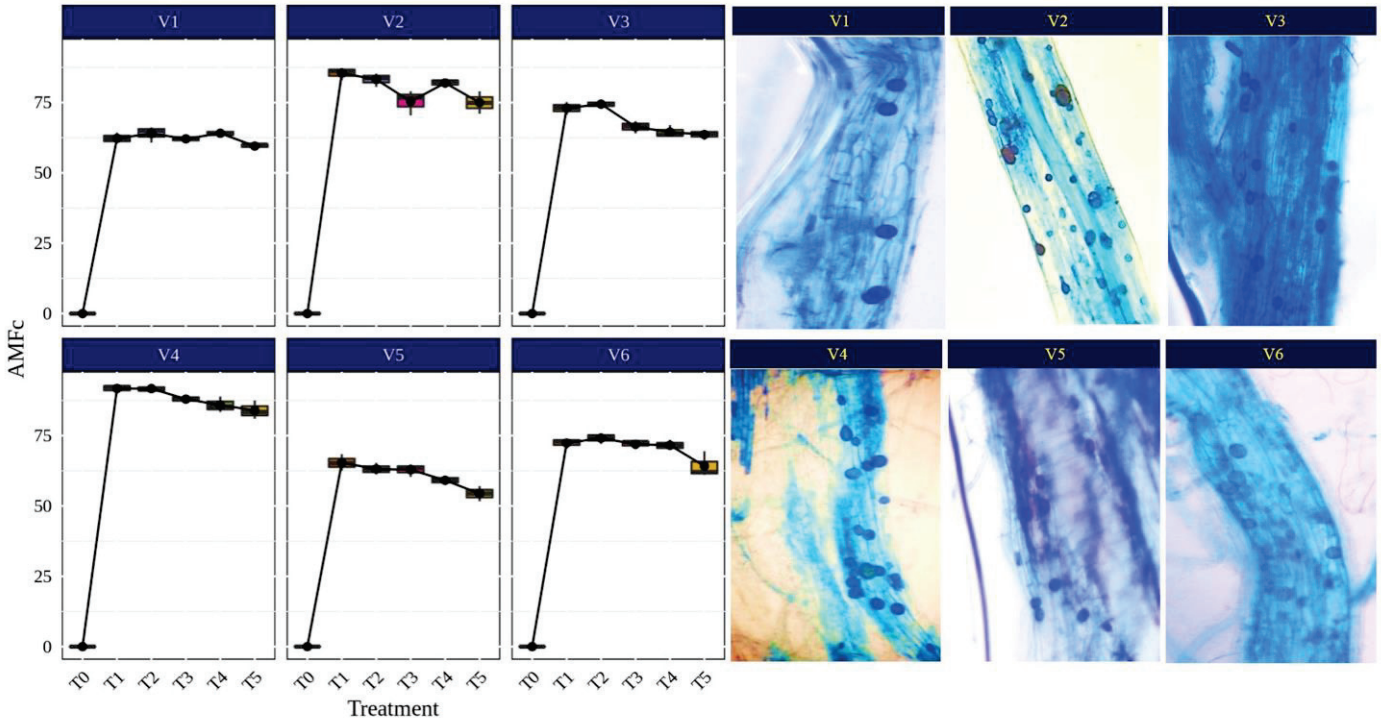


**Figure 3.** Percentage of AMF colonization (AMFc) in different rice varieties. Abbreviation: percentage of AMF colonization (AMFc).

### *3.4. Effects of AMF on Uptake of Plant P*

AMF both in aerobic and anaerobic rice cultivation increases nutrient concentration in the rice plant tissue; the bioavailability of nutrients increased in the soil solution due to mycorrhizae inoculation [52]. As shown in Figure 5, the P concentration in plants was higher in the rice variety CR Dhan 207 (14.796 mg. $pot^{-1}$), followed by Kasalath IC459373 (14.186 mg. $pot^{-1}$) and CR Dhan 204 (14.156 mg. $pot^{-1}$). Additionally, all the rice varieties inoculated with *Rhizophagus* sp., showed maximum P uptake, followed by *Funneliformis* sp., and *Glomus* sp. inoculation. The results deciphered 16.60–28.50% higher P uptake with AMF inoculation in all the rice varieties, compared to the uninoculated control.

### *3.5. Responses of AMF on Soil Enzyme and Microbial Properties*

Among the several AMF treatments, *Rhizophagus* sp. (56.59 g p-nitrophenol released $h^{-1}$ $g^{-1}$ soil) and *Funneliformis* sp. (31.99 g p-nitrophenol released $h^{-1}$ $g^{-1}$ soil) showed the highest levels of both acid (Figure 6a) and alkaline (Figure 6b) phosphatase activity in CR Dhan 207. Irrespective of the treatments, all rice varieties showed significantly higher acid and alkaline phosphatase activity in AMF-inoculated treatments as compared to the uninoculated control.

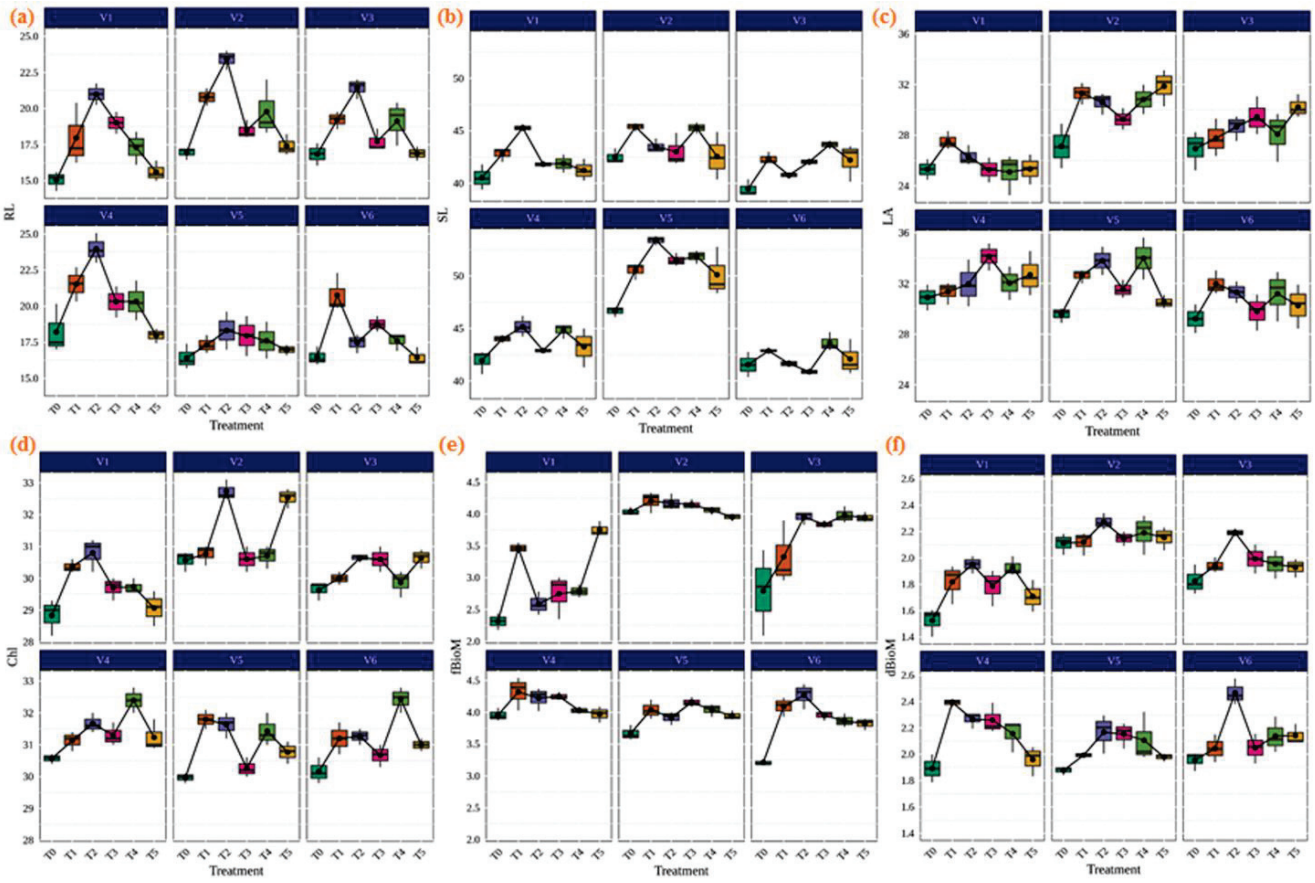


**Figure 4.** Enhancement of plant growth parameters due to AMF inoculation in different aerobic rice varieties. Abbreviations: (**a**) root length in cm. (RL); (**b**) shoot length in cm. (SL); (**c**) leaf area $m^2$ (LA); (**d**) chlorophyll SPAD (Chl); (**e**) fresh biomass in gm. (fBioM); (**f**) dry biomass in gm. (dBioM).

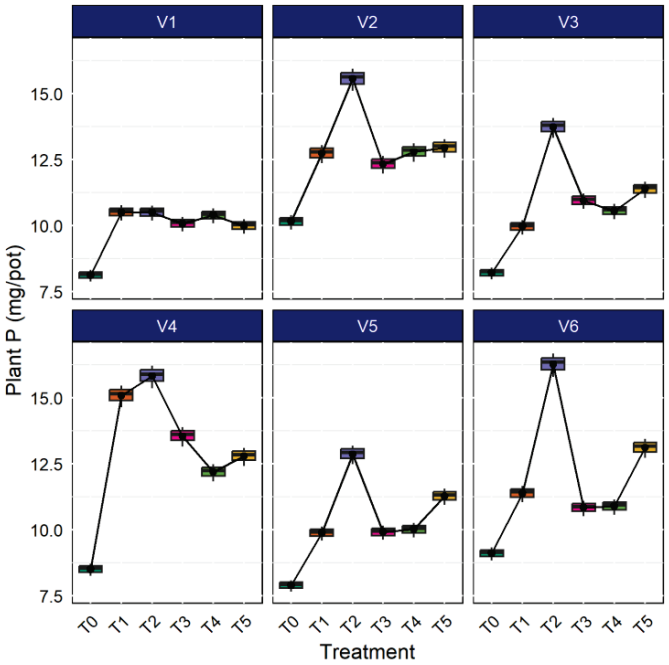


**Figure 5.** AMF inoculation on uptake of plant P in different aerobic rice varieties.

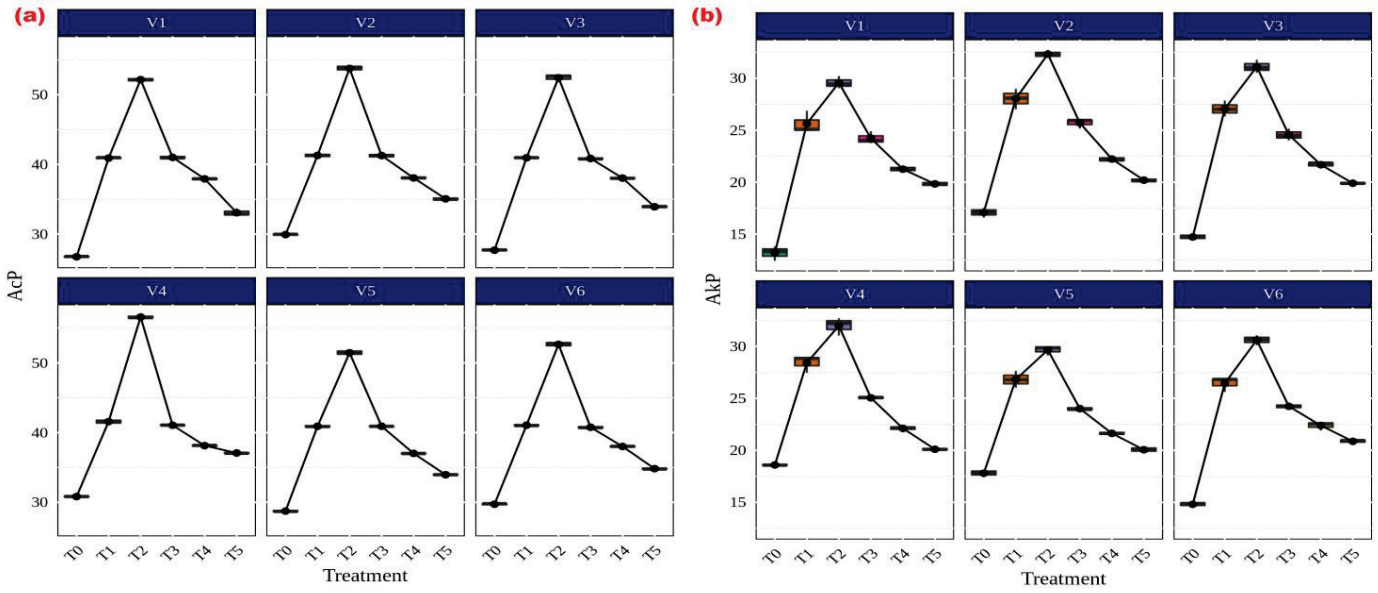


**Figure 6.** Enhancement of acid and alkaline phosphatase activities in different aerobic rice varieties. Abbreviations: (**a**) acid phosphatase (AcP) [µg p-nitrophenol released $h^{-1}$ $g^{-1}$ soil]; (**b**) alkaline phosphatase (AkP) [µg p-nitrophenol released $h^{-1}$ $g^{-1}$ soil].

In terms of microbial properties, *Funneliformis* sp., *Rhizophagus* sp., *Glomus* sp., *Acaulospora* sp. and *Claroideoglomus* sp. treatments significantly increased MBC in CR Dhan 204 and CR Dhan 207 (706.8 and 688.4 µg $g^{-1}$ soil) (Figure 7a). A similar trend was also noticed in DHA (29.43 and 31.82 µgTPF $h^{-1}g^{-1}$ soil) (Figure 7b) and FDA (15.37 and 16.13 µg fluorescein $h^{-1}g^{-1}$ soil) (Figure 7c).

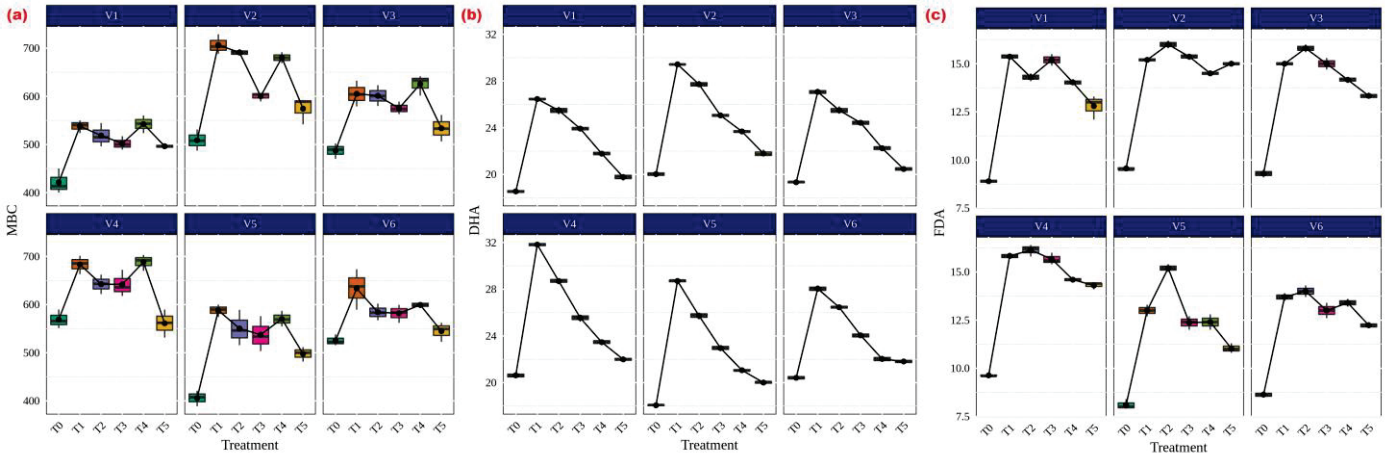


**Figure 7.** AMF and its influence on enhancement of microbial properties in different aerobic rice varieties. Abbreviations: (**a**) microbial biomass carbon (MBC) [µg $g^{-1}$ soil]; (**b**) dehydrogenase activity (DHA) [µgTPF $h^{-1}g^{-1}$ soil]; (**c**) fluorescein diacetate assay (FDA) [µg fluorescein $h^{-1}g^{-1}$ soil].

Through increasing microbial activity in the soil or by the exudation of enzymes by plants, AMF can also have an impact on soil enzyme activity as well as plant growth promotion [53–55]. Several studies have described how AMF intervention could stimulate soil enzyme activity through soil microorganisms [20,27,56,57]. Generally, soil enzymes are primarily produced by microorganisms; others, such as phosphatase [58], urease, and peroxidases, are also secreted by plant roots. Reports [59–61] have shown that the effects of AMF on various soil enzyme activities and growth-promoting compounds, which release the more biologically accessible nutrients from complex materials, were positively

correlated with increasing ratios of soil-available P and plant biomass as well as strongly abiotic context-dependent factors, with beneficial implications for plant growth. All of the aforementioned data made it very evident that AMF will increase soil enzyme activity, which could improve nutrient cycling.

### *3.6. Assessing the Mycorrhizal Responsiveness in Different Aerobic Rice Varieties*

Out of the selected rice varieties, mycorrhizal responsiveness was found highest in CR Dhan 207 followed by CR Dhan 204, CR Dhan 205 and Kasalath IC459373 with the application of *Funneliformis* sp. and *Rhizophagus* sp. under P-deficient conditions (Figure 8); however, the AMF responsiveness varies with different rice varieties.

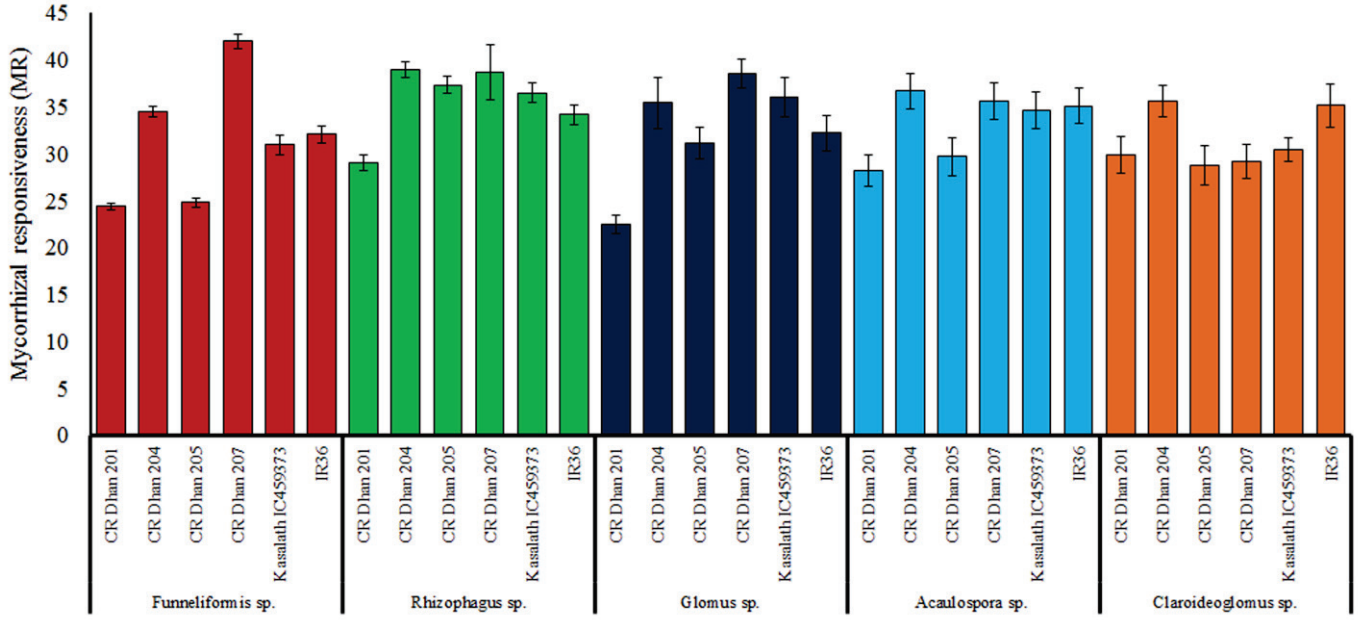


**Figure 8.** Mycorrhizal responsiveness in six aerobic rice varieties with five AMF inoculum effects.

### *3.7. Correlation of AMF Colonization with Soil and Plant Properties Using Linear Models*

The linear model was used to select the important parameters linked to AMF colonization and to calculate the correlation of the important variables (Table 2).

**Table 2.** Identification of important parameters using step regression model.

| Step | Variable | R-Square | Adj. R-Square | C(p) | AIC | RMSE |
|---|---|---|---|---|---|---|
| 1 | FDA | 0.8292 | 0.8275 | 22.3958 | 844.7126 | 11.862 |
| 2 | MBC | 0.8459 | 0.8429 | 12.0246 | 835.5882 | 11.320 |
| 3 | RL | 0.8532 | 0.8490 | 8.5771 | 832.3012 | 11.0993 |
| 4 | Plant P | 0.8608 | 0.8554 | 5.0000 | 828.6062 | 10.8628 |

C(p): Mallows' Cp constant; AIC: Akaike information criterion; RMSE: root mean square error.

The correlation analysis (Figure 9) showed that AMF colonization had a significant ($p < 0.001$) positive correlation with FDA ($R^2 = 0.911$), MBC ($R^2 = 0.707$) and plant-available P ($R^2 = 0.743$). The correlation between AMF colonization and FDA, the *Claroideoglomus* sp. ($R^2 = 0.797$) and *Acaulospora* sp. ($R^2 = 0.700$) treatments, showed a higher coefficient than other treatments. Similarly, with AMF colonization and MBC correlation, the higher coefficients were recorded in the treatment *Funneliformis* sp. ($R^2 = 0.880$) followed by *Glomus* sp. ($R^2 = 0.850$), *Acaulospora* sp. ($R^2 = 0.845$), —*Rhizophagus* sp. ($R^2 = 0.804$) and *Claroideoglomus* sp. ($R^2 = 0.744$) at $p < 0.011$ levels of significance. The correlation coefficient between AMF colonization and plant P was significantly ($p < 0.01$) at par for microbial treatments *Acaulospora* sp. ($R^2 = 0.919$), *Glomus* sp. ($R^2 = 0.919$), *Funneliformis* sp. ($R^2 = 0.908$), *Rhizophagus* sp. ($R^2 = 0.705$), and *Claroideoglomus* sp. ($R^2 = 0.632$). Similarly, many scientific

reports have well documented that AMF plays a crucial role in soils for improving microbial activity, nutrient cycling, soil structure and plant–soil microbe interactions [62–66].

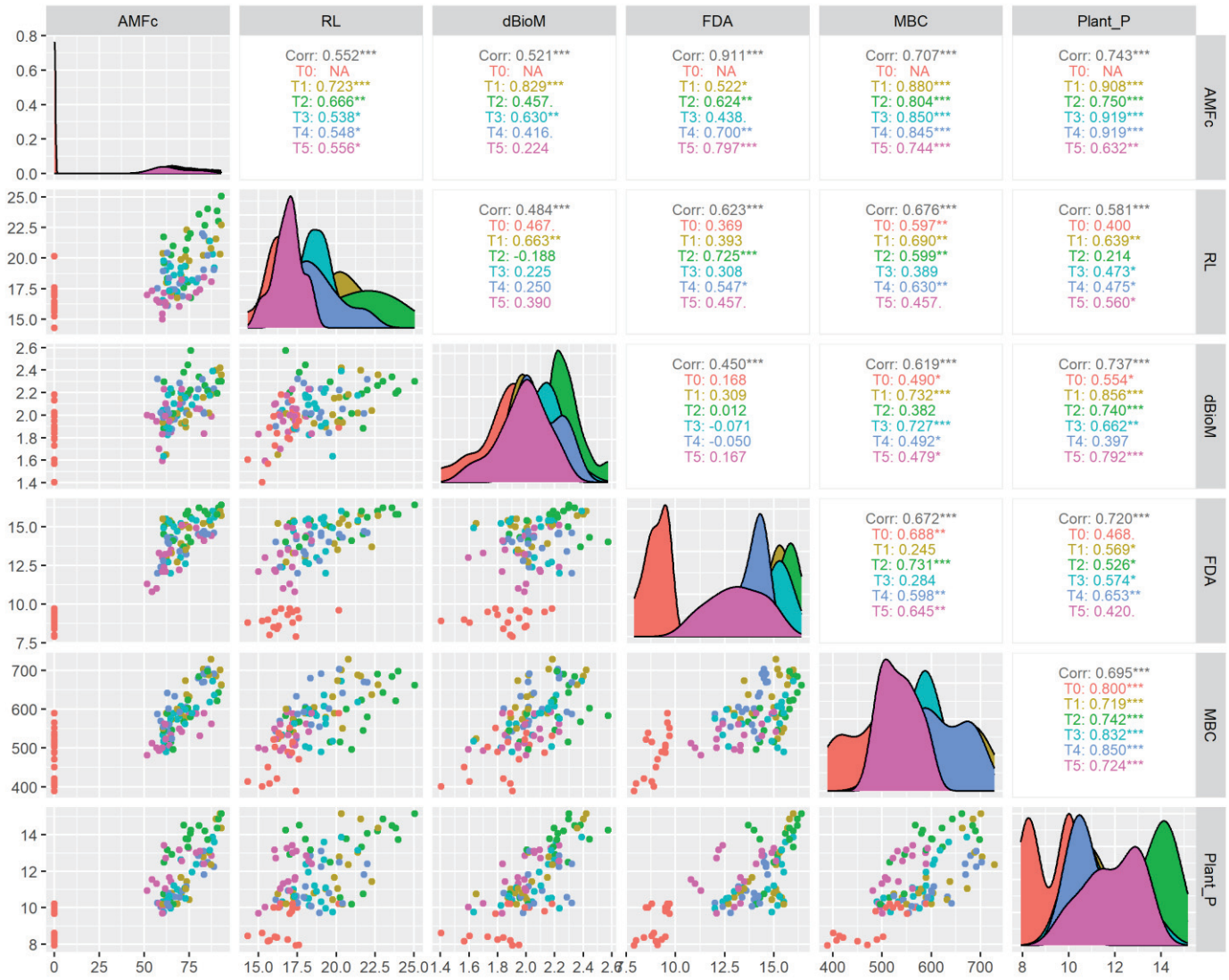


**Figure 9.** Correlation of AMF treatments in different aerobic rice varieties on plant P uptake and soil microbial properties. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Further correlation studies among varieties given in Figure 10 show that CR Dhan 207 ($R^2$ = 0.972), CR Dhan 204 ($R^2$ = 0.969), and Kasalath IC459373 ($R^2$ = 0.969) had the maximum coefficients between AMF colonization and FDA ($R^2$ = 0.911) among the different aerobic varieties. However, the correlation between AMF colonization and MBC ($R^2$ = 0.707) indicated that, among the varieties, IR 36 ($R^2$ = 0.884) and CR Dhan 201 ($R^2$ = 0.856) had the highest coefficient values, whereas CR Dhan 207 ($R^2$ = 0.560) and Kasalath IC459373 ($R^2$ = 0.653) registered the lowest coefficient among other varieties. Regarding the correlation between varieties and plant P uptake ($R^2$ = 0.743), the highest coefficient was found in CR Dhan 207 ($R^2$ = 0.927), at $p < 0.001$ significance. This finding clearly indicates that the response of AMF differs based on the type of variety. Thus, the selection of the right type of AMF is essential for exploring the maximum benefit from AMF symbiosis. Das et al. [67] reported that the application of *Glomus* spp. inoculation improved rice crop yields with better P availability in the rhizosphere under alternate wetting and drying irrigation.

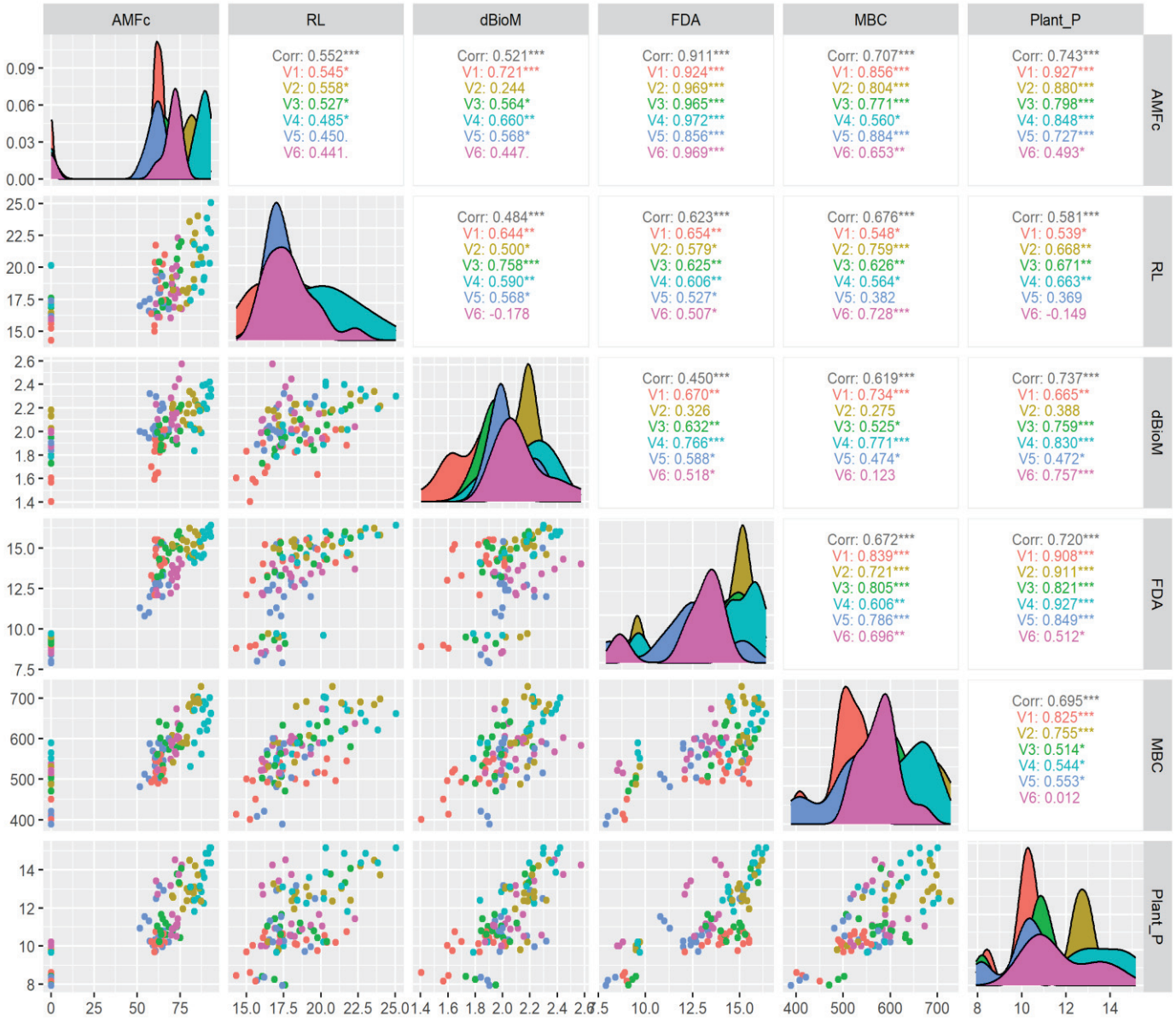


**Figure 10.** Response of aerobic rice varieties in AMF colonization correlation with plant P and soil microbial properties using Pearson correlation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

## 4. Conclusions

Soil phosphorus deficiency is one of the major problems in aerobic rice cultivation. The fixation of this element in the soil makes it unavailable for plant uptake. The present study revealed that AMF intervention could significantly increase the plant growth and enhance P uptake by 16.60–28.50% compared to the control. Among the four different aerobic rice varieties, the mycorrhizal responsiveness was found to be superior in CR Dhan 207, followed by CR Dhan 204, CR Dhan 205, and CR Dhan 201. The linear modelling approach found that the AMF colonization in all the rice varieties had significant ($p < 0.001$) positive correlation with FDA, MBC, and P uptake, deciphering the importance of AMF association in rice for the improvement of phosphate availability to plants. The present findings require further field validation. However, results suggest that the external application of suitable AMF is essential for improving the plant growth and enhancing the uptake of P in aerobic rice in P-deficient soil.

**Author Contributions:** Conceptualization, P.P., P.K.D.M. and D.M.; methodology, P.P., P.K.D.M. and D.M.; software, A.S. and D.M.; validation, P.P. and P.C.; formal analysis, D.M.; investigation, P.P., P.C., A.K.N. and P.K.D.M.; data curation, D.M.; writing—original draft preparation, D.M., P.P. and A.S.; visualization, D.M. and A.S.; supervision, P.P. and P.K.D.M.; project administration, P.P. and A.K.N. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data used to support the findings of this study are available from the corresponding author upon request.

**Acknowledgments:** The authors are grateful to the Honourable Director, ICAR—the National Rice Research Institute, India; the Honourable Vice-Chancellor, Raiganj University, India; the Department of Biotechnology, Government of India (BT/PR36476/ NNT/28/1723/2020) and Project no 2.7 (ICAR-NRRI, Cuttack) for support. DM is grateful to the Government of West Bengal, India, for a Swami Vivekananda Merit Cum Means Ph.D. Scholarship (WBP191584588825). The authors wish to extend special thanks to A. Anandan, Principal Scientist (Genetics and Plant Breeding), ICAR-NRRI, Cuttack, for providing seeds and support for this experiment. The research article is a part of Debasis Mitra's Ph.D. research programme, which was supervised by P. Panneerselvam, Principal Scientist, CPD, ICAR-NRRI, Cuttack, and Pradeep K. Das Mohapatra, Associate Professor and Head, Department of Microbiology, Raiganj University, India.

**Conflicts of Interest:** The authors declare no conflict of interest.

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*Review*

# Beneficial Microorganisms Improve Agricultural Sustainability under Climatic Extremes

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**Abstract:** The challenging alterations in climate in the last decades have had direct and indirect influences on biotic and abiotic stresses that have led to devastating implications on agricultural crop production and food security. Extreme environmental conditions, such as abiotic stresses, offer great opportunities to study the influence of different microorganisms in plant development and agricultural productivity. The focus of this review is to highlight the mechanisms of plant growth-promoting microorganisms (especially bacteria and fungi) adapted to environmental induced stresses such as drought, salinity, heavy metals, flooding, extreme temperatures, and intense light. The present state of knowledge focuses on the potential, prospective, and biotechnological approaches of plant growth-promoting bacteria and fungi to improve plant nutrition, physio-biochemical attributes, and the fitness of plants under environmental stresses. The current review focuses on the importance of the microbial community in improving sustainable crop production under changing climatic scenarios.

**Keywords:** PGPBs; abiotic stresses; growth-promoting fungi; crop productivity; plant tolerance

**Citation:** Jalal, A.; Oliveira, C.E.d.S.; Rosa, P.A.L.; Galindo, F.S.; Teixeira Filho, M.C.M. Beneficial Microorganisms Improve Agricultural Sustainability under Climatic Extremes. *Life* **2023**, *13*, 1102. https://doi.org/10.3390/life13051102

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 26 February 2023
Revised: 8 April 2023
Accepted: 24 April 2023
Published: 28 April 2023



## 1. Introduction

The severe impacts of transmutation with intense episodes of extreme weather can have significant consequences on agricultural outputs that should cause widespread food insecurity and affect survival of populations [1,2]. The severity, frequency, magnitude, and duration of extreme climatic events will become more highlighted and noticeable in the future [3]. The alterations in climate extremes have a direct or indirect influence on biotic and abiotic stresses with devastating impacts on agricultural crop production and food security [4]. Biotic stresses comprising phytopathogens and pests [5], as well as abiotic stresses including drought [6], soil salinity [6,7], heavy metals [8,9], flooding [10], high irradiance [11], low temperature [12] and high temperature [13], can cause intensified impacts on plant growth, physiology, metabolism, nutrient acquisition, and ecological desertification. The diverse effects of abiotic stresses on different mechanisms of plants are summarized in Figure 1.

In changing climate scenarios, intervention with microbes is considered a new sustainable strategy in agricultural production and mitigation of the resilient impacts of stresses [14]. The beneficial microbes and endophytes exhibit real-time amplifications to alleviate the devastating climatic impacts on plant health, physiology and biochemical aspects [14,15]. These microbial communities have several adaptations to abiotic stresses under different ecological processes, including facilitation of organic matter decomposition and nutrient acquisition in the rhizosphere of several plants [16]. Beneficial microbes, including plant growth-promoting rhizobacteria (PGPR), may have a controversial influence or no influence at all on plant growth and fitness under stressful environments, whereas other strains of PGPR have beneficial effects under climate-induced stressful extremes [17].

The PGPR engineered for agricultural practices boost plant growth, pathogen control, and microbial ecosystems by alleviating abiotic resiliencies [18,19].

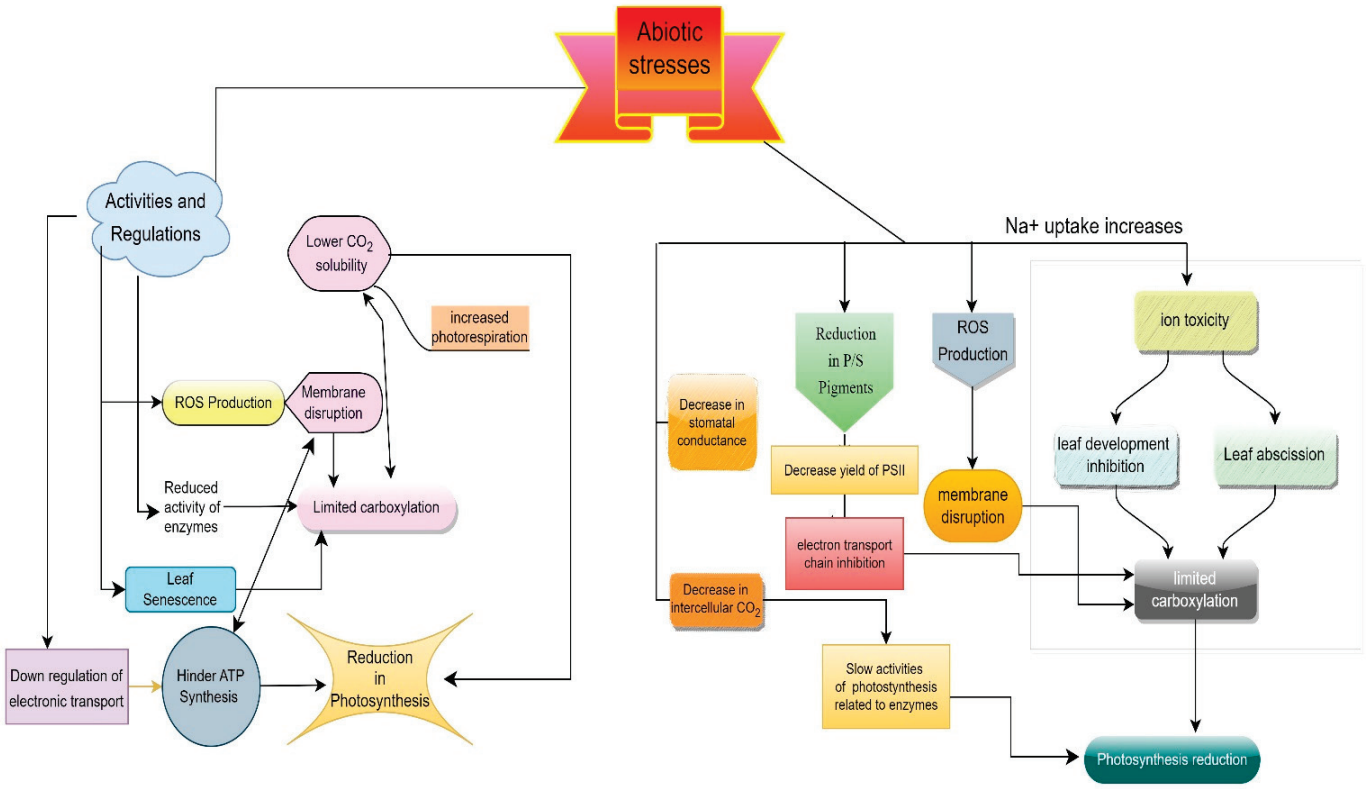


**Figure 1.** An overview of the effects of abiotic stresses on the different mechanisms of plants.

Plant growth-promoting rhizobacteria tackle abiotic stresses by boosting several physiological and biochemical processes (nutrient uptake, photosynthesis, and source–sink relationships), metabolism and the regulation of homeostasis, osmotic potential, protein function, phytohormone production (indole-3-acetic acid and 1-aminocyclopropane-1-carboxylic acid deaminase), enzymatic activity, and nutrient solubilization [20–22]. To combat the punitive impact of abiotic stresses, numerous PGPR strains (including *Bradyrhizobium* sp. SUTNa-2 [23], *Pantoea dispersa* IAC-BECa-132, *Pseudomonas* sp., Enterobacter sp. [24], *Bacillus amyloliquefaciens* EPP90, *Bacillus subtilis*, *Bacillus pumilus* [25], *Curtobacterium* sp. SAK 1 [26], *Burkholderia phytofirmans* PsJNT [27], *Pseudomonas putida* KT2440 [28], *Enterobacter* sp. [29], *Serratia marcescens*, *Microbacterium arborescens*, *Enterobacter* sp. [30], *Bacillus cereus* PK6-15, *Bacillus subtilis* PK5-26 and *Bacillus circulans* PK3-109 [31], *Azospirillum lipoferum* FK1 [32], and *Azospirillum brasilense* Sp7 and *Azospirillum brasilense* Sp245 [33] have been used to facilitate the management mechanisms of different cereal and legume crops under stressful environments. Plant growth-promoting rhizobacteria employ various strategies to endure harsh weather conditions (Table 1).

**Table 1.** Summary of the positive effects of microbial agents in mitigating unfavorable drought and salt stress conditions in plants (2012–2020).

| Microorganism | Stress | Plant Species | References |
|---|---|---|---|
| **Bacteria** | | | |
| *Azospirillum brasilense* | Drought | Marandu grass (*Urochloa brizantha*) | [34] |
| PGPRs strain IG 3, *Enterobacter ludwigii*, and *Flavobacterium* sp. | Drought | Wheat (*Triticum aestivum*) | [35] |
| *Bacillus* sp. | Drought | Sugarcane (*Saccharum* spp.) | [36] |

**Table 1.** *Cont.*

| Microorganism | Stress | Plant Species | References |
|---|---|---|---|
| *Bacillus megaterium*, *B. subtilis*, and *Bacillus thuringiensis* | Drought | Wheat (*Triticum aestivum* L.) and chickpea (*CicerArietinum*) | [37] |
| *Bacillus* sp. (12D6) and *Enterobacter* sp. (16i) | Drought | Wheat (*Triticum aestivum*) and maize (*Zea mays*) | [38] |
| *Actinobacterium* | Drought | Maize (*Zea mays* L.) | [39] |
| *Proteobacteria*, *Actinobacteria*, *Gemmatimonadetes*, *Chloroflexi*, *Cyanobacteria*, and *Acidobacteria* | Drought | Cotton (*Gossypium hirsutum*) | [40] |
| *Bradyrhizobium japonicum* and *Azospirillum brasilense* | Drought | Soybean (*Glycine max*) | [41] |
| *Acinetobacter calcoaceticus EU- LRNA-72 and Penicillium* sp. *EU-FTF-6* | Drought | Foxtail millet (*Setaria italica* L.) | [42] |
| *Pseudomonas lini*, *Bacillus*, and *Serratia plymuthica* | Drought | Jujube (*Ziziphus jujuba*) | [43] |
| *Rhizobium tropici* and *Azospirillum brasilense* | Drought | Common bean (*Phaseolus vulgaris*) | [44] |
| *Azotobacter chroococcum* | Salt | Tomato (*Solanum lycopersicum*) | [45] |
| *Microbacterium oleivorans*, *Brevibacterium iodinum*, and *Rhizobium massiliae* | Salt | Pepper (*Capsicum annuum*) | [46] |
| *Bacillus* spp. | Salt | Pepper (*Capsicum annuum*) | [47] |
| *Pseudomonas* sp. and *Hartmannibacter diazotrophicus* | Salt | Alfalfa (*Medicago sativa*) | [48] |
| *Pantoea agglomerans* | Salt | Rice (*Oryza sativa*) | [49] |
| *Arthrobacter aurescens*, *A. woluwensis*, *Microbacterium oxydans*, *Bacillus megaterium*, and *B. aryabhattai* | Salt | Soybean (*Glycine max*) | [50] |
| *Bacillus aryabhattai* and *B. mesonae* | Salt | Tomato (*Solanum lycopersicum*) | [51] |
| *Pseudomonas* sp. | Salt | *Arabidopsis thaliana* | [52] |
| *Pseudomonas fluorescens* | Salt | Barley (*Hordeum vulgare*) | [53] |
| *Arthrobacter nitroguajacolicus* | Salt | Wheat (*Triticum aestivum*) | [54] |
| *Bacillus cereus* and *B. aerius* | Salt | Safflower (*Carthamus tinctorius*) | [55] |
| *Pseudomonas* and *Azospirillum brasilense* | Salt | Rapeseed (*Brassica napus*) | [56] |
| *Pseudomonas geniculate* | Salt | Maize (*Zea mays*) | [57] |
| *Bacillus halotolerans* and *Lelliottia amnigena*, | Salt | Wheat (*Triticum aestivum*) | [58] |
| **Fungi** | | | |
| *Glomus mosseae* and *Glomus intraradices* | Drought | Rose geranium (*Pelargonium graveolens* L.) | [59] |
| *Trichoderma atroviride* strain (TaID20G) | Drought | Maize (*Zea mays* L.) | [60] |
| *Gaeumannomyces cylindrosporus* | Drought | Maize (*Zea mays*) | [61] |
| Arbuscular mycorhizal fungi (AMF) | Drought | Sweet potato (*Ipomoea batatas* (L.) Lam.) | [62] |
| AM fungus *Funneliformis mosseae* | Drought | Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] | [63] |
| *Trichoderma harzianum* | Drought | Tomato *(Solanum lycopersicum)* | [64] |
| *Rhizophagus intraradices*, *Funneliformis mosseae*, *F. geosporum* | Drought | Wheat (*Triticum aestivum* | [65] |
| *Arbuscular mycorrhizal fungi* | Drought | Chinese lyme grass (*Leymus chinensis*) and limpograss (*Hemarthria altissima*) | [66] |
| *Trichoderma harzinum 1*, *Trichoderma harzianum 2*, *Chaetomium globosum*, *and Talaromyces flavus* | Drought | Rice (*Oryza sativa* L.) | [67] |
| *Funneliformis mosseae*, *Glomus mosseae*, *G. intraradices*, *and G. etunicatum* | Salt | Desert grass (*Panicum turgidum*) | [68] |
| *Trichoderma harzianum* | Salt | Indian mustard (*Brassica juncea*) | [69] |
| *Trichoderma harzianum* | Salt | Tomato (*Solanum lycopersicum*) | [70] |
| *Trichoderma harzianum* | Salt | Rice (*Oryza sativa*) and maize (*Zea mays*) | [71] |
| *Klebsiella* sp. | Salt | Oat (*Avena sativa*) | [72] |
| *Glomus etunicatum*, *G. intraradices*, and *G. mosseae* | Salt | Cucumber (*Cucumis sativus*) | [73] |
| *Colobanthus quitensis* and *Deschampsia antarctica* | Salt | Lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*) | [74] |
| **Bacteria + Fungi** | | | |
| *Bacillus thuringiensis* + Arbuscular mycorrhizal fungus | Drought | French lavender (*Lavandula dentata*) | [75] |
| *Pseudomonas putida* + *Rhizophagus irregularis* | Drought | Calotrope (*Calotropis procera* Ait.) | [76] |
| *Micrococcus yunnanensis* + *Claroideoglomus etunicatum* | Drought | Moldavian balm *(Dracocephalum moldavica* L.) | [77] |

**Table 1.** *Cont.*

| Microorganism | Stress | Plant Species | References |
|---|---|---|---|
| *Pseudomonas fluorescens* + *Rhizophagus irregularis* or *Funneliformis mosseae* | Drought | Arizona cypress (*Cupressus arizonica* Green) | [78] |
| *Pseudomonas fluorescence* + *Glomus mosseae* | Salt | Bean (*Phaseolus vulgaris*) | [79] |
| *Methylobacterium oryzae* + *Glomus etunicatum* | Salt | Rice (*Oryza sativa*) | [80] |
| *Bacillus subitilis* + *Glomus. etunicatum*, *G. intraradices*, and *G. mosseae* | Salt | Acacia (*Acacia gerrardii*) | [81] |
| *Bradyrhizobium* sp. + *Trichoderma asperelloides* | Salt | Cowpea (*Vigna unguiculate*) | [82] |

In addition, root-associated microbes such as fungi can potentially influence different ecological processes to optimize plant health and growth, resulting in a great impact on plant physiology, nutrition, and survival ability that improves plant tolerance against environment-induced stresses [83]. These endophytic fungi confer abiotic stresses through the synthesis of various plant beneficial substances (ACC-deaminase, auxins, gibberellins, abscisic acid, siderophores) and solubilize nutrients for healthy plant growth [84,85]. The fugal endophytes form a mutualistic association with plants to promote photosystem activity, protein accumulation, primary metabolism that leads to higher growth, and tolerance under abiotic stresses [65,86]. Plants develop mutualistic relationships with several plant growth-promoting endophytic fungi, including *Piriformospora indica* [86], arbuscular Mycorrhizal fungi [65], *Trichoderma albolutescens*, *Trichoderma asperelloides*, *Trichoderma orientale*, *Trichoderma spirale*, and *Trichoderma tomentosum* [87], *Penicillium aurantiogriseum* 581PDA3, *Alternaria alternate* 581PDA5, *Trichoderma harzianum* 582PDA7 [88], and *Porostereum spadiceum* AGH786 [89], which can increase tolerance against abiotic stresses by improving the biochemical and physiological processes of different plants, as summarized in Figure 2.

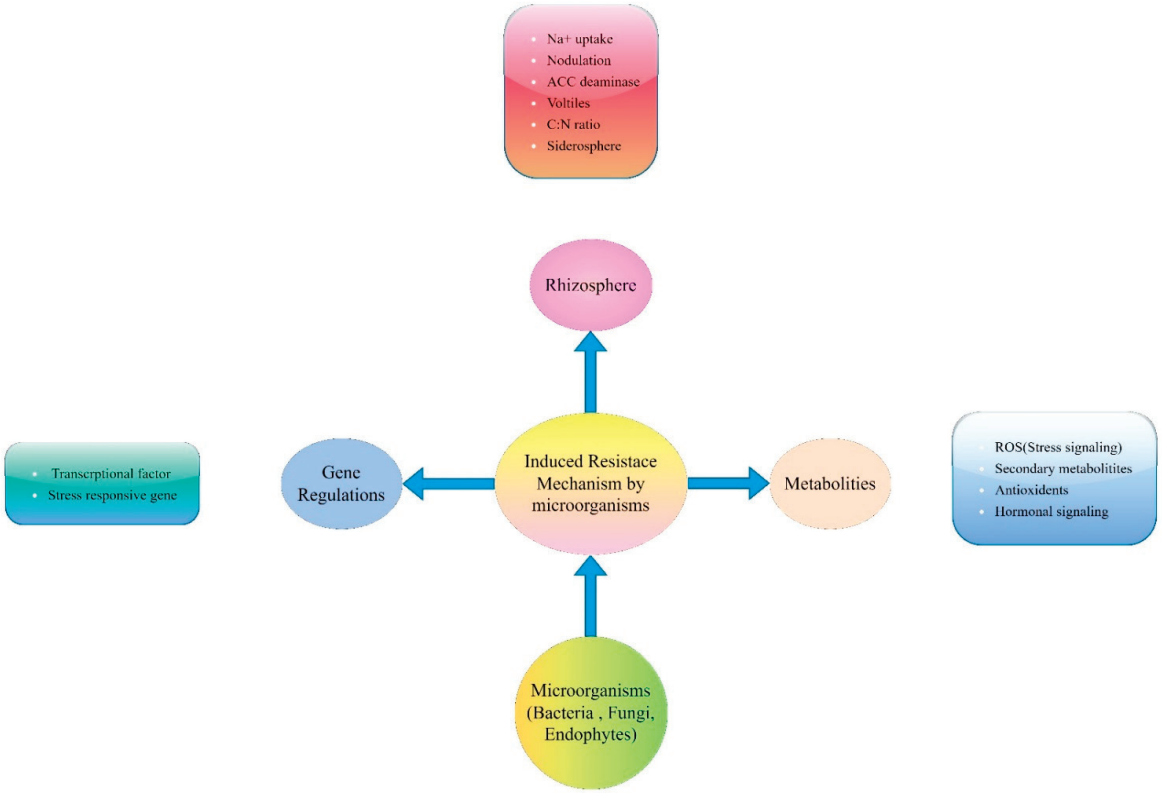


**Figure 2.** Mechanisms against abiotic stresses adapted from microorganisms.

The focus of this review is to highlight the mechanisms of plant growth-promoting microorganisms (especially bacteria and fungi) adapted to environmentally induced stresses such as drought, salinity, heavy metals, flooding, extreme temperatures, and intense light. The present state of knowledge focuses on the potential, prospective, and biotechnological approaches of plant growth-promoting bacteria and fungi to improve physiological and biochemical attributes and the fitness of plants under environmental stresses. Additionally, emphasis is placed on the significance of the role of microbial communities in promoting sustainable crop production amidst changing climatic scenarios.

## 2. Drought Stress

Disruption in the water cycle has become a serious challenge to overcome that is an alarming worry to farmers, horticulturists, and the world's population as it threatens the food needs of humans and animals. In this context, farmers have increased the amount of irrigation to improve the quantity and quality of agricultural crops; however, this strategy could increase the cost of production [90]. Drought can be described as an unfavourable environmental condition with an insufficient level of moisture that can affect normal development and growth cycle of plants [91]. It has been highlighted that drought can reduce yield and cultivation potential (ideal yield) of soybean by up to 70% [92].

Severe climatic variations with unstable precipitation can result in prolonged drought in certain crops depending on the duration and intensity of drought [93], which ultimately affects crop development and productivity [94]. The effect of drought on yield is a highly complex mechanism that could adversely influence fertilization, embryogenesis, seed development, and the physiological, biochemical, and molecular processes of plants [95], which includes cell dehydration, reduced leaf size, stem elongation, root proliferation, nutrient uptake, and their use efficiency [96,97]. Drought also alters the signal activity of nitrogen and carbon metabolism enzymes, as well as the level of antioxidants in plants [98]. Plant signal genes are responsible for the accumulation of abscisic acid (ABA) via distinct regulatory pathways under drought stress conditions [99]. Modulation of gene expression related to drought stress is achieved by critical signaling pathways such as strigolactone, reactive oxygen species (ROS), and lipid-derived signaling [100,101]. Moreover, soluble sugar, programed cell death [99], and qualitative trait loci (QTL) [102] are gene expression adjustments in response to drought stress.

Alterations in the time and duration of precipitation generate long-term drought, which prominently affects the activities of microbial communities. The availability of water in the changing climate scenario is one of the most important factors that influences soil microbial activity [103]. Microbes adapt different strategies to deal with short- and long-term drought in response to changing climatic patterns [104]. Beneficial engineering of microorganisms within the root rhizosphere and root endosperm is a strategic approach to attaining healthy and productive crops under drought stress conditions [105]. Microbial communities under changing climatic conditions improve crop production efficiency [106]. Inoculation with microbes such as plant growth-promoting bacteria, fungi, and algae, either alone or in combination [107] is considered as one of the best alternatives to fertilizers that can enhance plant growth [108], root growth, and nutrient availability via mobilization and mineralization [109] and can help in the alleviation of drought stress [35]. These endophytic and epiphytic plant growth-promoting microbial diversities have adapted several mechanisms, such as synthesis of exopolysaccharide, 1-aminocyclopropane-1-carboxylate deaminase, volatile compounds, osmolytes, and antioxidants that can up- or downregulate stress-responsive genes, change root morphology, and improve nutrient uptake against drought stress in different cereal crops under changing climatic conditions [42,110]. Several plant growth-promoting microbes improve phosphorous and zinc solubilization, nitrogen fixation, and siderophore production and act as antimicrobial agents against harmful microbes that could reduce tolerance in food crops against drought stress and extreme climatic conditions [111,112].

Some beneficial fungi (arbuscular mycorrhizal fungi—AMF) and algae (*Amphora ovals*) adapt several biochemical, physiological, and molecular strategies to overcome drought conditions and improve crop growth and productivity under changing climate scenarios [113,114]. Plant growth-promoting fungi such AMF, Trichoderma spp., and certain algae promote antioxidant enzymes, nutrient uptake, chlorophyll, proline content, and phytohormone production, which can promote growth and tolerance against drought stress in host plants [113,115]. Over the last decade, many studies have demonstrated the use of plant growth-promoting bacteria and fungi that can mitigate the unfavourable effects of drought stress in host plants as summarized in Table 1.

## 3. Salt Stress

Salinity is one of the major global and environmental concerns that limits agricultural productivity and is attributed to extreme episodes of climatic changes [116]. Water quality and irrigation management irrespective of source, such as dams, ponds, rivers, artesian wells, or high-depth aquifers, contains salt complexes [117]. These salt complexes include some of the important cationic species, such as calcium ($Ca^{2+}$), magnesium ($Mg^{2+}$), sodium ($Na^{2+}$), and potassium ($K^{+}$), and among the anionic complexes are chloride ($Cl^{-}$), carbonate ($CO_3{}^{2-}$), bicarbonate ($HCO_3{}^{-}$), sulfate ($SO_4{}^{2-}$), and boron (B) that all can have deleterious effects on agriculture ecosystems and plant productivity. Thus, the increased accumulation of these salts in low-quality irrigation water on arable land converts the land into non-usable and non-productive soil [118]. Soils irrigated with saturated water extract with an EC of 4.0 dS $m^{-1}$ (40 mmol $L^{-1}$ of NaCl) are considered to be saline and can cause osmotic pressure of 0.2 MPa that leads to a reduction in vegetable yields [119].

The expansion of salinity into formerly unaffected areas due to drastic climate changes can have adverse effects on plant growth through osmotic inhibition and phytotoxic effects on certain ions in the rhizosphere that trigger secondary oxidative stress in plants [116,120]. Salinity generates low water potential in the soil, thus restricting water availability for plants [121]. Plants with low osmotic potential under saline conditions often suffer from physiological drought that restricts nutrient mobilization to the aerial parts of plants. An excessive concentration of salt in the soil solution negatively affects plant physiology, photosynthesis, metabolism, protein and ATP synthesis, growth, and the productivity of crops [122]. The toxic effects of sodium ($Na^{+}$) and chlorine ($Cl^{-}$) ions are prevalent in saline soils, which disturbs enzymes and other macromolecules, thus damaging cellular organelles, disrupting photosynthesis and respiration, inhibiting protein synthesis, and causing ion-induced deficiencies [123].

Salinity negatively affects the photosynthetic rate of plants, which can impair crop productivity and cell membrane activity. Salinity also affects osmotic potential, which can reduce water availability, and further impacts $CO_2$ permeability and deactivates the transport of photosynthetic electrons via shrinking intracellular spaces [124]. Stomatal closure can decrease carbon fixation and the production of reactive oxygen species (ROS) such as superoxide and single oxygen, which disrupt cellular processes by damaging lipids, proteins, and nucleic acids [125]. The unbalanced concentration of salt within the cell causes ionic toxicity and inhibits cell metabolism and other functional processes. $Na^{+}$ can disrupt plant nutrition by inhibiting potassium ion ($K^{+}$) uptake, which leads to the disturbance of enzymatic activity ($K^{+}$ regulates more than 50 enzymes) within the cell [126]. The salt stress also triggers hormonal activity and alters assimilation and partition between sources and tissues [127]. Salinization alters phytohormones (abscisic acid, cytokinin, trans-Zeatin, indole-3-acetic acid, and carboxylic acid) in the tissues and nodules of the plant that cause leaf senescence and early tissue death [128]. It was demonstrated that carboxylic acid is the precursor of ethylene, which plays a vital role in the initiation of salt-induced senescence [129].

Plants adapt several strategies and evolutionary, physiological, and ecological processes to mitigate or tolerate salinity stress and improve productivity. The application of plant growth-promoting bacteria (PGPBs) is the most viable and effective alternative that

can mitigate toxicity and the adverse effects of salinity while improving crop health and productivity [130]. These microorganisms mainly act as producers of phytohormones such as auxins, cytokinins, and gibberellins, which contribute to the growth of root systems, stimulate water absorption, and inhibit the effects of salinity [131,132]. Plant growth-promoting bacteria of different *Pseudomonas* sp. can improve peroxidase enzymes, total polyphenol and proline content, which are being indicated to increase relative water content in the leaves of *Coriandrum sativum* under salinity stress [133]. Plant prolines are the most adaptable and sensitive amino acids to stress conditions and can act as protectors of enzymes and defend plant tissues against osmotic stress [47].

The association of PGPBs with beneficial fungi has synergistic effects on plant growth through induced tolerance against saline conditions [134]. Arbuscular mycorrhizal fungi can improve crop performance and tolerance to salinization by reducing $Na^+$ absorption while enhancing nutrient and water uptake and the antioxidant mechanisms of several plants [121,135]. Different species of ectomycorrhiza fungi (ECM), such as *Hebeloma*, *Laccaria*, *Paxillus*, *Pisolithus*, and *Rhizopogon*, can restrict Na+ transportation within plant tissues, thus improving mineral nutrition and water uptake and alleviating the effects of salination in host plants [136]. Trichoderma species are widely used as a biocontrol and plant growth-promoting agent in agriculture and can colonize in diverse substrates under different environmental conditions, therefore inducing tolerance against abiotic stresses [137].

Beneficial microorganisms are associated with increased water absorption, better use efficiency and uptake of nutrient, and improved soil fertility and structure, thus helping plants under salt stress conditions [138]. These microorganisms utilize nitrogen (N) for biological nitrogen fixation, nitrate reductase activity, and N use efficiency [139] while increasing phosphorous availability through phosphate solubilization [140]. In addition, these microorganisms can also increase the fertilizer use efficiency of NPK by 50% while alleviating the negative effects of salt stress in plants [141].

Over the past decade, numerous studies have highlighted the role of plant growth-promoting bacteria and fungi in mitigating the harmful effects of salt stress in plants (Table 1).

## 4. Heavy Metals

Heavy metals (HMs) are a serious threat to agriculture that can significantly harm different environmental, ecological, and nutritional factors of plants. The rising population has led to increased fertilizer use for higher food production, which can consequently lead to contamination of the environment and food chains [142]. The anthropogenic activities of humans, including mining, various industries, metallurgy, the use of chemical fertilizers containing HMs, and transportation, have led to a dramatic increase in HM accumulation in the ecosystem [143,144]. Heavy metals released into the air, environment, and soil can be absorbed by plants through roots and leaves, which can disrupt plant metabolism and cause several health risks to humans [143,145]. Edible plants are the major source of food in the human diet, and their contamination with toxic metals may result in catastrophic health hazards [143].

The term HMs refers to any metallic element that has a relatively high density and is either toxic or poisonous even at low concentration [142,143]. Heavy metals are generally categorized to belong to the group of metals and metalloids with high atomic density (density greater than 4 g $cm^{-3}$) and mass [142]. Heavy metals include non-essential plant elements such as lead (Pb), cadmium (Cd), aluminum (Al), chromium (Cr), mercury (Hg), arsenic (As), silver (Ag), and platinum group elements [143,146]. Some heavy metals, such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), nickel (Ni), and molybdenum (Mo), are essential micronutrients and are required for many of the biochemical functions of plants, including plant growth, oxidation and reduction reactions, electron transport, and many other metabolic processes; however, their high concentration can cause phytotoxicity [143,147].

Heavy metal toxicity in plants can cause leaf chlorosis, alter chlorophyll a and b ratios, decrease photosynthesis, inhibit root elongation, increase ROS production and membrane leakage, and change lipid composition through changing inter-cellular concentrations of nutrients [148,149].

Soils are a major sink for metal contamination in terrestrial ecosystems [131]. A diverse range of plants is used for the phytoremediation of toxic heavy metals and metalloids [150]. In addition, microorganisms such as PGPBs and PGPFs can enhance the effectiveness of phytoremediation [9,146,150] by producing organic acids, siderophores, bio-surfactants, bio-methylation, and redox processes that could transform heavy metals into soluble and bioavailable forms [9,150]. These microorganisms help the host plants by increasing biomass and phytoremediation attributes through synthesis of phytohormones such as indole-3-acetic acid (IAA) and enzyme like 1-aminocyclopropance-1-carboxylic acid deaminase (ACC), as well as through nitrogen fixation, P solubilization, and Fe sequestration [131,150]. These multiple traits improve the metabolic activity of microbes (Firmicutes, Proteobacteria, and Actinobacteria and most represented genera belong to *Bacillus*, *Pseudomonas*, and *Arthrobacter*) in heavy metal-contaminated sites [131,151].

Microbes play a key role in the remediation of HMs through phyto-stabilization, phyto-extraction, and phyto-volatilization [131,146]. Several studies have demonstrated the beneficial aspects of microbes in reducing HM toxicity in plant species over the past few decades (Table 2).

**Table 2.** Summary of the positive influence of microbes in mitigating heavy metal toxicity in contaminated sites (2010–2020).

| Microorganism | Heavy Metal | Reference |
|---|---|---|
| **Bacteria** | | |
| *Azotobacter chroococum* and *Rhizobium leguminosarum* | Pb | [152] |
| *Pseudomonas* sp. SRI2, *Psychrobacter* sp. SRS8, and *Bacillus* sp. SN9 | Ni | [153] |
| *Sporosarcina ginsengisoli* | As (III) | [154] |
| *Bacillus cereus* | Cr (VI) | [154] |
| *P. macerans* NBRFT5, *B. endophyticus* NBRFT4, *B. pumilus* NBRFT9 | Cu, Ni, and Zn | [155] |
| *Bacillus thuringiensis* GDB-1 | As | [156] |
| *Bacillus cereus* strain XMCr-6 | Cr (VI) | [157] |
| *Bacillus subtilis* | Cr (VI) | [158] |
| *Pseudomonas putida* | Cr (VI) | [158] |
| *Pseudomonas* sp. LK9 | Cd, Cu, and Zn | [159] |
| *Enterobacter* sp. And *Klebsiella* sp. | Cd, Pb, and Zn | [160] |
| *Kocuria flava* | Cu | [154] |
| *Pseudomonas veronii* | Cd, Cu, and Zn | [154] |
| *Bacillus pumilus* E2S2 and *Bacillus* sp. E1S2 | Cd and Zn | [161] |
| *Enterobacter cloacae* B2-DHA | Cr (VI) | [162] |
| *Planomicrobium chinense*, *B. cereus*, *P. fluorescens* | Co, Mn, Ni, and Pb | [163] |
| *B. cereus*, *P. moraviensis* | Mn and Cd | [164] |
| *B. safensis* FO-036b (T) and *P. fluorescens* | Pb and Zn | [165] |
| **Fungi** | | |
| *Pleurotus platypus* | Ag | [166] |
| *Rhizopus oryzae* (MPRO) | Cr (VI) | [167] |
| *Aspergillus versicolor* | Cu and Ni | [154] |
| *Aspergillus fumigatus* | Pb | [168] |
| *Rhizopus oryzae* | Cu | [169] |
| **Algae** | | |
| *Spirogyra* spp. and *Cladophora* spp. | Cu (II) and Pb (II) | [154] |
| *Spirogyra* spp. and *Spirullina* spp. | Cr Cu, Fe, Mn, and Zn | [154,170] |
| *Cystoseira barbata* | Cd, Ni, and Pb | [171] |
| *Hydrodictylon*, *Oedogonium*, and *Rhizoclonium* spp. | As | [172] |

## 5. High Temperature

High temperature is one the major abiotic stress in extreme climates that has deleterious impacts on crop yield, global production, human health, and socio-economic damage and wildfires [173,174]. The exposure of plants to unsuitable temperatures during crop cycles results in reduced growth and biochemical aspects. Prolonged heat stress has severe implications on different metabolic processes, including water relations, heat shock proteins, carbohydrate metabolism, and physiological disruptions that lead to cell death [91,175]. High temperature stress crucially affects the grain filling stage [176], grain quality [177], grain protein content [178], biomass, phenology, leaf senescence, grain yield [179], and the plant canopy in wheat [180]. High temperature stress also has drastic influences on several crops, including rice [181], sorghum [182], pearl millet [183], maize [184], and wheat [185].

High temperature stress induces the production of reactive oxygen species (ROS), which damage the cell membranes of plants and trigger stress responses [186]. The ROS molecules encompass free radicals from oxygen ($O_2$) metabolism, including superoxide radicals ($O_2^-$), hydroxyl radicals ($OH^-$), hydrogen peroxide ($H_2O_2$), and singlet oxygen ($1O_2$) [187]. Reactive oxygen species are produced via aerobic metabolism through the interaction of $O_2$ and escaped electrons from electron transport chains in the chloroplast and mitochondria under normal conditions [188]. However, under stress conditions, accumulation of ROS affects cellular components and causes damage to membranes through lipid peroxidation [186,189]. Plants adapt several mechanisms, including the induction of antioxidants and signaling processes to overlap ROS damage [190] and the use of metabolites, proteins, and membrane lipids to cope with temperature stress [191].

Plant–microbial association (bacteria and fungi) is an alternative and climate resilient strategy that promotes plant growth and improves tolerance against abiotic stress [192], especially high levels of temperature stress [193]. These microorganisms fight against induced climatic changes (abiotic factors) that impair the general performance of plants by improving phytohormone synthesis, the availability of nutrients, water absorption, and structure, therefore contributing to the successful adaptation of plants under stressful conditions [138]. Beneficial microorganisms are involved in various mechanisms, such as the stimulation of phytohormones (indole-3-acetic acid (IAA), ethylene, cytokinins, gibberellins) [194], polyamines (speridine, spermine, cadaverine) [195], and solubilization of phosphate [196–198], and zinc [199–201], as well as production of secondary metabolites that can improve the stability of leaf cell membranes and leaf abscission, and plant tolerance to abiotic stresses [44,202].

In addition, these microorganisms may induce plant oxidative stress, reducing the deleterious effects of ROS [203]. Beneficial microorganisms such as bacteria, actinomycetes, and fungi provide shelter to host plants against extreme climatic events and unfavorable environmental alterations [204]. Several studies have highlighted the ameliorative effect of PGPBs [205,206] and PGPFs [65,115,207], which can increase tolerance against the negative impacts of high temperature stress in different crop plants. Furthermore, PGPBs and PGPFs can compensate and mitigate the adverse impact of high temperature, as is evident from the past twelve years of study (Table 3).

**Table 3.** Summary of the positive effects of microbes in mitigating unfavorable high and cold temperature and flooding stress conditions in plants (2012–2020).

| Microorganism | Stress | Plant Species | Reference |
|---|---|---|---|
| **Bacteria** | | | |
| *Azospirillum brasilense* and *Bacillus amyloliquefaciens* | High temperature | Wheat (*Triticum aestivum*) | [175] |
| *Bacillus amyloliquefaciens* | High temperature | Rice (*Oryza sativa*) | [205] |
| *Bacillus amyloliquefaciens* | High temperature | Wheat (*Triticum aestivum*) | [208] |
| *Pseudomonas syringae* | High temperature | *Arabidopsis thaliana* | [209] |
| *Enterobacter* sp. | High temperature | *Arabidopsis thaliana* | [210] |

**Table 3.** *Cont.*

| Microorganism | Stress | Plant Species | Reference |
|---|---|---|---|
| *Bacillus velezensis* | High temperature | Wheat (*Triticum aestivum*) | [211] |
| *Bacillus cereus* | High temperature | Tomato (*Solanum lycopersicum*) | [212] |
| *Bacillus cereus* | High temperature | Tomato (*Solanum lycopersicum*) | [213] |
| *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Methylobacterium*, *Arthrobacter*, *Pantoea*, *Achromobacter*, *Acinetobacter*, *Exiguobacterium and Staphylococcus*, *Enterobacter*, *Providencia*, *Klebsiella and Leclercia*, *Brevundimonas*, *Flavobacterium*, *Kocuria*, *Kluyvera*, and *Planococcus* | Cold temperature | Wheat (*Triticum aestivum*) | [214] |
| *Arthrobacter*, *Flavimonas*, *Flavobacterium*, *Massilia*, *Pedobacter*, and *Pseudomonas* | Cold temperature | Tomato (*Solanum lycopersicum*) | [215] |
| Rhizobacterial isolates of *Bacillus* genera, Gu2 and 127b | Cold temperature | Wheat (*Triticum aestivum*) | [216] |
| *Pseudomonas fragi*, *P. chloropaphis*, *P. fluorescens*, *P. proteolytica*, and *Brevibacterium frigoritolerans* | Cold temperature | Bean (*Phaseolus vulgaris* L.) | [217] |
| *Bradyrhizobium japonicum* | Flooding | Soybean (*Glycine max*) | [218] |
| *Achromobacter xylosoxidans*, *Serratia ureilytica*, *Herbaspirillum seropedicae*, and *Ochrobactrum rhizosphaerae* | Flooding | Tulsi (*Ocimum sanctum*) | [219] |
| *Pseudomonas putida* | Flooding | Cucumber (*Cucumis sativus*) | [220] |
| *Azospira oryzae*, *Pelomonas saccharophila*, and *Methylosinus* sp. | Flooding | Rice (*Oryza sativa*) | [221] |
| *Pseudomonas putida* | Flooding | *Rumex palustris* | [222] |
| **Fungi** | | | |
| *Glomus deserticola* and *Glomus constrictum* | High temperature | Tomato (*Solanum lycopersicum*) | [223] |
| *Aspergillus japonicas* | High temperature | Soybean (*Glycine max*) and sunflower (*Helianthus annuus*) | [224] |
| *Thermomyces* sp. | High temperature | Cucumber (*Cucumis sativus*) | [225] |
| *Thermomyces lanuginosus* | High temperature | *Cullen plicata* | [226] |
| *Glomus mosseae* | Cold | *Elymus nutans* Griseb | [227] |
| *Trichoderma harzianum* | Cold | Tomato (*Solanum lycopersicum* L.) | [115] |
| *Glomus versiforme and Rhizophagus irregularis* | Cold | Barley (*Hordeum vulgare* L.) | [228] |
| *Rhizophagus irregularis* | Cold | Cucumber (*Cucumis sativus* L.) | [15] |
| *Rhizophagus irregularis* | Flooding | Tomato (*Solanum lycopersicum*) | [229] |
| *Glomus intraradices*, *G. versiforme*, and *G. etunicatum* | Flooding | Cattail (*Typha orientalis*) and rice (*Oryza sativa*) | [230] |
| *Trichoderma* | Flooding | Rice (*Oryza sativa*) | [231] |
| *Aspergillus fumigatus* | Flooding | *Arabidopsis* sp. | [232] |
| Bacteria and fungi | | | |
| *Bradyrhizobium* + arbuscular mycorrhizal fungi | High temperature | Soybean (*Glycine max* L.) | [233] |
| Proteobacteria, Actinobacteria, Chloroflexi, and Nitrospirae + Dothideomycetes, Sordariomycetes, and Ascomycota | High temperature and drought | Sorghum (*Sorghum bicolor* L.) and foxtail millet (*Setaria italica* L.) | [234] |
| *Bacillus* and *Pseudomonas* + *Penicillium* | Cold temperature | Potato (*Solanum tuberosum*) | [235] |
| *Paraburkholderia graminis* C4D1M and *Funneliformis mosseae* | Cold temperature | Tomato (*Solanum lycopersicum* L.) | [236] |

## 6. Low Temperature

Low temperature is also one of the most devastating environmental factors that affects plant growth and productivity. Occasional drops in the temperature of agricultural soils can affect the activity of terrestrial biota and plant growth. Low temperature corresponds to chilling (0–15 °C) that usually occurs in temperate regions and decreases plant productivity. These conditions stimulate the growth of saprophytic fungi that may disrupt soil nutrient cycling and compromise plant health [215]. Low temperatures disturb cellular homeostasis and some ROS, including hydrogen peroxide ($H_2O_2$), singlet oxygen ($O_2^-$), and $HO^{\cdot}$, and also disrupt some cellular functions related to proteins, lipids, carbohydrates, and DNA that may cause cell death in plants [217,227].

Several beneficial microorganisms have been reported to mitigate and alleviate the harsh impacts of abiotic stress, as indicated in Table 3. Different bacterial species, such as *Pseudomonas fragi*, *P. chloropaphis*, *P. fluorescens*, *P. proteolytica*, and *Brevibacterium frigoritolerans*, have been observed reducing freezing injuries and the content of lipid peroxides and ROS while stimulating some enzymatic activity (superoxide dismutase, catalase, peroxidase, and glutathione reductase) that could improve tolerance against cold stress in common bean seedlings [217]. Plant growth-promoting fungi such as *Trichoderma harzianum* and AMF (*Glomus mosseae*) are some of the most studied fungi in relation to improving resistance against cold stress conditions. These fungi could activate different enzymatic activity, discourage ROS production, and limit lipid peroxidation levels, which could decrease the damage caused by cold stress in tomato (*Solanum lycopersicum* L.) and *Elymus nutans* Griseb plants.

## 7. Flood Stress and Oxygen Deficit

Global agriculture is severely affected by climate change. Flooding is one of the most drastic conditions of climate extremes and has detrimental impacts on soil fertility and nutrients, causing disruption to the crucial processes of plants [237]. The intensity and frequency of flooding is increasing due to climate extremes that could be a serious threat to the stability and productivity of ecosystems [238]. Plants frequently experience stresses that are typically caused by insufficient water or a lack of oxygen in flooding conditions. Flooding leads to localized depletion of oxygen due to stagnant water and sediment deposition on the soil surface [239]. The inhibition of cellular respiration and the submersion of non-photosynthetic plant tissues or roots under flooding are some of the most serious plant stresses [240].

Plants under flood stress undergo several physiological and molecular changes that might be due to the lack of oxygen availability affecting roots. Plants demonstrate certain symptoms under oxygen deficiency, such as the closing of stomata and a reduction in the water conductivity and growth of roots. Plants develop different morphological functions to cope with oxygen/flood stress, such as increases in gas diffusion in the roots, the accumulation of lignin and suberin at the cellular level, and the promotion of aerenchyma and adventitious roots [229]. Aerenchyma are specialized tissues that transport gases ($O_2$) from aerial parts of the plant to the roots under oxygen deficit environments [240]. The aerenchyma are well developed in plants of aquatic and humid environments. Aerenchyma are developed in species of high economic importance, including plants such as sugarcane (*Saccharum* spp.), rice (*Oryza sativa*), barley (*Hordeum vulgare*), corn (*Zea mays*), wheat (*Triticum aestivum*), and soybeans (*Glycine max*) [240–244].

Plants undergo several metabolic alterations under flood stress, such as increased ethylene production and the signaling of stress hormones, which negatively interferes with plant morphology [222]. Flood stress causes anaerobic conditions that could reduce the microbial activity and enzymatic activity of plants in the rhizosphere [245]. Flood stress causes alterations in the structure of microbiota [246], which thus has consequences on the terrestrial biota and can enhance the role of bacteria and fungi in the decomposition of residues and nutrient cycling for the better performance of plants [247]. Understanding the behavior of potential soil microbiota in relation to flooding is one of the crucial discoveries that may confer stress tolerance in plants [240]. Several bacteria modulate the production of ethylene by plants through 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which is the immediate precursor for ethylene synthesis. Plant growth-promoting bacteria reduce ethylene production, which can lead to the reduction of plant damage [248], as shown by Grichko and Glick [249] who reported that the inoculation of tomato (*Lycopersicon esculentum*) seeds with different bacterial strains (*Enterobacter cloacae* UW4, *E. cloacae* CAL2, and *Pseudomonas putida* ATCC17399/pRKACC or *P. putida* ATCC17399/pRK415) produced ACC deaminase. Plants at the vegetative growth stage were exposed to flooding stress for nine consecutive days, which produced AAC, chlorophyll a and b, and adventitious roots, as well as develop stem aerenchyma of the host plants to withstand under flood

stress. Barnawal et al. [219] and Ravanbakhsh et al. [222] indicated that the inoculation of different plants with ACC deaminase-producing bacteria under flooded conditions increased plant growth by reducing ethylene production. The inoculation of *Cucumis sativus* with *Pseudomonas putida* UW4 under low available oxygen altered protein synthesis, nutritional metabolism, and antioxidant activity and promoted plant growth and defenses against stresses [220].

Beneficial microbes such as fungi prominently increase the tolerance of host plants under different environmental stresses [229]. Arbuscular mycorrhizal fungi applied to the roots of tomato plants under flooded and non-flooded conditions increased water relation and conductivity. It was also reported that indole-3-acetic acid (IAA) is one of the major phytohormones involved in the water conductivity of roots under low oxygen availability [229].

Several PGPBs and transgenic plants were studied under multiple stresses in field conditions. Farwell et al. [250] inoculated canola with *Pseudomonas putida* UW4 under nickel and flood stress and reported that *Pseudomonas putida* UW4 increased canola growth and biomass under flooding and heavy metal stresses. Cao et al. [239] indicated that flooding increased enzymatic activity in copper (Cu)-contaminated soil. In addition, the presence of Cu is inversely proportional to soil microbiota (bacteria and fungi), which could affect microbial communities and cause the immobilization of microelements under flooded and non-flooded conditions. The influence of beneficial microorganisms in improving tolerance to abiotic stresses (high and cold temperature and flooding) and regulating sustainable agricultural productivity under climatic extremes is summarized in Table 3.

## 8. Light Stress

Sunlight is one the major factors of photosynthesis that provides the necessary energy for plant growth and development. Despite this, intense light, especially its ultraviolet (UV) part, causes serious damage to DNA, proteins, and other cellular components of plants [251]. Sunlight damages photosynthetic machinery, primarily photosystem II (PSII), increases ROS production, and causes photo-inhibition that can hinder plant photosynthetic activity, growth, and productivity [252]. Excess light accelerates ROS production in PSI and PSII of chloroplasts, which may balance photo-inhibition and the repair of plant cells [252]. Light-triggered plant responses depend on the fluency, exposure time, and acclimation of plants before light exposure [251]. Reductions in the quantity and quality of light could signal plants to activate defensive systems by enhancing adaptive alterations in stem morphology [252]. The signaling pathways of light can balance the constructive and destructive impact of light on plant defense and growth mechanisms.

Microbes are less studied in the mitigation of light stress compared to other abiotic conditions. Some PGPBs have shown great potential by enhancing photosynthesis, chlorophyll content, and photosynthetic pigments that can reduce light damage [253]. The impact of light on the composition of rhizosphere communities, such as prokaryotes and fungi, can be increased or decreased under climatic extremes. There are several bacterial species, including *Pseudomonas* sp., *Massilia* sp., *Burkholderia* sp., and Acidobacteria, that are classified as beneficial microorganisms in the context of high light intensity. In addition, some fungal species, including *Geminibasidium* sp. and *Oidiodendron* sp., were also described as the most abundant species under intense light. The microorganism communities derived from soil under the influence of high light intensity are different in taxonomy and physiological characterizations. The impact of light on the soil rhizosphere includes the detection of *Pseudomonas* sp. that could consequently increase photosynthesis and carbon and nutrient assimilation [254]. Stefan et al. [255] verified that seed inoculation with *Bacillus pumilus* and *Bacillus mycoides* increased photosynthetic activity, water use efficiency, and chlorophyll content in runner bean (*Phaseolus coccineus* L.). Suzuki et al. [256] reported that *Acinetobacter calcoaceticus* could increase the chlorophyll content of lettuce (*Lactuca sativa* L.).

## 9. Conclusions

This review elaborated the importance of plant growth-promoting microorganisms (especially bacteria and fungi) that can mitigate the damage caused by environmentally induced stresses (drought, salinity, heavy metals, flooding, extreme temperatures, and intense light). This review determined the potential, prospective, and biotechnological approaches of plant growth-promoting bacteria and fungi for the alleviation of plants in response to environmental stresses. Some bacteria and fungi under abiotic stress conditions can improve physiological and biochemical processes, such as nutrient uptake, photosynthesis, source–sink relationships, metabolism and the regulation of homeostasis, osmotic potential, protein function, phytohormone production (indole-3-acetic acid and 1-aminocyclopropane-1-carboxylic acid deaminase), enzymatic activity, nutrient solubilization, and plant nutrition. Therefore, the use of plant growth-promoting bacteria (PGPBs) and fungi contributes positively to agricultural production in abiotic stress conditions.

Despite several studies demonstrating the benefits of beneficial microorganisms, there are still research gaps and restrictions on the molecular mechanisms of crops. A mechanistic understanding of the interactions of plants and microorganisms under abiotic stress should be developed to address agricultural difficulties and resolve the nutritional and production concerns that are brought by climatic extremes. Therefore, further studies involving microorganisms are recommended to enhance sustainable crop production and food security in the light of potentially unstable climatic conditions.

**Author Contributions:** Conceptualization, A.J. and M.C.M.T.F.; methodology, A.J. and C.E.d.S.O.; validation, A.J., F.S.G. and C.E.d.S.O.; formal analysis: A.J.; resources, M.C.M.T.F.; data curation, A.J., C.E.d.S.O. and P.A.L.R.; writing—original draft preparation, A.J., F.S.G. and C.E.d.S.O.; writing—review and editing, M.C.M.T.F., A.J. and F.S.G.; supervision, M.C.M.T.F.; project administration, A.J. and M.C.M.T.F.; funding acquisition, A.J. and M.C.M.T.F. All authors have read and agreed to the published version of the manuscript.

**Funding:** This review received funding from The World Academy of Science (TWAS) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the first author's doctoral fellowship (CNPq/TWAS grant number: 166331/2018-0) and the productivity research grant (award number 311308/2020-1) of the corresponding author.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors thank São Paulo State University (UNESP) for providing technology and support as well as CNPq for financial support.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Zn Fertilizer and Mycorrhizal Inoculation Effect on Bread Wheat Cultivar Grown under Water Deficit

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**Abstract:** During drought stress, many enzymes are inactivated in plants due to Zn deficiency. Zn application and arbuscular mycorrhiza fungi (AMF)–wheat symbiosis reportedly improve the tolerance of plants to drought stress. This study was done to investigate the effect of Zn and AMF on plant growth, yield attributes, relative water content (RWC), harvest index (HI), photosynthetic activity, solute accumulation, glycine betaine (GB) accumulation, antioxidant activities [(catalase (CAT) and superoxide dismutase (SOD)], and ionic attributes in a bread wheat cultivar (SST806) under drought-stress in plants grown under greenhouse conditions. Zn application and AMF inoculation, separately and combined, enhanced all plant growth parameters and yield. Root dry weight (RDW) was increased by 25, 30, and 46% for these three treatments, respectively, under drought conditions compared to the control treatment. Overall, Zn application, AMF inoculation, and their combination increased protein content, RWC, and harvest index (HI) under drought stress. However, AMF inoculation improved proline content more than Zn application under the same conditions. Regarding GB accumulation, AMF, Zn, and the combination of Zn and AMF increased GB under drought compared to well-watered conditions by 31.71, 10.36, and 70.70%, respectively. For the antioxidant defense, AMF inoculation and Zn application improved SOD and CAT activity by 58 and 56%, respectively. This study showed that Zn and/or AMF increased antioxidant levels and ionic attributes under abiotic stress.

**Keywords:** bread wheat; AMF; zinc; drought; growth parameters; osmolyte; osmoprotector; ionic attributes

**Citation:** Abdi, N.; Van Biljon, A.; Steyn, C.; Labuschagne, M. Zn Fertilizer and Mycorrhizal Inoculation Effect on Bread Wheat Cultivar Grown under Water Deficit. *Life* **2023**, *13*, 1078. https://doi.org/10.3390/life13051078

Academic Editors: Othmane Merah, Wajid Zaman and Hakim Manghwar

Received: 21 February 2023
Revised: 14 April 2023
Accepted: 20 April 2023
Published: 24 April 2023



## 1. Introduction

Abiotic stresses negatively affect crop production [1]. Water deficit is known to decrease plant growth, significantly reducing yield [2]. Less water is considered a key climatic problem that directly decreases crop production, such as cereals, globally [3]. Drought stress causes severe losses in wheat yield in different growing regions worldwide. As the largest contributor to total consumed calories by humans, wheat represents the principal dietary staple in the world [4]. Yield and its attributes are highly affected by drought in the different stages of the growing cycle of plants [5]. Drought stress decreased wheat yield by as much as 60% [6]. As a strategy for drought tolerance improvement, crops escape water deficit, especially in the climate change conditions currently being experienced [7]. Several physiological and biochemical alterations are induced by drought, causing plants to have many adaptation strategies as defensive survival mechanisms against drought stress. It was reported that different strategies could be followed to reduce food production decreases due to drought in the future [8,9]. The development of drought tolerance mechanisms in food crops is one such strategy. Plants have many options to escape drought stress effects, such as water uptake and flow in plant tissues, production of osmolytes and antioxidant activities, and photosynthesis mechanisms [10]. Moreover, plants were found to produce more osmolyte and soluble sugars and have increased antioxidant defense mechanisms (such as SOD and CAT) to combat the toxic

effects of the overproduction of reactive oxygen species (ROS) [11]. Due to the water deficit, genes encoding antioxidant enzymes were activated in tolerant genotypes. The wheat genome was known for some modification in terms of genes to control drought-stress conditions [12].

Zn fertilizer and AMF inoculation can contribute to plant survival and tolerance of water deficit conditions in many crops, such as wheat [13]. Zn application maintained nutrient balance and stomata reaction in different crops to reduce the effects of drought stress [14]. As an antioxidant reaction, SOD and CAT enzymes were enhanced due to Zn fertilization in response to water deficit.

Zn is classified as a necessary micronutrient for plant growth due to its involvement in carbon metabolism [15]. Zn plays an important role in plant nucleic acid metabolism. Several biomolecules as lipids and proteins, contain Zn as an essential component; also, it is a cofactor for many enzymes [15,16]. Many studies showed the plant responses to Zn application [17]. Physiological and biochemical processes such as plasma membrane functions and oxidative stress tolerance depend on Zn content [18].

Zn application also reduced the alteration of membrane permeability and the damage caused by oxidative and peroxidative reactions [19,20]. An adequate supply of Zn can reduce the effects of drought on different crops, such as wheat [21].

Many reports confirmed that Zn plays an important role as a strategic component for the root and shoot system and a cofactor of many enzymes [22]. Yield attributes of wheat were increased due to Zn application [23,24]. Soil Zn application increased grain yield by 29%, whole-grain Zn concentration by 95%, and whole-grain estimated Zn bioavailability by 74% [25].

Arbuscular mycorrhizal fungi (AMF) belong to the phylum Glomeromycota, are soil inhabitants, and can colonize 80% of the roots. Mycorrhizal characteristics are mutually beneficial. AMF provide the host plant with essential nutrients (especially P) and water, and photosynthates are transported into endosymbiotic AMF for its development. Mycorrhizal mycelium feeds plants with several secondary metabolites and carbohydrates. It also improves plants to fix nitrogen and increase osmotic adjustments during water deficit.

The effect of AMF colonization depends on the host-plant interaction [26]. To tolerate drought stress, for example, in wheat, symbiosis with AMF can increase plant tolerance against this stress [27,28]. Antioxidant reaction, osmotic adjustments, and root hydraulic conductivity are better regulated in AM-plant association [29]. Zn uptake by the plant increased in the presence of AMF; however, the assimilation depends on the crop–AMF symbiosis. Mycorrhizal association contributed to Zn uptake of up to 24.3% of the total aboveground Zn in wheat and up to 12% in barley. At low Zn application, the highest contribution by the mycorrhizal pathway was observed in barley. Besides this, the grain yield of bread wheat was increased by AMF [30]. The use of Zn and AMF as fertilizer is one of the most effective strategies that can reduce the effect of drought stress and improve yield and plant growth. In addition, the use of biocontrol and chemical fertilizers was increased to reduce the impact of stress factors on crops. Moreover, AMF colonization could improve the nutrient uptake of a crop such as wheat in different types of soil by enhancing the root surface absorption area [31]. In the case of soil containing heavy metals, it was reported that mycorrhizal colonization could reduce the uptake of these metals [32]. Many studies investigated the role of AMF under drought stress to improve plant nutrient uptake. The synergistic interaction of AMF and Zn could improve concentrations of different micronutrients. It was reported that AMF with extraradical mycelium in the soil improved immobile nutrient (such as P and Zn) uptake by the host plant [33], causing an increase in the exchange of photosynthesis products from the plant to the fungus.

Glycine betaine accumulation works as an osmolyte in protecting organisms against abiotic stresses via osmoregulation or osmoprotection. As an osmoregulator, GB enhances root water assimilation, reduces the damage caused by oxidative reactions, and increases drought tolerance [34]. Due to the Zn application, compatible solutes were increased under drought stress [35]. GB maintains water retention in plants owing to Zn application that

increases chlorophyll content and plant dry weight [36,37]. GB accumulation helpsplants to overcome drought and saline stress conditions. For example, in transgenic apples expressing the stress regulator gene, *Osmyb4*, accumulation of GB was linked to increased tolerance under drought and cold stress [38]. In chloroplast stroma, GB is produced by betaine aldehyde dehydrogenase (BADH). Under abiotic stress such as salinity, the enzyme choline monooxygenase (CMO) converts choline into betaine aldehyde and then an NAD+-dependent enzyme to improve tolerance against this stress [39].

To determine the effects of Zn application and/or AMF inoculation on bread wheat under drought stress, the regulation of various antioxidants, metabolites, and morphological traits was studied. It was hypothesized that Zn and AMF could improve bread wheat production under water deficit conditions.

## 2. Materials and Methods

### *2.1. Biological Materials and Growth Conditions*

Seeds of one commercial South African wheat cultivar (SST806, official standard for spring wheat quality) were planted in plastic pots containing 2 kg of soil collected from 1.5 m deep subsoil (Table 1). They were grown under glasshouse conditions at the University of Free State, Bloemfontein, South Africa, from May 2019, with day/night temperatures of 18 °C at night and 22–24 °C during the day. The relative humidity during the day and night was 78%. A soil meter (Efekto Ltd., Caledon, South Africa) was used in this study. A completely randomized block designwas replicated three times for each treatment;control (T0), Zn (T1) = 40 kg $ha^{-1}$, Arbuscular mycorrhizal fungi = AMF (T2), drought stress (T3), Zn+AM (T4), and Zn+AM+drought (T5).

**Table 1.** Soil, Zn, and AMF characteristics used in the trial.

| Soil | | AMF Characteristics and Zinc Application |
|---|---|---|
| pH | 6.8 | - 150 g per 150 kg of seed was applied |
| Sand | 50% | |
| Silt | 10% | - It is a commercial inoculum in powder form, registered and produced by Biocult (Pty) Ltd. 005333/07, Somerset West, South Africa |
| Clay | 40% | |
| Phosphorus (P) | 7.5 mg $kg^{-1}$ | - Active ingredient was mycorrhizae subspecies, 400 spores per gram (as indicated by the manufacturer. |
| Potassium(K) | 231.4 mg $kg^{-1}$ | |
| Calcium(Ca) | 564 mg $kg^{-1}$ | - The subspecies included *Glomus mosseae, Glomus intraradices, Glomus etunicatum,* and *Scutellospora dipurpurescens*. |
| Magnesium (Mg) | 147.6 mg $kg^{-1}$ | - Zn was applied at sowing at a depth of 5 cm (40 kg $ha^{-1}$) |

### *2.2. Growing Conditions*

Drought stress was applied at the three-leaf stage. When soil water content reached 25% field capacity, plants were allowed to receive water again; however, the well-watered conditions represented 100% field capacity. Before rewatering plants, a soil meter was used to measure soil water content.

### *2.3. Plant Biomass*

Different plant samples (roots, shoots, and seeds) were dried until they attained a constant weight following the method previously described [40]. Plants were separated at 80 days after sowing (DAS) in root and shoots for various physiological and biochemical analyses.

### *2.4. Chlorophyll Content*

Chlorophyll extraction was carried out from leaf discs of plants following the method previously described [41], and chlorophyll a, b, and total chlorophyll were computed from the extinction values following the equation of [42].

### *2.5. Total Protein, Relative Water Content, and Harvest Index*

Total protein was estimated following the method previously described by Bates et al. [43]. Leaf relative water content (RWC) was calculated by the method described by Grieve & Grattan [44]. For chlorophyll a and b extraction, leaf discs of plants were mixed with 5 mL of 80% acetone overnight. After centrifugation, the supernatant was used for absorbance reading at 645 nm (chl a) and 663 nm (chl b) using a spectrophotometer (Hitachi-U2001, Tokyo, Japan). Relative water content (RWC) was measured following Cavell [45], where selected leaves were rehydrated by soaking in deionized water for 24 h. Fully turgid leaves were weighed and, subsequently, oven-dried for 48 h at 80 °C. Here, FW is fresh weight, DW is dry weight, and TW is turgid weight. Plant yield efficiency in terms of the harvest index (HI) was computed according to Mehraban et al. [46]. The amount of aboveground biomass production invested into harvestable organs was calculated as follows:

$$\text{HI} = (\text{Seed dry weight}/\text{Aboveground plant biomass at harvest}) \times 100$$

### *2.6. Proline and Glycine Betaine Content*

Proline content was analyzed following absorbance of toluene soluble brick-red colored complex at 520 nm [47]. The concentration of proline was estimated by referring to a standard curve drawn from known concentrations of proline. GB was determined following the absorbance of the betaine−peridotite complex with iodide in an acidic medium at 360 nm as per the method of Dubois et al. [48]. Reference standards of GB were prepared as 50–200 μg $mL^{-1}$ for sample estimation.

### *2.7. Catalase and Superoxide Dismutase Estimation*

CAT and SOD were measured using 0.2 g fresh leaf samples. The obtained mixture (0.05 M Tris–HCl buffer (pH = 7.5) and samples) was centrifuged at 13,000 rpm for 20 min at 4 °C.

After centrifugation, the supernatant was used to estimate CAT according to a modified method of Kar and Mishra [49], and SOD was assayed by the method described by Beauchamp and Fridovich [50].

### *2.8. Nutrient Analysis and Zinc Content*

Nutrient extraction was done according to Carvalho et al. ([51], modified). Two g of flour for each sample was placed in labeled crucibles and ashed for 3 h in a furnace at 550 °C. Samples were digested with 2–2.5 mL of concentrated $HNO_3$,then placed into the furnace at 550 °C for 1h. After that, 10 mL of diluted $HNO_3$ ($HNO_3$:$H_2O$ 1:2 dilution ratio) was added to the sample and placed for 5 min in a sand bath. The mixture was filtered through Whatman paper for purification. The atomic absorption spectroscopy (AAS) (Varian AAS FS 240 Model, Varian Inc., Palo Alto, CA, USA) method was used to analyze the mineral concentration. Five replicates were done per sample.

### *2.9. Statistical Analysis*

Each parameter was investigated in its separate independent experiment. Analysis using variance (ANOVA) was performed, and subsequent comparison of the means was done using Duncan's multiple range test at $p = 0.05$. Treatment mean ± SE ($n = 12$) are for growth and yield attributes and ($n = 4$) for the other tested characteristics.

## 3. Results

### *3.1. Plant Growth, Yield, and Yield-Related Traits*

Drought stress significantly affected ($p < 0.05$) growth parameters, yield, and yield components (Table 2). Zn application and AMF inoculation significantly enhanced plant growth and yield components under well-watered conditions and drought stress. Zn application and/or AMF inoculation enhanced all growth parameters and yield attributes. For example, RDW increased by 25, 30, and 46%, respectively, for these three treatments,

compared to the control treatment. For 1000-grain weight, the increase was 9, 0.4, and 3% for the same three treatments (Table 2). Drought stress significantly decreased plant growth and grain yield attributes. The combination of Zn application and AMF inoculation alleviated the adverse effect of drought stress on all parameters except for grain weight per spike, which decreased by 45.9%. The decrease in 1000-grain weight was noticeably smaller after the application of Zn and AMF compared to drought stress only (Table 2). Under this constraint, Zn significantly enhanced 1000-grain weight. However, AMF or Zn did not affect grain number and grain weight per spike under drought stress (Table 2).

**Table 2.** Application effect of Zinc fertilizer and mycorrhizal inoculation on yield attributes of bread wheat.

| | SDW (g) | SL (cm) | RDW (g) | RL (cm) | Spike Number per Plant | Grain Number per Spike | Grain Weight per Spike | 1000 Grain Weight (g) |
|---|---|---|---|---|---|---|---|---|
| **Control** | 0.30 ±0.01 $^{cd}$ | 36.48 ±1.05 $^{b}$ | 0.29 ±2.13 $^{c}$ | 30.65 ±0.35 $^{e}$ | 1.75 ± 0.03 $^{b}$ | 35.43 ±0.76 $^{b}$ | 1.78 ±0.08 $^{a}$ | 46.19 ±0.32 $^{a}$ |
| **Zn** | 0.43 ± 0.20 $^{b}$ | 38.09 ± 0.54 $^{ab}$ | 0.39 ± 0.09 $^{bc}$ | 32.48 ± 0.08 $^{d}$ | 1.50 ± 1.25 $^{bc}$ | 34.52 ± 0.01 $^{c}$ | 1.59 ± 0.54 $^{b}$ | 51.07 ± 1.65 $^{b}$ |
| **AMF** | 0.48 ± 0.20 $^{ab}$ | 39.64 ± 0.32 $^{ab}$ | 0.42 ± 0.50 $^{b}$ | 38.52 ± 1.76 $^{b}$ | 1.75 ± 0.87 $^{b}$ | 35.19 ± 0.90 $^{b}$ | 0.34 ± 0.01 $^{d}$ | 46.38 ± 1.20 $^{b}$ |
| **AMF+Zn** | 0.54 ± 0.45 $^{a}$ | 42.71 ± 0.75 $^{a}$ | 0.54 ± 1.65 $^{a}$ | 42.06 ± 2.00 $^{a}$ | 2.00 ± 0.09 $^{a}$ | 36.10 ± 0.87 $^{b}$ | 1.77 ± 0.70 $^{a}$ | 47.68 ± 2.87 $^{ab}$ |
| **Drought** | 0.26 ± 0.43 $^{d}$ | 31.99 ± 1.43 $^{c}$ | 0.26 ± 0.07 $^{c}$ | 29.05 ± 0.98 $^{f}$ | 1.00 ± 0.06 $^{c}$ | 38.83 ± 0.39 $^{a}$ | 1.83 ± 0.90 $^{a}$ | 33.86 ± 1.33 $^{c}$ |
| **Zn+Drought** | 0.28 ± 0.97 | 32.15 ± 0.98 | 0.28 ± 0.01 | 29.99 ± 1.09 | 1.23 ± 0.09 | 36.90 ± 0.01 | 1.85 ± 0.99 | 44.65 ± 0.13 |
| **AMF+Drougt** | 0.37 ± 1.45 | 35.09 ± 0.09 | 0.33 ± 0.12 | 35.08 ± 0.23 | 1.44 ± 1.34 | 37.21 ± 1.23 | 0.98 ± 1.06 | 43.17 ± 0.05 |
| **AMF+Zn+Drought** | 0.39 ± 0.04 $^{c}$ | 33.10 ± 0.3 $^{c}$ | 0.37 ± 2.54 $^{bc}$ | 36.95 ± 1.00 $^{c}$ | 1.75 ± 0.12 $^{b}$ | 38.78 ± 0.56 $^{a}$ | 0.99 ± 1.03 $^{c}$ | 40.62 ± 1.05 $^{bc}$ |

Grown under water-stress conditions, SDW = Shoot dry weight, SL = shoot length, RDW = root dry weight, RL = root length. Values in columns followed by different letters are significantly different at $p \leq 0.05$. Means ± standard deviation.

### 3.2. Chlorophyll Content

There was significant variability of chlorophyll content due to Zn fertilization and AMF inoculation. Chlorophyll compounds were increased by Zn and AMF inoculation and their combination under both control and drought conditions. Chl a content increased by 69, 68, and 75%, Chl b content by 84, 87, and 90%, and Chl a+b content by 73, 74, and 80%, respectively, after the application of Zn and AMF inoculation and their combination compared with the control. However, there were nonsignificant effects on Chl a/b content under drought stress after Zn and AMF treatments. Overall, the highest chlorophyll content was observed in the plants treated with combined Zn and AMF under both control and drought-stress conditions (Figure 1).

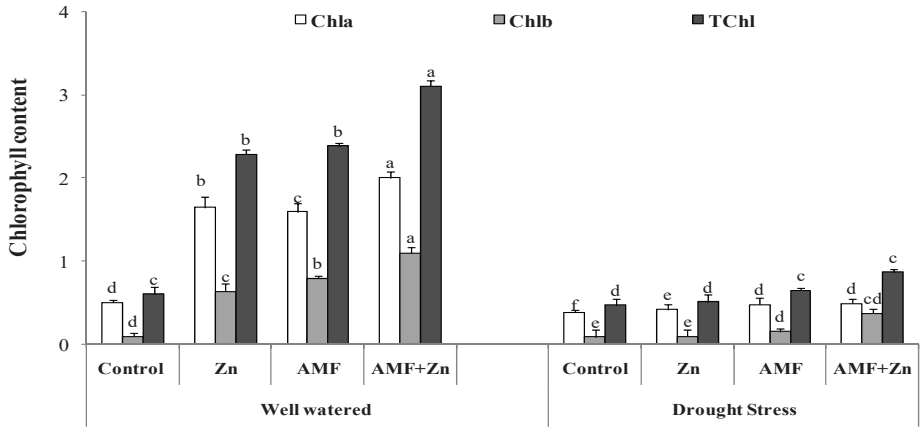


**Figure 1.** Individual and combined application of Zn and/or AMF effects on chlorophyll a, chlorophyll b, and chlorophyll (a+b) of a bread wheat cultivar under control (well-watered) and drought-stress conditions. Bars with different letters are significantly different at $p \leq 0.05$.

### 3.3. Protein, Relative Water Content, and Harvest Index

Protein, relative water content, and HI were significantly($p \leq 0.05$) affected by drought stress. However, AMF inoculation and/or Zn and their combination improved protein

content by about 15%. The highest level was 15.35% in plants that received combined Zn and AMF treatment under drought stress (Figure 2). Under well-watered conditions, Zn application, AMF inoculation, and their combination enhanced RWC by 14.10, 16.23, and 23.90%, respectively (Figure 2), although it decreased by 20.35, 20.15 and 21.66%, respectively, under drought stress. Under drought stress, Zn application and/or AMF inoculation enhanced HI by 45.91, 84.80 and 28.82%, respectively, compared to control conditions (Figure 2).

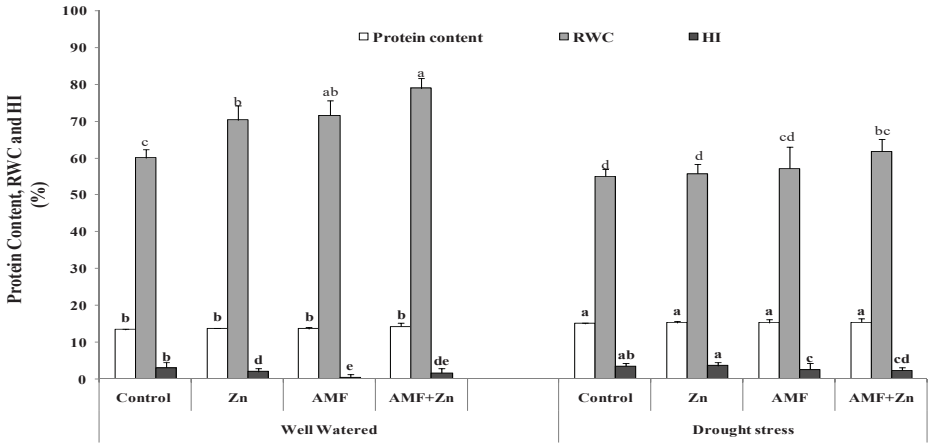


**Figure 2.** Zn application and/or AMF inoculation effect on protein content, relative water content (RWC), and harvest index (HI) of bread wheat cultivar under control (well-watered) and drought-stress conditions. Bars with different letters are significantly different at ($p < 0.05$).

### *3.4. Accumulation of Glycine Betaine and Proline Content under Drought Stress*

Treatment effects were significant for GB and proline. Under drought stress, the application of Zn and/or AMF inoculation increased GB compared to control conditions by 31.71, 10.36, and 70.70%, respectively. However, the level of GB was higher in the control under the same conditions (1.69 µmol $g^{-1}$). Regarding proline content, results showed significant variability ($p < 0.05$) under both control and drought conditions. AMF inoculation improved proline content more than Zn application. Generally, drought stress decreased proline content compared to control conditions (Figure 3).

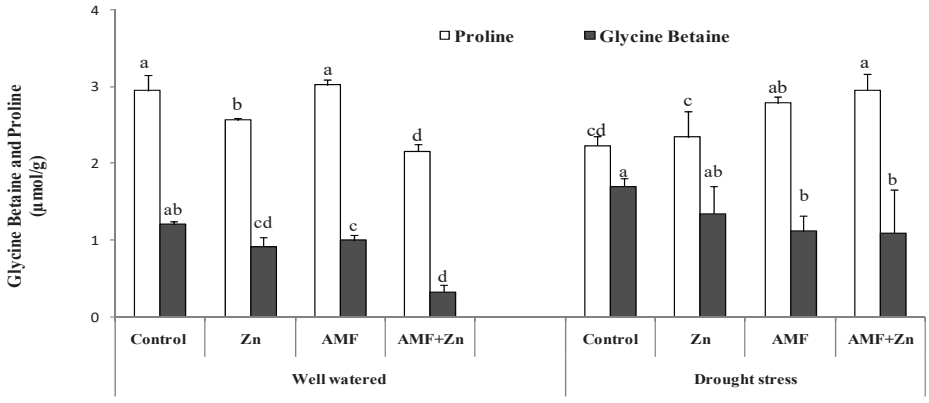


**Figure 3.** Zn application and/or AMF inoculation effect on glycine betaine and proline content in the bread wheat under control (well-watered) and drought-stress conditions. Bars with different letters are significantly different at $p \leq 0.05$.

### *3.5. Activities of Antioxidant Enzymes*

The antioxidant defense was enhanced significantly ($p < 0.05$) under drought stress mostly for peroxide dismutase activity, and the increase was outworn by 50% for all the treatments compared to the well-watered conditions. AMF inoculation and Zn application

improved SOD and CAT activity by 58 and 56%, respectively, under drought stress (Figure 4). Under well-watered conditions, Zn and/or AMF did not significantly a meliorate the enzymatic reaction (Figure 4).

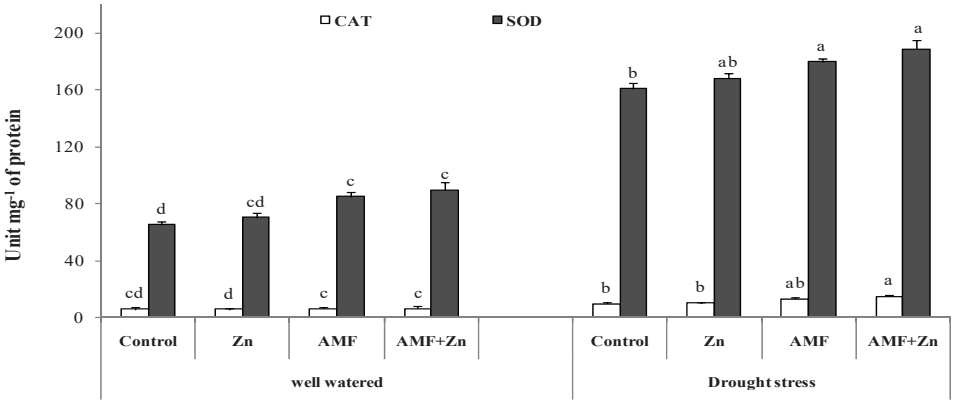


**Figure 4.** Zn application and/or AMF inoculation effects on catalase (CAT) and peroxide dismutase (SOD) in the bread wheat under control (well-watered) and drought-stress conditions. Bars with different letters are significantly different at $p \leq 0.05$.

### *3.6. Nutrient Composition of Wheat Flour*

Macro and/or microelements in wheat flour showed significant variability due to the combination of Zn application and AMF inoculation under drought stress (Figure 5). However, treatment effects were nonsignificant under control conditions for micronutrients. Drought stress significantly increased Na and Cu, compared to the control, by 21.68 and 36.13%, respectively. The microelements Fe, Mn, Zn, and Cu in the flour had very low concentrations (0.003–0.089%). On the contrary, macro elements were significantly affected by drought stress. Zn and/or AMF inoculation improved K, Ca, and P. For example, Zn combined with AMF increased K and P by 51.61 and 75%, respectively, under drought stress (Figure 5).

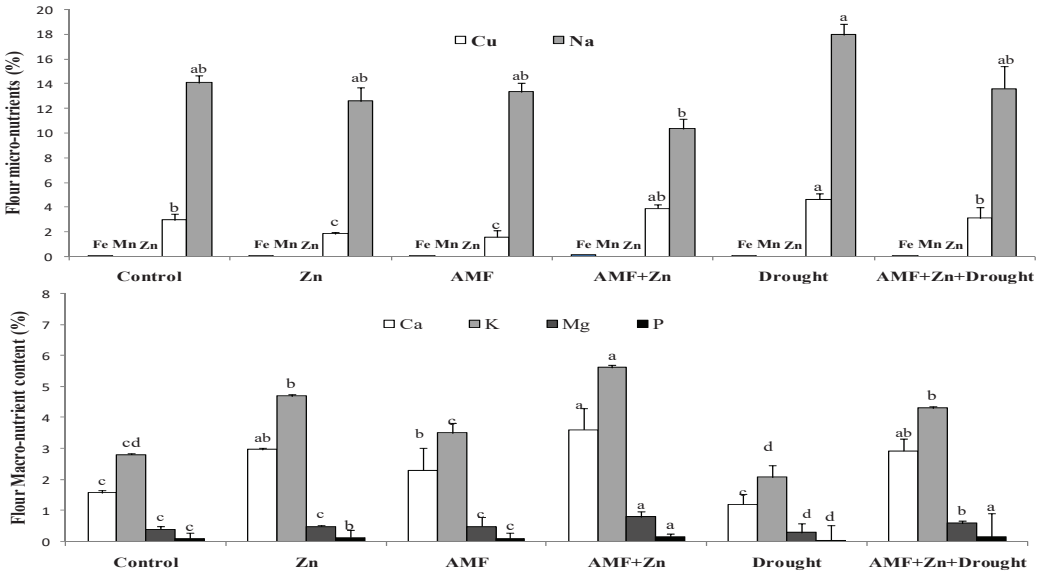


**Figure 5.** Zn application and/or AMF inoculation effect on macronutrient and micronutrient content in bread wheat cultivar under control (well-watered) and drought-stress conditions. Bars with different letters are significantly different at $p \leq 0.05$.

## 4. Discussion

The main objective of this study was to analyze the potential of Zn fertilizer and AMF for improving wheat performance under drought stress. Drought significantly affects wheat yield worldwide [52,53]. AMF improved water assimilation in many plants under drought stress as fungus mycelia can penetrate the soil and increase water absorption and transportation from roots to other plant parts as a tolerance mechanism to drought stress [54]. Fertilization using several nutrient sources increased plant vigor against environmental stress [55]. Zn fertilization and its co-application with AMF were evaluated by studying variability in different physiochemical mechanisms as described in a previous study [30]. AM fungus and/or Zn application positively affected morphological traits, increasing plant growth and yield attributes, as was reported previously [3]. Therefore, the efficiency of Zn and AMF application is confirmed in this study. Drought stress decreased plant dry weight and length. This was confirmed in another study [56]. Osmotic variability due to variations in osmotic potential caused a significant decrease in the fresh weight of plants due to a decrease in cellular division, consequently causing a decrease in total plant weight [57]. Zn combined with AMF treatment effectively improved plant growth under drought stress by sustaining higher water content in cells, thus ameliorating drought stress. All parameters were alleviated by Zn application and AMF inoculation, except for grain weight per spike, which decreased by 45.9% under drought stress. Zn improved chlorophyll synthesis, as it acts as a catalyst and cofactor of various enzymes [58]. This finding was confirmed in this study. Cell membranes, which cause improvement in the photosynthetic process, were protected by the application of Zn and AMF [59]. Similar findings were observed in rice and wheat plants. Zn increased all studied photosynthetic pigments [60].

Protein content was significantly enhanced only under stress conditions, and the effect was increased with Zn treatment. The potential effect of Zn on soluble protein in wheat under drought stress was previously reported [61]. Also, amino acid synthesis, which helps in protecting plants from drought stress, is related to Zn application [62]. Faced with drought stress, plant tolerance can be improved via drought escape by early flowering time in drier environments, avoidance by transpiration regulation, development of extensive root systems, trait flexibility, maintenance of water management in tissues, antioxidant scavenging, and secretion of plant growth substances by plant growth regulators and osmotic regulation [63]. Under drought stress, plants used stomatal closure to reduce the transpiration rate, causing an increase in leaf temperature. However, compared to the control, under the same conditions, Zn and/or AMF increased RWC and HI. These findings confirmed that Zn, at an optimum dose, maintained water status, stomatal conductance, and osmotic adjustment in many plants, such as chickpea, under drought stress [64]. For osmotic homeostasis regulation under stress conditions, proline as an osmolyte played an important role in protecting plants against drought [65]. The compatible solute accumulation leads to improved turgor potential and water content of plants, which contributes to enhanced plant growth performance under stress conditions. AMF was also reported to stimulate compatible solute and protein content under stress conditions [66]. The results of this study confirmed previous findings [67], mentioning that Zn and AMF acted synergistically to enhance proline and total protein content.

Drought stress reduces the assimilation of nutrients and inhibits the activities of important enzymes that are involved in the synthetic processes of energy for plant growth. For that, plants have an antioxidant defense against stress conditions, having different antioxidant reactions protecting plants under water deficit [68].This defense reaction was expressed by different enzymes which convert these harmful oxygen species to reduce their negative effect on plant growth [69]. In our study, drought stress increased levels of CAT and SOD compared to the control (well-watered). AMF inoculation or Zn application enhanced the activity of these antioxidant enzymes under drought-stress conditions, being more pronounced when applied together. This finding was confirmed by many reports mentioning enhancement in the enzymatic antioxidant defense system due to AMF and/or

Zn application in wheat under drought stress [70]. Zn reduced oxidative damage under stress conditions, which confers stress tolerance to plants [71]. The increase in antioxidant enzyme activity is assessed through decreased malondialdehyde content and $H_2O_2$ content as noted in many crops, for example, in sunflower (*Helianthus annuus*), chickpea (*Cicer arietinum*) [72], lentil (*Lens culinaris*) [73], and wheat leaves.

Moreover, nonenzymatic molecules, such as the accumulation of GB in wheat leaves, decreased the impact of drought stress as an antioxidant defense. Zn and AMF increased the activity of GB under drought stress, being more pronounced when applied together (Figure 3). It was reported [74] that enzymatic antioxidant defense systems were enhanced in wheat due to Zn application under drought-stress conditions.

In addition, as a strategy to tolerate stress, plants balance the concentrations of macro and micro elements. The results showed that drought stress significantly increased Na and Cu. However, Fe, Mn, Zn, and Cu were present in the flour at very low concentrations (0.003–0.089%). Many reports confirmed this finding explaining that a different nutrient supply as Zn and biofertilizer (AMF) can increase plant growth under water stress, depending on the severity of the drought, the concentration of the elements in the soil, and other conditions [75]. Application of Zn, AMF inoculation, and their combination increased K, Ca, Mg, and P. For example, Zn combined with AMF increased K and P by 51.61 and 75%, respectively, under drought stress.

## 5. Conclusions

This study investigated the effect of Zn application and AM fertilization. Wheat growth, yield, the antioxidant mechanism (enzymes, osmoprotectors, and osmolytes), and nutrient balance were improved. Root proliferation was significantly enhanced due to Zn and AMF fertilization under stress conditions. Zn fertilizer combined with AMF had larger impacts on measured traits. As a work perspective, deep research is needed to be done under field conditions to confirm these results on the effects of Zn and AMF and to be recommended to improve wheat production under drought stress. Moreover, extensive work on molecular studies as the contribution of differentially expressing endogenous genes encoding antioxidant enzymes should be established.

**Author Contributions:** N.A.—writing manuscript, methodology, data analysis; A.V.B. methodology and assistance; C.S.—methodology; M.L.—data analysis, writing-review, visualization, and editing. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was financially supported by the National Research Foundation through the South African Research Chairs Initiative (UID 84647).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy and ethical reasons.

**Acknowledgments:** This research was funded by the National Research Foundation through the South African Research Chairs Initiative.

**Conflicts of Interest:** The authors declare no conflict of interest.

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*Review*

# Breeding and Genomic Approaches towards Development of Fusarium Wilt Resistance in Chickpea

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**Abstract:** Chickpea is an important leguminous crop with potential to provide dietary proteins to both humans and animals. It also ameliorates soil nitrogen through biological nitrogen fixation. The crop is affected by an array of biotic and abiotic factors. Among different biotic stresses, a major fungal disease called Fusarium wilt, caused by *Fusarium oxysporum* f. sp. ciceris (*FOC*), is responsible for low productivity in chickpea. To date, eight pathogenic races of *FOC* (race 0, 1A, and 1B/C, 2-6) have been reported worldwide. The development of resistant cultivars using different conventional breeding methods is very time consuming and depends upon the environment. Modern technologies can improve conventional methods to solve these major constraints. Understanding the molecular response of chickpea to Fusarium wilt can help to provide effective management strategies. The identification of molecular markers closely linked to genes/QTLs has provided great potential for chickpea improvement programs. Moreover, omics approaches, including transcriptomics, metabolomics, and proteomics give scientists a vast viewpoint of functional genomics. In this review, we will discuss the integration of all available strategies and provide comprehensive knowledge about chickpea plant defense against Fusarium wilt.

**Keywords:** Fusarium wilt; conventional breeding; molecular makers; QTLs; genomics; transcriptomics; metabolomics and proteomics

**Citation:** Yadav, R.K.; Tripathi, M.K.; Tiwari, S.; Tripathi, N.; Asati, R.; Patel, V.; Sikarwar, R.S.; Payasi, D.K. Breeding and Genomic Approaches towards Development of Fusarium Wilt Resistance in Chickpea. *Life* **2023**, *13*, 988. https://doi.org/10.3390/life13040988

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 7 March 2023
Revised: 27 March 2023
Accepted: 29 March 2023
Published: 11 April 2023



## 1. Introduction

Chickpea (*Cicer arietinum* L.) is a self-pollinating, annual diploid (2n = 2x = 16) species with a genome size of 738 Mb [1]. It is also referred to as gram, Bengal gram, Egyptian pea, garbanzo, or garbanzo bean [2]. It encourages biological nitrogen fixation, which boosts soil fertility. The family Fabaceae (Leguminosae), subfamily Faboideae (Papilionaceae), and tribe Cicereae make up the taxonomic hierarchy of chickpeas. There are nine annual species and roughly 34 perennial wild species [3]. The only annual species that is grown commercially is *Cicer arietinum* [4,5].

There are two varieties of grown chickpea: Kabuli and Desi. The Desi (microsperma) varieties of plant contain thick seed coats, pink blooms, and stems that are anthocyanin-pigmented [6], while the Kabuli (macrosperma) varieties of plant have white blooms, white- or beige- colored seeds with a ram's head shape, a smooth seed surface with a thin seed coat and an absence of anthocyanin coloration on the stem [5]. Every year, more than 2.3 million tons of chickpeas are imported to supplement the needs of many nations of the world that are unable to produce a large enough quantity to satisfy their domestic demand [7]. The top exporters are Australia, Argentina, and Canada. The Kabuli variety of chickpea is grown extensively in West Asia, North Africa, North America, and Europe [7].

Chickpea seeds are nutrient-dense foods that have a high protein content and include dietary elements such as calcium, iron, and phosphorus [8]. The seeds include modest amounts of thiamin, vitamin $B_6$, magnesium, and zinc, as well. They are beneficial in the management of various serious human diseases such as diabetes, cardiovascular disease, and digestive disorders [9,10]. Excluding sulfur-containing amino acids, chickpea seeds contain several important amino acids. On the surface, chickpea grains contain: 17.1% protein, 60.9% carbs, 5.3% fats, 3% minerals, and 3.9% crude fiber [11]. The measurement of free proline levels is a helpful indicator for assessing plant physiological condition and stress [12]. Despite having just trace levels of lipids, chickpea contains unsaturated fatty acids such as linoleic and oleic acids [13]. Essential sterols, viz., stigmasterol, campesterol, and sitosterol, are also found in chickpea oil [14]. Despite these benefits, numerous biotic factors, such as Fusarium wilt and Ascochyta blight diseases and the insect pest known as the pod borer, along with abiotic challenges, such as drought, salinity, and heat, have a significant influence on yields of chickpea [15]. By alleviating these challenges, chickpea productivity can be increased. While efforts have been made using an array of conventional methods [16–18], there is significant potential for advancement when they are combined with molecular methods, such as genomics-assisted breeding [19,20]. Chickpea breeding aims to increase production by pyramiding genes for drought, cold, salinity, fungal, and pod borer resistance / tolerance into superior chickpea genotypes [21].

Since chickpeas are self-pollinated, the target feature, i.e., wilt resistance, may be easily incorporated in the desired genotype after successful introgression [22]. Backcross, recombination breeding, and other traditional approaches are equally effective in developing cultivars with wilt resistance [23]. Several Fusarium wilt (FW) resistant donors and cultivars have been identified and released in chickpea as a result of straightforward field screening and selection under wilt-diseased plots [24]. Numerous crosses may be generated to develop segregating populations, which is a crucial prerequisite for undertaking a successful crop improvement program [25]. However, the mapping of populations in chickpea for the purpose of identifying targeted genes and constructing linkage maps is challenging due to the requirement of large numbers of plants in the mapping population [26,27]. To overcome these challenges, researchers are using advanced breeding technologies to identify targeted genes and the mechanisms of their interaction with each other or with environmental conditions [28]. The combination of modern approaches with traditional breeding technology is useful in the analysis of the mechanism of Fusarium wilt resistance, as well. The prime goal of traditional breeding in legumes is to increase yield.

As a result, modern breeding techniques can be employed to enhance crop yields [29]. However, this notion has begun to change in the last decade due to improved novel techniques and the associated decreasing cost [24]. As a result of the crop's economic importance, research on chickpea genomics has recently surged, and a wealth of genomic materials, including molecular markers and linkage maps, ESTs, and NGS-based transcriptomes, have become readily available [28].

Among advanced technologies, marker-assisted selection (MAS) has helped in targeting desirable genes [30]. Markers have demonstrated their role in enhancing selection efficiency and creating novel cultivars [31,32]. Recently, the integration of several "omics" methods has been developed into effective solutions for plant systems with the development of superior cultivars [33,34]. In order to address a variety of biological concerns, second-generation sequencing [35–37] is currently extensively employed. The genetic resources for chickpeas have, however, significantly enhanced in recent years with the applications of next-generation sequencing initiatives and their application in genomics research [38–40]. The current review aims to summarize all the advancements made, obstacles encountered thus far, and prospects for future advancements in chickpea Fusarium wilt resistance.

## 2. Fusarium Wilt

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. ciceri, is important due to its severe effects on the yield of chickpea [41,42]. It is most common in hot, dry regions and can result in annual output losses of up to 10% to 15%, with epidemics leading to yield losses of up to 100% [43,44]. According to Verma et al. [23], it has eight different types of pathogenic races and pathotypes, which may be a reason for its pathogenic diversity. Based on their ability to produce unusual symptoms, the races are categorized. Major plant symptoms associated with Fusarium wilt disease infection (Figure 1) include yellowing and wilting [45]. The ability of the races to evoke separate reactions that result in two different sorts of symptoms—yellowing and wilting—sets them apart from one another. More dangerous than yellowing syndrome is with erring syndrome [46].

**Figure 1.** Fusarium wilt-infected chickpea plants.

In six continents, 32 countries are affected by chickpea wilt [47]. Butler originally described this disease in India in 1918, but it was not until Padwick accurately identified its cause in 1940 that it was fully understood [48]. Different levels of yield losses have been documented in chickpea due to FW (40% [49] and 77–94% [50]). In the case of "late wilt", dropping petioles and leaf yellowing symptoms appear during the podding stage, resulting in yield losses of 24–65 percent. The yellowing pathotype of *F. oxysporum* f. sp. ciceris causes a disease condition in chickpeas that is comparable to that of *F. redolens* (*FOC*). Because it is challenging to distinguish between *Fusarium redolens* and *F. oxysporum* using morphology-based diagnosis, and because the two species affect chickpea in ways that are similar, the use of molecular techniques may be required in the efficient identification of the Fusarium pathotype in chickpea [50,51].

The amount of yield loss due to wilt disease in chickpea depends on the agro-climatic conditions of the region. Sometimes, the wilt disease becomes more dangerous, resulting in severe damage (Figure 2) and yield failure [52]. Fusarium wilt is a disease that spreads through the soil. It has an array of mechanisms of transmission, such as through contaminated plant wastes (leaf, root, and stem), soil and seeds, macroconidia, mycelium, and most frequently, chlamydospores [50,53].

The Indian subcontinent and areas where crops are cultivated in the spring and more regularly manifest under warm, dry growing circumstances are more troubled by Fusarium wilt [27]. Fungicidal seed coats provide protection against infection transmitted by seeds, but because the pathogen is persistent in soil, the best way to eradicate the infection is through host resistance. The pathogen gains access to the vascular bundles of the chickpea plants and blocks or lowers water intake to the foliage. The infected plants eventually wilt and die [28]. The causes include a buildup of fungus mycelium in the xylem and/or the production of toxins, host defense mechanisms such as the production of gels, gums, and tyloses, and vessel crushing brought on by the expansion of nearby parenchyma cells [54].

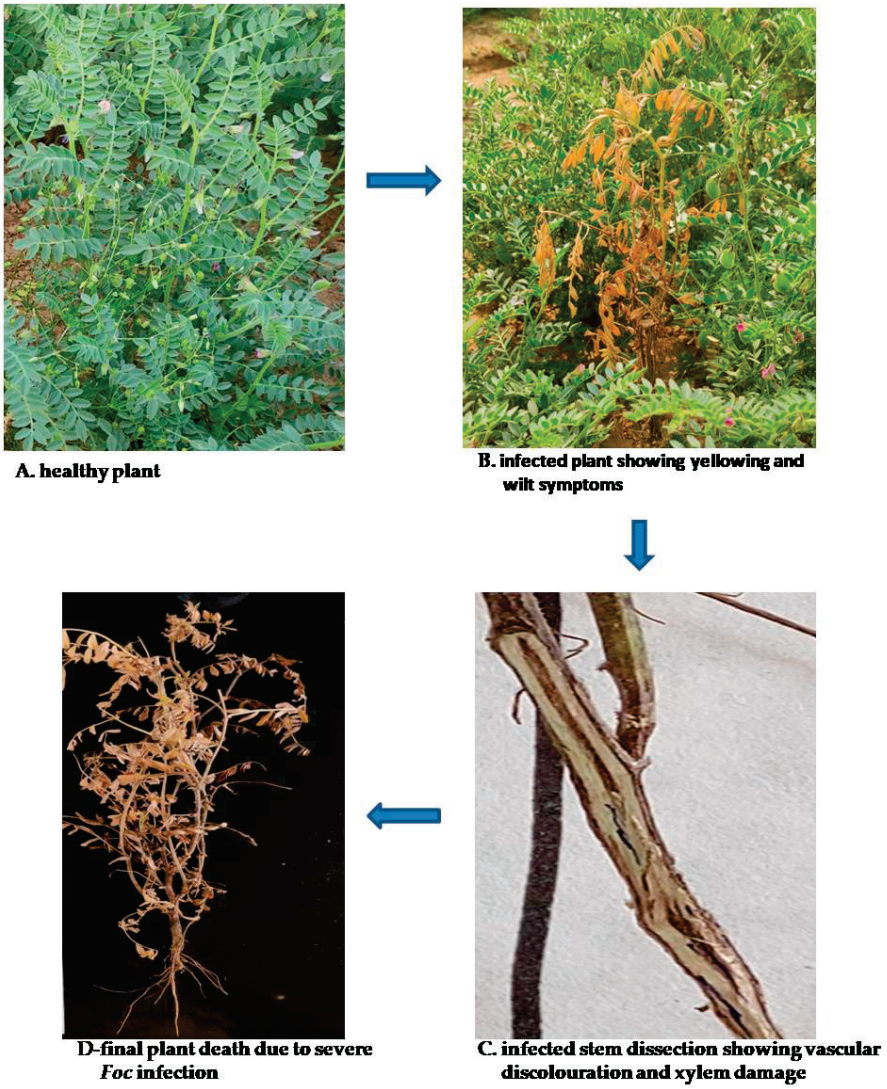


**Figure 2.** Sequence of Fusarium wilt infection in chickpea plants.

## 3. Genetics of Resistance to Fusarium Wilt

The *Fusarium oxysporum* f. sp. ciceris (*FOC*) pathogenies known to possess great pathogenic diversity that is classified into different pathogenic races, including races 0 and 1A, 1B/C, 2, 3, 4, 5, and 6. Additionally, two categories of FW symptoms have been identified: early yellowing and late wilting [55,56]. Additionally, researchers have also looked at the genetics of races 1A, 2, 3, 4, and 5 [57]. The symptomatic wilting pathotype induces quick and severe chlorosis, flaccidity, vascular discoloration, and early plant death, mostly in races 1A, 2, 3, 4, 5, and 6 [55], whereas the symptomatic yellowing pathotype instigates slow foliar yellowing, vascular discoloration, and late plant death in races 0 and1B/C [56,57].

It has been documented that chickpea resistance to Fusarium wilt can be either monogenic or oligogenic (Table 1) depending on the source or race of the resistance [57]. Three distinct genes ($h_1$, $h_2$, and $H_3$) independently govern resistance to race 1A, according to early investigations on *FOC* [58]. Late wilting resistance can be conferred by any one of these three genes, but total resistance can be conferred by any two of these genes ($h_1h_2$, $h_1H_3$, or $h_2H_3$) [59]. While resistance to race 3 has been proven to be monogenic, resistance to race 2 is controlled by a single recessive gene [60,61]. As stated in earlier studies, race 4 resistance is recessive and digenic, but race 5 resistance is governed by a single gene [62].

Geographical classifications of the pathogenic races of *FOC* have been made. Indian, Mediterranean, and American populations of race 1A have been documented [63]. In addition, race 4 has been documented in Ethiopia, India, and Iraq [64,65]. Races 0,1B/C,5, and 6 are most common in the Mediterranean Basin and California (USA) [66], while races 2 and 3 have been observed in Ethiopia, India, and Turkey [50].

**Table 1.** Genetics of resistance to races of the chickpea wilt *Fusarium oxysporum* f. sp. ciceris.

| Fusarium Race | Name of Resistance Gene | Number and Nature of Wilt Resistance Gene | Effect of Resistance Gene on Wilting | Symptoms | References |
|---|---|---|---|---|---|
| 0 | *FOC-01/FOC-01*<br>*FOC-02/FOC-02* | Monogenic or digenic | Complete resistance | Yellowing | [26] |
| 1A | *h1 (syn FOC-1)*<br>*h2*<br>*H3* | Trigenic | Late wilting<br>Late wilting<br>Late wilting | Wilting | [57] |
| 1B/C | - | - | - | Yellowing | [63] |
| 2 | *FOC-2* | Monogenic | Complete resistance | Wilting | [27] |
| 3 | *FOC-3/FOC-3* | Monogenic | Complete resistance | Wilting | [62] |
| 4 | *FOC-4* | Monogenic recessive | Complete resistance | Wilting | [27] |
| 5 | *FOC-5/FOC-5* | Monogenic | Complete resistance | Wilting | [67] |
| 6 | - | - | - | Wilting | [63] |

## 4. Breeding Methods Employed for Fusarium Wilt Resistance in Chickpea

Higher and more consistent yields are the main objectives of chickpea breeding programs [15]. According to an investigation conducted by Srivastava et al. [68], chickpea resistance to Fusarium wilt may be either monogenic or oligogenic, depending on the resistance source or race. The selection of plants for characteristics and disease resistance is the second most important step in a breeding program involving evaluation of the plant for commercial production.

Breeding programs are dependent upon the magnitude of genetic variation present in the population. The type and degree of diversity influence a breeding strategy's efficacy. Even though the disease is soil-borne, chemical control is ineffective and impractical to use [69]. Utilizing host plant resistance is the most reliable strategy for solving the problem. Several sources of chickpea resistance to Fusarium wilt have been found in the past. These resistance sources have been identified using different methods, including a wilt-diseased plot in the field and hot spot location screening, as well as greenhouse and laboratory procedures [70–72]. The majority of these methods were employed in resistance breeding programs at the National Agricultural Research System (NARS) and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), which significantly increased chickpea productivity in semi-arid parts of Africa and Asia [73,74]. However, in these areas, substantial genetic diversity in the pathogen and GxE interaction have an impact on resistance durability. A variety of strategies, including the GGE billet technique, have been utilized in different studies to investigate the GxE interaction [75]. Utilizing biplot analysis of GxE data, it is now possible to graphically address many important aspects to develop a better understanding, including genotype stability, mean performance, discriminating ability, mega-environmental investigation, representativeness of the environment, and who-resistant-where pattern [76–78].

The process of using plants as a strategy involves gathering and analyzing genotypes from different sources in order to find suitable genotypes that are adapted to the local environment and have high productivity or any other desired specialized attribute [79]. As a result, the type of material introduced determines whether plant introductions are successful. Genes must be fixed in breeding lines in order to create pure-line cultivars. The

initial selection process that uses landraces is the simplest and is known as mass or pure-line selection. Crossover programs and several iterations of pedigree and bulk approaches were employed to manage segregating generations [79,80]. Through pure-line selection, the JG315 chickpea cultivar evolved resistance to Fusarium wilt in Madhya Pradesh, India. The JG 62 cultivar, in addition to race 0, is a variety that is very vulnerable to FW, whereas ICCV 05530 is a cultivar that is highly resistant to FW [81].

Most breeding operations for chickpeas use single-cross hybridization. Hybridization almost occurs within the same species of the genetically distinct Desi and Kabuli varieties [82]. To promote genetic diversity and introduce beneficial genes from wild *Cicer* spp. into cultivated species, interspecific crosses have been attempted. *FOC* race resistance has largely been found in the Desi germplasm and in wild *Cicer* spp. In fact, accessions of *C. bijigum*, *C. cuneatum*, and *C. judaicum* showed combined resistance against races 0 and 5, but accessions *C. canariense* and *C. chorassanicum* were found to be resistant to race 0 whenever vulnerable to race 5. Additionally, the *C. pinnatifidum* accessions evaluated were found to be vulnerable to race 5, whereas some were resistant to race 0 [83].

Various chickpea breeders have used traditional methodologies and breeding techniques, and the population has improved in terms of increased output, different resistance, and desired plant types. Regarding FW response, genetic heterogeneity in chickpea genotypes has been recorded [84]. In accordance with the earlier findings, resistant sources were identified against FW in both Kabuli (ICCV 2 and UC 15) and Desi types (FLIP 85-20C, FLIP 85-29C, and FLIP 85-30C). Numerous chickpea Fusarium wilt-resistant genotypes, including ICCV 98505, ICCV 07105, ICCV 07111, and ICCV 07305, were identified by Sharma et al [85] using GGE biplot analysis. Four Kabuli chickpea genotypes resistant to FW, including ICCV 2, ICCV 3, ICCV 4, and ICCV 5 (Table 2), were previously generated using the pedigree method. Crop breeders now have a range of more effective tools for resistance breeding owing to recent developments in legume genomic technologies. As a result, legume crops can now be improved using genomics to better withstand different biotic and abiotic challenges [86,87].

**Table 2.** Important cultivars/donors (genetic resource) contributing to Fusarium wilt resistance.

| Important Varieties/Donors | Country | Reference |
|---|---|---|
| Surutato-77, Sonora-80, UC-15, UC-27, and Gavilan | Mexico | [27] |
| BG-312, ICCVs 98505, 07105, 07111, 07305, 08113, and 93706, ICCVs 08123, 08125, 96858, 07118, 08124, 04514, 08323, and08117(moderately resistant) | India | [85] |
| WR 315, JG 315, CPS 1, JG 74, Avrodhi, and Phule G | India | [84] |
| ICCV 2,3,4,5 and ICC 11322, 14424, and 14433 (against race I) | India | [88] |
| Digvijay | India | [89] |
| ICC 14194, ICC 17109, and WR 315 | India | [90] |
| Three lines derived from MABC-based C 214 and WR 315 cross | India | [91] |
| ICCV 09118, ICCV 09113, ICCV 09115, ICCV 09308, ICCV 09314, ICCV 05527, ICCV 05528, and ICCV 96818 | India | [73] |
| Super Annigeri and improved JG74 (resistant against *FOC*4) | India | [92] |
| ICC 7537 resistant to all races (except race 4) | Ethiopia | [27] |
| FLIP 84-43C (against race 0), ILC-5411, FLIP 85-20C (against race 5), FLIP 85-29C, FLIP 85-30C, ILC-127 (against race 0), ILC-219 (against race 0), ILC-237, ILC-267, and ILC-513 (against race 0) | Santaella, Córdoba, Spain | [93] |
| Annigeri | India | [27] |
| ICC-7520 | Iran | [27] |
| Andom1 and Ayala | - | [63] |

Pande et al. [70] found twenty-one accessions free from FW disease and twenty-five that were resistant during their study on the screening of chickpea genotypes against FW. In a separate study, genotypes JG 315, Avrodhi, DCP 92-3, JG 74, BG 372, and KWR 108 were found to be resistant to Fusarium wilt [87], while ICCV 05530 maintained its resistance against two FW races, viz., 1 and 3. Among these genotypes, JG 62 showed 89–100% wilt incidence against both FW races.

The use of nested association mapping (NAM) and multi-parent advanced generation intercross (MAGIC) populations is being developed in chickpea to make inter-crosses between multiple (4, 8, or 16) parental lines that originate from diverse regions. The creation of these crosses is possible through the balanced funnel crossing method, which recombines mosaics of founder parents, resulting in novel genotype and haplotype combinations [89]. At ICRISAT, a MAGIC population was created by mating cultivars and elite breeding lines, including ICC 4958, ICCV 10, JAKI 9218, JG 11, JG 130, JG 16, ICCV 97105, and ICCV 00108, with eight varied founder parents [73,85,88].

## 5. Screening Strategies to Identify Wilt-Resistant Genotypes

The utilization of host plant resistance (HPR) begins with the development of trustworthy and reproducible disease screening techniques to assess many germplasm accessions and breeding materials. It has been claimed that screening in the field and under controlled conditions (such as in greenhouse and lab settings) may help to identify resistant genotypes against FW [94]. However, there are some problems associated with maintaining uniform conditions for each plant during the screening of genotypes. So, it is important to develop a simple and efficient technique to screen chickpea genotypes for the identification of FW-resistant cultivars for future breeding programs. Generally, the following methods are applied for the screening of Fusarium wilt-resistant chickpea genotypes.

### *5.1. Field Screening*

The most frequent and recurrently applied technique for identifying FW-resistant genotypes is the wilt-diseased plot (WDP) strategy. The primary advantage of the WDP technique is that it makes it possible to screen a vast array of genetic materials under field conditions [95]. Effective wilt-diseased plots for field and hot spot location screening, as well as greenhouse and laboratory methodologies and successful breeding programs, have all been created [96]. Assessing inoculum homogeneity in a plot involves planting test genotypes next to susceptible cultivars as an indicator line or checking susceptibility after every 2–4 test entries. The widely applied susceptibility checks for races 1 to 4 in India include "JG 62", a twin-podded chickpea type that is extremely susceptible to all *FOC* races except race 0. The cultivar "JG 74" and the germplasm line "WR 315" (ICC 11322) of chickpeas are the two main sources of resistance. While the latter is resistant to all races but race 2, the prior is resistant to all *FOC* races except for race 3. The stepwise identification of host plant resistance to diseases has recently been revised by Pande et al. [89]. In order to screen many germplasm lines against FW, WDPs have been created at the International Center for Agricultural Research in the Dry Areas (ICARDA), ICRISAT, and NARS of countries that cultivate these crops.

Chickpea wilt has been investigated globally since the last decade of the 20th century using several methods. These efforts have involved the creation of multiple disease grading scales to calculate disease incidence and prevalence when evaluating new chickpea germplasm lines. Disease reactions are categorized based on the proportion of dead plants, whereas physiological maturity represents the reaction score of each genotype. To determine phenotypic resistance and susceptibility for race identification, different disease scoring scales are applied.

The six-point scale makes scoring simple (Table 3). Interpretation of the scale is as follows:

**Table 3.** Details of scoring scale to calculate Fusarium wilt disease incidence in chickpea.

| Rating | Wilt/Mortality (%) | Field Observation |
|---|---|---|
| 1 | 0% | No lesions visible |
| 2 | <10% | Few scattered lesions, usually seen after careful examination |
| 3 | 11–20% | Lesions and defoliation on some plants; little damage |
| 4 | 21–50% | Lesions very common and damaging; 25% plants killed |
| 5 | 51–80% | All plants with extensive lesions, causing defoliation and drying of branches; 50% plants killed |
| 6 | >81% | Lesions extensive on all plants; defoliation and drying of branches; more than 75% plants killed |

### *5.2. Screening under Controlled Conditions*

#### 5.2.1. Greenhouse Screening

Conducting screening under controlled conditions using a greenhouse can be a useful technique to verify the outcomes of evaluating wilt-diseased plots (WDP). This is crucial for researching the molecular mapping and tagging of a specific disease race, as well as the inheritance of pathogens [85]. Furthermore, pathogenic diversity studies can be carried out under controlled circumstances to learn the disease's genotypic information [89]. To screen the chickpea germplasm in greenhouses, the pot culture method has been standardized [97]. Another method that is frequently used for growing chickpea is root dip inoculation under greenhouse screens [94]. The identification of ninety percent of wilt in susceptible lines is guaranteed using the pot screening technique, although soil compaction from repeated irrigation may impair the association between pot and field performance. The chickpea seedlings are raised in autoclaved soil, dipped in inoculum at the roots, and then, transplanted into pots containing autoclaved soil, and the disease incidence is then measured [97]. There are some limitations to the greenhouse screening method, as well. It is very difficult to maintain uniform density of the inoculums in each diseased plot. So, it is not possible to differentiate the wilted plants in to early, late, and resistant categories.

#### 5.2.2. Laboratory Screening

Laboratory screening methods include various technologies, such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), quantitative PCR (qPCR), etc., for the accurate detection of *FOC*. In chickpea, artificial screening methods have been created by ensuring uniform inoculum load at the same vegetative stage of each test plant. This method guarantees that all inoculated plants have a roughly equal chance of infection by injuring the roots prior to inoculation [98]. Using this method, 25 resistant genotypes and 21 asymptomatic genotypes were identified. The method was applied to 211 genotypes from a core collection that included more than 16,000 unique chickpea germplasm accessions [70]. It has been suggested that pollen bioassays be employed as a quick and effective screening method to distinguish between resistant, late wilting, and susceptible genotypes [99]. One of the poisons produced by the fungus, fusaric acid (FA), is used as a selection agent to examine the genotypes of chickpeas.

## 6. Management of Fusarium Wilt in Chickpea

Management techniques to treat the disease are always adopted after a thorough disease evaluation. The management of Fusarium wilt in chickpea cannot be fully accomplished using a single control measure [100]. Elimination of the pathogen, as well as a reduction in the quantity and/or effectiveness of the main inoculums, are necessary for disease management [101]. The ideal control measure for such a goal should include the efficient application of one or a combination of the following management strategies:

### *6.1. Utilization of Pathogen-Free Planting Material*

Fusarium wilt can be spread by infected seeds and plant waste [102]. Using infected propagation material, the pathogen is transferred into productive areas or soils that are pathogen-free. Therefore, the significance of monitoring the health of the item through certification programs under quarantine legislation and phytosanitary inspection should be taken in to consideration. The right choice of planting site is aided using *F. oxysporum* spp.-free planting material in non-infested soils [102].

### *6.2. Chemical Control*

Chemical control is one of the finest disease management strategies for diseases that are spread through soil. FW can be controlled using organic chemical methyl bromide, which is a very effective fumigant. This chemical was used by Animisha et al. [100] to control FW. In addition to this, some popular fumigants, including dazomet, chloropicrin, carbendazim, and 1,3-dichloropropene, were also employed to combat FW in pea and chickpea, respectively [101].

### *6.3. Biological Control*

An integrated disease management strategy can easily include biological control and plant resistance as a cost-efficient and environmentally beneficial method of disease control [102]. An effective cure for chickpea wilt disease has been demonstrated using an arbuscular mycorrhizal consortium to control the biological processes of Fusarium wilt [103]. Numerous biocontrol agents have been used effectively and have led to a significant decrease in both pathogenic fungal growth in vitro and disease development in plants [104]. These bacteria and fungi include non-pathogenic and non-host Fusarium species [105]. The *Pseudomonas fluoresces* formulation treatment has increased chickpea production in the field and can be applied as a seed treatment to prevent chickpea wilt. Additionally, Fravel et al. [106] linked higher plant defensive responses to root colonization by the non-pathogenic strain of *Fusarium* spp. with disease reduction [107]. In a study, it was discovered that pre-treating chickpea seedlings with *Rhizobium* isolates before subjecting them to *FOC* increased the levels of total phenolics, constitutive is flavonoids, for mononetin, and biochanin [108]. The protection of chickpea against Fusarium wilt by non-pathogenic and non-host Fusarium species has been linked to the induction of the phytoalexins medicarpin and maackiain, as well as the related isoflavones formononetin and biochanin A [109].

### *6.4. Cultural Control*

Fusarium wilt disease in numerous crops was successfully controlled using the soil solarization method [110]. The heat produced by solarization may not kill the pathogen, but it may weaken it, reducing its host's sensitivity and increasing its susceptibility to assault by other soil microflora members [111]. The risk of disease in the following crop could be reduced by clearing away the debris from a field that has been afflicted by Fusarium wilt and igniting or burning it to destroy the *FOC* chlamydospores. Temperature has a big impact on chickpea's ability to resist Fusarium wilt. When there is a rise in temperature of 2–3 °C, different races of *Fusarium oxysporum* f. sp. ciceris (*FOC*) become more vulnerable to pathogens [112].

According to an investigation by Orr and Nelson [113], the Fusarium wilt pathogen in chickpea can live in the soil for up to 6 years, and 3 years of crop rotation is ineffective in lowering the incidence of the disease. In a 1998 study in southern Spain, Navas-Cortes found that planting date had the greatest impact on epidemic development. Sowing chickpea crops later in the year, from early spring to early winter, can slow the spread of Fusarium wilt epidemics and boost chickpea seed production [112].

### *6.5. Use of Resistant Cultivars*

The most practical and cost-effective technique for controlling Fusarium wilt is the use of resistant cultivars. However, several factors that affect disease resistance, such as genetic and pathogenic variability, the evolution of the pathogen, the availability of resistance sources, the co-infection of plants with other pathogens, genetics, and the penetrance of resistance (i.e., reduced expression as a result of the interaction between host genotype and inoculum load, temperature, and seedling age), etc., can seriously limit its use and effectiveness [112,113]. A crucial element of the integrated disease management (IDM) program is the use of resistant chickpea cultivars and additive or synergistic combinations of biotic, cultural, and chemical control strategies [112]. The use of resistant cultivars has been restricted because certain novel materials have undesirable agronomic characteristics. Furthermore, the effectiveness and widespread use of current resistant cultivars may be constrained by the considerable pathogenic diversity of *FOC* populations [114].

Recent years have seen significant challenges in achieving the desired yield of chickpea due to various factors. In most chickpea-growing regions, studying different stressors is important [90]. Future work should therefore concentrate on creating cultivars that are multi-stress-resilient. A thorough comprehension of significant pressures and the genetics of resistance ought to result in more methodical methods of resistance breeding. It is important to breed wild Cicer species for resistance because they have a lot of potential [99].

## 7. Advanced Breeding Techniques

The study of an organism's entire genome is referred to as genomics. Recombinant DNA, DNA sequencing techniques, and bioinformatics are all combined in genomics to sequence, assemble, and analyze the structure and function of genomes [115]. Genomic science is the study of how genes and genetic data are structured inside the genome, the procedures for gathering and evaluating these data, and how this organization influences their biological usefulness. The three key fields of genomic biology are structural, comparative, and functional (Figure 3) genomics [116]. With the goal of understanding evolutionary linkages and how genes and genomes function to produce complex phenotypes, such as gene regulation and environmental signaling, genomics is a branch that aids in comprehending the sequencing of genes and genomes [117].

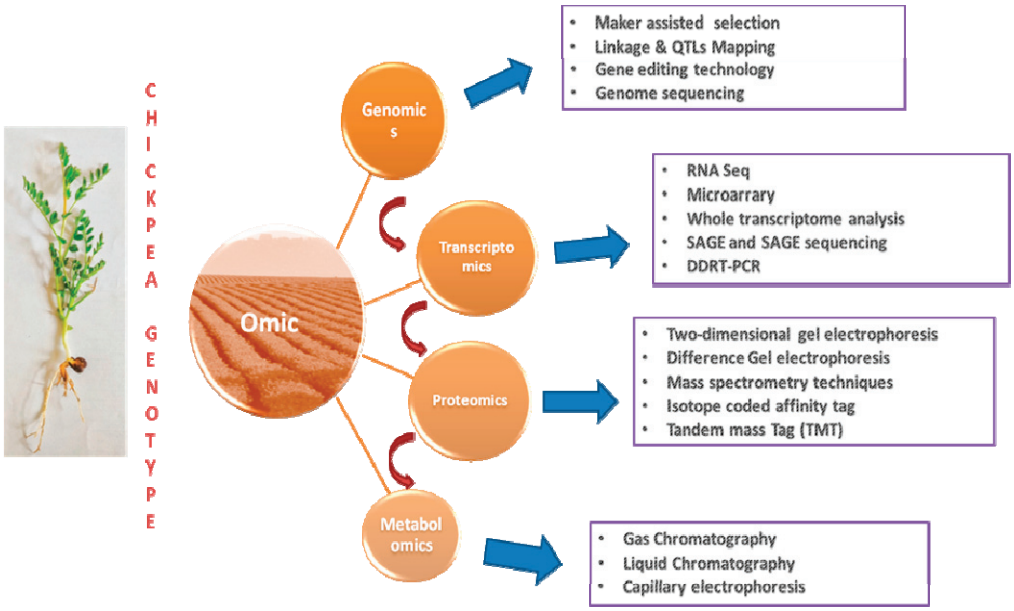


**Figure 3.** Omics approaches and their role in chickpea breeding.

### *7.1. Marker Technology*

There are three types of markers generally used in crop improvement programs including phenotypic, biochemical, and molecular markers [118]. Among these markers, molecular markers are more authentic due to their neutral behavior in different environmental conditions. Nucleotide sequences make up molecular markers, and the variation in nucleotide sequences among different individuals makes it possible to study these sequences [119,120]. The use of molecular markers that are closely related to the genes or QTLs controlling Fusarium wilt resistance allows for quicker and more accurate breeding. Although they are created through insertion, deletion, point mutations, duplication, and translocation, these polymorphisms are not always connected to the activity of the genes [121,122].

The genetic marker is a gene or DNA sequence with a known chromosome location that regulates a certain gene or characteristic. Genetic markers are closely related to the target gene and act as warning indications or flags [118]. Meanwhile, in contemporary genetics, genetic polymorphism describes the relative variation in the genetic loci of the genome. Genetic markers can be used to aid in the study of heredity and variation. Recent advances in molecular breeding, including the use of PCR-based techniques, such as simple sequence repeats (SSRs), insertion/deletion mutations (Indels), single-nucleotide repeats (SNPs), genomic sequencing (GS), genotype by sequencing (GBS), etc., have been widely used in crop improvement programs worldwide [119].

In contrast to multi-locus markers, including random amplified polymorphic DNA (RAPD), arbitrarily primed polymerase chain reaction (AP-PCR), inter-simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP), and sequence-specific amplification polymorphism (S-SAP) markers [120], the single-locus markers—including fragment length polymorphisms (RFLPs), variable number tandem repeats (VNTRs), simple sequence length polymorphisms (SSLPs), sequence-tagged microsatellite sites (STMSs), simple sequence repeats (SSRs), sequence tagged sites (STSs), single-nucleotide polymorphisms (SNPs), cleaved amplified polymorphic sequences (CAPSs) and sequence-characterized amplified regions (SCARs)—are frequently used in plant breeding in a variety of studies. In modern plant breeding, single-locus markers are used for various purposes, including germplasm characterization and protection, gene tagging, genome mapping, linkage map construction and analysis, evolution studies, parental selection, $F_1$ hybrid testing, genetic purity testing of seeds, genes, QTL mapping, etc. [121,122]. Employing marker loci that are strongly connected to vital genes that regulate features with economic relevance, such as disease resistance, male sterility, self-incompatibility, and seed qualities (including form, size, color, and texture) can help in selection.

#### 7.1.1. Molecular Markers and FW Resistance in Chickpea

The identification and creation of genetic maps of the segregating population are breeder's top priorities. Utilizing molecular markers for labeling traits and site-specific genes of interest, chickpea genetic maps have been created [123]. Using isozymes from the $F_2$ population resulting from interspecific crosses, the first maps were produced [124]. Numerous studies have discovered genes that influence floral color, wilt resistance (Fusarium), double pods, and growth behavior [123,125]. Higher numbers of maps connected to features were derived using multiple markers, crosses from *C. reticulatum*, and other techniques. Microsatellite markers, however, were used to create populations from interspecific crosses, which take advantage of more genetic variations among chickpea genotypes [126]. The first transcriptome study of the chickpea genome was finished after the development of next-generation sequencing [127]. With the development of transcriptome information, detailed genetic maps were created using large-scale molecular markers [128–130]. The genetic population utilized to map and find QTLs in the chickpea genome may benefit from having access to draught genome sequencing in the Desi and Kabuli types [131]. Omics methods gathered genomic data and sparked the development of tightly connected QTLs in molecular markers [132].

The diseases for which significant resistance genes have been backcrossed into elite cultivars are the ones for which MAS in plant breeding is most effective [133]. Chickpea provides some evidence of the application of MAS to facilitate efficient and accurate breeding. The SSR markers, namely, TR19, TA194, and TA660, which were discovered to be polymorphic between the parental lines, have already been used for foreground selection via marker-assisted backcrossing in order to introduce *FOC*1 in a superior chickpea cultivar [134]. As part of marker-assisted introgression, the SSR markers TA110 and TA37 in chickpea LG2 were also used to introduce *FOC*-2 into the background of a superior cultivar [135]. To develop virtually isogenic lines with disease resistance, TA59, one of the several markers discovered to flank the *FOC* race 5 resistance gene, was used [136].

The use of molecular markers is an essential method for classifying, characterizing, and screening infections and diseases. To categorize and filter fungi, internal transcribed spacer (ITS) markers are often used. Even though information on pathogen variety is required to comprehend pathophysiology and development for management strategies, SSR markers are employed in unique backcross generation to aid in the selection against Fusarium resistance. The importance of resistant molecular markers in identifying disease-causing genes and resistance mechanisms has been acknowledged. Numerous crops have additionally demonstrated a substantial association between microsatellite markers and resistance genes, such as Fusarium wilt resistance genes, in chickpea, and many others.

Initial efforts to map resistance genes using restriction fragment length polymorphism (RFLP), RAPD markers, and isozymes failed. Only modest polymorphism was detected in chickpea using the resistant gene analogue (RGA), ISSR, and RAPD [137]. Nevertheless, *FOC*1 was mapped at 7.0 cm on the same side of the gene using two markers, viz., CS27700 and UBC170550. The resistance genes *FOC*3, *FOC*4, and *FOC*5 were later mapped using ISSR, RAPD, and SSR markers [138].

The first WR gene discovered was $H_1$ against race 1 in chickpeas [139]. Two primers, UBC-170550 and CS-27700, respectively, amplified susceptibility and the DNA region linked to FW resistance [140]. However, after transforming these two markers into allele-specific associated primers (ASAPs), only CS-27700 was shown to be specific to the susceptible allele, whereas the other one (UBC-170550) appeared to be locus-specific. The same RAPD markers were later shown to be connected to the gene controlling race 4 resistance at 9 cm [141,142]. ISSR markers were also applied to tag the WR gene in a population that was inter-specific to the mapping method. The authors discovered two ISSR markers associated with the resistance gene for race 4: UBC-855500 and UBC-8251200.

SSR markers are the preferred markers for plant breeding or for plant breeders owing to their multi-allelic and co-dominant properties [143]. The development of SSR markers has made the application of genomic and transcript databases feasible. Several hundred SSR markers have been developed from genomic DNA libraries [144]. The "ICRISAT Chickpea Microsatellite" (ICCM) markers are a set of 311 distinct SSR markers that were created by Nayak et al. [144] using information from an SSR-enriched genomic library of the chickpea accession ICC 4958. Additionally, SSR markers (ESTs) have been mined using expressed sequence tags [144,145]. Primer pairs were created by Varshney et al. [145] for 177 unique EST-SSR markers, and 3728 SSR markers were found.

Using DNA markers, marker-assisted selection can expedite conventional breeding [146,147]. The resistant genotypes of chickpea that were discovered in this investigation may be employed in breeding programs to breed resistant cultivars. Previously, resistance to *FOC* races 1, 2, and 3 was delivered through genes 3, 2, and 1, respectively. The marker CS27 was first associated with *FOC* 1 at 7.0 cm by Mayer et al. [139], and later, this marker was modified to become an allele-specific related marker (CS27A). The *FOC*2 resistance gene was found at 2.7 cm and 0.2 from the SSR markers H3A12 and TA96. The formerly discovered DNA markers proved useful in establishing relationships to phenotypic data and connections to *FOC* 2 resistance genes. This was accomplished by using molecular markers, such as the ASAP marker (CS27700) and several STMS markers [139,148,149]. Utilizing the primers TA110, TR19, TS82, and CS27, a total of 28 genotypes were screened, and it was

found that these genotypes were strongly related with *FOC* 2 resistance genes [138,150]. Resistance gene analogue, DNA amplification, fingerprinting, and other later-developed chickpea markers demonstrated more polymorphism compared to isozymes, RAPDs, and RFLPs. Nevertheless, the development of polymorphic markers led to substantial advancement in the discovery of STMS markers [151].

#### 7.1.2. Marker-Assisted Breeding

Marker-assisted selection (MAS), among other genomic methods, can significantly improve chickpea breeding programs [152]. How well MAS performs depends on the degree of association between the marker and the gene locus determining the target feature. The positioning of the marker in a genomic area with higher levels of polymorphism and simplicity of interpretation can affect the MAS technique [153]. The main advantage of MAS over traditional selection is the capacity to choose features that are difficult or inconvenient to assess directly, eliminating complicated and time-consuming evaluations. This is true when breeding for disease resistance is performed. By pyramiding different resistance genes in a single genotype, MAS also enables quicker variety release and development [154]. An effective technique for utilizing the potential of genes for agronomic traits is marker-assisted selection [155].

For orphan pulse crops, the success of MAS in cereal crops serves as a model. Many genetic resources have recently been invented and employed in marker-trait association research in pulses [156]. Under the auspices of the Indo-US Agricultural Knowledge Initiative (AKI) program, the Government of India, and the Indian Council of Agricultural Research (ICAR) launched the chickpea genomics initiative program.

##### Variations in MAS

The numerous molecular methods used in MAS include marker-assisted backcrossing (MABC), gene pyramiding, marker-assisted recurrent selection (MARS), and genomic selection (GS). In order to characterize genetic material and select individuals in the early segregating generation, these techniques have been applied in plant breeding, speeding up and improving the precision of the breeding cycle [157–159]. The genomics-assisted breeding (GAB) techniques MABC, MARS, and GS have recently been applied to breeding superior chickpea varieties with increased yield and resistance/tolerance to adverse climatic conditions [160].

##### Marker-Assisted Backcrossing (MABC)

MABC, a backcrossing technique, is made possible by molecular markers [161]. It expedites both the selection process and the genetic recovery of the recipient parents. By transferring the gene of choice or quantitative trait loci (QTLs) from the donor parent, this method is frequently used to eradicate undesirable features, such as disease and pest susceptibility, anti-nutritional factor, etc. from high-yielding cultivated varieties [162]. Foreground selection, background selection, and recombinant selection are the three steps of MABC.

Two high-yielding Desi cultivars viz., Annigeri 1 and JG 74, were employed in a collaborative effort between the University of Agricultural Sciences (UAS-Raichur) and Jawaharlal Nehru Krishi Vishwa Vidyalaya (JNKVV), Jabalpur, India, to increase FW resistance using the MABC method. In Central and South India, both grown species demonstrated high susceptibility to Fusarium wilt race 4 (*FOC* 4) and decreased production. This led to the development of two novel resistant varieties, namely, "Super Annigeri 1" and "enhanced JG 74", by introgressing a genomic region that imparts resistance to *FOC* 4, utilizing MABC and WR 315 as the donor parent [163].

The two primary factors limiting the output of chickpeas are Ascochyta blight (AB) and Fusarium wilt (FW). Using a step-by-step MABC strategy, a superior chickpea cultivar, C 214, was given dual resistance [164]. The *FOC* 1 gene for FW and two quantitative trait loci (QTL) regions, ABQTL-I and ABQTL-II, were targeted for introgression to produce

resistant lines. Employing foreground selection with six markers related to *FOC*1 and eight markers linked to both QTLs, it is now possible to choose plants with desirable alleles in several segregating generations. To find a plant with high recurrent parent genome recovery, background selection employing 40 uniformly distributed SSR markers was performed, in addition to foreground selection. After three backcrosses and three rounds of selfing, 22 $BC_3F_4$ lines for FW and 14 MABC lines for AB were acquired [165]. Three resistant lines for FW and seven resistant lines for AB have been identified phenotypically using this line.

Marker-Assisted Gene Pyramiding (MAGP)

One of the contemporary MAS methods used to produce MAGPs is the pyramiding of different genes. Two or more genes are picked for pyramiding simultaneously in MAGP. Gene pyramiding has been performed using an array of methods, including backcrossing, recurrent selection, complicated crossing, and multiple-parent crossing [166].

Marker-Assisted Recurrent Selection (MARS)

Recurrent selection, where two genes are chosen at a time for pyramiding, is an effective method used in plant breeding to improve quantitative traits through continuous crossing and selection processes [167]. The breeding cycle is slowed down by environmental changes, which have a negative effect on the breeder's ability to select. At each generational level, molecular markers are employed for the intended features in MARS. Every cycle of crossing and selection in this case involves selectively crossing specific plants. The selection is made utilizing phenotypic data and marker scores. As a result, it accelerates the breeding or selection cycle and boosts the efficiency of recurrent selection. MARS is a forward breeding approach that has been extensively used for polygenic traits such as agricultural production and resistance to different biotic and abiotic stresses [168].

### *7.2. Genetic Mapping and QTL Technique*

Studying the genetics of quantitative traits is crucial in the field of plant biotechnology. Complex quantitative features can be found in many plant species in nature. We now have better knowledge of these complicated traits. The section of the genome known as a QTL is linked to a quantitative trait's influence [169]. Quantitative trait loci are made up of a single gene or a group of linked genes that affect phenotypes. One or more genes that influence quantitative traits have been identified using molecular markers and advanced statistical methods, together with specific chromosome loci. These identified loci are known as QTLs. For the attributes of yield, quality, insect-pest resistance, abiotic stress tolerance, and environmental adaptation in chickpea, QTL mapping is conducted. When identifying connected QTLs in a population with segregating traits, it is essential to select parents with a variety of genetic backgrounds and to hybridize parental lines that differ in one or more of their quantitative traits [170].

Genetic maps are created by employing the segregation and recombination principles of Mendelian genetics. They may demonstrate how close together chromosomes and DNA producers are within an organism. This level of parental differentiation in the population is crucial for the creation of genetic maps. Crop breeding and genetic mapping are closely related, and many crop breeding populations have already undergone genetic mapping [171]. Building genetic maps based on molecular markers that are easy to produce, highly repeatable, co-dominant, and specific to recognized linkage groups is greatly desired for breeding purposes. Because the length of each marker is the most crucial component, maps created using AFLPs, RAPDs, and ISSRs have limited transferability between populations and pedigrees within a species [171,172].

The identification and mapping of genes that impact chickpea resistance to different races of *FOC* have been made easier thanks to the use of DNA marker technology. In two mapping populations, CA 2156-JG 62 and CA 2139-JG 62, Halila et al. [172] discovered a second gene, *FOC*02/*FOC*02, which is flanked by markers TS47 and TA59 on LG2. Jendoubi

et al. [173] used nearly isogenic lines (NILs) to finely map the *FOC*01/*FOC*01 gene on LG5 within a 2 cM interval. An SSR-based QTL analysis of the $F_{2:3}$ population (C 214 × WR 315) identified two QTLs on LG6 for *FOC*1 resistance: FW-Q-APR-6-1 and FW-Q-APR-6-2 [174].

The first genetic maps of the chickpea were created using isozymes from $F_2$ populations resulting from interspecific crosses. Following this, additional maps were created by various study groups. One of these maps included QTLs related to flowering time, agronomic traits, and Ascochyta blight resistance [175]. Other characteristics included double pod, growth habit, and Fusarium wilt resistance [176]. To map the *FOC*-3 resistance gene and connect it to the *FOC*-1, *FOC*-3, and *FOC*-4 resistance genes, RAPD, STS, ISSR, and STMS markers were used. At 0.6 cm from the *FOC*-3 gene, the STMS marker TA96 was found, but the STMS markers TA27 and CS27A co-segregated with TA96. Additionally, the authors found a link between *FOC*-3, *FOC*-1, and *FOC*-4. While *FOC*-1 and *FOC*-4 were mapped close together at 1.1 cm, *FOC*-3 appeared to be associated with them at distances of 9.8 cm and 8.7 cm, respectively.

Using the SSR marker TA103, *FOC*1 was introduced from WR 315 to C 214. Earlier, scientists discovered *FOC*1 flanked by the SSRs TA110 and H3A12 on LG2. On LG2, the genes for *FOC*2 (TA96-H3A12) and *FOC*3 (TA194-H1B06y) were also discovered. However, according to Jingade and Ravikumar [177], a major QTL (GSSR 18-TC14801) on LG1 for *FOC*1 resistance can account for up to 71% of phenotypic variance (PV). Moreover, a sizable QTL (FW-Q-APR-2-1) was found on CaLG02, and two smaller QTLs (FW-QAPR-4-1 and FW-Q-APR-6-1, respectively) were found on CaLG4 and CaLG6, indicating resistance to *FOC*1 and *FOC*3 [178]. It has been determined that the resistance loci on LG2 are either monogenic or oligogenic with respect to *FOC* 5. With the help of SNP and SSR markers, the possible LG2 genomic area was recently reduced to 820 kb [179].

Moreover, two distinct genes that provide race 0 resistance have been identified and labeled. The first resistance gene, *FOC01*/*FOC01*, was flanked by two markers, i.e., OPJ20600 and TR59, on linkage group 3 (LG3), which corresponds to LG2. In an $F_{2:3}$ mapping population of "C 214" × "WR 315", Sabbavarapu et al. [174] recently revealed two unique QTLs for race 1A (*FW-Q-APR6-1* and *FW-Q-APR-6-2*). The second gene (*FOC02*/*FOC02*) was located on LG2, and the STMS markers TS47 and TA59 were located on each side of it (Table 4). All additional wilt pathogen resistance genes were found in linkage group 2, except for *FOC*-01 and two QTLs for race 1A.

Numerous studies have shown that four genes, including *FOC*-1, *FOC*-3, *FOC*-4, and *FOC*-5, should be in the same linkage group [180]. Five resistance genes, viz., *FOC*-1, *FOC*-2, *FOC*-3, *FOC*-4, and *FOC*-5, were found to be clustered in chickpea. On LG2, a cluster of five genes covering 8.2 cm was discovered. The resistance gene cluster was 2.952 Mb in size, where 1 cm equals 360 kb. Among the five genes, *FOC*-1 and *FOC*-5 were separated by 2.0 cm, but *FOC*-5 was separated from *FOC-3* by 3.4 cm. It was determined that 5.4 cm separated *FOC*-1 from *FOC*-3. There was a 1.0 cm distance between *FOC*-3 and *FOC*-2 and a 1.8 cm distance between *FOC*-2 and *FOC*-4. At the extremities of the cluster, 8.2 cm separated two genes (*FOC*-1and *FOC*-4). It was observed that gene order and map distances were more accurate because only one source of resistance to five genes was utilized, and the mapping population descended from an intraspecific cross.

The discovered QTLs for various traits can be utilized in genomics-assisted breeding using modern techniques, such as marker-assisted backcrossing, the introgression of superior alleles from wild species through advanced backcross QTL, marker-assisted recurrent selection, and genome-wide selection. Garg et al. [178] constructed a genetic map for resistance to Fusarium wilt on 188RILs gene rated from a cross between JG 62 and ICCV 05530, and identified five QTLs for resistance, with explained phenotypic variance ranging from 6.63 to 31.55 percent. Out of the five QTLs found, three QTLs onCaLG02 and one minor QTL each on CaLG04 and CaLG06 were mapped for race1.

**Table 4.** List of various QTLs contributing to Fusarium wilt in chickpea.

| Fusarium Race | Name of Population | QTLs | Marker Identified | Linkage Group | References |
|---|---|---|---|---|---|
| Race 1<br>Race 4 | C-104 × WR-315 | - | CS-27700,<br>UBC-170550 (RAPD) | - | [140] |
| Race 3 | WR-315 × C-104 | *FOC-3* | TA96 and TA27, TA196 (STMS) | - | [26] |
| Race 1<br>Race 4 | - | *FOC-1* (syn. h (1)) and *FOC-4* | CS27A (STS/SCAR)<br>TA194 (STMS) | - | [61,138] |
| Race 5 | - | *FOC-5* | TA59 and TA96 (SSR) | - | [174] |
| Race 2 | - | *FOC-2* | TA96 and H3A12 (STMS) | - | |
| Race 4<br>Race 5 | *C. arietinum* × *C. reticulatum* | - | STM S and a SCAR | - | [138] |
| Race 1 | F9 | *FOC-1* | H3A12, TA110 (STMS) | - | [61] |
| Race 0 | CA 2139 × JG 62 | *FOC01*/*FOC01* | OPJ20(600) (RAPD)<br>TR59 (STMS) | LG3 | [138] |
| Race 0 | CA 2139 × JG 62 | *FOC02*/*FOC02* | TA59 (STMS) | LG2 | [174] |
| Race 1A | C 214 × WR 315 | *FW-Q-APR-6-1* (*FOC-1*) and *FW-Q-APR-6-2* (*FOC-1*) | CaM1402 and<br>CaM1101 (flanking)<br>CaM1125-TA22 | LG6 | [176] |
| Race 5 | - | *FOC-5* | TA59 (STMS) | LG2 | [59] |
| Race 1 | JG 62 × WR 315 | - | TA27-TA59 (STMS) | LG2 | [4] |
| Race 1<br>Race 3 | C 214 × WR 315 | *FOC-1* and *FOC-3* | GA16, TA110, and TS82 | LG2 | [134] |
| Race 1 | JG 62 × ICC V05530 | 3QTL (race 1),<br>*FW-Q-APR-2-1*<br>*FW-Q-APR-4-1*<br>*FW-Q-APR-6-1* | TR19 and H2B061, TA132 and TA46 (STMS) | CaLG02,<br>CaLG04, and<br>CaLG06 | [178] |
| Race 3 | JG 62 × ICC V05530 | 2QTLs (race 3)<br>*FW-Q-APR-2-1* and *FW-Q-APR-4-1* | CKAM1256 and TS72 | CaLG02 and<br>CaLG04 | [178] |
| Race 0 | CA 2156 × JG 62 | *FOC01*/*FOC01* | H2I20 and TS43 (STMS) | LG5 | [58] |
| Race 5 | WR 315 × ILC 3279 | *FOC-5* | TA59, CaGM07922, and SNPs | LG2 | [179] |
| Race 4 | Annigeri1 × WR-315 | *FOC-4* | TA59, TA96, TR19, and TA27 | LG2 | [164] |
| Race 4 | JG 74 × WR 315 | *FOC-4* | GA16andTA96 | | [164] |
| Race 5 | - | *FOC-5*/*FOC-5* | TA27 and TA59<br>TA96<br>$CS27_{700}$ (RAPD)<br>UBC170550 (RAPD) | LG2 | [57,138] |
| Race 5 | - | *FOC-5*/*FOC-5* | ECAMCTA07<br>OP-M20-21045<br>OP-M20-31103 | LG2 | [164] |

According to molecular mapping investigations, resistance genes for pathogen races 0, 1, 2, 3, and 4 have been found on LG2 of the chickpea map. Due to the grouping of six resistance genes, LG/2 is a hotspot for Fusarium wilt resistance. In order to employ MAS and better understand the molecular mechanism of resistance, strongly related markers for some of the genes have been found and verified in various genetic backgrounds [181]. Race 5 resistance gene near-isogenic lines have been created, which can be used for map-based cloning and fine mapping.

*FW-Q-APR-2-1*, a significant QTL for race 1, was identified on CaLG02. Additionally, minor QTLs on CaLG04 (*FW-Q-APR4-1*) and CaLG06 were detected (*FW-Q-APR-6-1*). For race 3 of an FW discovered in Ludhiana, a significant QTL was discovered on CaLG02 (*FW-Q-APR-2-1*) and CaLG04 (*FW-Q-APR4-1*). Since the primary QTLs for races 1 and 3 on CaLG02 shared flanking markers, i.e., TR19 and H2B061, it is possible that the same genomic regions regulate resistance to these two races.

### *7.3. Genome Sequencing*

A few decades after the Sanger DNA sequencing method was created, deep, high-throughput, in-parallel DNA sequencing techniques known as next-generation sequencing (NGS) were created. Amplification libraries, also known as amplified sequencing libraries, are required for second-generation sequencing methods. It is now possible to perform single-molecular sequencing by employing third-generation sequencing, without the time-consuming and expensive amplification libraries. Research teams may now create de novo draught genome sequences for every organism of interest, with the help of bioinformatics tools and the synchronized rapid advancement of NGS technology. These technologies can be applied to whole-transcriptome shotgun sequencing (WTSS, also known as RNA sequencing (RNA-seq)) [182], targeted (TS) or candidate gene sequencing (CGS) [183,184], whole-exome sequencing (WES) [185], and methylation sequencing (MeS) [186].

Genome sequencing is being transformed due to advances inhigh-throughput technology. The intense rivalry among new sequencing techniques has led to some incredible advancement. The essential concepts of the best-known sequencing platforms are: ABI/SOLiD sequencing, Roche/454 Life Sciences sequencing, and Solexa/Illumina sequencing.

Prior to 2013, the chickpea was recognized as an orphan crop due to a lack of genetic data. However, the first draughts of the genomes of the Desi and Kabuli chickpea investigations were released in 2013 [187]. The development of high-throughput sequencing and next-generation technologies laid the foundation for the sequencing of the chickpea genome. A thorough map of deviation in 3171 cultivated and 195 wild accessions was produced by Varshney et al. [188] to provide resources for breeding and research on chickpea genomics.

The creation of genetic resources is still crucial for molecular or genomics-assisted breeding. Unfortunately, there has been delayed development of genetic resources for this important crop of legumes. Chickpea genomic resources have significantly increased in recent years due to next-generation sequencing (NGS) initiatives and their use in genomics research [188]. The discovery of the candidate gene(s)/genomic regions controlling disease resistance may be made possible by the availability of whole-genome sequence information in different plant species, including chickpea. Williams et al. [189] and Srivastava et al. [190] reported on the virulence-related genes *FOC* (*FOC*-38-1) and Fop (Fop-37622), which have provided fresh information that has increased our comprehension of the pathogenicity of FW and the evolution of the host–pathogen interaction in legume species.

The use of NGS technology has led to the creation of numerous molecular markers for the advancement of chickpeas [188]. In the past, millions of SNP markers, 2000 SSR markers, and more than 15,000 feature-based diversity array technology (DArT) platform markers have been produced for chickpea. The NGS revolution has made it possible to perform sequencing at different depths, including whole-genome re-sequencing, skim sequencing, and low-depth sequencing (genotyping via sequencing, RAD-Seq).

## 8. Multi-Omics Approaches

Several interesting omics technologies have evolved during the past few decades. The information gathered using these omics techniques may be combined with genetic information to alter a variety of biological processes involved in chickpea breeding. These omics-based techniques have been proven to be useful for examining the molecular and genetic foundations of crop development by modifying DNA, proteins, metabolites, transcript levels, and mineral nutrients against negative environmental and physiological stress responses [191]. Numerous omics methods have disclosed each corresponding molecular bi-

ological aspect integrated with plant systems, including metagenomics, genomics, transcriptomics, metabolomics, proteomics, ionomics, and phenomics [192]. High-throughput and speedy data creation for transcriptomes, genomes, proteomes, metabolomes, epigenomes and phenomes has been made possible by the development of next-generation sequencing (NGS) technology [193]. The integration of different omics techniques under physiological and environmental stress could reveal gene networks and activities [15]. The use of omics provides a systems biology approach to comprehending the intricate relationships between genes, proteins, and metabolites within the phenotype. In order to preserve and develop crops, this integrated approach largely relies on computational analysis, bioinformatics, chemical analytical procedures, and many different biological disciplines [194]. For the purpose of finding possible candidate genes and their pathways, the integration of various omics methods may prove useful. Omics allows for a deeper understanding of the processes behind the complex architecture of numerous phenotypic features with agricultural importance. Thus, the integration of multi-omics approaches may be beneficial to identify the mechanisms behind the expression of simple and quantitative traits such as higher yield and disease resistance. Omics approaches are also important for understanding the inheritance of these traits [195]. This information is significant in the development of biotic stress-resistant cultivars through the introgression of desired traits to maintain the sustainable production of different crops, including chickpea. For example, metabolomics may help in the identification of the up-and down-regulation of different metabolites that are important for defense systems in plants [196].

### *8.1. Transcriptomics/Gene Expression Studies*

Differential gene expression in chickpea plants infected with Fusarium wilt, as well as plants without infection, comparatively offers a wealth of resources for the functional analysis of resistance-related genes and their application in breeding for long-lasting wilt resistance. In chickpea, various studies have been conducted to identify differentially expressed genes. Using cDNA-RAPD and cDNA-AFLP techniques, Nimbalkar et al. [182] identified differentially expressed genes in chickpea during root infection by *Fusarium oxysporum* f. sp. ciceri race 1. Based on a cDNA template and decamer primers, the former discovered nine transcripts that were differently expressed in the infection-resistant chickpea variety. In total, 273 of the 2000 transcript-derived fragments (TDFs) displayed differential expression in infected chickpea stems. Only 13.65% of the TDFs were differentially expressed during the pathogen infection process in chickpea roots, while the remaining 86% did not vary in expression (Table 5). In a study, Saable et al. [196] identified 162 DEGs that belonged to defense signaling pathways. Using this sequence, other studies have also been carried out to discover differentially expressed genes (DEGs). Ashraf et al. [197] discovered 6272 DEGs that belonged to stress-responsive genes in chickpea through RNA blot analysis during wilt infection with race 1. Gupta et al. [198] race 1 induced redox state alterations in chickpea. Recently, Priyardashni et al. [199] analyzed the expression of NBS-LRR and WRKY genes in chickpea infected with Fusarium wilt, causing a fungal pathogen.

**Table 5.** Differentially expressed genes (DEGs) contributing to FW resistance in chickpea.

| Genotype Used in Study | Platform/Technology | Differentially Expressed Genes (DEGs)/Candidate Gene | Study Based on | References |
|---|---|---|---|---|
| WR 315 and JG 62 | cDNA-RAPD and cDNA-AFLP | 273 DEGs related to stress response, gamma-glutamyl-cysteine synthetase, and *NBS-LRR* | Race 1 | [182] |
| | RNA blot analysis | 6272 ESTs belonged to stress-responsive genes and cell signaling, transcription, RNA processing, modification, cellular transport, homeostasis, and hormone response-related genes | Race 1 | [197] |
| | Suppression subtractive hybridization | 162 ESTs belonged to genes responsible for defense signaling pathways, energy metabolism, cell rescue, and superoxide dismutase | Race 4 | [196] |
| | qPCR, Microarray analysis | Stress-responsive and other defense-associated genes, including aquaporin, ATP synthase, immunity-associated genes, cystatin and DnaJ, pectinesterase and xyloglucosyl transferase, actin- and profilin-like genes, cytochrome P450, and peroxidase | Race 1 | [197] |
| | qPCR | Transporter gene, transporter like gene, redox regulatory respiratory burst oxidase homolog F (RBOHF), thioredoxin 3 (TRX3), cationic peroxidase 3 (OCP3), flavodoxin-like quinone reductase 1 (FQR1), iron superoxide dismutase 1, NADH cytochrome b5 reductase (CBR), Fe (II) oxidoreductase 7 (FRO7), genes related to intracellular transportation ABC transporter-like gene, polyol transporter gene, translocase, heavy metal transporter (detoxifying protein) (FRS6), bZIP, homeodomain leucine zipper, MYB, helix loop helix, zinc finger (CCHC type), heat shock family protein, sucrose synthase (SUS4), b-amylase (BAM1), serine threonine kinase (CDKB1.1), and vacuolar ATPase (TUF) | | [198] |
| | Expression analysis | NBS-LRR and WRKY genes | | [199] |
| Digvijay and JG 62 | qPCR | Stress-responsive genes | | [200] |
| | qRT-PCR and LongSAGE | 3816 DEGs and G protein b subunit gene lignification, hormonal homeostasis, plant defense signaling, ROS homeostasis, and R-gene mediated defense | | [201,202] |
| | qRT-PCR | 5 DEGs related to stress-responsive category, glycosyltransferase gene, *GroEs2*, *60srp*, and *Betvi E* | Races 1, 2, and 4 | [203] |
| ICC4958 | Illumina (NGS) and Poly(A)-based qRT-PCR | 122 conserved miRNAs, 59 novel miRNAs, and defense gene encoding Toll/Interleukin-1 receptor–nucleotide binding site leucine-rich repeats miR2111 targets a Kelch repeat-containing F-box protein | | [204] |
| NILs—RIP8-94-5/RIP8-94-11 | qPCR | 22 potential defense-related genes encoding a MADS-box transcription factor, and TMV resistance protein | Race 5 | [205] |
| WR315 and BG256 | Sequencing (Roche 454 GS FLX system) | 202 DEGs related to polyubiquitin, chlorophyll a-b binding protein, ferredoxin-NADP, translation factor sui1, carbonic anhydrase, ribulose bisphosphate carboxylase, oxygen evolving enhancer, elongation factor 1-alpha, and post-translational modification genes | | [206] |
| JG 62, WR 315 and JAKI9218 | qRT-PCR | 6 DEGs, including transcription factors such as extracellular calcium-sensing receptor, Nitric oxide reductase, growth hormone-releasing hormone receptor, Cytochrome C oxidase Cbb-3 type subunit I, Hydroxynitrite lyase, Tir chaperone, and ionotropic glutamate receptor | Races 2 and 4 | [207] |

The transcriptome, or the complete collection of RNA transcripts produced by an organism's genome in a cell or tissue, is the subject of the study of transcriptomics [208]. To study how genes are expressed in response to various stimuli over an extended period, a dynamic technique called transcriptome profiling has grown in popularity [209,210]. By enabling the researcher to examine the differential expression of genes in vitro, this method aids in the clarification of a gene's basic function. To analyze transcriptome dynamics, at first, conventional profiling approaches, such as differential display-PCR (DD-PCR), SSH, and cDNAs-AFLP, were used; however, these methods had poor resolution [211]. The use of microarrays, digital gene expression profiling, NGS, RNA seq, and SAGE for RNA expression profiling was soon made possible through the development of truthful techniques [212,213]. A breakthrough technique for advancing transcriptomics uses in situ RNA-seq, often referred to as in situ ligation, to sequence RNA in living cells or tissues [214]. A second method called spatially resolved transcriptomics uses spatial information to detect gene expression within cells or tissues in order to provide a detailed molecular description of physiological processes in living things [215]. One of the better techniques for creating genic-SSR markers that can be connected to phenotypic features associated with candidate genes is RNA-seq.

Before the discovery of digital transcriptome profiling, expressed sequence tags (ESTs), cDNA-AFLP, and cDNA-RAPD were mostly employed to identify the gene(s) involved in plant defense mechanisms and plant–pathogen interactions [216–220]. In recent years, the transcriptome analysis of the four chickpea cultivars, viz., JG 62, ICCV 2, K 850, and WR 315, allowed the genomic regions regulating FW resistance to have "big effect" SNPs and Indels [221,222]. The chickpea from the cross ILC 3279 × WR 315 was functionally validated for the genomic area determining *FOC* (race 5) resistance [223]. In this experiment, resistant and sensitive NILs were generated. Three novel candidate genes, i.e., LOC101495941, LOC101509359, and LOC101510206 (encoding the MATE family protein, MADS-box transcription factor, and serine hydroxymethyl-transferase, respectively) and two previously known candidate genes, i.e., LOC101490851 and LOC101499873 (encoding chaperonin) were related to defense activity against FW, recognized via differential gene expression analysis at twenty-four hours post inoculation (hpi) [224]. Numerous transcripts associated with distinct TFs were found to be differently expressed in JG 62 and WR 315 in response to FW (race 1) infection. Through sugar metabolism and cellular transporters, defense signaling against FW was activated in chickpea [224].

### *8.2. Proteomics and Metabolomics*

Proteomics is a method used to profile all the proteins that are expressed in an organism. It is broken down into four separate categories: sequence, functional, structural, and expression proteomics [225,226]. Traditional proteomics includes size exclusion chromatography (SEC), exchange chromatography (IEC), and affinity chromatography. Western blotting and an enzyme-linked immune sorbent assay can be utilized to analyze specific proteins (ELISA). Additionally, more advanced methods for the separation of proteins have been developed and employed, including SDS-PAGE, 2-DE, and 2-D differential gel electrophoresis (2D-DIGE).

The numerous proteins involved in host–pathogen interaction and their function in protecting the host plant from pathogen attacks can be uncovered using a proteomics method [227,228]. Many proteins have been linked to significant host–pathogen interactions, including the establishment of the pathogen in a host plant that is vulnerable to it, as well as the host plant's defense against pathogen invasion [229–232]. These proteins range from syntaxins to subtilin-like proteases in different plant species in response to FW infection. They include chitinases, -1,3-glucanases, xylem proteinases, proteinase inhibitors, leucine-rich repeat proteins, proline-rich glycolproteins, pathogenesis-related (PR) proteins, cellulose synthases, ankyrin repeat-containing protein, and PR-5b [228,233–235].

The genotypes JG 62 (FW-susceptible) and Digvijay (FW-resistant) of chickpea were both found to contain a variety of defense-related proteins against FW infection [228].

Several ROS-activating enzymes, including glutaredoxin, glutathione peroxidase, ascorbate peroxidase, glutathione S-transferase, and peroxiredoxin, were identified in higher concentrations in Digvijay than in JG 62. This is similar to how Digvijay was able to reduce FW pathogen assault compared to the FW-sensitive cultivar JG 62 due to the genotype's excess of PR proteins [228]. Proteomics may therefore improve our understanding of the unknown proteins linked to numerous signal transduction pathways that cause host innate immunity in grain legumes to be triggered in response to FW attack.

Metabolomics is the complete study of metabolites that participate in many cellular processes in a biological system. The total collection of metabolites generated by metabolic pathways in the plant system is referred to as the "metabolome", instead [236,237]. The early metabolic system of an organism can be employed to predict its genome sequencing using metabolomics and NGS technology [238]. In one study, information was combined using the genome sequencing method (NGS) and metabolite measurement method (MS) to generate crop enhancement methods [239]. This can improve our understanding of how plants respond metabolically to stress via contact with pathogens or under stress.

Our understanding of many metabolites, hormonal interactions, and signaling components associated with plant defense systems against FW infection in agricultural plants, including grain legumes, may facilitate the development of resistant cultivars [228]. Hexokinase, trehalose, invertase, sucrose synthase, -amylase, and glucose-6-phosphate are examples of sugars that are generated in the reaction to FW [240]. These sugars act as an oxidative burst substrate, supplying energy, generating ROS, acting as a signaling molecule in coordinate on with various phytohormones, and enhancing lignification of the cell wall in order to activate plant innate immunity, and plays a crucial role in plant defense against pathogen attacks [241,242]. There are many different proteins that are involved in the TCA and glycolysis processes in Digvijay, as well as defense-related metabolites such as endo beta-1,3-glucanase, caffeic acid O-methyltransferase, chitinases, and caffeoylCoA O-methyltransferase; phytoalexins such as luteolin, genistein, and quinone; and phenolic compounds, including flavonoids [228]. A considerable decrease in specific amino acids and carbohydrates, like sucrose and fructose, in a vulnerable crop enables FW pathogens to enter and hasten the development of disease [228].

The function of PR proteins, chitinases, ROS activating enzymes, flavonoids, phenolic compounds, and phytoalexins in conferring wilt resistance is further supported by thorough analyses of plant transcriptomes, metabolomes, and proteomes in response to FW disease [243–245].

## 9. Genomic Selection (GS)

A promising method called genomic selection (GS) uses molecular genetic markers to create new breeding programs and new marker-based models for genetic valuation [246]. It offers chances to boost the genetic gain of complex traits per unit of effort and expense in plant breeding. For GS, weighing the pros and cons of working in crop plants is crucial. The most crucial elements for its successful and efficient application in crop species are the availability of genome-wide high-throughput, affordable, and flexible markers, and its low as certain bias, suitable for large population sizes, as well as for both model and non-model crop species with or without the reference genome sequence [247]. However, in order to achieve evaluable genetic gain from complex traits, these marker technologies may be paired with high-throughput phenotyping.

Most of the molecular markers, which have both large and small marker effects, are what determine the GS. Molecular markers are chosen based on their total genome coverage, and all QTLs should be in linkage disequilibrium with at least one marker [248]. The training population and the testing population are two separate sorts of populations that are employed in GS. The testing population, which is related to the breeding population, is used to estimate the genomic selection model parameter. A testing population is a population group in which genetic selection is employed.

One important issue with marker-assisted selection is that it can only target significant QTLs or genes. It is now commonly acknowledged that a multitude of genomic regions, each of which has just a tiny amount of genetic control, are involved in many complex traits, such as yield or broad-spectrum disease resistance. In many situations, it is highly advantageous to select for all or a few QTLs linked to the desired characteristic [249]. In this case, genomic selection, which has the capacity to capture several genes with minor additive effects, could prove beneficial for crop breeding. Genomic prediction, which relies heavily on the availability of high-throughput genotyping, along with accurate phenotyping data, is the key to success in GS breeding [90]. GS + de novo GWAS and haplotype-based GS + de novo GWAS approaches, together, have potential for developing capable chickpea genotype(s) [90].

## 10. In Vitro Selection against Fusarium Wilt Disease Tolerance/Resistance in Chickpea

Both biotic and abiotic stressors have a significant impact on legume crops. Therefore, it is essential to undertake efforts to cultivate plants that are tolerant to stress in order to increase agricultural yield. Growing stress-tolerant plants using tissue culture-based in vitro selection has become a practical and economical approach in recent years [250–252]. Applying selective agents to the culture media, such as pathogen culture filtrate [253], fusaric acid phytotoxin [254] or the pathogen itself (for disease resistance)—NaCl (for salt tolerance), and PEG [255] or mannitol for drought tolerance—may aid in the development of plant tolerance to both biotic and abiotic factors. Many efforts have been made in this respect for the screening and development of chickpea cultivars [256].

The optimal outcome depends on the availability of an appropriate selection agent. Fungal culture filtrate or a well-known toxin, such as oxalate acid or fusaric acid, are typically utilized as the selection agents [257]. In vitro pathogen resistance selection is possible by including a phytotoxin, such as fusaric acid, that is unique to the host. Fusaric acid ($C_{10}H_{13}O_2N$), a metabolite generated by many strains of *Fusarium oxysporum*, is employed as a "selecting agent" for cell culture and callus culture to stop the germination of fungus. In comparison to plants derived from tissue culture without selection, several pathogen-produced non-specific phytotoxins, such as deoxynivalenol (DON), crude pathogen culture filtrate, or sometimes, the pathogen itself, have been shown to increase the frequency of resistant/tolerant plants [258]. Because there is a link between toxin tolerance and disease tolerance, toxin or filtrate can be used to make an agent decision based on reality. By exposing somatic embryos, shoots, embryogenic calli, or cell suspensions [259,260] to pathogen toxins, pathogen culture filtrate, or the pathogen itself, these selections can be made.

*Fusarium oxysporum* cultural filtrate affected the levels of total peroxidase, phenol, and beta 1, 3 glucanase in chickpea and reduced callus growth [261]. Resistance was apparent in chickpea plants that had grown back after being exposed to culture filtrate (*Fusarium oxysporum*) [262]. According to research conducted by Hamid and Strange [257] on the relationship between disease and the susceptibility of chickpea shoots to toxins (Solanapyrone A, B, and C) and the culture filtrate of *Fusarium oxysporum* (*Ascochyta rabiei*), the enzyme glutathione s-transferase may prove useful for boosting resistance.

## 11. Speed Breeding in Chickpea Improvement

Crop varieties that are resistant to disease can be developed using plant breeding techniques [258,259]. In order to protect global food security, it is urgently necessary to increase the existing pace of genetic gain in key food crops [260,261]. This may be helpful in the fast transfer of desired genes [262]. Lengthy breeding cycles/generations are mostly to blame for the poor advances in crop improvement [263]. Traditional/conventional breeding methods may not be sufficient to meet the demands of future generations. Speed-breeding approaches are increasingly applied at large/small scales to obtain rapid genetic gain in several crop species in order to overcome the limitations associated with traditional methods and to ensure food security [264]. Crop varieties can be developed more quickly through speed breeding. This involves a synthetic habitat that has longer daylight hours

to extend the growing season and aid in the manipulation of photo insensitive crop life cycles [265].

The rapid generation cycling methods of double haploids [266], the in vitro culturing of immature embryos [267], the embryo rescue technique [268], and other methods have not been successful in the chickpea. Three generations per year in short-season conditions were supported in the first report on chickpea rapid generation development [269]. It may be advantageous to increase production and reduce life cycles using the recently established "speed breeding" technique in chickpea, which could let researchers conduct more generations per year [270,271]. In the pigeon pea plant, a rapid generation advancement approach, which showed 100% germination from immature seeds taken from 35-day-old plants, opened new possibilities for developing three to four generations in a year [272].

The induction of early blooming and the germination of immature seeds in a more recent study on chickpeas resulted in a shorter seed-to-seed cycle period [273]. A system for growing chickpeas in greenhouses with artificial light but no growth regulator has been developed. In this technique, the photoperiod must be extended to 22 h using a temperature-controlled greenhouse with working high-pressure lamps. This greenhouse provides for precise control of temperature, humidity, and lighting. Immature seeds were germinated at 20–23 days after flowering (DAF) to further shorten the generation cycle, and the photoperiod was increased to encourage early flowering. There were six accessions used, with two each from the early, medium, and late maturity groups. Six or seven generations occurred annually. This method may also be adopted for the screening of wilt-resistant plants, as it may save time.

According to Fikre and Tulu [274], a unique field-based rapid generation cycle strategy may increase breeding effectiveness and hasten the release of improved chickpea varieties for the farming community. Compared to other rapid generation progress technologies that require expensive infrastructure, the strategy is easy to use, effective, and requires little investment. Importantly, the field-based rapid cycle technique for chickpeas is best suited for breeding operations located in tropical and subtropical areas, where the climate allows for chickpea development all year round. However, because this strategy includes propagating plant generations outside, it is crucial to implement risk management procedures to safe guard priceless breeding resources from severe weather conditions and wildlife. Speed breeding strategies may also be applied to the development of Fusarium wilt-resistant chickpea varieties.

## 12. Conclusions

Biotic stressors significantly decreased the yield of the leguminous crop. After yield improvement, resistance to FW one of the most important breeding goals of crop improvement programs for chickpea. The development of efficient, innovative, conventional, and molecular breeding technologies can be used to strategically control breeding for FW resistance. This review has covered the many approaches that may be utilized to locate and incorporate novel wilt resistance gene in chickpea. The capacity to apply a QTL mapping strategy for the genetic study of stressors in chickpeas was made possible by recent advancements in the utilization of molecular marker technologies and the availability of high-density genetic maps. Draft chickpea genome sequences have since been made public. Due to the significantly increased chickpea genomic repertoire, we have a fantastic opportunity to examine the unique genetic determinants/haplotypes governing this stress across the full genome level using genome-wide association studies (GWAS). Several marker-assisted breeding methods, including MABC and MARS, are now being applied in chickpea breeding programs. To understand functional analyses, the molecular mechanisms of genes, and gene networks, these omics approaches—genomics, transcriptomics, proteomics, metabolomics, ionomics, and phenomics—have been employed. There is an urgent need for the identification of transcription factors that play an important role in limiting the pathogen activity of *Fusarium oxysporum* in the soil, as well as in chickpea. This review outlines approaches that can be used to manage the effect of FW on chickpea

production and suggests recommendations for improving chickpea wilt-resistant breeding programs. The adoption of these approaches is anticipated to be given more prominence in future breeding programs. This review includes information on the biotic limitations of chickpea production and future directions.

**Author Contributions:** Conceptualization, M.K.T., R.K.Y., and S.T.; writing—original draft preparation, R.K.Y., R.A., and V.P.; writing—review and editing, M.K.T., N.T., R.S.S., and D.K.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Current Epidemic Situation and Control Status of Citrus Huanglongbing in Guangdong China: The Space–Time Pattern Analysis of Specific Orchards

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**Abstract:** Huanglongbing (HLB) is the most harmful bacterial disease in citrus production in the world, and has been seriously ravaging the citrus groves of South China since the 1930s. The surveillance of the epidemiological characteristics of HLB is of utmost priority for citrus production in this region. In order to explore the effects of disease control measures, analyses on the space–time statistical features of the HLB epidemic, from 2019 to 2021, within six orchards in the Guangdong province are presented. Overall, the number of citrus plants in the orchards usually slightly decreased year by year. The reduction was mainly related to the level of plant susceptibility, which is correlated with citrus varieties. The maximum disease severity (incidence and race increment) was correlated with the awareness of this disease and the management intensity applied by the manager. A higher disease index was found in the conventional management orchards than in the comprehensive prevention and control orchards. Proper insect-protective screen houses can effectively prevent the epidemic of HLB, without affecting the fruit quality, and can also aid with higher yields. A high correlation was found between the geometry and topography of orchards and the HLB epidemic due to the wind direction from May to September and the Asia citrus psyllid activity characteristics. For flat orchards, the incidence of HLB in the north and entrance areas was higher than that in the southwest. In the mountain area, the incidence of the windward side in the south was higher than that of the leeward side in the north. Diseased trees tended to have an edge effect in the grove, whereas the trees of the same disease scale were found clustered in their distribution. These results allow a better understanding of HLB epidemiology and provide guidance for the early warning of HLB in new groves in areas that are severely affected by this disease. Furthermore, they also provide a scientific basis for the comprehensive prevention and control of HLB in old groves.

**Keywords:** surveillance; *Candidatus* Liberibacter asiaticus; comprehensive control; distribution; screen house

**Citation:** Zhang, J.; Liu, Y.; Gao, J.; Yuan, C.; Zhan, X.; Cui, X.; Zheng, Z.; Deng, X.; Xu, M. Current Epidemic Situation and Control Status of Citrus Huanglongbing in Guangdong China: The Space–Time Pattern Analysis of Specific Orchards. *Life* **2023**, *13*, 749. https://doi.org/10.3390/life13030749

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 2 February 2023
Revised: 4 March 2023
Accepted: 5 March 2023
Published: 10 March 2023



## 1. Introduction

Citrus, including oranges, tangerines, grapefruits, pomelos, lemons, limes, etc., is a perennial grafted crop of the most planted and produced fruits in the world (https://www.statista.com, accessed on 5 January 2023). Moreover, citrus Huanglongbing (HLB) is one of the most crucial and devastating diseases in the citrus industry worldwide. It was included as one of the top ten crop diseases by the Ministry of Agriculture and Rural Affairs of China in 2020. HLB causes the yellowing of new shoots, the mottling of fully mature old leaves, or a zinc-deficiency-like symptom in mature fresh leaves; however, these symptoms occur with no programmed cell death (PCD), such as necrosis. Among these, the mottling leaf is the most typical symptom to identify HLB. Compared to normal healthy fruits, the most reliable diagnostic symptoms of affected fruits are being improperly colored (greening or

'red nose fruit'), being lopsided with a curved columella, and developing mostly aborted seeds. These phenomena of the various symptoms mentioned are particularly prevalent in Guangdong, as HLB ravages the citrus industry there [1–3].

Huanglongbing is caused by phloem-limited bacteria, specifically, the '*Candidatus* Liberibacter spp.' of α-proteobacterium [4]. Three distinct Liberibacter species are related to HLB. The HLB caused by '*Candidatus* Liberibacter asiaticus' (CLas) have a wider geographical spread, are more severe, present in lower elevations, and possess higher temperature tolerance [5]. Further, it is vectored by Asia citrus psyllid (*Diaphorina citri* (Kuwayama)) [6] in a persistent and propagative manner. The HLB caused by '*Ca*. L. africanus' (CLaf) and '*Ca*. L. americanus' (Clam) are more restricted, less severe, and more temperature-sensitive [7]. CLas is the only species detected from HLB-affected citrus in China [8]. After infection, CLas quickly colonizes the root system before canopy symptoms develop. The upward movement of CLas from roots to canopy is linked to seasonal flushes and the CLas population [9]. Globally, HLB has been distributed in 64 countries within Asia, Africa, North America, South America, and Oceania (data accessed on 20 December 2021 from Center for Agriculture and Bioscience International (CABI)). The pathogen, CLas, is widely distributed in Asia and North America, and partially distributed in Africa [10–12]. Though CLam is only partially detected in Brazil [13], there has been a shift in the prevalence of CLam to CLas, which has been recently observed [7].

Citrus cultivation was highly developed early last century in the Pearl River Delta and the Hanjiang Delta in the Guangdong province of China [14]. As determined from the tonnage data of citrus production, the citrus industry peaked in 1933; however, a significant drop occurred in 1934 in several areas, including the Chaoshan area in Hanjiang Delta. According to Lin Kong-Hsiang's publications [1], the HLB epidemic in the Chaoshan area could be traced back to as early as the 1870s, despite its, then, slight prevalence status. Whereas the first report of this disease was most likely recorded by Reinking [15] after a disease survey along the Pearl River Delta and West River, it became a serious disease from the 1930s onwards in Guangdong, with the citrus production cycle becoming shorter as a result [1,14–19]. The report and incidence of HLB in the Fujian and Guangxi provinces was a little bit later than those found in Guangdong. Nonetheless, the HLB in these three provinces has been considered the most widely distributed and the most serious since the mid-20th century. From the late 1970s until the 1980s, HLB became a serious issue in Sichuan, Jiangxi, Yunnan, Hainan, and Taiwan. It then spread into Hunan, Guizhou, and Zhejiang [19] after 1980. Until now, 11 of the 19 citrus cultivation provinces and regions in China, accounting for more than 80% of the total citrus cultivation area, had been damaged by HLB [19].

In recent years, the damage caused by HLB in the Guangdong Province has been increasing; further, it is most likely that the shortened citrus production cycle from planting to replant in the whole orchard is a reflection of this fact. The citrus producers summarized a famous 'ten-year cycle', which means that the trees in the orchards would be replaced every 10 years. This would be performed because most trees were affected and, thus, were producing limited valuable fruits. Take the famous Yangcun citrus farm in Huizhou, which is the largest (2000 $hm^2$) citrus farm in Asia and was established in 1951–1953, as an example. The farm experienced two large-scale disease tree eradications from 1979 to 1982 and during 1996–1999. The production of the fresh fruits reached its peak in 1977 and 1991, but dropped significantly in 1982 and 2000 [17]. Since the ravages of HLB from the 1970s, the citrus production in this area cannot avoid the 'ten-year cycle'. After that, the most famous local varieties, e.g., the *Citrus reticulata* 'Shatang' tangerine in Sihui, the *C. sinensis* Osbeck cv. 'Hongjiang' orange in Zhanjiang, and the *C. reticulata* Blanco 'Tankan' in Puning, were also almost destroyed at the beginning of this century. A recent survey in 2016–2017 concluded that citrus groves in all 17 cities and 65 counties in the Guangdong Province were affected by HLB, and around 59,700 hectares of citrus were affected, accounting for about 25% of the total citrus planting area [20]. Currently, HLB still seriously restricts the development of the citrus industry in Guangdong. However, with more attention being

paid recently via government policy toward the comprehensive prevention and control of HLB, the resumption of citrus production has been promoted. More growers prefer large-scale groves, where standardized management with higher technical specifications is adopted.

Recorded systematic investigation on the occurrence of HLB in the field were mainly concentrated around the 1950s and 1980s [17]. Most of those studies focused on the annual increase and removal of diseased trees in orchards. A few of these investigations analyzed the correlation between geographical environment conditions and the prevalence of HLB. Quite a few studies have analyzed the relationships between HLB prevalence results, and cultivation and management measures. With the development of molecular biology technology, after the pathogen of HLB was identified as a type of bacteria [4], the research hotspots have shifted from disease epidemic to the molecular interaction among the pathogen–host–vector. Although HLB has been ravaging the citrus industry of Guangdong Province continuously, no studies have systematically investigated the annual prevalence of HLB within specific orchards in the last 30 years.

In recent decades, mathematical models play an important role in understanding the epidemiology of HLB [21–24]. However, most of the dynamic behaviors of these models are studied by only using computer simulations or are only understood by professional persons. In this study, space–time dynamic (year-by-year) point pattern measures were applied to highlight the HLB progression over time in the groves of Guangdong. All the screened orchards were larger than 5 hectares and with different management levels. A 'two-to- three year' survey was conducted for the appearance and degree of HLB symptoms on each tree within these groves for the appearance and degree of HLB symptoms. In this study, the aims are to provide guidance for the early warning of HLB, to enhance the prevention awareness of growers, and to provide suggestions on the specific effective measures for the prevention and control of HLB in areas where HLB is severely endemic.

## 2. Materials and Methods

### *2.1. Orchard Information and Survey Methods*

To reduce the impact of spatial heterogeneity, the research area is located in South China, specifically in the province of Guangdong. Six orchards in Zhaoqing City, Huizhou City, Guangzhou City, or Meizhou City were selected as the survey sites. Only one Liberibacter species (CLas) was detected in these areas. The tree density of the orchards was similar in all orchards, except for orchard 1, wherein the cultivar within it was usually planted at a high density. The information on the selected orchards is shown in Table 1. The A1 region of orchard 4 was under semi-natural conditions in a screen house. Orchard 5 and orchard 6 contain both sloping plots and non-sloping plots. In the conventional management orchards, the diseased trees were not timely monitored or rouged, and attention was not paid to killing the psyllid. Comprehensive control is taken to mean integrated management concepts, which combine cultural, chemical, and biological control measures that are conducted between 9 and 12 times in a timely manner with pesticide application.

Investigations were carried out via visual inspection in Autumn and Winter from 2019 to 2021, when the diseased citrus varieties showed their apparent symptoms (Figure A1). During each survey, 12 experienced Citrus Huanglongbing Research Laboratory members participated, with 2 persons in a group. Each investigator collected at least 20 leaf samples which were visually inspected as HLB–affected. Further, another 20 samples evaluated as healthy were collected each day. DNA was extracted from the randomly collected samples and amplified by RT-PCR for CLas detection [25]. If the PCR results of 95% of the collected samples were confirmed to be consistent with the visual inspection, the data from the survey were used for further analysis. Otherwise, the surveyed plot would be assigned to another pair of investigators. In addition, the CLas-positive samples were assigned for genetic diversity analysis by conventional PCR and RT-PCR based on the phage types (three type-specific prophage loci), a miniature inverted-repeat transposable element (MITE) region (CLIBASIA_05620 ~ CLIBASIA_05625), and their short tandem

repeat genes (CLIBASIA_03080 and CLIBASIA_01215) [26]. After collecting the band information of conventional PCR or Ct values of RT-PCR according to Zheng et al. [26], data were further used for diversity analysis of the CLas strains. Cluster analysis of the CLas populations in the six orchards was completed by Popgene v. 1.32 (https://www.ualberta.ca/~fyeh/popgene.html, accessed on 3 March 2023) based on Nei's (1972) genetic distance. The cluster map was generated in MEGA v. 11.0 [27].

**Table 1.** Information of the surveyed citrus groves.

| Location | Orchard No. | Management Methods | Cultivar | Survey Area (ha) | Geographical Coordinates |
|---|---|---|---|---|---|
| Deqing County, Zhaoqing City | 1 | Conventional management | *C. medica* L. var. *sarcodactylis* Swingle 'fingered citron' | 5 | 112°12′ E, 23°19′ N |
| | 2 | Conventional management | *C. reticulata blanco* var. 'Gongkan' | 10 | 113°5′ E, 23°13′ N |
| | 3 | Comprehensive control | *C. reticulata blanco* var. 'Gongkan' | 20 | 111°48′ E, 23°15′ N |
| Boluo County, Huizhou City | 4 | Conventional management or Comprehensive control | *C. reticulata* Blanco 'Shatangju' *C. maxima* 'Mi Yu' | 8 | 114°28′ E, 23°29′ N |
| Conghua District, Guangzhou City | 5 | Comprehensive control | *C. reticulata* Blanco 'Wokan' | 16 | 113°29′ E, 23°38′ N |
| Dapu County, Meizhou City | 6 | Comprehensive control | *C. maxima* 'Shatian Yu' and 'Mi Yu' | 10 | 116°41′ E, 24°22′ N |

The scales (0, 1, 2, and 3) of the diseased trees were recorded based on the severity of the disease, wherein scale 0 indicates non-HLB-affected trees and scale 1 indicates that 1/3 of the canopy is affected by HLB, etc. The 'two-step-path' app (Shenzhen 2bulu Information Technology Co., Ltd., Shenzhen, China) was applied to record the path/track of the survey, to map the diseased trees in space and to record the time of the surveys. The data from the 'two-step-path' app were viewed and exported by a LocaSpaceViewer4 PC (Zhongke Tuxin Technology Co., Ltd., Suzhou, China). These procedures resulted in the development of space–time point pattern survey maps of the different scaled symptomatic citrus. Counting was carried out in each survey for the total number of trees, the number of those which were removed, and the number of infected trees. The incidence of the latter three was calculated by normalizing the counts to the total number of plants in the orchard.

### *2.2. DNA Extraction and RT-PCR*

The midribs of collected leaf samples were cut into small pieces with sterilized blades. In addition, 0.05 g of them were ground by a FastPrep tissue homogenizer (MP Biomedicals, Irvine, CA, USA). A subsequent extraction of DNA was performed using the E. Z. N. A. HP Plant DNA Kit (Omega Bio-tek., Norcross, GA, USA), according to the manufacturer's protocol. The concentration and purity of the DNA samples were determined using a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific, Shanghai, China).

The RT-PCR assays for the CLas detection were performed with a primer set (CLas4G/HLBr) and a probe (HLBp) according to a previous study of our lab [28]. The 20 µL of PCR mixture contained 1 µL of a DNA template (~25 ng), 10 µL of a Bestar qPCR Master Mix (DBI Bioscience, Shanghai, China), 0.2 µL of PCR Probe (10 µM), 0.4 µL of each forward and reverse primer (10 µM), and 8 µL of DNase/RNase-Free dd$H_2O$. Standard TaqMan thermocycling conditions were used: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 58 °C for 30 s, with fluorescence signal captured at the end of each 58 °C step. All PCR assays were run in triplicate in a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). The data were analyzed using Bio-Rad CFX Manager 2.1 software with the automated baseline settings and threshold.

### *2.3. Measurement of Citrus Canopy, Yield, and Juice Quality*

In order to assess the effects of a protective screen on the growth of citrus plants, the A1 and A2 areas in orchard 4, with and without the coverage of an insect-proof screen, were divided into 5 blocks (i.e., south, west, north, east, and center). Two healthy and two HLB-affected trees were selected, respectively, from each block of the two regions. The height and crown diameter were measured monthly using a tape, for a year. The height measurement of each tree was performed three times in three directions, i.e., the south–north, 45° to the south–east, and 45° to the south–north. The diameter of each tree was represented by the average data extracted from the two measurements in the directions of south–north and east–west. The crown surface area (S) was estimated via using the formula of $S = 4\pi R^2$. In the formula, R was calculated by the average height and diameter.

To analyze the influences of the HLB and the semi-natural cultivation model on the yield, a total of 20 healthy trees and 20 diseased trees were selected from different blocks. Fruits from every selected tree were collected and weighted with ten replications. As the HLB symptoms are often sectored within a tree, only visually symptomatic fruits from the diseased trees were selected for fruit quality assessment. The following morphological characterizations were conducted: single fruit weight (FW); the fruit transverse and longitudinal diameters (FTD and FLD); outer pericarp thickness (OPT); outer pericarp weight (OPW); fruit shape index (FSI); fruit firmness (FF); concentration of vitamin C; total soluble solids content (TSS); and the total titratable acids (TA). The FSI was calculated by the following formula: FSI = FTD/fruit surface area × 100. Puncture and compression tests were based on a texture analyzer, which were used to assess FF. The fruit mass rate and outer pericarp rate were also obtained.

Collected fruits were transported to the laboratory and temporarily stored at 4 °C. After peeling, the hand-pressed juice was filtered through four layers of sterile gauze pieces, and then collected into sterile containers. The content of vitamin C was measured in the freshly squeezed juices via a 2,6-dichlorophenol indophenol redox titration method [29]. The TSS and TA were determined by a sugar acid digital display refractometer (PaL-BXIACID F5, Atago Co, Tokyo, Japan).

The color values of fruits on the surface were evaluated using a chromameter tristimulus color analyzer, which was calibrated with a white porcelain reference plate. Each fruit was measured six times in the upper, middle, and lower parts. The apparatus calculated and returned the three color parameters from the spectra. The color coordinates of the uniformed color space L, a, b, and hue angle (H°) were determined [30]. The L values (ranging from 0.09 to 107.26) represent the luminosity. Both a and b values represent different colors, with a values ranging from −60 (green) to 60 (red), whereas b values range from −60 (blue) to 60 (yellow). The H° = hab = arctg (b/a) is the qualitative attribute that graded any color as reddish, greenish, etc.

### *2.4. Statistical Analyses*

The morphological characterization (FW, FTD, FLD, OPT, etc.) and the quality data analyzed were collected and averaged using Microsoft Excel software (Microsoft, Redmond, Washington, DC, USA). The minimum, maximum, and mean values; the standard deviations (SD); and the coefficient of variation (CV) were calculated separately for the measured traits among the individuals of the different groups' fruits. F-test, one-way ANOVA, and S Shapiro–Wilk tests were performed for the relevant data sets. To test whether the traits of the healthy and diseased fruits, both inside and outside the screen house, differed significantly, we ran independent-sample *t*-tests. A one-way analysis of variance (ANOVA) test was used to determine the significant differences in the measured traits. A Pearson correlation coefficient was then used to determine the relationships between the traits and the infection status or the screen house coverage. The analysis of variances between the two different experimental groups was conducted with Tukey's post hoc comparison test. The data were analyzed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The bar chart was generated using the software of Origin 2021 (OriginLab Corp., Northampton, MA, USA).

## 3. Results

### *3.1. The Epidemic Characteristics of HLB Were Correlated with Management Level*

All collected DNA extracted from six orchards' CLas–positive samples were used to analyze the prophage types, short tandem repeat genes, and MITE region by conventional PCR or RT-PCR. The clustering result of these CLas strains is shown in Figure 1. The CLas population from orchard 5 (Guangzhou, China) was different from populations of other orchards, whereas the CLas populations collected from orchard 4 (Huizhou, China) and orchard 6 (Meizhou, China) were similar. Similarly, the CLas populations from orchard 1, 2, and 3 in Deqing county were also highly similar.

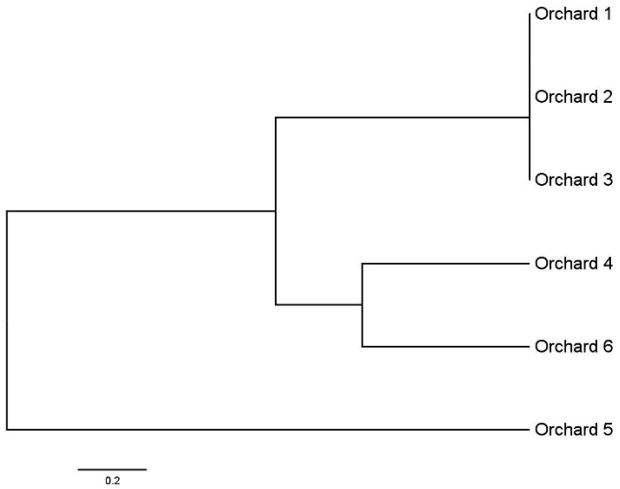


**Figure 1.** Clustering of *Candidatus* Liberibacter asiaticus population in six orchards of Guangdong Province based on six gene loci. The clustering relationship is based on Nei's (1972) genetic distance.

Of the six surveyed orchards, two were under conventional management, and three received comprehensive prevention control practices. The number of citrus plants decreased yearly in all orchards except for orchard 3. This reduction was found related to the severity of symptoms caused by HLB rather than to the management level. For example, after being affected by HLB, *C. medica* 'Fingered' and *C. maxima* (Burm.) Merr. will undergo a long asymptomatic stage and subsequently create a hidden epidemic of this disease. Thus, in three years, the number of citrus plants in the two orchards reduced from 17,964 to 17,447, and from 5893 to 5748. By contrast, the diseased trees of *C. reticulata* Blanco 'Shatangju' (from 7032 to 5277), *C. reticulata* Blanco var. 'Gonggan' (from 5326 to 4205), and *C. reticulata* Blanco 'Wokan' (from 15,171 to 14,065) were eradicated mainly due to remarkable symptoms. The affected trees of these three cultivars evidently declined in Autumn and Winter, with the leaves yellowing and falling off, and the fruits being small and deformed. The epidemic characteristics of HLB in the two 'Gonggan' orchards under conventional management (orchard 3) and comprehensive control (orchard 4) in Deqing were subsequently compared. The disease incidence rates of these two orchards differed distinctly (Figure 2). The HLB rate at the former farm rose from 15.58% (830/5326) to 30.65% (1289/4205), whereas that of the latter rose from 0% to 0.18% (21/11,623). Due to the conventional methods implemented in the former orchard, the population of *D. citri* was observed to be at a stable level during the year. Unfortunately, the owner did not have the intensive awareness to prevent and control the psyllid. As a result, the orchard was fully replanted in 2022. The latter orchard was managed by a famous company. The location of the grove was carefully selected, and the nursery stocks at the beginning of the orchard establishment were strictly inspected as to whether they were free of CLas and other viruses. Additionally, cultivation management in the later orchard was carried out according to certain technical regulations for the prevention and control of HLB (T/SHSTJ 002—2020).

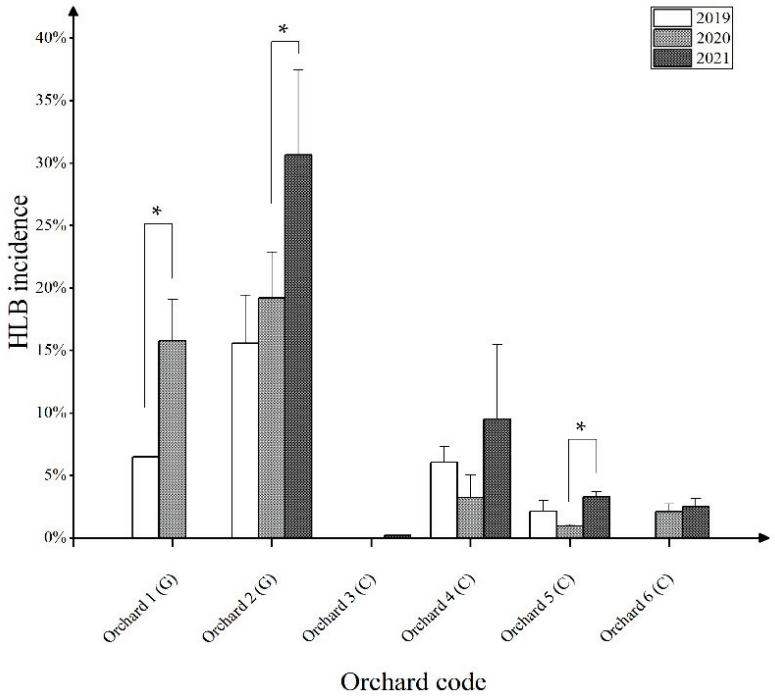


**Figure 2.** Statistical chart of Huanglongbing (HLB) incidence in the six orchards of the Guangdong Province from 2019 to 2021. G: general management; C: comprehensive control management. The data represent the means ± SD. * $p < 0.05$; (ANOVA with Tukey's post hoc test).

For the other three orchards (orchards 1, 4, and 5), which possessed a certain HLB incidence base (2–6.5%) in 2019, the disease epidemic situations differed during the investigation period (Figure 2). From 2019 to 2021, the incidence of the fingered citron orchard in Deqing (orchard 1) increased from 6.49% (1165/17,964) to 15.78% (2753/17,447); it was finally destroyed due to the damage of HLB and freezing. By contrast, the incidence of the comprehensive control demonstration orchard in Yangcun (orchard 4) decreased from 6.06% (436/7032) to 3.22% (220/6834), and then increased to 9.51% (502/5277) from 2019 to 2021. In this orchard, the sudden HLB incidence increase was related to the removal of all pomelos trees that were planted at the edge of the orchard. The *D. citri* could easily invade inward, thus leading to the quick spread of HLB; this phenomenon is called the 'marginal effect'. For the comprehensive control orchard in Conghua, the 3-year incidence of HLB was 2.13% (323/15,170), 0.94% (142/15,171), and 3.29% (478/14,065), respectively. Chemical and physical methods were used to control *D. citri* in the two comprehensive control orchards from 2019 to 2020. However, instead of chemical control, the Conghua orchard began to use biological control methods in 2021, which led to an increase in the *D. citri* population and to a spread of HLB.

### *3.2. Screen Houses Effectively Prevent the HLB Epidemic without Affecting Fruit Quality, but with an Increased Yield for Diseased Trees*

Insect-protective screen houses can effectively prevent the epidemic of HLB. Region A of orchard 4 was divided into A1 and A2, with A1 under semi-natural conditions in a screen house. In the first survey, the incidence of HLB in these two regions was 4.48% (19/424) and 5.79% (20/345), respectively. Although there were still 424 citrus plants in A1, 22 (5.18%) were affected, with a disease index of 4.36 the next year. By contrast, 38 (11.01%) of the trees were affected in the A2 region, and the disease index was as high as 8.70. Specifically, the severity of HLB and the increased rate of the diseased trees in the insect-proof net area were generally lower than those found in natural conditions. In the two regions from 2019 to 2021, the three-year average HLB incidence was 1.05 ± 0.48% and 6.58 ± 2.85%, respectively. In addition, the tree number reduction in this net-covered region was also lower (0.05% and 0.14% from 2019 to 2020, and from 2020 to 2021, respectively).

Additionally, the fruit quality indexes of both healthy and HLB-affected trees inside (Region A1) and outside (Region A2) the screen house were compared. There was no significant difference in the yield of the healthy trees in these two areas (39.27 ± 7.27 kg for A1 and 33.63 ± 4.89 kg for A2, $p = 0.149$). In contrast, the trees in A1 had a significantly higher crown surface area (103.08 ± 3.84 $m^2$ for trees in A1 and 91.09 ± 3.35 $m^2$ for trees in A2, $p = 0.016$). By contrast, the yield of the HLB-affected trees in the A1 region was significantly ($p < 0.001$) higher than those found in the A2 region (15.37 ± 0.86 kg/tree and 4.56 ± 0.21 kg/tree, respectively). For the fruits with economic value picked from the two regions, there were no significant differences in fruit quality parameters (i.e., the contents of vitamin C, TSS, TA, TSS/TA ration, and fruit juice rate) and the morpho-physiological parameters (i.e., the FW, FSI, OPT, FF, pericarp rate, and residue rate). However, the red value of the fruit peel outside the screen house (36.96 ± 0.57) was significantly higher ($p = 0.003$) than that of the fruits inside the screen house (32.51 ± 1.02), whereas there was no significant difference noted in the yellow value, color saturation, and brightness of the fruit peel between fruits inside and outside. No *D. citri* was found via the three-year monitoring in the screen house region with the yellow plate method and the knocking method. Collectively, the abundance and types of other citrus pests, as well as their natural enemies, in A1 were less than those found in A2.

### *3.3. Field HLB Incidence Is Influenced by the Direction and Latitude of the Field*

#### 3.3.1. Regional Distribution Characteristics of HLB Trees in the Flat Groves

According to the terrain, path, water channels, etc., the orchards were subdivided into multiple regions, as shown in Figure 3. Orchard 1 and orchard 2 are two conventional managed orchards in the Deqing County (Figure 3—1,2), where the citrus is densely planted on flat terrain. Regarding orchard 1, the HLB incidences of the sub-regions in 2019–2020 are shown in Table 2. The geographical position from region A to region C was from north to south, with incidences of HLB decreasing from 14.01% to 3.38% in 2019. Similarly, the incidences in region D to region E also decreased from 4.93% to 2.18%. In 2020, the HLB incidences in the northern segment were still higher than those in the southern segment. Surprisingly, in region B, which was the entrance of the orchard, the incidence increased to the highest recorded of 19.80%. In addition, the incidence also rapidly increased to 17.02% in the center (region D). However, whereas the growth rates of all other regions were higher than 10%, the incidence in region A only increased by 0.65%. Orchard 2 had a similar disease incidence pattern to orchard 1. Region A, located in the southwest, had a three-year average of 16.2% in HLB incidence, lower than those in the other regions. The annual disease growth rate of region D in the northeast was also significantly lower than those found in other regions. The disease incidence growth rates of regions B and C in the center of the orchard were higher than those in the other regions. As the entrance of orchard 2, region B still had the highest annual incidence in the whole orchard.

Although orchard 4 was also built on flat terrain, the practice of comprehensive management was still enacted. 'Shatangju', the main cultivar in orchard 4, was planted from region A to region D. However, the pomelo planted in region E was removed in the winter of 2020, due to the serious effects of HLB. There was no doubt that region D, adjacent to region E, had the highest three-year average disease incidence rate (14.8 ± 12.2%) among the whole orchard. Even when the pomelo trees had been completely removed, the HLB incidence of region D increased by 15.89%, whereas other regions did not show a significant increase.

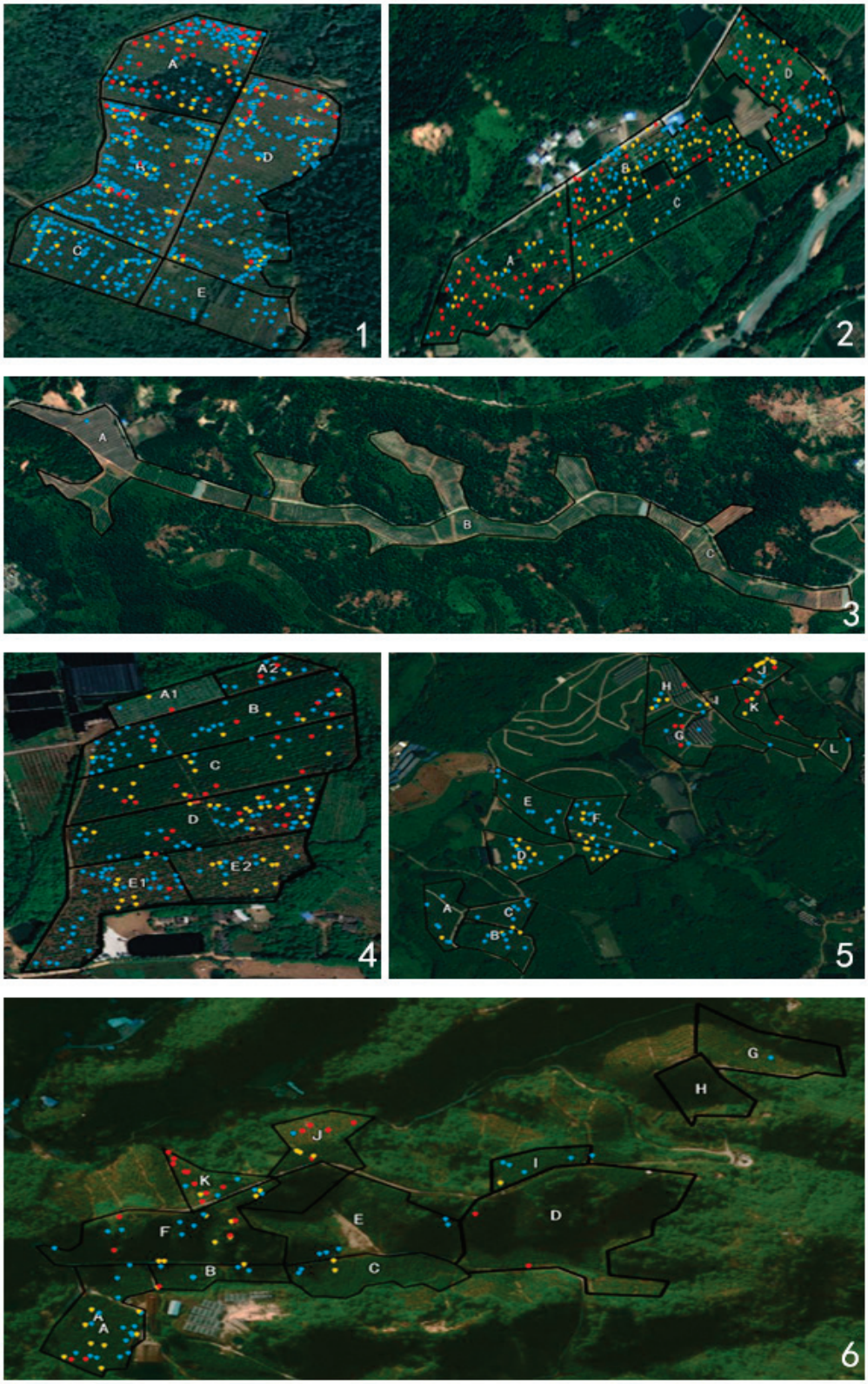


**Figure 3.** Distribution of the diseased trees in the orchards. The subfigure **1–6** correspond to the topographic of orchard 1–6 respectively. Letters A to L were different regions of the groves. The blue dots indicate scale 1 trees, that with 1/3 canopies of each tree affected by HLB. The yellow dots mean scale 2 trees, where more than 1/3 and less than 2/3 canopies of each tree were symptomatic. The red dots represent scale 3 trees, with more than 2/3 canopies affected by HLB. The codes of the groves are in accordance with those in Table 1.

**Table 2.** Cumulative annual change in the tree number and regional incidence rate of each orchard.

| Orchard and Block | 2019 | | 2020 | | | 2021 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Affected Plants | Affection Rate (%) | Affected Plants | Affection Rate (%) | Compare 1 | Affected Plants | Affection Rate (%) | Compare 2 | Compare 3 |
| 1-A | 400 | 14.01 | 413 | 14.66 | 0.65 | - | - | - | - |
| 1-B | 344 | 8.04 | 665 | 19.8 | 11.76 | - | - | - | - |
| 1-C | 142 | 3.38 | 524 | 13.64 | 10.26 | - | - | - | - |
| 1-D | 38 | 2.18 | 217 | 11.19 | 9.01 | - | - | - | - |
| 1-E | 241 | 4.93 | 934 | 17.02 | 12.09 | - | - | - | - |
| 2-A | 105 | 7.03 | 291 | 21.19 | 14.16 | 255 | 20.38 | −0.81 | 13.35 |
| 2-B | 265 | 22.23 | 268 | 29.32 | 7.09 | 409 | 49.7 | 20.38 | 27.47 |
| 2-C | 227 | 15.05 | 151 | 10.55 | −4.5 | 367 | 32.97 | 22.42 | 17.92 |
| 2-D | 233 | 20.58 | 269 | 21.3 | 0.72 | 258 | 25.34 | 4.04 | 4.76 |
| 3-A | - | - | - | - | - | 7 | 0.22 | - | - |
| 3-B | - | - | - | - | - | 14 | 0.22 | - | - |
| 3-C | - | - | - | - | - | 0 | 0 | - | - |
| 4-A1 | 3 | 0.76 | 3 | 0.79 | 0.03 | 6 | 1.6 | 0.81 | 0.84 |
| 4-A2 | 30 | 8.82 | 10 | 3.38 | −5.44 | 20 | 7.55 | 4.17 | −1.27 |
| 4-B | 43 | 2.64 | 55 | 3.44 | 0.8 | 46 | 2.94 | −0.5 | 0.3 |
| 4-C | 89 | 4.4 | 29 | 1.44 | −2.96 | 80 | 4.38 | 2.94 | −0.02 |
| 4-D | 180 | 12.23 | 59 | 4.08 | −8.15 | 350 | 28.09 | 24.01 | 15.86 |
| 4-E | 81 | 6.88 | 64 | 5.82 | −1.06 | - | - | - | - |
| 5-A | 25 | 2.21 | 9 | 1.17 | −1.04 | 16 | 1.7 | 0.53 | −0.51 |
| 5-B | 14 | 1.31 | 14 | 1.16 | −0.15 | 77 | 7.03 | 5.87 | 5.72 |
| 5-C | 7 | 0.66 | 7 | 0.88 | 0.22 | 46 | 4.44 | 3.56 | 3.78 |
| 5-D | 28 | 1.22 | 30 | 1.37 | 0.15 | 163 | 7.93 | 6.56 | 6.71 |
| 5-E | 19 | 1.15 | 14 | 0.84 | −0.31 | 42 | 2.6 | 1.76 | 1.45 |
| 5-F | 4 | 0.26 | 20 | 1.12 | 0.86 | 26 | 1.67 | 0.55 | 1.41 |
| 5-G | 16 | 1.41 | 10 | 0.84 | −0.57 | 12 | 1.04 | 0.2 | −0.37 |
| 5-H | 45 | 2.57 | 8 | 0.57 | −2 | 21 | 1.43 | 0.86 | −1.14 |
| 5-I | 21 | 1.81 | 1 | 0.05 | −1.76 | 28 | 2.62 | 2.57 | 0.81 |
| 5-J | 89 | 14.59 | 19 | 2.98 | −11.61 | 31 | 4.96 | 1.98 | −9.63 |
| 5-K | 30 | 2.56 | 9 | 0.77 | −1.79 | 11 | 1.07 | 0.3 | −1.49 |
| 5-L | 25 | 4.17 | 1 | 0.26 | −3.91 | 5 | 1.21 | 0.95 | −2.96 |
| 6-A | - | - | 27 | 2.45 | - | 29 | 2.57 | 0.12 | - |
| 6-B | - | - | 7 | 1.89 | - | 11 | 2.84 | 0.95 | - |
| 6-C | - | - | 4 | 0.63 | - | 7 | 1.05 | 0.42 | - |
| 6-D | - | - | 3 | 0.38 | - | 7 | 0.86 | 0.48 | - |
| 6-E | - | - | 24 | 2.49 | - | 25 | 2.65 | 0.16 | - |
| 6-F | - | - | 25 | 3.41 | - | 29 | 3.84 | 0.43 | - |
| 6-G | - | - | 1 | 0.81 | - | 2 | 1.57 | 0.76 | - |
| 6-H | - | - | 0 | 0 | - | 2 | 0.89 | 0.89 | - |
| 6-I | - | - | 6 | 2.47 | - | 8 | 3.42 | 0.95 | - |
| 6-J | - | - | 10 | 2.93 | - | 14 | 3.87 | 0.94 | - |
| 6-K | - | - | 13 | 5.6 | - | 12 | 4.98 | −0.62 | - |

The orchard number is consistent with Table 1, and the partition number is shown in Figure 3; Compare 1 refers to the difference between the incidence rate in 2019 and 2020; Compare 2 refers to the difference between the incidence rate in 2020 and 2021; and Compare 3 refers to the difference in incidence rate between 2019 and 2021. The dashes mean no deteced diseased trees.

#### 3.3.2. Regional Distribution Characteristics of HLB Trees in the Groves Established along Mountains

Field HLB incidence is influenced by altitude. For orchard 5 and 6, the citrus was planted in both the mountainous and flat areas. Orchard 5 was divided into 12 regions (Figure 3), among which the plot consists of four regions (A, B, C, and D) that were in the southwest direction and had the lowest altitude. Accordingly, the three-year average HLB incidence of these four blocks was relatively higher (2.81 ± 2.67%), and the yearly growth rate was also noted as the highest. Regions G, H, I, and J are the steep slope at the north-east of the orchard, with the most elevated altitude. The highest HLB incidence was found in region J (7.51 ± 6.21%). The terrain of orchard 6 was complex, but generally showed a higher disease incidence in the west than in the east. This orchard was divided into highlands (I, J, and K), slopes (D, E, and F), and lowlands (A, B, and C). The HLB incidences of these three parts had a rising trend from north to south: specifically, region C at 0.63% to region A at 2.45%, region D at 0.38% to region F at 3.41%, and region G at

0.81% to region K at 5.60%. Regarding the survey in 2021, the incidences did not increase significantly when compared to those found in 2020.

### *3.4. Disease Scales Are Related to the Management Measures Intensity*

The severity of the diseased trees was found to be related to the management levels. Diseased plants in each comprehensive control orchard were mainly on scale 1, followed by scale 2, and then scale 3. However, the numbers of diseased trees at all three levels in the general management orchard were similar (Table 3) ($p > 0.05$). On the map generated by LocaSpace Viewer 4 (pc) (Figure 3), plants of scale 1 and scale 2 were found to be concentrated, whereas the scale 3 trees were mainly distributed at the edge of the region. Interestingly, there were only a few diseased plants of other grades around these plants of scale 3.

**Table 3.** Number of HLB-affected plants with different severities in the different orchards.

| Orchard Code | Disease Severity Classification | | | Mean ± SD |
|---|---|---|---|---|
| | L1 | L2 | L3 | |
| 2 | 310 | 276 | 305 | 297 ± 18.4 a |
| 4 | 136 | 56 | 28 | 73.3 ± 56.0 b |
| 5 | 67 | 62 | 13 | 47.3 ± 30.0 b |
| 6 | 59 | 33 | 28 | 40 ± 16.6 b |
| Mean ± SD | 143 ± 116.6 a | 106.8 ± 113.5 a | 93.5 ± 141.2 a | |

L1 represents trees with one third of the branches being affected by Huanglongbing; L2 represents trees with more than one-third, but less than two-thirds, of the branches being diseased; L3 means more than two-thirds of the branches of the diseased tree show Huanglongbing symptoms. Values with the small letters, a and b, are significantly different across the line columns. The data represent the means ± SD. ANOVA with Tukey's post hoc test, at a significance level of 0.05.

HLB spreading characteristics were generated by three years of data acquired from the orchards. Diseased plants were mainly densely distributed at the edge of the field (e.g., beside the canals and along the roads), but were also sporadically distributed in the center of the block (Figure 3). We speculate that HLB spreads from the edge of the field to the center of different blocks. For example, in orchard 4, regions D and E are at the edge of the field. Diseased trees were clustered in both blocks and were considered as two edge disease centers from where HLB spreads outwards. Likewise, HLB has also spread from the end of the block. The disease trees gathered to the end where most trees were disease-free. For example, in area B, HLB trees were gathered in the north end in 2019. A spread of diseased trees from north to south was found there the following year. Similarly, this also happened in the east of block C.

## 4. Discussion

Yellowing and mottling are two characteristic leaf symptoms that occur after being affected by HLB. In China, the yellowing symptom was more prevalent before the 1960s, whereas mottling became more and more common since the 1970s. This may be explained by cultivar conversion, cultural practice changes, and varied environmental factors [17]. In addition, a shift in the prevalence of CLam to CLas has been observed in citrus orchards in Brazil since 2010 [7,31,32]. These HLB-related shifts (symptoms and pathogen species) could be traced and explained by data generated by yearly surveys, such as in this study of a sufficiently long period, which is also a common means of plant disease epidemiology.

Analyzing the genetic diversity of CLas populations based on polymorphic gene loci will provide important information to guide HLB control, as some CLas strains were found newly imported with the seedlings [33]. In this study, only one CLas was detected in the six orchards. In addition, we previously indicated that the pathogens from these cities were genetically similar based on six gene loci [34]. Here, we specifically analyzed the six CLas populations from the six orchards. CLas strains from the same city were clustered in a bunch. However, although Guangzhou (orchard 5) is next to Huizhou (orchard 4),

there is a certain genetic distance between the two CLas populations. Moreover, the CLas populations from Huizhou (orchard 4) and Meizhou (orchard 6) were genetically similar, whereas these two cities were geographically far apart. We suggest that the epidemic of HLB needs to consider more factors, such as the source of the seedlings.

Regardless of the incidence or severity of HLB, the orchards that were implemented with comprehensive methods were in a much better situation than those which were implemented with conventional methods on the premise that the planting scale did not change. This proves the importance of scientific and effective prevention and control methods. Comprehensive management could effectively decrease the rate of novel infection as the average relative control efficacy reached 95.53–99.34% in an assay carried out by Wang et al. [35] for 3 years in Shunchang County, Fujian. Similarly, Yu et al. [36] were involved in an investigation regarding the incidence of citrus HLB in groves under integrated management measures, and they conducted this without any technical measures from 2002 to 2019 in the Zhejiang province. The results show that HLB could be effectively controlled with 6 years of comprehensive management. The continuous removal of HLB trees and the replacement of new trees were noted as the most economical and effective way to control this disease for almost ten years [37]. Nonetheless, even at a time when no affected trees were presented in the orchards, this does not summarily mean that the disease is under control. Instead, it may also be the case that there will be a breakout soon [38]. However, most growers still have no awareness of the scientific prevention and control of this disease. In fact, they still insist on partially or temporarily keeping the infected trees, even if for its only, in actuality, quite limited economic value. There is no doubt that these orchards were destroyed, which resulted in confusion for some other managers and growers with respect to doubting the continuous effectiveness of the 'three-pronged' measures (i.e., planting disease-free seedling, timely removing the diseased trees, and killing *D. citri* in the large region) [39]. Consequently, the HLB-affected citruses were not timely removed in orchard 1 and orchard 4 (region E) in this study, thus resulting in the destruction of the orchards. The above facts prove that the 'three-pronged' measures are still the most effective prevention and control measures for HLB in recent years. Moreover, a comprehensive quarantine can more effectively control the outbreak of disease [38]. However, as insecticide resistance has a vital negative impact on psyllid control, frequent insecticide application is not recommended. Collectively, constant reproduction and saturated reproduction are of pivotal importance [24].

As one of the new citrus production measures accepted by some growers, citrus under protective screen (CUPS) can efficiently exclude the *D. citri* vector of HLB, thereby producing HLB-free healthy fruits [40]. In actuality, this measure is efficient in insect prevention, as it consequently regulates the epidemics of vector-borne diseases. Ferrarezi et al. [41] also found that screen houses, rather than open-air planting, could also provide a better growing environment for young citruses to accelerate their growth. Moreover, this study also found that the yield of the tree and the economic value of the fruits in the screen house were significantly higher than those found in open-air planting areas. Although the red value of the fruit peel in the screen house was significantly lower than those found in open-air planting areas, which may be due to reduced solar radiation accumulation and greater air temperature [41], there was no obvious difference in the fruit quality and morpho–physiological characteristics. However, CPUS can also alter the microclimate inside the screen house, hence increasing the mite population, and also affecting plant growth to a certain extent [42]. On the ground that the mites were well controlled, the anti-psyllid screen house coverage is suggested to be an acceptable new environmental platform by which to cultivate high-value fresh citrus.

HLB trees usually occur in aggregates or clumps in the field. Furthermore, the direction or within-and-across row effects of HLB appearance have also been noted in China, the Philippines, Reunion Island, and São Paulo [43–47]. Our investigation showed that the occurrence of HLB had a certain regularity in the direction of north to south in the flat land orchard, and the incidence was highest in the north end. Before this study,

Gottwald et al. [46] found that the trees infected by CLas tended to gather in the same direction in the field of Shantou, Guangdong. In addition, the occurrence of HLB was influenced by multiple other factors. The natural ACP population was found to be the highest from May to September. During this period, the wind blows from the southeast and southwest. This is relevant due to the fact that *D. citri* passively migrates with the wind. Similarly, the distribution characteristics of HLB-affected trees can be explained by the wind direction. Bassanezi et al. [47] found that the degree of diseased tree aggregation was also positively related to disease incidence in 36 plots from 8 farms in the central region of the São Paulo state. Meng et al. [48] used the aggregation index method to analyze 2900 citrus trees sampled from different locations. They found that the aggregation intensity increased with the rise of the positive rate. In our study, the diseased trees were also clustered at certain points in the different orchards, and most of the clustered diseased trees had the same severity. Once citrus trees were infected with CLas in the orchards, the adult *D. citri* also tended to be clustered there first [49]. This behavior of *D. citri* explains the accumulation of citrus plants that were infected with HLB in the orchards. Most infected trees appeared at the edge of the orchard. Moreover, this phenomenon has been mentioned in some survey articles. Data from China, Reunion Island, Brazil, and Florida all indicated occasional higher-than-expected incidence rates of HLB-positive trees at the periphery of the plantings [43–47,50]. A closer scrutiny of the distribution patterns revealed that the interface of zones with non-citrus crops at its perimeter should be avoided. In addition, planting that was created by roads, canals, ponds, and other features all contribute to HLB epidemics as potential linear and/or curvilinear foci of disease. This is because HLB infections tend to accumulate in proportionally higher incidences at these respective interfaces [46,50–54].

## 5. Conclusions

The distribution and epidemic of HLB in orchards have a certain regularity, which is influenced by the planting environment and conditions, the altitude, orientation, wind direction, varieties of citrus, etc. The question regarding how to fully use comprehensive management in order to curb the spread of the disease will be the key problem in the future. As such, this study provided a reference and basis for the formulation of orchard management strategies for tackling the impact of HLB.

**Author Contributions:** Methodology, J.Z., Y.L., J.G., C.Y., X.Z., X.C., M.X. and Z.Z.; software, J.Z., Y.L. and J.G.; validation, J.Z. and J.G.; investigation, J.Z., Y.L., J.G., C.Y., X.Z., X.C., M.X. and Z.Z.; resources, X.D.; data curation, J.Z., Y.L. and J.G.; writing—original draft preparation, J.G. and J.Z.; writing—review and editing, M.X. and J.Z.; visualization, J.Z., Y.L. and J.G.; supervision, X.D. and M.X.; funding acquisition, X.D. and M.X. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by Natural Science Foundation of Guangdong Province, grant number 2022A1515010889; and the open competition program of top ten critical priorities of Agricultural Science and Technology Innovation for the 14th Five-Year Plan of Guangdong Province, grant number 2022SDZG06.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data are available in the manuscript.

**Acknowledgments:** The authors thank all members in the Citrus Huanglongbing Research Laboratory from 2019 to 2021 for the field investigations. We also thank Ye Hu from Zhejiang Normal University for her linguistic assistance while preparing this manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## Appendix A

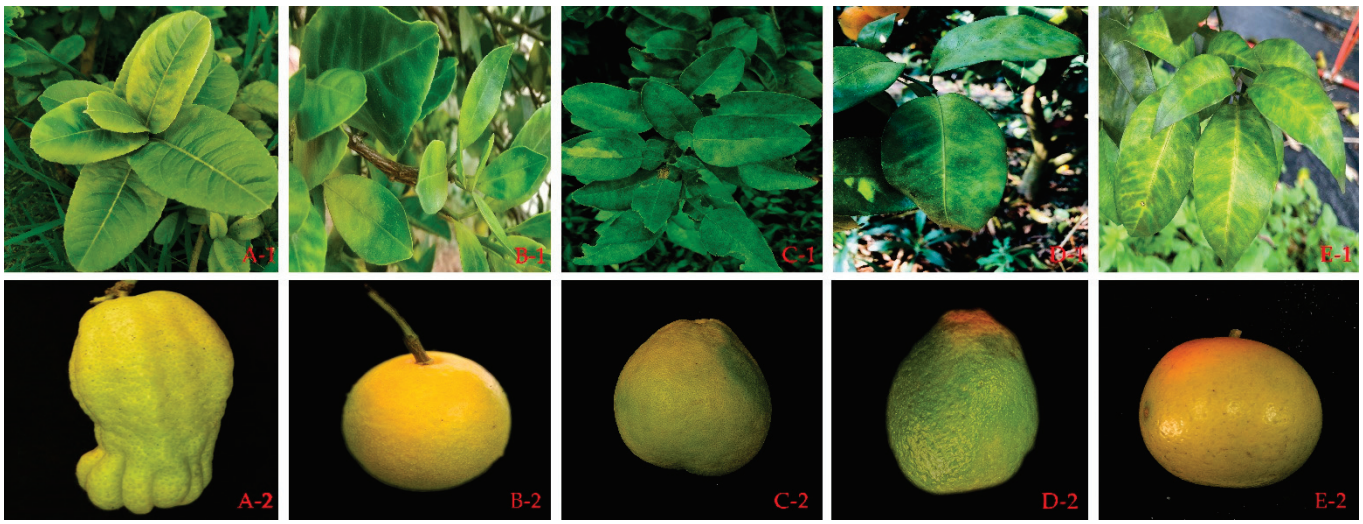


**Figure A1.** Leaf and fruit symptoms of different citrus cultivars affected with HLB in the different orchards. The number '1' and '2' represent the leaves and fruits, respectively; (**A**) *C. medica* L. var. *sarcodactylis* Swingle 'fingered citron'; (**B**) *C. reticulata* blanco var. 'Gongkan'; (**C**) *C. maxima* 'Mi Yu'; (**D**) *C. reticulata* Blanco 'Shatangju'; (**E**) *C. reticulata* Blanco 'Wokan'.

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# EDTA and IAA Ameliorates Phytoextraction Potential and Growth of Sunflower by Mitigating Cu-Induced Morphological and Biochemical Injuries

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**Abstract:** As an essential micronutrient, copper is vital for normal growth and development of plants, however, its accumulation in soil exerts a severe negative impact on the agronomic characteristics and yield of the crop plants. Phytoextraction is a low-cost method for restoring soil fertility and avoiding losses due to heavy metal contamination. We found that using EDTA and IAA together improved sunflower hyperaccumulation capacity. Sunflowers were cultivated under various levels of Cu (0 (control), 25, 50, and 75 mg/kg of soil) and treated with EDTA alone or combined with IAA. The results revealed that the amended treatment significantly enhanced the absorption and accumulation of Cu in the sunflowers. Furthermore, the various doses of Cu significantly reduced the root and shoot growth of sunflowers in a concentration-dependent manner by impairing the chlorophyll content, hormones (indole 3-acetic acid, salicylic acid, and gibberellic acid), flavonoids, phenolics, and antioxidant response. The injurious effect of Cu was reduced by the addition of EDTA alone, and the supplementation of IAA led to a significant restoration of shoot growth (~70%) and root growth (~13%) as compared to the plant treated with Cu alone. Moreover, significantly higher levels of chlorophyll content, $GA_3$, endogenous IAA, and flavonoids were recorded, indicating the effectiveness of the treatment in ameliorating plant health. The results also showed considerable restoration of the catalase and ascorbate peroxidase activities in plants treated with EDTA and IAA. These results are suggestive that application of EDTA and IAA enhances the Cu absorption potential of sunflower and increases its tolerance to copper, which may not only serve as a better technique for phytoextraction of Cu, but also to bring Cu contaminated soil under cultivation.

**Keywords:** Copper hyperaccumulation; stress mitigation; EDTA and IAA; sunflower

**Citation:** Shah, N.; Irshad, M.; Hussain, A.; Qadir, M.; Murad, W.; Khan, A.; Awais, M.; Alrefaei, A.F.; Ali, S. EDTA and IAA Ameliorates Phytoextraction Potential and Growth of Sunflower by Mitigating Cu-Induced Morphological and Biochemical Injuries. *Life* **2023**, *13*, 759. https://doi.org/10.3390/life13030759

Academic Editors: Kousuke Hanada, Wajid Zaman and Hakim Manghwar

Received: 4 January 2023
Revised: 8 February 2023
Accepted: 9 March 2023
Published: 10 March 2023



## 1. Introduction

Heavy metals are part of the natural soil system; however, their high concentration accumulated by plants and animals due to agricultural malpractices and other anthropogenic activities poses a serious concern [1]. In plants and algae, copper (Cu) is a crucial micronutrient. Plants utilize copper, particularly in ATP synthesis, photosynthesis, $CO_2$ assimilation, and as a vital component of several proteins. However, the overuse of Cu in industry and mining has increased its concentrations to toxic levels in ecosystems [2]. Cu above the threshold level can cause kidney and liver dysfunction, anemia, intestine and stomach irritation, hypertension, and nervous system problems. Cu-induced phytotoxicity leads to physiological stress and causes stunted growth and leaf chlorosis [3]. Due to the presence of malondialdehyde (MDA), which promotes bilayer lipid and protein

peroxidation, Cu seriously harms plants through oxidation. Reactive oxygen species, such as hydroxyl radicals (OH), superoxide radicals ($O_2$) and singlet oxygen ($^1O_2$), exhibit increases due to toxic Cu levels; however, OH, SOD, and POD are crucial antioxidants for the scavenging of ROS [4,5].

The copper content in the soil should be reduced in order to avoid Cu-induced phytotoxicity. In general, heavy metal removal from polluted soil involves physical, chemical, and biological methods [6]. Most chemical and physical methods, such as stabilization, solidification, vitrification, electrokinetics, soil washing, and vapour extraction, are, however, costly and ineffective [7–10]. Due to its extensive use and economic viability, phytoextraction, a green technique that allows the removal of soil contaminants, has emerged as a viable alternative. Fast-growing plants are used in an efficient and eco-friendly manner to remove heavy metals and other dangerous substances from contaminated soils and accumulate in harvestable portions. Exposure duration also impacts the reclamation of metal under certain conditions, i.e., metal accumulates actively as plants grow; however, after a certain growth period, the reclamation remains the same and the plant is unable to accumulate more metal [11]. More than 400 plant species from 45 distinct plant families, ranging from tropical to temperate regions, have been documented and claimed to be able to withstand and absorb heavy metals from soil. Heavy metals are absorbed from soil to shoots through roots, which depends on the species of plant, availability of heavy metals, growth stage and use of fertilizers [12].

Chelates, such as EDTA, lower the pH of soil solutions by forming complexes with heavy metals, thus increasing metal bioavailability and facilitating metal translocation from soil to root and then shoot. Limited amounts of chelators are required to boost metal absorption by plants [13].

Plant hormones have a crucial role in several important physiological processes [14]. Indole-3-acetic acid (IAA), a naturally occurring auxin, has the power to control a variety of aspects of plant growth, including vascular tissue differentiation, growth, and elongation, the production of lateral roots, apical dominance, and fruit formation and ripening. There have been numerous publications on the phytoextraction of various heavy metals from a variety of plant species using EDTA and IAA; however, there is limited literature on the synergistic effects of EDTA and IAA assisted phytoremediation [13,15].

As a novel study, this work was aimed at finding out the phytoextraction capacity of sunflowers in Cu-contaminated soils in the presence of EDTA and IAA. Moreover, the biochemical signatures of the sunflowers exposed to Cu and all other treatments were also investigated.

## 2. Materials and Methods

### *2.1. Preparation of Soil*

The soil used throughout the experiment to grow the sunflowers was a sandy loam soil with composition of sand and clay was used with particle size of approximately 0.5 mm and 0.002 mm, respectively. The sand and clay were mixed with manure with the approximate ratio of 2:1:1 to make the plant growth medium, which was then used to prepare a sandy loam for improved sunflower development. Pots containing 5 kg of soil were maintained in the green house at the department of Botany, Abdul Wali Khan University in Mardan.

### *2.2. Experimental Design*

Viable and healthy sunflower seeds were purchased and the surface of the seeds was disinfected by 70% ethanol, and the ethanol was then washed off with sterile distilled water. The experiment was in three factorial combinations, i.e., Cu concentrations (25, 50, and 75 mg/kg of soil in the form of $CuCl_2$ as a bioavailable form of Cu), EDTA (5 mM (1.45 g/kg)), and foliar application of IAA (2.5 µM sprayed at intervals of 5 days until harvest), EDTA and IAA (in the same concentrations as used in the separate treatments) were used in combination with different levels of the selected metal. Each treatment consists of three replicates, and every replicate had three plants. The pots received a thorough

watering of tap water every morning and evening. Several biochemical tests were run to determine the effect of copper concentration, IAA, and EDTA treatment on the agronomic and physiological characteristics of the host plant, as well as metal accumulation.

### *2.3. Morphological Parameters*

The sunflowers roots and shoots were measured in centimeters (cm). The fresh and dry weights of the root and shoot were calculated and represented in grams using an analytical weight balance. To obtain their dry weight, the samples were oven dried at 80 °C.

### *2.4. Estimation of Chlorophyll Contents*

A UV-visible spectrophotometer was used to quantify chlorophyll (Biochrom Libra S22) [16]. For the purpose of extracting chlorophyll, 0.5 g of fresh leaves were crushed with 80% acetone, and Whatman filter paper No. 42 was used for the filtration of mixture. Additional acetone was used to dilute the solution by about 1 mL (approximately 2 mL of acetone). Two wavelengths, 663 and 645 nm, were used to measure the optical density (OD) in comparison to a blank surface.

### *2.5. Estimation of Phytohormones*

For estimation of IAA, the Salkowski reagent technique [17], and salicylic acid was measured using 1% iron chloride [18]. The gibberellic acid content was determined using a wheat endosperm assay [19].

### *2.6. Metabolite Determination*

Total flavonoids were determined by the $AlCl_3$ method [20]. Leaf samples of 0.5 g were homogenized using 80% ethanol (5mL) and kept for incubation for 24 h to achieve full flavonoid extraction in the shaker. After that, the mixtures were centrifuged for 15 min at 10,000 rpm at 25 °C.

For the determination of total phenolics, 16 mL of ethanol was added to 1 g of crushed plant leaves. Centrifugation of the homogenates were performed at 10,000 rpm after being incubated at an increased temperature (between 20 °C and 80 °C) for 3 h. The supernatants were concentrated to 1 mL at 40 °C by using a rotary evaporator after being filtered through filter paper (Whatman No. 42). Resolving the concentrations in 10 mL distilled water allowed for the measurement of phenolics [20].

In order to extract proline content, the solutions were centrifuged at 10,000 rpm after being incubated for 24 h at 4 °C for 5 min following the protocol of Bates et al. [21].

### *2.7. Determination of Antioxidant Response*

Catalase activity (CAT) and ascorbate peroxidase (APX) were used for the determination of antioxidant responses. The cleavage of $H_2O_2$ was determined for CAT activity using the procedure of Radhakrishnan and Lee [22]. Approximately 0.1 mL of supernatant, 0.4 mL of 3% $H_2O_2$, and 0.1 mM EDTA were added to 2.6 mL of 0.05 M phosphate buffer (pH 7). The drop in $H_2O_2$ was accompanied by a reduction in absorbance at 240 nm, which was quantified as M $H_2O_2$ $min^{-1}$ cleavage.

To estimate APX in the leaves, the Asada [23] procedure was used. The reaction mixture consists of 0.1 mL ascorbic acid (0.5 mM), 0.6 mL PBS (50 mM, pH 7.0), 0.1 mL $H_2O_2$ (0.1 mM), and 0.2 mL leaf extract. A decrease in optical density was measured at 290 nm. Protein content was calculated for each extract using the technique of Bradford [24].

### *2.8. Estimation of the Copper in Plant Biomass*

Oven dried 0.5 g samples were mixed with perchloric acid ($HCLO_4$) and nitric acid ($HNO_3$) in the ratio of 1:4 in order to prepare samples for metal analysis using an atomic absorption spectrophotometer. After cooling, the mixture was filtered, and the final volume of the mixture was raised to 25 mL by adding distilled water. Control plant samples were treated using the same method as the positive control solution. With the exception of the

inclusion of sample, the blank solution was created in the same manner as the sample solution. For quantification of the copper contents in the biomass, Amin et al.'s [5] method was followed using atomic absorption spectrophotometer (Perkin Elmer 700) to determine selected heavy metals.

### *2.9. Data Analysis*

The trials were carried out three times, with the treatment conditions for copper, copper/EDTA, copper/IAA, and copper/EDTA/IAA being separated from the data acquired from the factorial testing. The significance level of $p \leq 0.05$ was determined using one way ANOVA followed by DMRT using SPSS Statistical Package version 21 (IBM, Armonk, NY, USA).

## 3. Results

### *3.1. Effect of Copper on the Growth of Sunflower*

Various copper concentrations led to a considerably decreased root and shoot length of the sunflowers as compared to the untreated control group (Figure 1a). At the highest copper concentration (75 mg Cu/kg soil), the greatest reduction in root and shoot length and of the fresh or dry weight of root, stem, and leaf was observed. A reduction of about 15%, 22%, and 25% in shoot length and 48%, 51%, and 53% in root length was recorded for 25, 50, and 75 mg Cu/kg of soil, respectively. In a concentration-based manner, fresh weight decreased by 57%, 68%, and 71%, and dry weight decreased by 34%, 43%, and 50%, respectively. Fresh and dry weight of different plant parts showed improvement with the application of EDTA along with different Cu concentrations (Figure 1b–d).

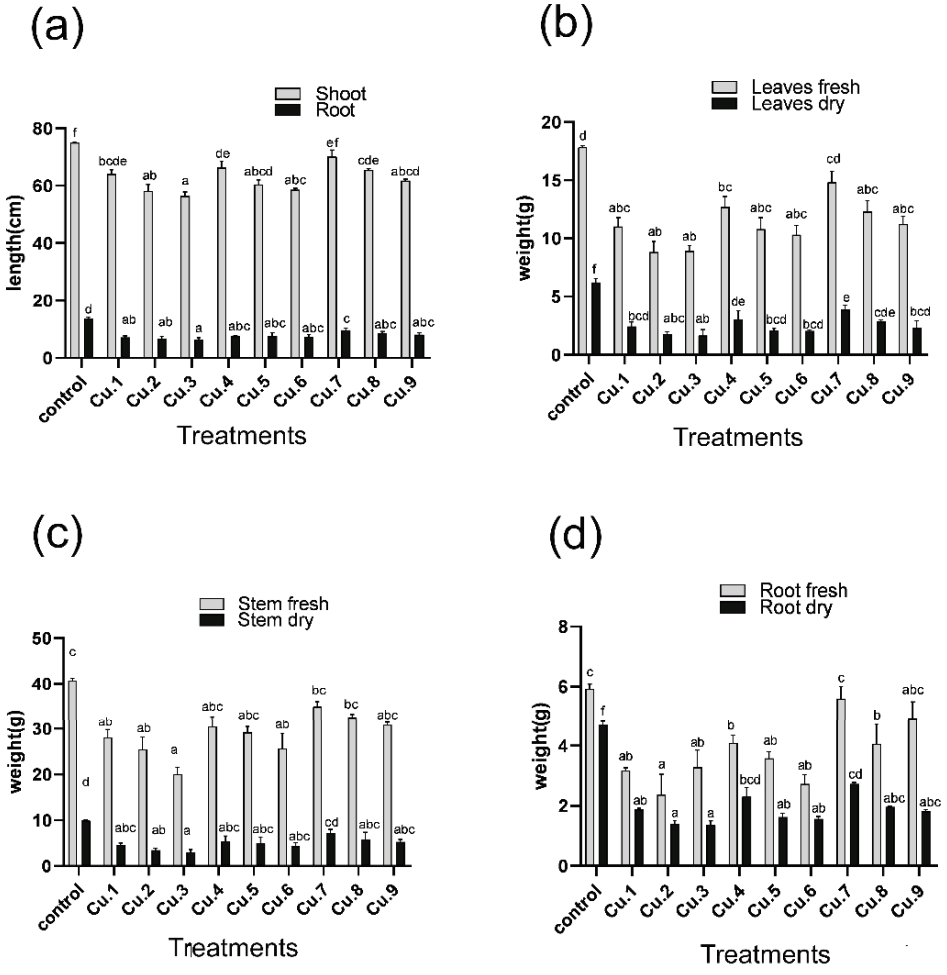


**Figure 1.** *Cont.*

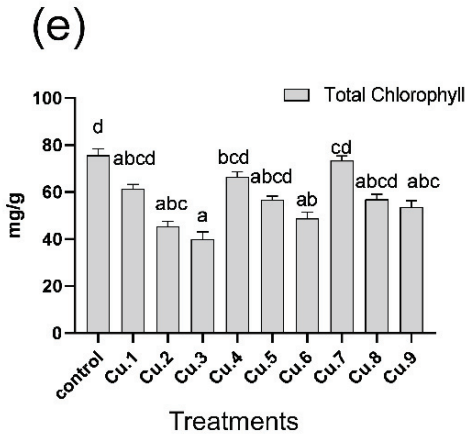


**Figure 1.** Effect of copper concentrations, EDTA and IAA application on (**a**) Root/shoot length, (**b**) leaves fresh/dry weight, (**c**) stem fresh/dry weight (**d**) root fresh/dry weight, and (**e**) total chlorophyll contents of the sunflower. Bars represent the means of the triplicates with standard error (±), and the letters indicate significant difference among treatments at the levels of $p \leq 0.05$. Cu.1, Cu.2, and Cu.3: 25, 50 and 75 mg Cu/kg of soil, respectively; Cu.4, Cu.5 and Cu.6: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA; Cu.7, Cu.8 and Cu.9: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA and IAA.

A simultaneous addition of IAA and EDTA to different copper concentrations, exhibited an approximate increase of 8%, 10%, and 7% in shoot length and above 18%, 14%, and 13% in root length. Besides, an increase in root and shoot length was detected upon the foliar application of IAA. However, the positive results obtained after treating biomass with EDTA and IAA (23%, 19%, and 16%) show a declining pattern as the Cu level increases.

### *3.2. Effect of Copper on Total Chlorophyll Contents*

According to the results, the total chlorophyll contents in the leaves of sunflowers decreased by 19%, 41%, and 48% up to 75 mg Cu/kg soil (Figure 1e). However, improvement was noticed with the application of EDTA alone and in combination with IAA by 16%, 15%, and 18%, respectively.

### *3.3. Metal Accumulation and Translocation to Aerial Parts of the Host*

The total copper content of various sunflower plant parts was determined using different concentrations of copper with EDTA alone and in combination with IAA. Copper accumulation in sunflowers increased as the levels of copper in the growth medium increased from 0 to 75 mg Cu/kg soil (Figure 2a–d). A similar pattern was observed when EDTA was applied to the soil, reflecting a rise with the elevation of Cu in the soil, which remained lower than that of plants untreated with EDTA. In the case of IAA application, high accumulation was noted with a rise in soil Cu.

Similarly, the accumulation was directly proportional to exposure duration i.e., longer exposure times resulted in higher accumulation levels, and vice versa (Figure 2a). Cu accumulation was lower in the 15-day-old plant. An increase was recorded after 30 days of exposure. After 60 days of copper supplementation, significant accumulation was observed. Nonetheless, EDTA application in the soil improved copper accumulation, demonstrating a concentration-based increase with increased copper supplementation.

The same patterns were observed after the transfer of metals to aerial parts of the plant (Figure 2b). The findings suggested that enhanced copper concentrations in the soil medium resulted in increases of copper hyperaccumulation in the aerial parts of the sunflowers. The distribution of copper in the aerial parts of the sunflower shows a pattern of stem > leaf > seed. EDTA application to the soil enhances the translocation capability of the stem, except for 50 mg Cu/kg soil, which recorded a higher accumulation than 75 mg Cu/kg of soil. Foliar IAA application also increases copper translocation; however, a declining

trend was observed with a dose-dependent increase of the metal supplement in the soil. Contrarily, a concentration-based decline was noted in the translocation of the metal in the leaves (lowest in the case of 75 mg/kg). EDTA application in the soil and IAA foliar spray enhanced the translocation of copper to the leaves. In the case of translocation to the seeds, an increasing trend was recorded in copper accumulation, peaking at 75 mg Cu/kg soil supplementation. EDTA application resulted in a similar increasing pattern up to 50 mg Cu/kg soil; however, a dip was observed at 75 mg Cu/kg soil of metal spiked soil. With the foliar application of IAA, maximum accumulation was observed between 25 and 50 mg Cu/kg soil; however, a decline was recorded at 75 mg Cu/kg soil.

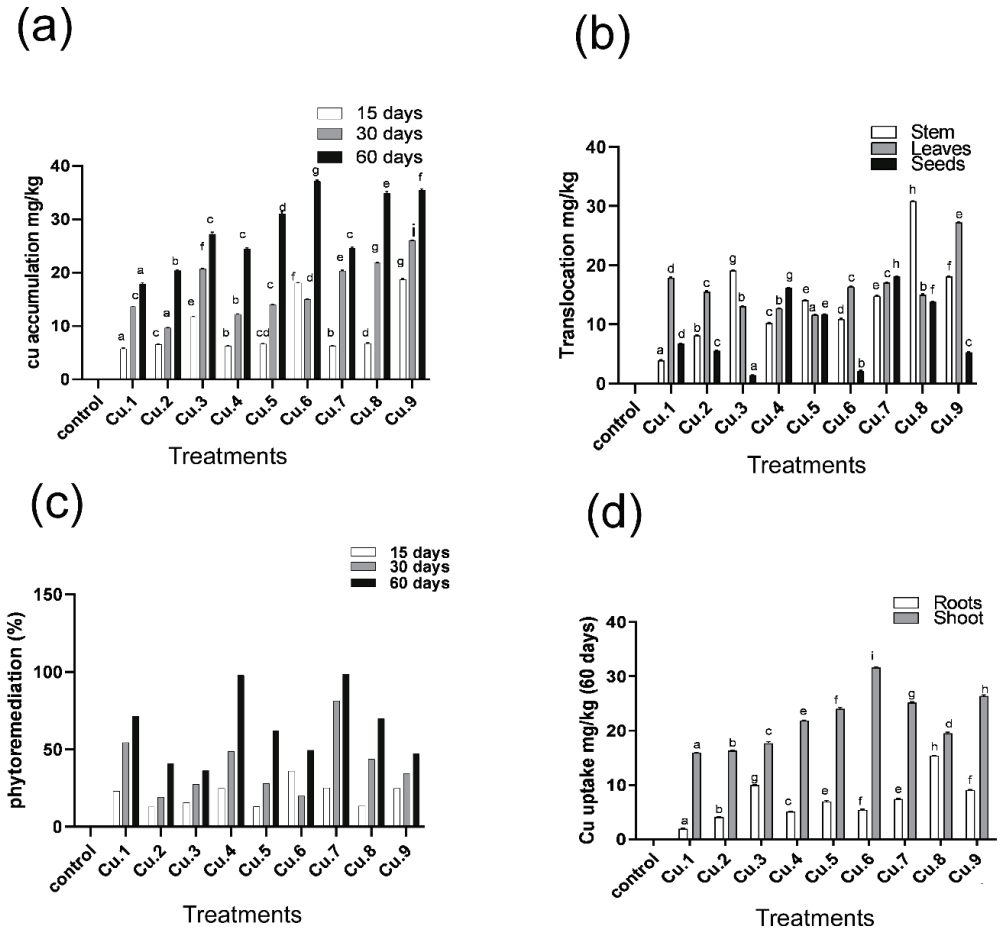


**Figure 2.** Effect of Cu as supplement, IAA and EDTA application on (**a**) Cu bioaccumulation, (**b**) Cu translocation, (**c**) phytoremediation %, and (**d**) root/shoot Cu absorption by sunflowers. Bars represent the means of the triplicates with standard error (±) and various letters indicates significant difference among treatments at the levels of $p \leq 0.05$. Cu.1, Cu.2, and Cu.3: 25, 50 and 75 mg Cu/kg of soil, respectively; Cu.4, Cu.5 and Cu.6: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA; Cu.7, Cu.8 and Cu.9: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA and IAA.

A decrease in the phytoremediation potential of copper was recorded with the concentration-based increase in metal supplementation (Figure 2c). The phytoremediation of the host increased and, in some cases, became twice as high after treating the soil with EDTA in the soil as compared to the control plants. A similar response was recorded with the foliar application of IAA, showing a similar increase with increasing metal concentration. A similar incline was recorded with the exposure duration. A higher percentage of phytoremediation of Cu was recorded in the plants exposed to 60 days of 25 mg Cu/kg soil of supplementation with EDTA amendment in the soil. A similar decline pattern was recorded with the inclination of soil metal supplement. EDTA application in the soil improved the metal uptake and its bioaccumulation, whereas the higher percentage of phytoremediation

was noticed in most cases of Cu supplementation in comparison to the stressed treated control plants. Phytoremediation was improved, showing a similar declining trend, with the foliar spray of IAA.

Similarly, increasing the metal in a dose-dependent manner caused an increase in the root to shoot copper ratio (Figure 2d). The supplementation of IAA and EDTA resulted in an increase in metal accumulation in the roots as well as a similar increase in translocation. When compared to the roots of the sunflowers, maximum copper was accumulated in the shoots for all doses of copper with the application of EDTA alone and in combination with IAA.

### *3.4. Production of Phytohormones*

Supplementing sunflowers with the aforementioned levels of copper resulted in a dose-dependent, significant production of endogenous IAA in the host plant, as documented after raising the Cu level in soil (Figure 3a). The use of EDTA and IAA also increased endogenous IAA production. The production of $GA_3$ was severely reduced at all copper supplemented concentrations, indicating a concentration base decline (Figure 3b). $GA_3$ production increased by applying EDTA and IAA as compared to untreated control plants. SA production displayed a contrary tendency to IAA production. No significant increase/decrease was reported at lower concentrations of the metal; however, an abrupt increase, an approximately 2200 µg/g of salicylic acid production, was recorded at 75 mg Cu/kg soil (Figure 3b). A similar effect was shown using either EDTA or IAA. Nonetheless, the improvement was much lower in comparison to stress control plants, i.e., only Cu supplemented plants. GA production was also reduced as the Cu levels increased (Figure 3c). The use of IAA and EDTA increased GA production, but the amount was lower when compared to untreated control plants with no Cu, EDTA, or IAA.

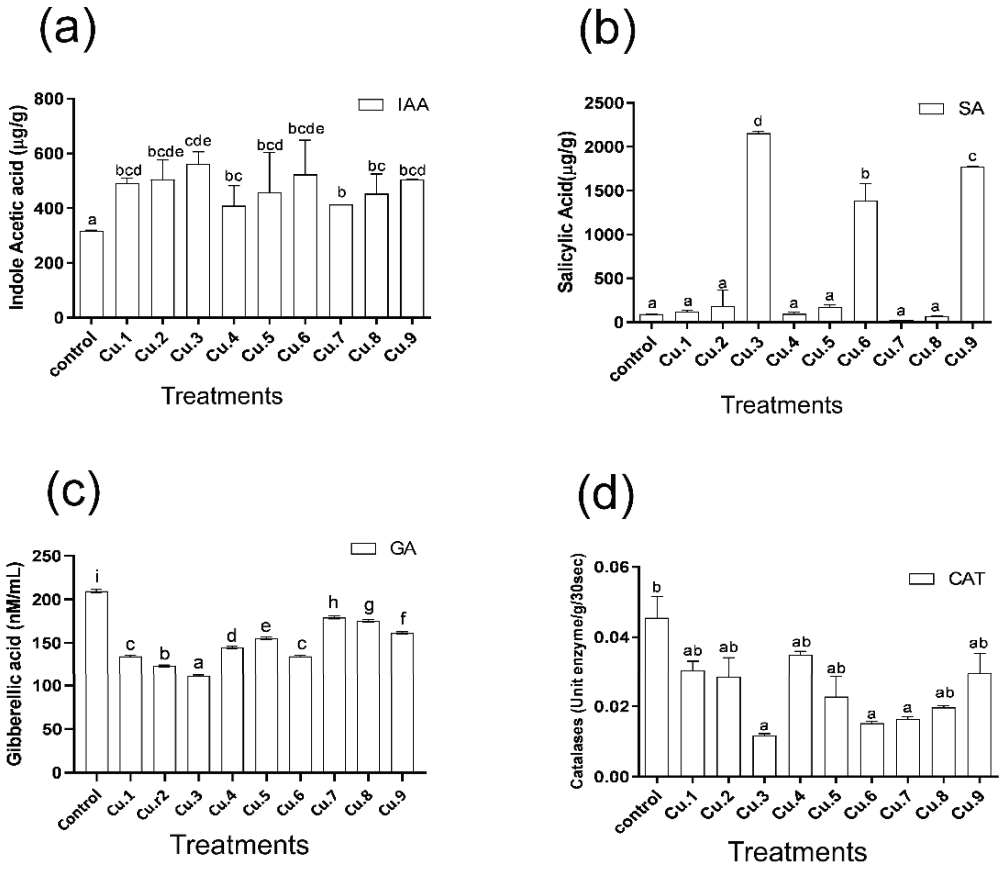


**Figure 3.** *Cont.*

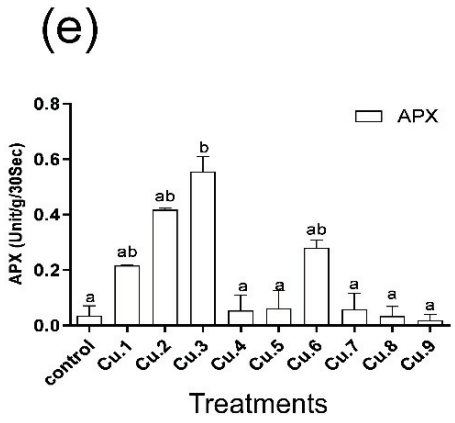


**Figure 3.** Effect of Cu supplementation, EDTA and IAA application on endogenous IAA (**a**), salicylic acid (**b**), $GA_3$ (**c**), catalases (**d**), and ascorbate peroxidase (**e**) activity of sunflowers. Bars represent the means of the triplicates means with standard error (±), and the various letters indicates significant difference among treatments at the levels of $p \leq 0.05$. Cu.1, Cu.2, and Cu.3: 25, 50 and 75 mg Cu/kg of soil, respectively; Cu.4, Cu.5 and Cu.6: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA; Cu.7, Cu.8 and Cu.9: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA and IAA.

### *3.5. Response of Antioxidants*

The plant antioxidant system increases in both primary and secondary stressful conditions. In the present study, plants treated with Cu supplements showed a decline in catalase production of 33%, 38%, and 74%, displaying the lowest value at 75 mg Cu/kg soil treatments (Figure 3c), whereas an improving rate of 10% was recorded at 25 mg Cu/kg soil with the supplementation of EDTA in the soil. However, the number of enzymatic units were less than that of the control plants. A different result was observed in the condition of ascorbic peroxidase, which increased multifold with the increase of metal in the soil, with higher enzyme units recorded at 75 mg Cu/kg soil (Figure 3d). Interestingly, an abrupt dip was recorded in the production of ascorbate peroxidase with EDTA application in soil, except for 75 mg/kg, which showed a significant increase. IAA foliar application with the previously mentioned Cu supplementation in the soil yielded the lowest value.

### *3.6. Production of Metabolites*

Cu treatment has a negative impact on endogenous flavonoid production in plants (Figure 4a). Decreases in the concentrations of the endogenous flavonoid contents were recorded (63%, 71%, and 76%) in a concentration-dependent manner, and the lowest was noted at 75 mg Cu/kg soil. EDTA applications in the soil boost flavonoid production by over 9%, 12%, and 4%, respectively, and foliar IAA applications boost flavonoid production in the host plant; however, the amount is lower when compared to untreated control plants. Furthermore, after supplementation with Cu, endogenous phenolics concentrations increased in the plants, indicating a positive relationship with Cu levels (Figure 4b). Similarly, EDTA application significantly increased phenolic production when compared to untreated control plants. For example, foliar application of IAA resulted in a decrease in total phenolics; however, endogenous phenolics were higher than in control plants. The higher the Cu levels, the greater the proline accumulation, and thus they show a direct relationship with Cu supplementation, similar to phenolic contents (Figure 4c). Supplementation of EDTA in the soil significantly decreased endogenous proline accumulation, but it was still greater than in the untreated plants. Foliar IAA resulted in a further dip, with the lowest level recorded at 75 mg Cu/kg soil.

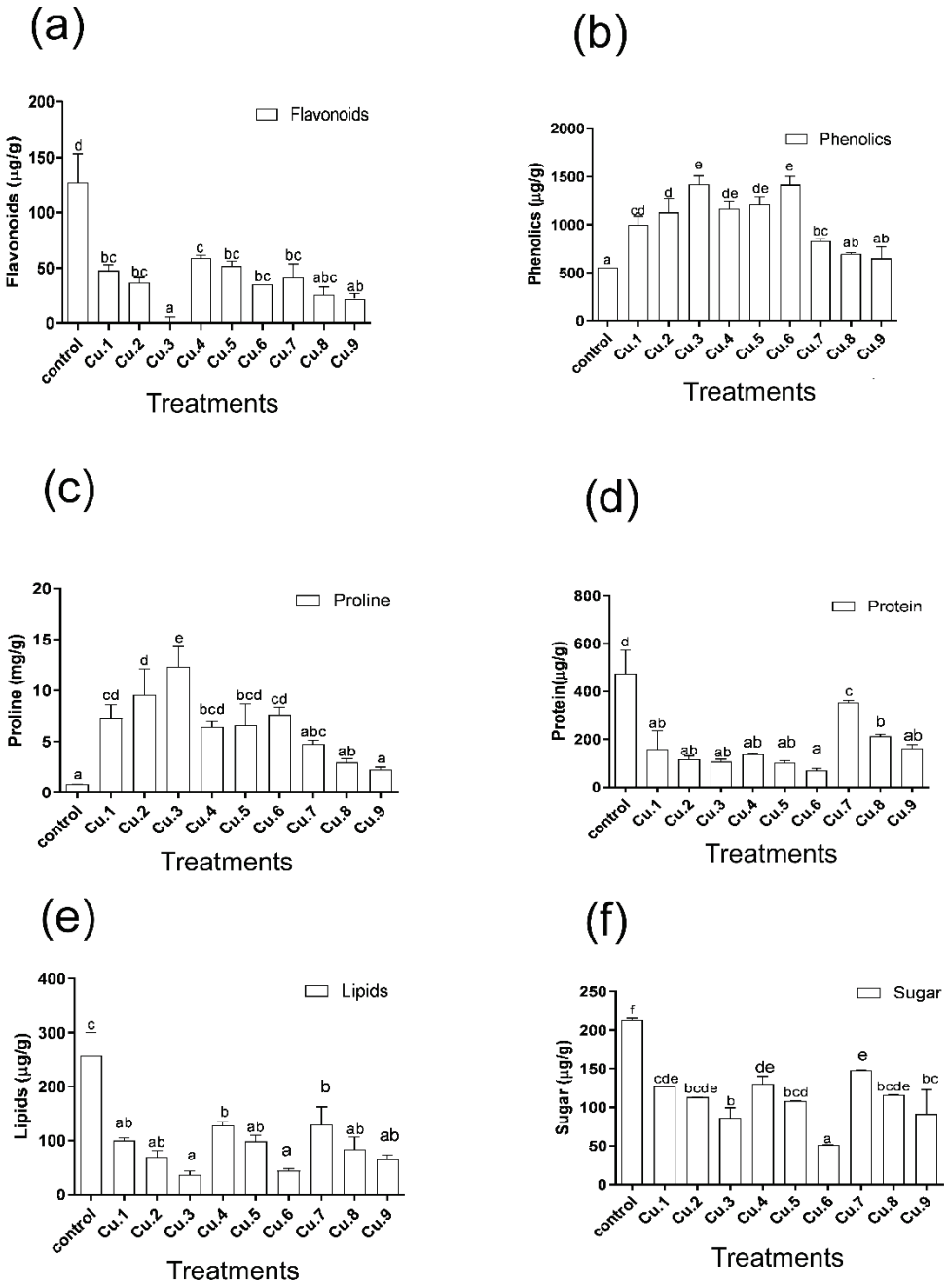


**Figure 4.** Effect of Cu supplementation, EDTA, and IAA application on flavonoids (**a**), phenolics (**b**), proline (**c**), protein (**d**), lipids (**e**) and sugar contents (**f**) of sunflowers. Bars represent the means of the triplicates with standard error (±) and various letters indicates significant difference among treatments at the levels of $p \leq 0.05$. Cu.1, Cu.2, and Cu.3: 25, 50 and 75 mg Cu/kg of soil, respectively; Cu.4, Cu.5 and Cu.6: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA; Cu.7, Cu.8 and Cu.9: 25, 50 and 75 mg Cu/kg of soil, respectively, with EDTA and IAA.

Total protein decreased significantly by 66%, 76%, and 77% and the lipids by 61%, 73%, and 86% in host plants under Cu stress (Figure 4d,e). The application of EDTA enhanced the protein content by 41%, 21%, and 11%, and the lipid content by 11%, 12%, and 12%; nevertheless, the quantity was much lower in relation to Cu treated plants, but an increase was noted with the IAA application. The same patterns were observed in the flowers endogenous total sugar contents, with decreases of 40%, 47%, and 60% with increasing Cu supplementation (Figure 4f). Increases of about 2%, 1%, and 2% were observed with EDTA

in the soil and IAA foliar application, respectively; nevertheless, the quantities were lower when compared to the plants with no copper supplementation.

## 4. Discussion

Pollution of the agroecosystem occurs because of anthropogenic activities, including the industrial use of certain chemicals and industrial effluents. The use of industrial water for irrigation of agricultural land leads to the buildup of certain hazardous chemicals, including cadmium (0.05 mg $kg^{-1}$), chromium (98.94 mg $kg^{-1}$), lead (1.35 mg $kg^{-1}$), copper (8.44 mg $kg^{-1}$), and others [25,26]. Among the metals, copper is an essential heavy metal that is extensively used in pesticides and insecticides, leading to the buildup of copper in agroecosystems. In the current study, different copper concentrations were used to assess the phytoextraction potential of sunflower in a controlled environment [27]. Excessive copper supplementation significantly reduced the sunflowers growth attributes in terms of root and shoot length, and fresh and dry biomass ($p \leq 0.05$). Copper toxicity induces nutritional imbalances in plants and constrains their growth. This may have a paramount importance in the case of an essential plant macronutrient—phosphorus (P) [28]. Foliar application of IAA improved the agronomy of the plants in the presence of the mentioned copper concentrations. IAA has a 2-fold function: firstly, it acts as plant growth-promoting hormones and enhances host agronomic features and biomass production, while secondly, it was recently discovered that the IAA (and GA) have a role in stress mitigation. IAA and $GA_3$ have long been known for their plant growth promotion and stress mitigation potential, and as a result of plant growth promotion, the size of the plant increases and the metal is distributed over a larger area, leading to stress mitigation [29]. Hence, in the current situation, IAA not only improves host growth and biomass production but also efficiently mitigate the copper stress in the host plant [30,31]. Similarly, results were also recorded in the case EDTA, showing improvements in the fresh and dry mass of the plants grown in the soil amended with copper supplementation. In a study with *Brassica napus*, the application of EDTA improved biomass of *B. napus* in a Cu-amended hydroponic system [32]. Our findings are in positive correlation with the previous studies, as the synergistic combination of IAA and EDTA significantly reduced Cu-induced toxicity in sunflower seedlings, possibly through reducing Cu-induced oxidative damage, and thus enhanced the morphological features of the tested plants [33–36]. In the case of chlorophyll contents, a decline was recorded up to 75 mg/kg of copper stress. The application of EDTA and IAA supplementation greatly increases the chlorophyll contents, which are, however, lower than those of untreated control plants [37,38].

By increasing the Cu concentration in the soil, the endogenous IAA content increased, which had a positive impact on plant growth promotion and stress mitigation. On the other hand, interesting results were recorded in the case of endogenous salicylic acid production. No significant increase or decrease was recorded at lower concentrations of the metal whereas a significant increase (approximately 2200 µg/g of salicylic acid production) was displayed at 75 mg Cu/kg soil. Similarly, either EDTA or IAA has no effect, except in the case of the 75 mg Cu/kg soil treatment, showing an abrupt increase in both cases. Plants release significant quantities of salicylic acid in order to mitigate the stress by activating several stress response genes, including heat shock protein, chaperon proteins, lower molecular weight osmolyte production, and several other mechanisms, in order to cope with the stressful environment [39]. Higher SA production helps the host grow normally in stressful conditions by maintaining its normal vigor. Because of the lower GA production, the host growth attribute was negatively regulated, resulting in lower biomass production and yield. The addition of EDTA and IAA regulated GA production positively, improving the host's agronomic attributes [40,41]. Aside from growth and stress phytohormones, the release of flavonoids and phenolics, such as proline, lower the molecular weight, and sugar and protein maintain cellular viability and homeostasis [27,30,31,42]. With regards to metabolites, flavonoids are of prime importance, and in the presence of competitive inhibitors, all flavonoids are able to chelate copper; however, some compounds, particularly

those containing the 3-hydroxyl group in association with the 4-keto group and the 2,3-double bond or possessing the 5,6,7-trihydroxyl substitution (baicalein), were very potent even in a highly competitive environment [41,43]. In this study, different concentrations of Cu negatively compressed the endogenous production of flavonoids in the host plant. Dose-dependent declines in the endogenous flavonoid contents were recorded, and the lowest was recorded at 75 mg Cu/kg soil. EDTA and IAA applications significantly enhanced flavonoid production.

Contrarily, the endogenous phenolics were increased in soil that was supplemented with Cu, showing a positive relationship to Cu levels. Previously, Cu stress stimulated the production of phenolics in *Colobanthus quitensis* [44], *Zea mays* [45] and *Phaeodactylum tricornutum* [46]. Similarly, *Dunaliella tertiolecta* was found to excrete almost double polyphenol concentration in the 790 nM $L^{-1}$ copper enrichment experiment [47]. These studies show that in response to Cu stress, plants may stimulate the production of antioxidant secondary metabolites, mainly phenylpropanoids, which have a quenching effect against heavy metals. These phenolic compounds act as nonenzymatic antioxidants and directly quench the reactive oxygen species, thereby reducing their attack on biological membranes, and hence cells remain viable, ensuring their normal growth and development [48,49]. When compared to untreated control plants, EDTA application increases phenolic production even more. For example, foliar application of IAA resulted in a decrease in total phenolics; however, endogenous phenolics were higher when compared to control plants, i.e., plants without Cu treatments. Endogenous proline production was significantly reduced with EDTA application, but it remained higher than in control plants. In the case of IAA application, a further decrease was observed, with the level reaching its lowest point at 75 mg Cu/kg soil. The amounts of proline content at 75 mg Cu/kg soil were comparable to those of untreated control plants. Our results are in positive correlation with the findings of Saleem et al. [50] who reported improved *Corchorus capsularis* L. biomass and phenolics, and enhanced uptake of copper in response to EDTA treatments in Cu-augmented hydroponic solutions.

When the soil was enriched with Cu, the total lipids and proteins in the plants decreased significantly. The copper binds to certain enzymes and acts as a competitive inhibitor of the enzymes, inducing conformational changes that result in the decrease in protein content. The configurational changes make the enzyme either lower its optimal activity or, in some cases, cease its normal functions [51]. The abnormalities of these enzymes lead to severe physiological changes, including altered metabolite production, and abnormal glucose and lipid metabolism [52]. This altered glucose and lipid metabolism leads to lower glucose and lipid production [53]. Moreover, the plant absorbs these metals with an active expenditure of energy, and probably the majority of these energy compounds are wasted in the uptake of the metal, leading to their reduction and lower conversion to storage molecules, i.e., lipids [54,55]. EDTA application and foliar spray of IAA enhanced the protein and lipid contents; however, the amount was much lower in comparison to untreated control plants. The same patterns were observed in the host's endogenous entire sugar content, which decreased as Cu supplementation increased. An increase in sugar was observed with EDTA and IAA foliar applications, nevertheless, the quantity was lower in comparison to plant with no supplementation of copper in the soil.

Plants produce a gamut of enzymatic antioxidants to cope with secondary oxidative stress due to environmental constraints [33,56]. Treating plants with Cu supplements reduces catalase production as metal supplementation increases in the soil; the lowest level was recorded at 75 mg Cu/kg soil treatments. This is probably due to the fact that catalases are more sensitive to copper stress, leading to conformational changes and degradation [57]. Enhancements were noted with EDTA and IAA-assisted applications; however, the number of enzyme units was lower as compared to control plants. An interesting contrast was observed with APX, showing a direct proportional and several-fold increase with copper supplementations in the growth medium, and higher enzyme units were observed at 75 mg Cu/kg soil, implying that Cu stimulates the oxidative capacity,

which is responsible for the conversion of $H_2O_2$ to water and $O_2$. APX is a component of the ascorbate-glutathione pathway, which plays a role in scavenging $H_2O_2$ [34,57]. Interestingly, an abrupt dip was recorded in the production of ascorbate peroxidase with the supplementation of EDTA in growth medium, except for 75 mg Cu/kg soil, which showed a significant increase. The lowest value was obtained with the foliar application of IAA in conjunction with the mentioned Cu supplementation in the soil [23].

As an essential micronutrient, plants tend to accumulate higher quantities of copper in their tissue [58]. Higher quantities of copper are taken up by plant roots with increases in metal levels and exposure times. The total copper content in plant biomass boosts with the inclination of metal concentration in the growth medium, showing a multifold accumulation from 0 to 75 mg Cu/kg soil. Similar results were previously recorded in the case of sunflower, brassica, and soybean treated with cadmium, chromium, and arsenic, showing a direct relation between metal levels and exposure duration [52,55,59,60]. A similar pattern was observed when EDTA was applied to soil. In the case of the IAA application, higher accumulation was also noted as soil metal supplementation increased. The plant spiked with 50 mg Cu/kg soil and treated with IAA foliarly had the highest accumulation. For instance, the copper accumulation was lower in the case of application of EDTA and IAA as compared to untreated plants with EDTA and IAA supplemented with the mentioned levels of copper [38].

Similarly, the accumulation increases with an increase in exposure time [61]. Plants exposed for 15 days to Cu stress showed lower accumulation, with the exception of 75 mg/kg of copper, which showed a decline with the passage of time. Except for the plants exposed to 50 mg Cu/kg soil with foliar application of IAA [59], for which an increase was observed after 30 days of copper exposure. A comparable rise was also documented in the case of 60 days of exposure to copper supplementation, showing an increase up to 50 mg Cu/kg soil; however, a decline was noted in the case of 75 mg Cu/kg soil [62]. Nonetheless, EDTA application in the soil further improved the copper accumulation, showing a concentration-based increase with the increase of copper supplementing [63]. Foliar application of IAA, for example, increases copper accumulation, although a declining pattern was observed with the incline of metal in the soil [64]. The decline recorded with the duration was due to more copper accumulation and the subsequent harm due to metal stress and successive oxidative stress; it negatively affects the physiological attributes of the host by altering the physiology of the host, lowering the biomass, and, therefore, lowering the phytoremediation potential of the host plant [35,36,59,60]. Similar arrays were observed in the transfer of metals to the aerial parts of plants and were also documented in the case of the translocation of metals to the aboveground parts of the plants. Higher copper translocation was noted to the shoots of the plants, showing an increase up to 50 mg Cu/kg soil of the metal; however, a decline was recorded at 75 mg Cu/kg soil. EDTA and IAA applications promote the translocation capability of the host, resulting in higher accumulation with increasing copper in the soil, with the maximum accumulation recorded at 75 mg Cu/kg soil. The higher translocation to leaves and subsequent accumulation were probably due to the fact that plants shed their leaves early to get rid of the metal and its phytotoxicity. This is a strategy that plants mostly use to avoid the toxicity of the metal by translocating it to the leaves [6,65,66].

## 5. Conclusions

From the current study, it was evident that the *Helianthus annuus* L. is a hyperaccumulator that accumulates higher quantities of copper from polluted soil and effectively translocates it to the above ground plant parts, particularly leaves. Early shedding of the leaves was a strategy to get rid of the metal and subsequent toxicity. Moreover, the amount of copper supplementation and duration of exposure also increased the accumulation of metal which was positively improved with the application of EDTA and IAA.

**Author Contributions:** Data curation, Formal analysis, Investigation: N.S. Methodology, Writing original draft: N.S., S.A., M.Q., M.A. and A.K. Supervision: M.I. and A.H. Lab facility, resources: W.M., A.H. and M.I. Supervision review and fund acquisition: S.A. and A.F.A. All authors have read and agreed to the published version of the manuscript.

**Funding:** We extend our appreciation to the Researchers Supporting Project No. RSP2023R218, King Saud University, Riyadh, Saudi Arabia.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets generated and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

**Conflicts of Interest:** The authors declare no conflict of interest.

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*Review*

# Plant Metabolomics: An Overview of the Role of Primary and Secondary Metabolites against Different Environmental Stress Factors

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**Abstract:** Several environmental stresses, including biotic and abiotic factors, adversely affect the growth and development of crops, thereby lowering their yield. However, abiotic factors, e.g., drought, salinity, cold, heat, ultraviolet radiations (UVr), reactive oxygen species (ROS), trace metals (TM), and soil pH, are extremely destructive and decrease crop yield worldwide. It is expected that more than 50% of crop production losses are due to abiotic stresses. Moreover, these factors are responsible for physiological and biochemical changes in plants. The response of different plant species to such stresses is a complex phenomenon with individual features for several species. In addition, it has been shown that abiotic factors stimulate multi-gene responses by making modifications in the accumulation of the primary and secondary metabolites. Metabolomics is a promising way to interpret biotic and abiotic stress tolerance in plants. The study of metabolic profiling revealed different types of metabolites, e.g., amino acids, carbohydrates, phenols, polyamines, terpenes, etc, which are accumulated in plants. Among all, primary metabolites, such as amino acids, carbohydrates, lipids polyamines, and glycine betaine, are considered the major contributing factors that work as osmolytes and osmoprotectants for plants from various environmental stress factors. In contrast, plant-derived secondary metabolites, e.g., phenolics, terpenoids, and nitrogen-containing compounds (alkaloids), have no direct role in the growth and development of plants. Nevertheless, such metabolites could play a significant role as a defense by protecting plants from biotic factors such as herbivores, insects, and pathogens. In addition, they can enhance the resistance against abiotic factors. Therefore, metabolomics practices are becoming essential and influential in plants by identifying different phytochemicals that are part of the acclimation responses to various stimuli. Hence, an accurate metabolome analysis is important to understand the basics of stress physiology and biochemistry. This review provides insight into the current information related to the impact of biotic and abiotic factors on variations of various sets of metabolite levels and explores how primary and secondary metabolites help plants in response to these stresses.

**Keywords:** metabolomics; tolerance; metabolic responses; biotic stress; abiotic stress; metabolites variation

**Citation:** Salam, U.; Ullah, S.; Tang, Z.-H.; Elateeq, A.A.; Khan, Y.; Khan, J.; Khan, A.; Ali, S. Plant Metabolomics: An Overview of the Role of Primary and Secondary Metabolites against Different Environmental Stress Factors. *Life* **2023**, *13*, 706. https://doi.org/10.3390/life13030706

Academic Editors: Othmane Merah, Kousuke Hanada and Balazs Barna

Received: 8 December 2022
Revised: 2 January 2023
Accepted: 28 February 2023
Published: 6 March 2023



## 1. Introduction

Comprehensively, biotic and abiotic stresses negatively affect crop production and cause a marked decrease in annual crop yield, i.e., qualitative and quantitative [1,2]. Recently, biologists, especially agriculturists, need to find an alternative way to deal with biotic and abiotic stresses such as herbivores, insects, and pathogens, as well as salinity,

trace metals (TM) contamination, drought, and extreme temperatures [3,4] respectively. All these stresses affect the physiological and morphological aspects, such as the hindering of the functional groups of important molecules, e.g., enzymes, polynucleotides, transport systems for substantial ions and nutrients, as well as the growth and metabolic activities of plants [5,6]. However, to cope with these stresses, plants adopt several mechanisms, including metabolomics, transcriptomics, proteomics, and genomics, individually or in combination. The plant metabolome consists of the following two kinds of metabolites: primary and secondary metabolites. Primary metabolites are essential for the proper growth and development of plants and microorganisms. On the contrary, secondary metabolites are formed near the stationary phase of growth and have no direct role in growth, reproduction, and development. The metabolic profiling of primary and secondary metabolites provides extensive knowledge of biochemical processes that occurs in plant metabolism [7].

Modern research endorsed the purpose of several important genes, metabolites, proteins, and molecular systems that induced plant reactions to drought, salt stress, cold, TM, heat, and certain other biotic and abiotic factors [8,9]. Metabolomics analyses have become an influential tool to monitor plants' responses to different environmentally stressed conditions [10]. Therefore, the findings of such studies give an understanding of the working of plants in definite circumstances, which are considered an important part of enlightening the molecular processes in responses to various stress conditions [11]. An appropriate data analysis, detection, identification, and evaluation of these metabolites are possible with the help of advanced metabolic tools such as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) nuclear magnetic resonance (NMR) [12].

Furthermore, it is estimated that biotic and abiotic stresses are responsible for more than 50% of crop losses in the world [13,14]. The findings of Bayer in 2008 demonstrated that crop losses caused by abiotic stressors were significantly higher than by biotic factors [15]. However, the exact loss of crop yield depends on the plant's developmental stage and the intensity and duration by which various stresses occur [16]. Among other stresses, salinity affects more than 800 million hectares of land—nearly 50% of the total irrigated area, which provides about 33% of the world's food [17,18]. In the same way, drought also causes a loss of more than 50% of the average yield of crops [19]. Subsequently, other studies indicated that abiotic factors, such as temperature (low or high), salinity, and drought, significantly decreased plant production if existing alone or in combination [20]. Interestingly, another concern is the aggregation of reactive oxygen species (ROS), which is produced by excessively stressed accumulators of cadmium (Cd), chromium (Cr), lead (Pb), zinc (Zn), and copper (Cu) that can cause oxidation and dysfunction of biological molecules, hence disturbing certain physical and biological processes in plants [3,21]. Optimizing metabolic flux by the organellar electron transport chain (ETC) is essential in reducing oxidative stress [3]. Consequently, keeping the redox state of a cell is another essential issue that provides the decreasing power necessary for the foraging of ROS [22].

Therefore, there is a need for novel, easy, inexpensive, ecologically friendly, and robust crop types that can be conceived by cross-breeding or genetic engineering [23]. For example, recently, different wheat, rice, barley, maize, and other economically crucial varieties of crop plants have been considered very necessary than model plants [24,25]. However, the development of some modern 'omics tools, such as genomics, proteomics, transcriptomics, and metabolomics, has rationalized the research of crop plants and abetted the complete study of acquaintances concerning biological components and plant breeding [26]. In this concern, metabolomics gives the possibility to accelerate the selection of superior breeding stock and the screening of elite crop types [27]. Primary and secondary metabolites, with their functional diversity, play an important role in fine-tuning the environmental stress tolerance and productivity in crops. Understanding plant behavior under multiple environmental stressors is one of the ways to deal with agricultural sustainability [20]. In this piece of work, more than 200 published works were considered to provide an overview of the role of primary and secondary metabolites against several abiotic and biotic stressors.

## 2. Instrumentation Applied in Metabolomics Studies

The identification of different classes of metabolites in plants is largely based on using hyphenated mass spectrometric methods to chromatographic equipment and electrophoretic approaches [28]. Choosing an appropriate ionization technique and analyzer type for metabolite analysis is important in a mass spectrometer [29]. Through the study of mass spectrometry (MS), ionized molecules are calculated. Similarly, mass-to-charge ratio values ($m/z$, m-mass, or z-charge) of the produced ions are assessed with the precision of one mass unit and to the fourth decimal point, small or high-resolution mass spectra, after elimination in the MS analyzer. The use of a high-resolution mass analyzer permits the accomplishment of the elemental composition of the identified ions existing in mass spectra. At first, it is probable to estimate the elemental composition and molecular mass of the molecules from enumerated $m/z$ values for protonated $[M + H]^+$ and deprotonated molecules $[M - H]^-$. The clear documentation of compounds is highly dependent on the applied MS system. MS machines designed with electrospray ionization (ESI) and matrix-assisted laser desorption or ionization (MALDI) source could be utilized. The ionization of MALDI can be joined to one or two unified times of flight analyzer (TOF and TOF/TOF). The source of ESI works well through quadrupole (Q), ion trap (IT), time of flight (TOF) analyzer, and a mixture of them. The maximum resolution in the mass analyzer could be attained by ion cyclotron resonance through Fourier Transformation Instruments (FT ICR MS) when the ESI is employed as an ionization system.

Moreover, designing the experiment according to the Metabolomics Standard Initiative (MSI) is also crucial, which endorses defined measures for the right biological materials preparation, procedures of metabolite extraction, and analytical protocols [30]. Following the regulations that have been stipulated, a sufficient number of sample replications and the conditions under which plant development should occur to be investigated and defined [31]. Similarly, the control of MS parameters in mass spectra registration is necessary. Such data deliver environments for suitable documentation and quantification of metabolites and consistent statistical quantification [32].

After employing this method, different statistical calculations could be performed to determine the metabolites' capacities that allow the defining changes of a specific compound in definite situations [33]. The number of primary and secondary metabolites in a single organism may range from several hundred to tens of thousands, with little variation across orders of magnitude in concentration. Some strategies developed for metabolites analysis include metabolic profiling, metabolic fingerprinting, and target analysis [34].

Metabolic profiling is expected a simultaneous measure of a set of metabolites in a sample. Several analytical techniques can be used for metabolic profiling, such as (GC-MS), (LC-MS), and (NMR). To date, GC-MS is the most advanced analytical approach to metabolic profiling in plants [35]. Using GC-MS, it is possible to recognize several hundred compounds belonging to various classes, including sugar, organic acids, amino acids, alcohols, amines, and fatty acids. Similarly, LC-MS provides a better alternative for non-volatile compounds. The importance of LC-MS is increasing in metabolomics, especially after the adoption of ultra-performance liquid chromatography technology that can increase separation efficiency and decrease analysis time [36]. Substantially, NMR spectroscopy offers an entirely different analytical technique compared to MS-based approaches. The sensitivity of the NMR technique is much lower than MS-based techniques; however, the structural content information, reproducibility, and computable aspect could be superior to them [37]. Moreover, the preparation of the sample is simple, more convenient, and non-destructive measurement may possible. These properties of NMR make it an ideal tool for the identification of metabolites through metabolic profiling [38].

## 3. Workflow of Plant Metabolomics Analysis

The metabolomics of plants is very complex and varied in their chemical structure. Extensive identifications and a wide range of metabolic depictions could be attained with the arrangement of two or more metabolomics approaches and analytical methods, with the

difference in extraction protocols [39]. Metabolomics analyses comprise the following three key tentative methods: (1) sample preparation, (2) data gaining, and (3) the identification of compounds by using the statistical analysis of the data. The preparation of the sample is a key step because it can contribute to the identification of a wide range of metabolites, which is comprised of tissue collecting, drying, or quenching, and metabolite extraction for analysis (derivatization) [40]. Thus, care should be taken in this step to avoid engaging in undesirable variation that can significantly disturb the analysis results. Many methods of enzyme quenching, such as drying, enzyme inhibitors and acids, and high meditations of organic solvents, could also distress the analysis and identification [41].

Plant metabolites are structurally different with high complications, such as dissimilar size, solubility, explosive nature, separation, amount, and stability [42,43]. The extraction method of metabolites relies on varied factors such as the type of plant organs, physical and chemical properties of the targeted metabolites, chemical structure, and the solvent used [44]. Generally, metabolite extraction methods include solvent extraction, supercritical fluid extraction, solid-phase extraction, and sonication [45]. Moreover, other methods are used to extract the essential oils, such as hydrodistillation, vapor-hydrodistillation, vapor-distillation, hydro diffusion, organic solvent extraction, and cold pressing [46]. Though, it is critically essential to evaluate metabolite extraction methodologies for a precise metabolite extraction study because a solvent composition that is good for one chemical class may not be suitable for another chemical class. Moreover, this could not be appropriate for extracting large numbers of metabolites from a specific tissue. So, it is important to understand and monitor the effects of the applied solvent treatment on the sample's metabolic content and profile obtained [47].

The measurement of complex metabolites needs an advanced analytical platform for sample analysis. Every platform's range has a particular constraint, maybe in selectivity or sensitivity [48]. The selection of the analytical platform relies on the study initiated, the group of compounds, and their physiochemical properties, such as polarity, solubility, volatility, and concentration levels [49]. Additionally, one issue is that metabolites occur in a wide dynamic range of concentrations such as nanomolar and millimolar in the plant body. Subsequently, another problem is that not every metabolite is present in each tissue [50].

However, the most applied metabolomics approaches in analytical studies are liquid or gas chromatography synchronized with mass spectrometry (LC/GC-MS) and nuclear magnetic resonance spectrometry (NMR) [51]. Subsequently, another report [52] demonstrated an integrated technique that combines metabolites extraction and analysis with proteomic and RNA from a single sample that permits the immediate inquiry of all molecular levels and examines their interrelation and co-variance structure [53]. Consequently, biochemical regulation could result in the co-variance design of molecular dynamics in a cellular system [54]. In the context of metabolomics, the block diagram (Figure 1) of a typical experiment shows the following key steps:

1. Sample collection and organization;
2. Metabolites extraction;
3. Derivatization and separation;
4. Data acquisition;
5. Data analysis;
6. Metabolites identification;
7. Data submission to public repositories.

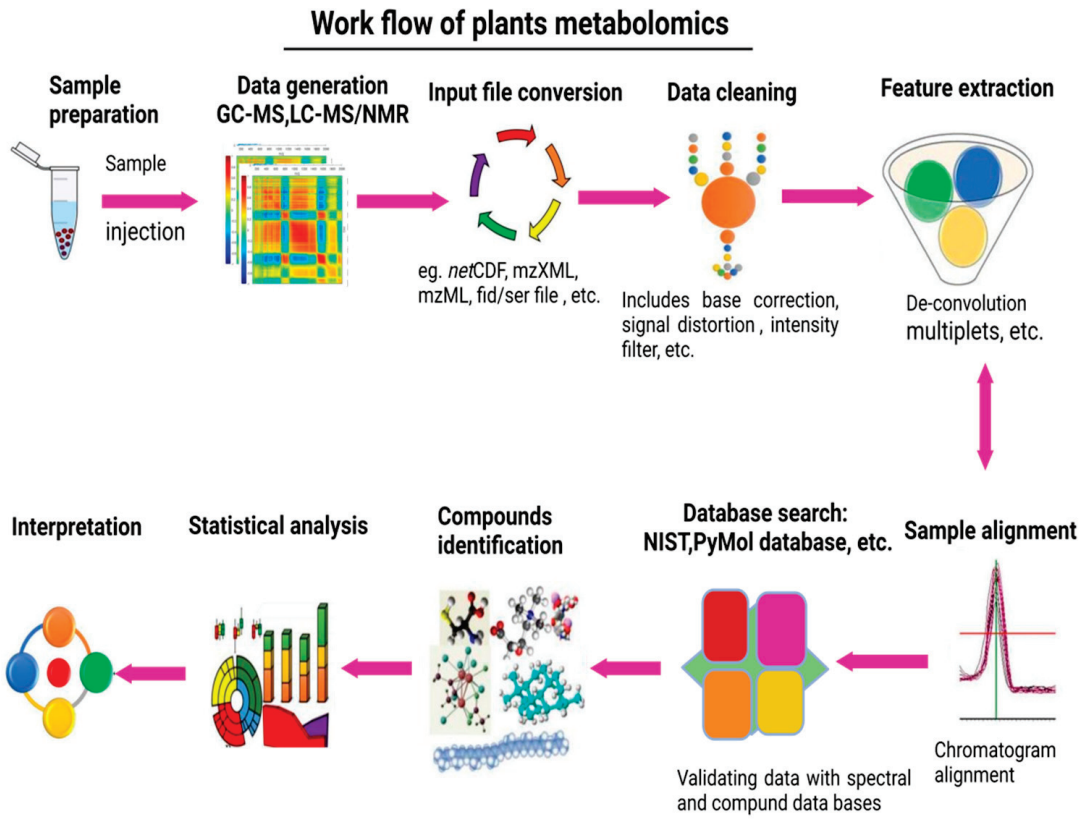


**Figure 1.** Respective illustration of the processes involved in plant metabolomics analysis of GC–MS, LC-MS, CE-MS, and NMR-based chromatography.

## 4. Metabolomics for Plant Stress Responses

Metabolomics is the scientific study of the set of metabolites present within an organism, plant cell, or tissue [55]. However, plant stress is any amendment in the growth and developmental conditions that distracts metabolic homeostasis and needs to modify the metabolic pathways in a process generally designated as acclimatization [35]. Over the last decade, metabolomics has developed promptly and is recognized as the prevailing technology in changing climatic conditions and assessing or elucidating testing phenotypes in assorted living systems [56]. Substantially, it may contribute to studying stress biology in plants or other organisms by recognizing various molecules, such as by-products of stress metabolism and compounds of stress signal transduction and related to the plant acclimation responses [52]. Their application has been driven in several fields, including medicinal, imitation biology, or analytical molding of plants, animals, and microscopic organisms [57].

Additionally, to the applicability of other fields, nowadays metabolomics could also be used on a large scale in the assortment procedure of plants and resistant to the varying environmental states. Different findings revealed that drought stress, salinity, extreme temperature, and soil flooding could cause significant instabilities in the pattern of plant metabolome [22]. Metabolomics signifies the ultimate omic's level in a living system or reveals modifications in the traits of an organism or function. Different findings show the study of metabolomics under several environmental abiotic stresses, such as temperature [58], salinity and drought [59], and soil flooding [60]. In the same way, various metals and metalloids including, sulfur [61], phosphorus [62], oxidative stress [63], TM [64], and the combination of other several stress factors [65] in plants (Table 1). Various environmental factors that could negatively disturb the homeostasis and growth of plants are shown below (Figure 2).

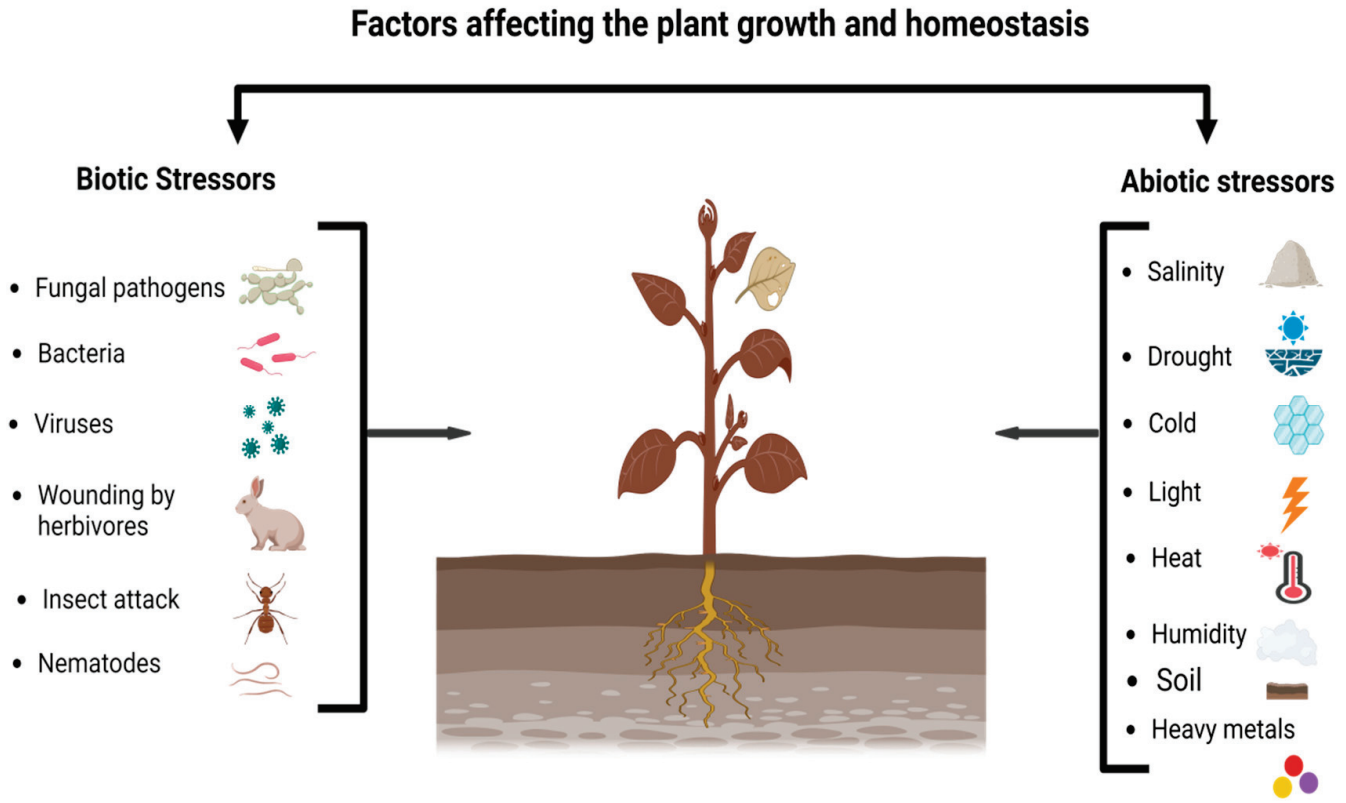


**Figure 2.** Environmental stresses of biotic and abiotic factors affecting the growth and homeostasis of plants.

### *4.1. The Response of Primary Metabolites to Abiotic Stresses*

Plants established several adaptive mechanisms to endure abiotic factors, containing variations of metabolism in various directions, to confirm their existence in combative environmental situations [66] (Table 2). Several plant metabolites could assist and reduce the effect of the harsh stress of salt, drought, and water by acting as osmolytes and osmoprotectants [67]. Examples of such metabolites include dimethylsulfoniopropionate (DMSP) and glycine betaine; sugars, such as sucrose, trehalose, and fructan; amino acids, such as proline and ectoine, as well as some metabolites of polyols, sorbitol, and mannitol [68,69]. In plants, a wide range of waxy layers known as epicuticular wax keeps water balance during water shortage and acts as a mechanical stoppage to encounter disease-causing agents. Additionally, ascorbic acids, glutamine, alpha-tocopherol, anthocyanins, and carotene shield plant tissues by foraging the intermediates of bustling oxygen produced during oxidative stress [70]. Similarly, several other smaller compounds guard plants against oxidation damage related to various constrictions [65].

Besides, the plant's defense system is related to generating phytoalexins, stimulating the common phenylpropanoid pathway and producing lignin biosynthesis [71]. Further, phytochemicals and hormones such as salicylic acid and methyl salicylate, methyl jasmonate and jasmonic acid, as well as other small molecules formed due to stress, play a significant role against environmental stresses [72–74]. All of these may also function as signaling compounds by stimulating the resistance system and reactions of acclimation [75]. Among the defense systems of plants, osmotic regulation is one of the broadly pronounced responses to the water shortage that needs the accretion of harmonious solutes, such as sugars, amino acids, polyols, and glycine betaine [76]. These chemical compounds do a significant job in sustaining cell turgor and stabilizing cell membranes and protein. Moreover, other studies designate the importance of these compounds in rehabilitating redox stability through the scavenging of ROS, which could adversely affect cellular structures and metabolism [68,77].

**Table 1.** List of species, various metabolomics approaches, and applications cited in this review under diverse abiotic stresses.

| Species | Abiotic Factors | Method | Application | References |
|---|---|---|---|---|
| *Arabidopsis thaliana* L. | Temperature | GC-MS | Exploring the temperatures stress metabolome | [58,78] |
| *Populus euphratica* Oliv. | Water and salinity | Metabolite profiling | Changes in early and late transcription and metabolite profiles | [79–83] |
| *Thellungiella halophila* (C.A.Mey.) O.E.Schulz | | Metabolic fingerprinting | Identify metabolic changes in fruits | |
| *Solanum lycopersicum* L. | | Metabolic fingerprinting | To classify control as well as salt-treated groups of tomatoes | |
| *Arabidopsis thaliana* L. | | GC/MS and LC/MS | To reveal the short-term responses to salt stress | |
| *Arabidopsis thaliana* L. | Drought and flooding | Metabolic profiling | When defense pathways collide | [22] |
| | | | To identify the responses of plants to abiotic stresses | [84] |
| *Arabidopsis thaliana* L. | Sulfur | Multi-parallel, high-throughput analysis | To reveal novel findings | [85] |
| *Phaseolus vulgaris* L. | Phosphorus | Transcript profiling | To investigate global gene expression and metabolic responses | [86] |
| *Arabidopsis thaliana* L | Oxidative | GC-MS | To characterize the dynamics of metabolic | [87] |
| *Arabidopsis thaliana* L | Heavy metals Caesium (Cs) Cadmium (Cd) | NMR | Change metabolic consequences of stress | [88] |
| *Silene cucubalus* Wibel | | | Metabolomics analysis of the consequences of cadmium exposure | [89] |
| *Glycine max* L. | Salinity | GC-MS | Metabolomics analysis in the roots of different soya been varieties, under salinity levels | [90] |
| *Glycine. max* L. | CE-MS | CE-MS | Proteomic profile investigation of different soya bean varieties, under Cd stressed conditions | [91] |

#### 4.1.1. Amino Acids

Amino acids are considered a precursor for protein and other organic molecules, e.g., nucleic acids, which designate an active part in the responses of a plant under several stress factors. Amino acids could also play a significant role in signaling and controlling molecules [92]. Various studies showed that many amino acids stored in plants are apparent to different abiotic stresses [93,94]. Moreover, the exposure of plants to such stresses appearance an accumulation of proline and other amino acids. In plants, the role played by stored amino acids differs after acting as an osmolyte to adjust ions passage, reducing stomatal opening and reclamation of TM [95]. Moreover, amino acids can also disturb the synthesis and activity of several enzymes, gene expression, and redox state of homeostasis [96]. The accumulation of proline and ectoine is considered the most extensively dispersed osmolytes, as they act as osmoprotectants to protect plants from harmful effects and exciting environmental stresses, including low and high temperature, salinity, UVr, water, and osmotic stresses [68,97].

Primarily, proline is produced from a glutamate and proline metabolizing enzyme, pyrroline-5-carboxylate synthetase (P5CS), which reduces glutamate to pyrroline-5-carboxylate (P5C). At last, from the reduction of P5C, this stress-responsive amino acid forms by pyrroline-5-carboxylate reductases (P5CR) [98]. In transgenic plants, the significant role of proline was established during osmotic stress. For example, overexpression of the *P5CS* gene in soybean increased proline content and, thus, tolerance to salt stress in transgenic plants [99]. Besides osmolytes, proline is thought to accomplish many other important functions related to plant resistance, e.g., ROS scavenging, redox balancing, cytosolic pH buffer, molecular chaperon, and a stabilizer of protein structure [98]. Subsequently, in response to abiotic factors, the enlarged levels of proline were observed for several years to be the stress-responsive feature in plants. The relationship between the accumulation of proline as osmolytes and stress tolerance had a great share because of its applicability to different crops [100,101].

Remarkably, some of the metabolites were related to drought resistance and drought vulnerability of the considered hybrids [102]. Additionally, studies on drought responses at metabolomics levels indicated that Andean potatoes with a phenotype designating greater stress exposure have more proline related to the genetically assembled plant that was a higher dearth-tolerant [103]. It was established that the cultivar with a sensitive phenotype has high-level certain amino acids, containing proline and Gamma-aminobutyric acid (GABA) when barley exposed to salinity stress [104]. It may well advocate a greater liability of these plants to such stress. According to [96], this accretion could be associated with the deterioration of the leaf and slowing the development of a more subtle genotype. Furthermore, studies on Arabidopsis revealed that proline could be a lethal compound under heat stress [105], while Charlton et al. found that water deficiency was the cause of the decrease in isoleucine concentration in *Pea* and *Arabidopsis* plants [106].

#### 4.1.2. Polyamines

Plants are tested by different stress factors and adversely affect their growth, yield, and geographical circulation [107]. To survive the combative environmental stress circumstances, plants have developed many adaptive strategies, amongst which the accumulation of metabolites plays an important defensive role [108]. Metabolites strongly involved in stress resistance are the low-molecular-weight (LMW) acyclic polyamines [109]. Polyamines are the LMW nitrogen-containing organic compounds with more than two amino groups with a positive charge at the cellular pH, allowing them to link with negatively charged molecules, such as nucleic acids, phospholipids, and proteins [110]. Usually, polyamines are polycations essential for plant growth and development and play an important role in abiotic stress resistance in higher plants. Triamine spermidine, tetraamine spermine, and their diamine predecessor, putrescine, are the general polyamines [111]. Because of their cationic nature, these compounds have often been correlated to environmental stresses, such as drought, chilling, heat, TM, and salinity [112].

The results of Khan et al. [95] and Capell et al. [113] showed that the accumulation of spermidine with the up-regulation of spermidine synthase of *Cucurbita ficifolia* augmented several stress responses in a recombinant Arabidopsis plant, such as waterlogging and salinity stresses. It was shown that spermidine acts as a signaling molecule and controls the assertion of intricate genes in drought resistance. Furthermore, it has been demonstrated that polyamines are attributed to being involved in maintaining membranes shielding from damage under stressful environments [114] and controlling the formation of nucleic acid as well as enzyme activity [115]. Additionally, different findings revealed that polyamines play a significant role in oxidative stress by mitigating the balance state of ROS through their direct contact or indirectly regulating the antioxidant system and suppressing ROS production. Moreover, some authors hypothesized that polyamines could act as a cellular signal in plants throughout the stress responses [116].

#### 4.1.3. Carbohydrates

Carbohydrates produced during photosynthesis are the main building units that provide energy and support to the plant biomass [117]. Extensive studies revealed that non-living factors lead to the assemblage of non-structural saccharides, such as sucrose and lactose, simple sugars, or polyhydric compounds (alcohols and phenols), amongst various species of plants [118]. Particularly, there is a robust association between carbohydrate accretion and osmotic stress resistance, including oxidative stress (ROS) conditions, salt stress, and the scarcity of water [95]. As a source of carbon and energy in a cell, soluble carbohydrates may take a significant part in the metabolic processes of plants. Several stress factors may impact the level of these soluble carbohydrates because the accumulation of carbohydrates is associated with photosynthesis [119]. Rosa et al. [120] demonstrated that certain soluble sugars, such as sucrose and hexoses, improved stress tolerance by down-regulating the stress-related genes and up-regulating growth-related genes. Though, the contents of certain carbohydrates, such as raffinose, glucose, fructose, and maltose, are highly sensitive to environmental stresses and increase. However, the contents of myoinositol were reduced in barley roots during water-scarce conditions [121]. The findings of Sperdouli and Moustakas [122] revealed an increase and contact of augmented soluble carbohydrates, sustaining a great antioxidant defense in the leaves of *Arabidopsis thaliana* under dry environmental stress conditions. Studies showed renovation of carbon metabolism under salt-related stress (paraquat) in *A. thaliana* tissues and inferred by the researchers as a substitute approach to staying alive [122].

In water-deficit conditions, soluble sugars function as osmoprotectants, decreasing the harmful impact of osmotic stress and helps in sustaining the turgidity of cell and cell membrane stability by keeping plants from humiliation [123]. Under stress conditions, the increase in sugar quantity is generally the result of carbohydrate hydrolysis that needs enzymes with hydrolytic usage [124]. Moreover, carbohydrates that are soluble, such as disaccharides (sucrose and trehalose), oligosaccharides (raffinose and stachyose), and polymer of fructose molecules (fructans) next to their linked metabolic enzymes are essential compatible osmolytes associated with the scavenging of unstable molecules (ROS) during their assortment in plant tissues [125]. In low-temperature stress, sugar alcohols, such as polyols, function as osmoprotectants and shield cell membranes against ice adhesion [77]. Moreover, carbohydrates may act as signaling molecules [126]. The demonstrated data advocate a specific response of carbohydrates in plants. However, it should be noted that the accumulation of carbohydrates depends on the kind of stress to which it bared [127].

#### 4.1.4. Glycine Betaine

Glycine betaine (GB) is a widely studied quat compound, which is active in retaining the water balance between the plant cell and the environment during drought conditions. Moreover, GB playing a significant role in stabilizing the macromolecules, shielding photosynthesis, detoxification of reactive oxygen radicals, and as an osmoprotectant [128,129]. Several studies indicated their importance in improving plant tolerance under various abiotic factors. It has been shown that plants are distinguished according to the formation of GB, such as barley, spinach, maize, and wheat, produce and accumulate a higher quantity of GB in their chloroplast. However, some plant species cannot obtain substantial amounts of GB during stress, such as *A. thaliana*, rice, and tobacco [130]. Furthermore, it has been shown that transgenic plants could mitigate the impact of abiotic stresses. Therefore, efforts have been made to improve tolerance through glycine betaine biosynthesis to achieve transgenic plants. In transgenic plants, such as *Arabidopsis*, the *cyanobacteria* genes, such as glycine sarcosine methyltransferase, and in transgenic maize, a greater amount of GB accumulates. As a result, in transgenic *Arabidopsis*, resistance to drought and salt is greater; nevertheless, a recombinant plant of maize retained well in cold-related to non-transgenic cultivars [131,132].

Moreover, through genetic engineering, other transgenic plants with a GB-producing capacity have been achieved, including *Brassica juncea* and tobacco with greater tolerance

to salt and chilling, indicating a progressive ability to propagate and grow well related to wild-type in abiotic environmental conditions [133,134]. Besides, transgenic tomatoes with GB synthesis were more resistant to cold stress and produced fruit at a rate from 10 to 30% higher than the wild type. [135]. Though, the meditations of GB produced in every transgenic plant were scarce to control the osmotic stress to which plants were exposed. Similarly, previous studies showed that GB could enhance root growth and reduce oxidative stress. Additionally, the exogenous application of GB improves the stress tolerance of Cr in chickpea plants [136] and salinity stress in wheat [137]. Consequently, further protecting approaches of GB, such as defense against ROS and heavy metals stress, should be considered, which may enhance the tolerance level [138].

#### 4.1.5. Lipids

Lipids are a fundamental component of biological membranes, particularly the plasma membrane, which serves as the contact between the cell and its surroundings [72]. Lipids can be grouped into eight major types based on the chemical structure in conjunction with distinctive hydrophobic and hydrophilic components, such as fatty acids, glycerides, phosphoglycerides, sphingolipids, steroids, isoprenoids, glycolipids, and polyketides [139]. Being sessile organisms, plants are subjected to a wide variety of biotic and abiotic factors, such as temperature, drought, heavy metals, salinity, and pathogen attack. However, lipid-mediated signaling occurs in response to all these stressors (Figure 2). The plasma membrane, which is typically the signaling source of lipids, is commonly used by plants to sense these stimuli and transform the signal into subsequent biochemical metabolism. Generally, these are acclimating enzymes that have all been proposed as signaling lipids, such as phospholipases, lipid kinases, and phosphatases [140]. Commonly, lysophospholipid, fatty acid, phosphatides, triacylglycerol, inositol phosphate, oxylipins, sphingolipids, and nacylethanolamine are considered the major contributing signaling lipids molecules [141]. The conformation and activity of cellular proteins and metabolites are influenced by signaling lipids because they have the ability to temporarily attract molecular markers to the membrane.

The enzyme phospholipase A (PLA) is very important in the formation of fatty acids and lysophospholipids. Usually, lysophospholipids are present in very limited amounts in plant tissues; however, in stressed conditions such as freezing their quantity increases [142]. Some reports revealed the physiological role of lysophospholipids against various environmental stresses. Similarly, the phospholipase A2 (PlA2) has been shown to increase the production of some elicitors in poppy plants [143], while lysophosphatidyl-choline and lysophosphatidyl-ethanolamine act as signals transducers in arbuscular symbiosis in potato [144].

Fatty acids have also been demonstrated as stress-responsive lipids in plants. Oleic acids modulate nitric oxide-related proteins, thereby regulating nitric oxide and mitigating tolerance in *Arabidopsis* [145]. Moreover, fatty acids also regulate drought, salt, and heavy metals tolerance, as well as the wound-induced responses of pathogens/herbivores in plants [146]. Likewise, the responsive role of phosphatidic acid (PA), inositol polyphosphates, oxylipins, sphingolipids, and some other lipids have been studied in various plant species [147–149]. Some of the environmental stress factors under which the plant lipid responses were reported to include chilling, freezing, and wounding [150], pathogens [151], low-temperature stress [152], salt stress [153], and water and drought [154] stress response.

### *4.2. The Response of Secondary Metabolites to Abiotic Stresses*

Primary metabolites are compounds that are related to important physiological functions in organisms. Hence, they are generally found in all plant species and are directly involved in growth, development, and reproduction [155]. Compared to primary metabolites, secondary metabolites are very definite in their function, as they are not directly involved in plant growth, development, and reproduction of organisms. Generally, they are species-specific that could be redundant in different situations [156]. Usually, they

are made under particular conditions for a definite purpose, such as defense against pathogens infection, enhanced resistance to abiotic stresses, and protect the harmful effect of UVr [157]. Furthermore, secondary metabolites produce different compounds important for several biochemical and biophysical processes in plant cells and tissues (Table 2). However, they have no common familiar physiological functions in plants, such as photosynthesis, respiration, translocation, transportation of solute, acclimatization of nutrients, and differentiation [158].

In addition, the specified plant species produce these natural products, and their concentration level is controlled to some extent with the growing period, environments, and adjustment progress [159]. Substantially, they attracted insects and animals for fertilization and seed spreading. The accumulation of phenyl amides in beans to the impact of abiotic factor (heat) was described, proposing an antioxidant role of these secondary metabolites [160]. Modern research tries to identify the key roles the secondary metabolites play in plants as indicators, antioxidants, and for other purposes. Secondary metabolites are also important in plants used by humans [161]. Besides, the compounds of secondary plant metabolites are distinctive means of food essences, medicines, flavorings, and other industrial materials [162]. In plants, the accretion of certain metabolites frequently occurs exposed to different stress factors, such as several phytohormones, elicitors, TM, and signal transduction compounds [163–165].

Some famous examples of secondary plant metabolites with medicinal properties include the anesthetic and antipyretic compounds salicin taken from *Salix* sp., which is used to make aspirin [166]. Similarly, other pharmacological secondary metabolites, such as taxol (anticancer), sequestered from pacific yew (*Taxus brevifolia*), and the strong obsessive compound morphine removed from opium (*Papaver somniferum*). Secondary metabolites have the following three major groups: phenolics, terpenes, and S and N comprising compounds (Figure 3) [167,168].

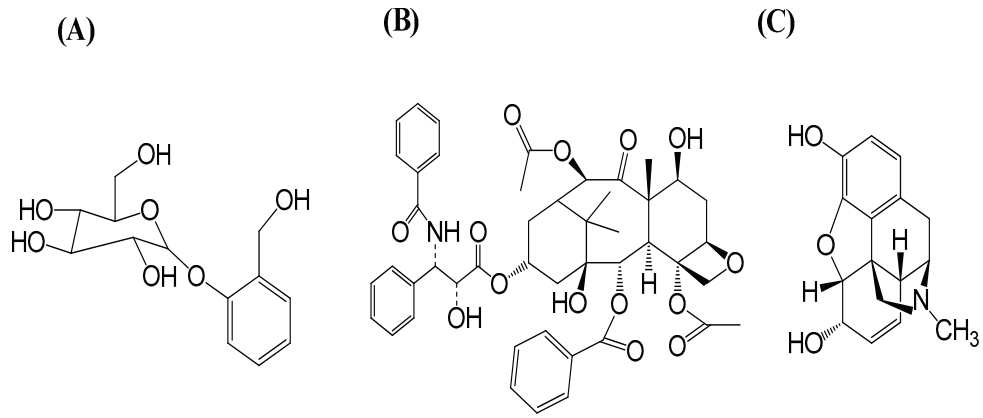


**Figure 3.** Some eminent examples with medicinal properties of secondary plant metabolites are (**A**) salicin, (**B**) taxol (paclitaxel), and (**C**) morphine.

#### 4.2.1. Phenolic Compounds

In plants, phenolic compounds are recognized as the largest and essential group of secondary metabolites changing from simpler aromatic rings to more complicated ones, such as lignin, and play a significant physiological role in increasing the resistance and adaptableness suboptimal circumstances during the life cycle of plants [169,170]. Phenolics are produced in optimum and sub-optimum environments in plants and play a major role in various developmental mechanisms, such as cell division, balancing hormones, photosynthetic processes, and reproduction, as well as in the mineralization of nutrients [75]. These compounds constitute secondary metabolites, including lignins and tannins, flavonoids, isoflavonoids, anthocyanins, and coumarins [171]. Moreover, all these chemical compounds are produced in plants by the phenylpropanoid pathway, in that phenylalanine compound

is the main substratum that can do significant work in the resistance mechanism of plants against various stress factors in the environment [172].

The pathway of phenylpropanoid is regulated by biotic and abiotic factors, including drought, salt stress, TM, low or high temperature, wounding, pathogen attack, herbicide treatment, nutrient deficiencies, and UV radiations causing the accumulation of different phenolic compounds [75,173]. Consequently, the aggregations of phenolic compounds in plant materials are considered an important sustaining strategy of plants in harsh environmental situations. Hence, respond to these stresses and contribute to the removal of ROS, catalyzed-oxygenated reaction with the establishment of metabolic structures and obstructing the processes of oxidative enzymes, thus increasing evolutionary aptness [174,175]. Besides, phenolic accumulation is also considered a reliable feature and key defense mechanism under stress, leading to the enhanced creation of free radicals and other oxidative species in plants [176].

Moreover, to survive in oxidative stress conditions, plants had established two diverse biological ways, such as escaping ROS creation and eliminating it through enzymatic and non-enzymatic processes, such as the deposition of LMW antioxidants [177,178]. Further, studies revealed that the accumulation of LMW antioxidants results due to the activities of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and other essential enzymes [179]. Various physiological processes of plants related to growth and development in plants comprising seed germination, cell division, and synthesis of photosynthetic pigments, are influenced by phenolic acids and flavonoid accumulation for persistence and adaptation to environmental conflicts [180,181]. In particular, phenolic compounds consult greater tolerance in plants such as TM stress, which enhances the production of ROS and reduced growth [182], and phenolic compounds (flavonoids), in response, protect plants from oxidative stress damage through the chelation process [183,184]. Similarly, when plants are exposed to other abiotic factors can also affect their life cycle. Under drought conditions, the concentration of ferulic acid decreased, while the p-coumaric acid and caffeic acid increased in maize xylem sap, which could be supportive in stiffening and lignification of the cell wall [185]. Spatially confined fluctuations in cell wall phenolics were presented to be engaged in the advanced inhibition of wall extensibility and root growth, which can enable root acclimation to drought [186].

Various environmental stress factors mediated the synthesis of flavonoids, isoflavonoids, and anthocyanins. In plants, flavonoids play a defensive role due to their antioxidant properties when exposed to a water-deficit situation [187]. Moreover, Nakabayashi et al. [188] indicated that flavonoid significantly improves resistance in *A. thaliana* in water scare conditions. Similarly, phenolic acids and flavonoids as antioxidants and sunshades are involved in plants' response to a dry environment [189]. Other studies reported that different polyphenolic was associated with gene expression reforms in an account of potato plants under drought conditions, though the fluctuations were greatly specific to the cultivar [190]. Rodziewicz et al. [96] and Parida et al. [191] suggested that polyphenols are involved in conserving osmotic potential in cells and confiscating free radicals during drought stress. Besides, polyphenols affect the source and movement of organic and inorganic soil nutrients existing for plants and microbes and indicate a reply to nutrient insufficiency, therefore offering a way for identifying nutrient ailments earlier to the occurrence of evident symptoms [192]. Stress conditions of drought and waterlogging increased the flavonoids quercetin and rutin in the herbaceous pharmaceutical plant *Hypericum brasiliense*, whereas cold stress caused a different reaction [193]. Comparatively, a greater decrease in flavonoids was noticed in the sensitive genotypes; thus, they could show that the flavonoid content was imperative in sustaining the greater antioxidant activity in water-stressed conditions [96,194].

Furthermore, anthocyanins were identified to increase their content in plant tissues against drought and cold stress because of their antioxidant and ROS scavenging properties, which cause protection to plant cells [195,196]. In red-fleshed apple callus culture, low temperature (16 °C) tempted an increased level of anthocyanin [197].

Additionally, the assembly of phenolics rises into the cell wall either as suberin or lignin under low-temperature stress [198]. Though, suberin deposition and lignification increase the adaptability and resistance to cold stress [192]. Similarly, to respond to the negative effects of Uvr, endogenous phenolic compounds (flavonoids) accumulate in plant cells and make a shield under the epidermal layer, which protects the plant and the component of the cell from these harmful radiations [199]. Moreover, flavonolignan silymarin has been reported to accumulate in in-vitro cultures of *Silybum marianum* upon application of abiotic stress treatments, such as NaCl, polyethylene glycol, and gamma irradiation, because of the defensive mechanisms that cells perform to counteract the stress of these factors [200]. Therefore, stress elicitation successfully produces high-value phytoconstituents from medicinal crops [201,202].

#### 4.2.2. Terpenoids

Plants have developed a complex resistance system that depends on the swift perception and instigation of secondary metabolites to adopt different environmental stress factors in an ecosystem [203]. Among all, terpenoids establish a broad and structurally diverse group of lipophilic secondary metabolites, which are produced in plants from isoprene units ($C_5H_8$) [204]. Physiologically, terpenoids play an important role as phytohormones, such as the sesquiterpenoid abscisic acid (ABA) and the diterpenoids gibberellic acid (GA), against biotic and abiotic stresses. Studies have shown that the phytohormone abscisic acid (ABA) triggers defense mechanisms, such as facilitating responses to drought and water stress by adapting the membrane properties [205]. Moreover, terpenoids show antioxidant and antibiotic activity that maintains lipid membranes and increases environmental stress tolerance against herbivores [206].

Terpenoids also function as phytoalexins (LMW antimicrobial compounds), prepared as part of the plant defense mechanism in response to abiotic and biotic factors. For example, many diterpene phytoalexins have been reported in *Oryza sativa* [207]. Similarly, in cotton plants, sesquiterpenoid phytoalexins, such as gossypol, hemigossypolone, and heliocides, as defensive metabolites accumulated both above and below the ground against pathogens and herbivores [208]. Moreover, in maize and rice leaves and roots, diterpene phytoalexins are produced, including zealexins, kauralexins, and oryzalexins that exhibit antimicrobial properties and respond against pathogenic fungal blast diseases, such as rice blast caused by *Magnaporthe grisea* [207,209]. Additionally, UVr and TM stress induced the accumulation of rice phytoalexins. According to Vaughan et al. [209], the accumulation of phytoalexins in response to drought is root-specific and does not affect the level of phytoalexins aboveground. However, the reduced content of the terpenoid compound was described in cotton species in drought conditions [191,210]. Yusuf et al. [211] noticed that the increased content of soluble alcohol tocopherol with antioxidant properties shows a significant role in the mitigation of stress by stabilizing the cell membranes induced by salinity, TM, and osmotic potential in *B. juncea*. Furthermore, the content of saponins in soybean plants was recognized as one of the crucial secondary metabolites related to the resistance of salt stress [212].

**Table 2.** The response of various types of metabolites against different abiotic stresses.

| Metabolomics | Stress | Mode of Action | References |
|---|---|---|---|
| **Primary Metabolites** | | | |
| Amino acids: (proline) | Drought, salinity, temperature, and cold | Acts as osmoprotectant | [77,96,97] |
| Polyamines: (triamine spermidine, tetraamine, spermine) | Heavy metals | Regulating antioxidant systems, suppressing ROS production | [168] |
| Carbohydrates: a. (sugar, sucrose) | Water deficit | Osmoprotectant, maintain turgor, cell membranes stability | [95] |

**Table 2.** *Cont.*

| Metabolomics | Stress | Mode of Action | References |
|---|---|---|---|
| b. alcohols (sorbitol, ribitol, and inositol) | Cold stress | Cryoprotectants protect cell membranes against ice adhesion | [97,124] |
| c. disaccharides, raffinose | ROS | ROS scavengers, control ROS signaling | [39,106] |
| Glycine betaine | Drought, ROS, salt, and low temperature | Osmoprotectant detoxification of ROS, | [128] |
| Lipids | Heavy metals stress | Scavenge the ROS production | [136,138] |
| | Secondary Metabolites | | |
| Phenolic compounds: p-coumaric acid, caffeic acid; flavonoids, anthocyanin, suberin, or lignin | Heavy metals/ ROS | Scavenging of ROS and chelation process | [151,159] |
| | Water stress | Antioxidant mechanism | [154] |
| | Drought, UV | Stiffening and lignification of the cell wall, antioxidant, and sun shields properties | [191] |
| | Drought, nutrient deficiency | Scavenging of ROS, maintenance of osmotic potential in cells, and identifying nutrient ailments | [77] |
| | Cold and drought | Increase resistance and protect plant cell | [161] |
| | Cold or low temperature | Lignification and submarine deposition increase adaptability and resistance | [213] |
| TerpenoidsAbscisic acid (ABA), gibberellic acid (GA), phytoalexins (gossypol, hemigossypolone and heliocides), momilactones, oryzalexins, tocopherol, saponins | Biotic and abiotic factors | Physiological function, ameliorate heavy metal stresses, antioxidant, and antibiotic activity | [170,171] |
| | Heavy metal, drought, UV, pathogens, and herbivores | Improve stress tolerance, drought, heavy metals, and enhances antimicrobial properties | [207–210] |
| | Fungal blast | Stabilizing the cell membranes | [212] |
| | Salinity, heavy metal, potential osmotic | Salt stress tolerance | [212] |
| Nitrogen-containing metabolites<br>Alkaloids<br>Glucosinolates<br>Non-protein amino acids | Drought, herbivores | Increase tolerance level and defense against herbivore attack | [158,214] |
| | Drought, waterlogging | Osmoprotectants increased phytochemical contents | [215,216] |

#### 4.2.3. Nitrogen-Containing Secondary Metabolites

Plants have developed several defense mechanisms against invading enemies, such as microbial pathogens and herbivorous animals, as well as abiotic factors, e.g., drought, waterlogging, and salinity, which are considered for the high loss of crop production worldwide [217]. However, plants have developed a complex defense system of secondary metabolism against these stressors, including the nitrogen-containing secondary metabolites, such as alkaloids, cyanogenic glycosides or glucosinolates, and non-protein amino acids (Figure 4) [158,217]. Previously, nitrogen-containing secondary metabolites were considered unwanted materials of plants and are known now for their resistivity towards different stress factors [119]. Among the phytochemicals, alkaloids are heterogeneous groups of secondary metabolites consisting of one or more nitrogen atoms produced under abiotic stress conditions. It has been found that alkaloids perform a significant role against microbial pathogens and herbivorous animals. Besides, more alkaloid contents and derivatives are produced in abiotic stress conditions. For example, poppy plants make more alkaloids when there is a drought period as well as under salinity stress [214]. In lupins (*Lupinus termis*) cultivars, the content of alkaloids was also influenced by the drought and activated yeast extract treatment [216].

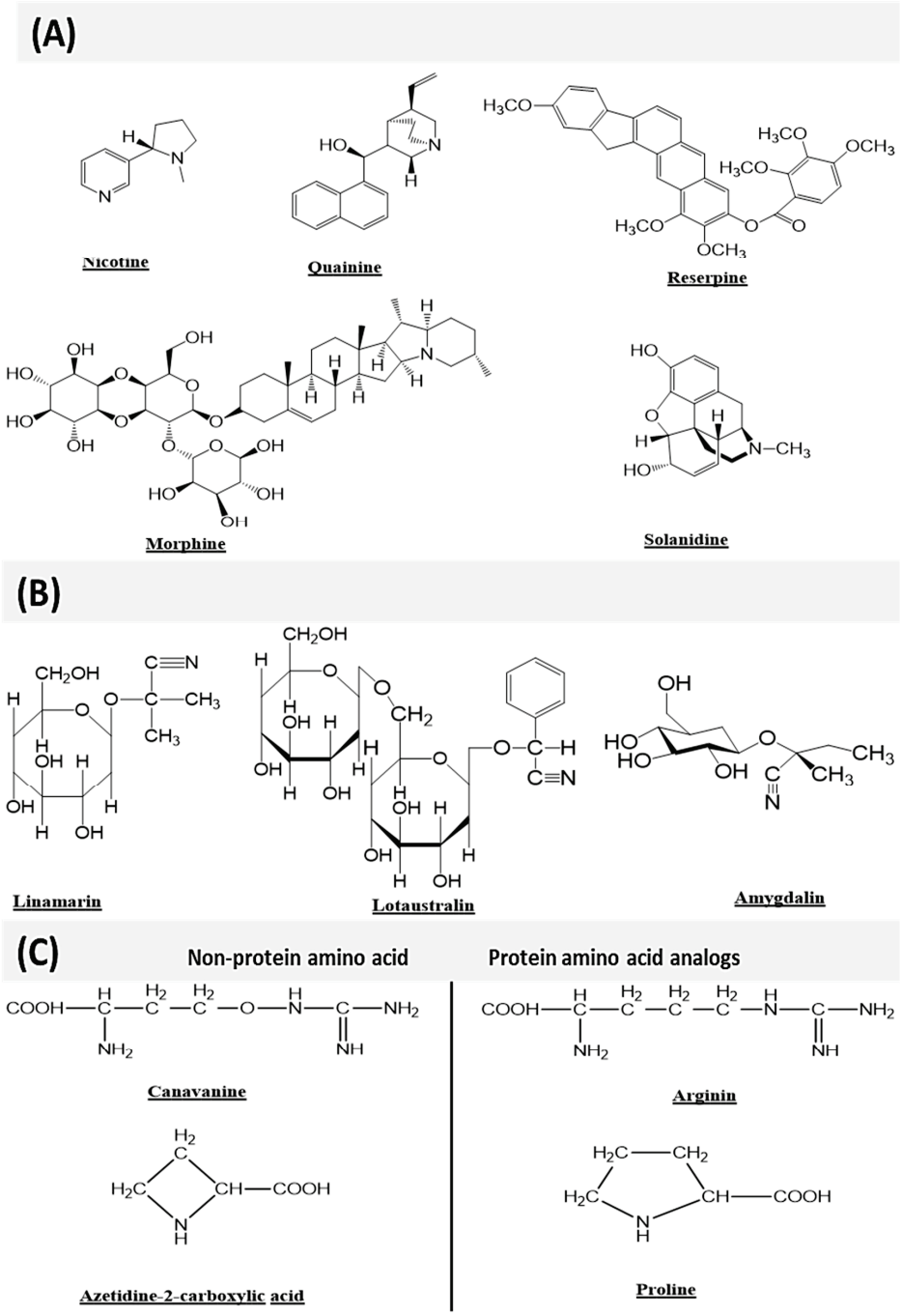


**Figure 4.** Chemical structures of some plants derived primery and scndary metabolites with key importance in different era of lfe. Among all, some commonly known alkaloids (**A**), cyanogenic glycosides (**B**), and (**C**) non-protein amino acids along with their protein amino acids analogues.

Additionally, glucosinolates and cyanogenic glycosides are sulfur and nitrogen-containing secondary metabolites derived from glucose and amino acids. Similarly, Rodziewicz et al. [96] demonstrated that all these natural compounds play a significant role against different environmental factors (biotic and abiotic). Mewis et al. [215] showed that in *A. thaliana*, under drought and water logging conditions tend to increase aliphatic compounds of glucosinolate and flavonoids. Moreover, in *B. juncea*, the increased level of glucosinolate was observed during the vegetative stage under water deficit conditions. In plants, apart from the essential 20 amino acids, there are more than 200 free plant cell amino acids that

are not assimilated into proteins. These free amino acids are called non-protein amino acids. Their major function in plants is to respond to various environmental stresses [158,217].

## 5. Conclusion and Future Perspectives

The acceleration of climate change increases the severity of damage to crop productivity under environmental stress. Understanding the role that primary and secondary metabolites play during stress resistance mechanisms is important for developing crop species and improving their stress resistance, ensuring that the need for food security is met for a growing global population. However, less has been understood about the function of these metabolites against environmental stresses in plants, especially abiotic stresses. In the current review, we have provided an overview of the role of primary (amino acids, polyamines, carbohydrates, glycine betaine, and lipids) and secondary (phenolics, flavonoids, terpenoids, alkaloids, and glycosides) metabolites against several abiotic factors, such as drought, salinity, temperature, UVr, and TM. Analysis of more than 200 articles allowed us to describe the main responses of primary and secondary metabolite products of different plant species to abiotic stresses. Metabolomics has occupied a prominent place in plant stress physiology and biology research. Metabolic change due to abiotic stress is complex to describe the variability between different plant species. Nevertheless, metabolomics needs more extensive research in data annotation, assessment, processing, and evaluation. Progress in "omics" tools and bioinformatics and enhanced assimilation of the data from varying molecular levels is needed. Hence, to expose the full picture of sustaining mechanism, which will lead to new biomarkers of resistance towards biotic and abiotic stresses. Affirmation of the impact of environmental stresses on plants and their metabolite level responses recorded valued genes about the mechanism underlying such acclimation. However, the balancing mechanism between the gene expression and the subsequent metabolic phenotype is a big challenge nowadays. Therefore, comprehensive research of the dynamic behavior of metabolic systems is a great task for researchers in systematic biology. Furthermore, identifying the genetic background behind the diversity of primary and secondary metabolites produced by plants will help in improving and developing stress tolerance. Manipulating and overexpressing genes related to the biosynthetic pathway of secondary metabolites could be a solution for plant tolerance to environmental stress conditions.

**Author Contributions:** Conceptualization: S.A. and U.S.; Investigation: Z.-H.T. and S.A.; Resources: S.U., U.S., A.A.E. and Y.K.; Software: S.U. and A.A.E.; Supervision: Z.-H.T.; Writing—original draft: S.U.; Review and editing: A.A.E., J.K., and Y.K.; Equal contribution: S.U.; References: A.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** The key Research and Development project of Heilongjiang Province, China (JD22A008).

**Acknowledgments:** The authors are thankful to the China Scholarship Council (CSC) for support and to the Key Laboratory of Plant Ecology, Northeast Forestry University, Harbin 150040, China, for providing an excellent research environment. Moreover, Uzma Salam and Shakir Ullah are thankful to Zhong-Hua Tang for his continuous support and assistance during their studies.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Characterization of AtBAG2 as a Novel Molecular Chaperone

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**Abstract:** Bcl-2-associated anthanogene (BAG) family proteins regulate plant defense against biotic and abiotic stresses; however, the function and precise mechanism of action of each individual BAG protein are not yet clear. In this study, we investigated the biochemical and molecular functions of the *Arabidopsis thaliana* BAG2 (AtBAG2) protein, and elucidated its physiological role under stress conditions using mutant plants and transgenic yeast strains. The T-DNA insertion *atbag2* mutant plants were highly susceptible to heat shock, whereas transgenic yeast strains ectopically expressing *AtBAG2* exhibited outstanding thermotolerance. Moreover, a biochemical analysis of GST-fused recombinant proteins produced in bacteria revealed that AtBAG2 exhibits molecular chaperone activity, which could be attributed to its BAG domain. The relevance of the molecular chaperone function of AtBAG2 to the cellular heat stress response was confirmed using yeast transformants, and the experimental results showed that overexpression of the *AtBAG2* sequence encoding only the BAG domain was sufficient to impart thermotolerance. Overall, these results suggest that the BAG domain-dependent molecular chaperone activity of AtBAG2 is indispensable for the heat stress response of Arabidopsis. This is the first report demonstrating the role of AtBAG2 as a sole molecular chaperone in Arabidopsis.

**Keywords:** BAG (Bcl-2-associated anthanogene) family proteins; abiotic stress; molecular chaperone

**Citation:** Kang, C.H.; Lee, J.H.; Kim, Y.-J.; Kim, C.Y.; Lee, S.I.; Hong, J.C.; Lim, C.O. Characterization of AtBAG2 as a Novel Molecular Chaperone. *Life* **2023**, *13*, 687. https://doi.org/10.3390/life13030687

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 22 November 2022
Revised: 31 December 2022
Accepted: 31 December 2022
Published: 3 March 2023



## 1. Introduction

B cell lymphoma 2 (Bcl2)-associated athanogene (BAG) family proteins are conserved across a wide range of eukaryotes including animals, yeast, and plants. BAG1, the first BAG protein to be discovered, was identified by screening for human Bcl-2-interacting proteins in a mouse embryo cDNA library [1–3]. To date, six BAG proteins (BAG1–BAG6), harboring a conserved BAG domain (BD) at the C-terminus have been reported in humans [4]. The three-dimensional structure of the BD, which contains approximately 110 amino acids (aa), comprises three α-helices, each containing 30–40 aa [5,6]. Through the BD, BAG proteins modulate the refolding activity of the heat shock-inducible heat shock protein 70 (HSP70) and the constitutively expressing heat shock cognate protein 70 (HSC70) by interacting with their ATPase domain [5]. In mammals, BAG proteins interact with members of diverse protein families to regulate various cellular phenomena such as protein degradation and cell apoptosis, migration, and proliferation [7,8].

In plants, *BAG* genes are associated with development and stress responses [9]. An advanced bioinformatics analysis revealed that the model plant *Arabidopsis thaliana* contains seven BAG proteins (AtBAG1–AtBAG7) [10,11]. Among these, AtBAG1–AtBAG4 carry

an N-terminal ubiquitin-like (UBL) domain, whereas AtBAG5–AtBAG7 are plant-specific proteins harboring a calmodulin (CaM)-binding motif near the BD [10,11]. AtBAG proteins are reportedly involved in various processes related to plant development and environmental stress response. For example, AtBAG1 plays a critical role in the Hsc70-mediated proteasomal degradation of misfolded and unimported plastid proteins in the cytosol, and an optimal level of this protein is important for normal plant growth. AtBAG6, a CaM-binding protein, induces programmed cell death (PCD) in Arabidopsis and yeast, and its 134 aa stretch, which encompasses both the CaM-binding IQ motif and BD, is sufficient to induce cell death [11]. Additionally, AtBAG4, AtBAG6, and AtBAG7 are involved in the plant response to pathogen attack as well as the tolerance to cold, heat, ultraviolet (UV), and endoplasmic reticulum (ER) stresses [9,10,12]. Recently, *AtBAG2* and *AtBAG6* were reported to be involved in the tolerance to multiple abiotic stresses in Arabidopsis [13]. However, information on the biochemical and molecular functions of BAG proteins that affect plant physiology is extremely limited. In this study, we explored the intrinsic biochemical and molecular properties of AtBAG2 and characterized this protein as a novel molecular chaperone.

## 2. Materials and Methods

### *2.1. Plant Material and Growth Conditions*

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild type (WT) in this study. The homozygous T-DNA mutant *atbag2* (SALK_030295; Col-0 background) was obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus, OH, USA). Seeds were sown in Petri dishes containing full-strength Murashige and Skoog (MS) medium (Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 2% (*w*/*v*) sucrose and 1% (*w*/*v*) agar, and cold-stratified in the dark at 4 °C for 3 days. The plates were then transferred to an environmentally controlled growth chamber maintained at 22 °C temperature, a 16 h light/8 h dark photoperiod, and 100 µmol $m^{-2}$ $s^{-1}$ light intensity. The chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical, Saint Louis, MO, USA).

### *2.2. Heat Shock Treatment and Phenotypic Analysis of Plants*

To examine the thermotolerance of the WT and *atbag2* mutant, seedlings were grown on solid nutrient medium containing 2% (*w*/*v*) sucrose, and incubated under the above-mentioned conditions [14]. Plates containing 7-day-old seedlings were sealed with plastic electrical tape and submerged for 30 min in a water bath maintained at 44 °C. The plates were then transferred to normal growth conditions, and the thermotolerance of seedlings was determined by measuring their survival rate. To measure electrolyte leakage, the seedlings were submerged in 5 mL of deionized water and placed on a shaker at 22 °C. The initial conductivity of the solution was measured using an Orion 3-Star Benchtop Conductivity Meter (Thermo Electron Cooperation, Rosemount, MN, USA). The seedlings were then autoclaved for 15 min, and conductivity was measured again (final conductivity) to determine the total amount of ions in solution. Percent ion leakage was estimated as the ratio of initial conductivity to final conductivity.

### *2.3. Yeast Strain, Survivability Assay, and TB Exclusion Assay*

The yeast (*Saccharomyces cerevisiae*) strain W303 (SVL82; *MaTα*, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *can1*) was grown in Yeast Extract-Peptone-Dextrose (YPD) medium at 27 °C [15]. Cells transformed with the glutathione S-transferase (GST)-only vector (control) and various GST-AtBAG2 fusions (G-AtBAG2, G-AtBAG2-Nt, G-AtBAG2-M, and G-AtBAG2-Ct) were grown in the YPD medium overnight. The cells were then transferred to fresh YPD medium and incubated at a concentration of 5 × 107 cells/mL at 27 °C or 55 °C. Cells were sampled at 0, 10, 20, 30, 40, 50, and 60 min after incubation, and suitable dilutions were plated on YPD agar plates. The number of viable cells or colony forming units (CFU) was counted after 2–3 days of incubation at 27 °C.

Samples of vector, *G-AtBAG2*, *G-AtBAG2-Nt*, *G-AtBAG2-M*, and *G-AtBAG2-Ct* transformants collected at the 60 min time point were subjected to trypan blue (TB) staining and spot assays. The TB staining assay was performed as described previously [16]. To conduct spot assays, the concentration of each culture was adjusted to an optical density ($OD_{600}$) of 1.0. Next, 6 µL aliquots of 10-fold serial dilutions were spotted onto YPD agar plates, incubated at 27 °C, and examined after 2–3 days.

### *2.4. Construction of Expression Plasmids*

The *AtBAG2* coding sequence was PCR-amplified using the following primer pair: AtBAG2(*Bam*HI)-F (5′-GGATCCTAATGATGAAAATGAGTATCGGA-3′) and AtBAG2(*Xho*I)-R (5′-CTCGAGTTAATTGAATAATTCCCATTTA-3′). The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and the *Bam*HI/*Xho*I fragments were subcloned into the pGEX-2T vector (GE Healthcare, Piscataway, NJ, USA). To identify the amino acid sequence required for the molecular chaperone activity of AtBAG2 and the thermotolerance of yeast cells, deletion fragments of *AtBAG2* were generated by PCR using the following primers: AtBAG2(*Bam*HI)-F and AtBAG2-Nt(*Xho*I)-R (5′-CTCGAGTTAGATTGCTTTAGATGATTT-3′) for *AtBAG2-Nt* (1–133 aa); AtBAG2-M(*Bam*HI)-F (5′-GGATCCTAATGAAAGAGAAATCATCTAAA-3′) and AtBAG2-M(*Xho*I)-R (5′-CTCGAGTTACTGCATCTTCTTCTTCAA-3′) for *AtBAG2-M* (131–195 aa); and AtBAG2-Ct(*Bam*HI)-F (5′-GGATCCTAATGAAGAAGAAGATGCAGAAT-3′) and At-BAG2(*Xho*I)-R for *AtBAG2-Ct* (191–296 aa). The amplified products were cloned into the pGEM-T Easy vector, and subcloned into the pGEX-2T expression vector digested with *Bam*HI and *Xho*I (for expression in *E. coli*) or into the pYES2-GST fusion vector digested with *Bam*HI and *Xho*I (for expression in yeast).

### *2.5. Recombinant Protein Production and Purification*

The pGEX-AtBAG2, pGEX-AtBAG2-Nt, pGEX-AtBAG2-M, and pGEX-AtBAG2-Ct vectors were transformed into *Escherichia coli* BL21(DE3) pLysS cells. Recombinant protein production and purification were performed according to the procedure described previously [17]. The AtBAG2 deletion variants were further purified using the TSK heparin-5PW HPLC column (7.5 mm × 75 mm), as described previously [17,18]. DnaK, a possible co-purifying contaminant on GSH columns, was removed using the ATP-agarose column (Amersham Pharmacia Biotech, Piscataway, NJ, USA), according to the manufacturer's instructions. The purified AtBAG2 proteins were dialyzed against 20 mM HEPES (pH 8.0) before use.

### *2.6. Protein Quantification and Chaperone Activity Assay*

Mixtures containing a given recombinant AtBAG2 protein (30 µg/mL protein in 20 mM HEPES [pH 8.0]) and 10 µM 4,4′-bis (1-anilinonaphthalene 8-sulfonate) (bis-ANS; Sigma-Aldrich) were incubated at various temperatures for 30 min. The fluorescence of each mixture was then measured using a SFM25 spectrofluorometer (Kontron, Zurich, Switzerland) at an excitation wavelength of 380 nm and emission wavelengths ranging from 400–600 nm.

The chaperone activity of recombinant proteins was measured using citrate synthase (CS), malate dehydrogenase (MDH), and insulin (Sigma-Aldrich) as substrates, as described previously [19,20]. Turbidity caused by substrate aggregation was monitored using the DU800 spectrophotometer (Beckman Coulter, Indianapolis, IN, USA) equipped with a thermostatic cell holder.

## 3. Results

### *3.1. AtBAG2 Enhances Heat Shock Tolerance in Arabidopsis*

To confirm the physiological significance of *AtBAG2* in plant stress response, we selected homozygous lines of the *atbag2* T-DNA insertion mutant originally obtained from ABRC (OH, USA) (Figures 1A,B, S1 and S2). Transcript levels of *AtBAG2* were compared

between WT (Col-0) and *atbag2* mutant plants by semi-quantitative reverse transcription PCR (sqRT-PCR) (Figures 1C, S3 and S4). Although the *AtBAG2* signal was clearly detected in WT plants, it was barely detectable in the *atbag2* mutant, as expected (Figures 1C and S3). By contrast, the signal for the *Actin-2* (*ACT2*) gene (*AT3G18780*), which was used as a control, was nearly identical in both WT and *atbag2* samples (Figures 1C and S4). Next, we investigated the physiological function of *AtBAG2* in 7-day-old WT and *atbag2* seedlings under heat shock conditions (Figure 1D–F). Under normal conditions (22 °C), the two genotypes exhibited no difference in seed germination and seedling survival rates (Figure 1D,E). However, when incubated at 44 °C for 30 min, the 7-day-old *atbag2* seedlings exhibited outstanding thermosensitivity (Figure 1D,E), as indicated by their low survival rate (31.4 ± 5.6%) compared with the WT (56.6 ± 5.2%) (Figure 1E). Because heat shock causes considerable damage to the channel and transporter proteins, fluidity, and other properties of the cell membrane, resulting in electrolyte leakage [21,22], we compared ion leakage between the WT and *atbag2* plants. Following incubation at 44 °C for 30 min, the seedlings of both genotypes exhibited an increase in ion leakage; however, the level of ion leakage was higher in *atbag2* seedlings than in the WT (Figure 1F). The WT and *atbag2* seedlings took 53 and 37 min, respectively, to reach 50% ion leakage. Overall, these results indicate that *AtBAG2* plays a major role in plant survival under heat stress.

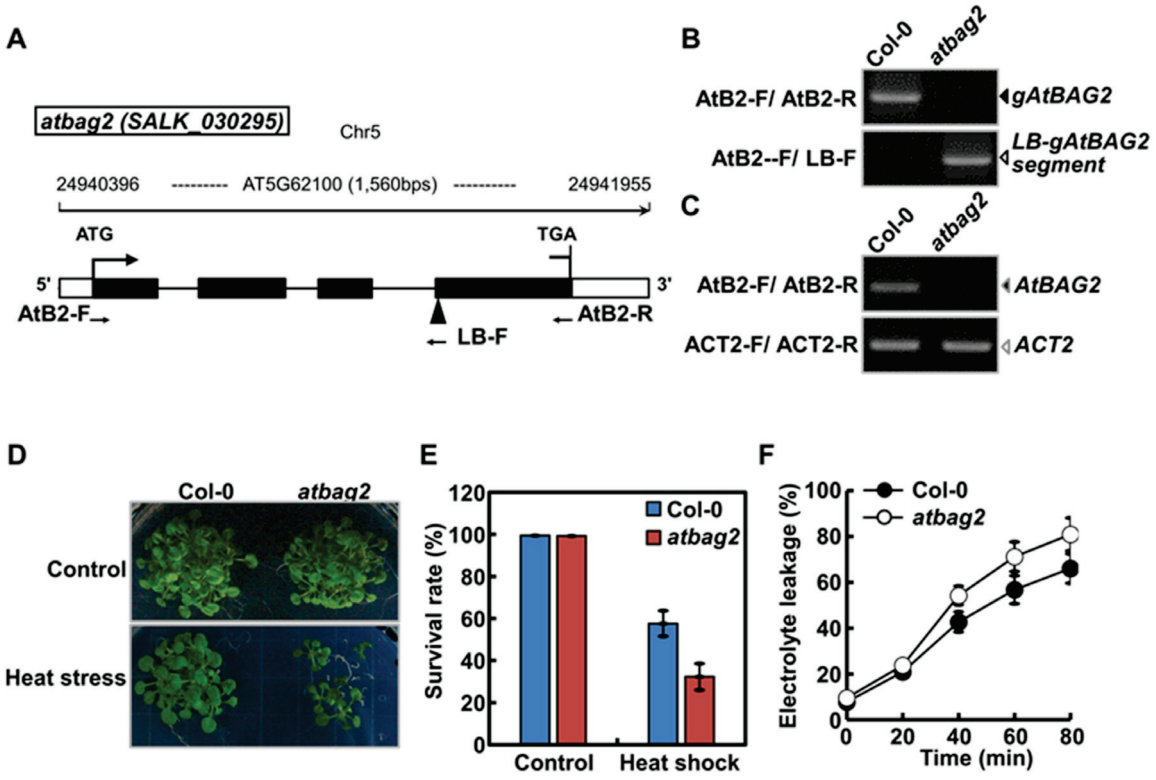


**Figure 1.** *AtBAG2* regulates heat stress tolerance in Arabidopsis. (**A**) Schematic representation of the predicted gene structure of *AtBAG2*. Black elbow arrows represent the start codon (ATG). Filled boxes and horizontal black lines indicate exons and introns, respectively. Empty boxes at either end of the gene represent 5′ and 3′ untranslated regions (UTRs). A black arrowhead indicates the T-DNA position in *atbag2*. Black arrows represent the binding sites of primers used for genotyping. (**B**,**C**) Confirmation of homozygous *atbag2* mutant lines by genomic DNA-based PCR (**B**) and sqRT-PCR (**C**). (**D**) Photographs of WT (Col-0) and *atbag2* seedlings grown at optimal temperature (22 °C) for 14 days (control) or subjected to heat stress conditions (7-day-old seedlings treated with heat shock [44 °C] for 30 min, and then grown at 22 °C for 7 days). (**E**,**F**) Comparison of survival rate (**E**) and ion leakage (**F**) between 7-day-old WT (Col-0) and *atbag2* seedlings subjected to the heat shock treatment, as described in (**D**). In (**E**), the survival rates of heat-stressed Col-0 and *atbag2* seedlings

(heat shock) were compared with those of unstressed plants (control). Data represent the mean ± standard deviation (SD) of at least three independent experiments. To measure the ion leakage of Col-0 (-●-) and *atbag2* (-○-) seedlings shown in (**F**), samples were collected at the indicated times and submerged in deionized water for 1 day. The conductivity of at least 10 seedlings was measured before autoclaving (initial conductivity) and after autoclaving (final conductivity). Data represent the mean ± SD of at least three independent experiments.

### *3.2. AtBAG2 Overexpression Enhances Thermotolerance in Yeast*

Because *atbag2* plants were more sensitive to heat shock than WT plants, we investigated whether *AtBAG2* imparts heat stress tolerance at the cellular level by monitoring the effects of *AtBAG2* overexpression in yeast cells. To conduct this experiment, we cloned the *GST-AtBAG2* gene fusion under the control of the *GAL1* promoter to generate the *pYES2-G-AtBAG2* construct. We then transformed *pYES2-G-AtBAG2* and the *GST (G)*-only construct (*pYES2-G*) separately into yeast W303 cells for the conditional overexpression of recombinant proteins. To confirm the physiological significance of *AtBAG2* overexpression in yeast, we compared the viability of heat-shocked *G* and *G-AtBAG2* transformants. Cultures of the two transformants were adjusted to equal cell densities at the mid-exponential growth stage, and aliquots were incubated at 27 °C or 55 °C. Viable cell counts were measured at regular intervals (Figure 2A). The *G*- and *G-AtBAG2*-transformed cells showed no significant difference in percent survival upon incubation at 27 °C for up to 60 min. However, at 55 °C, the survival rate of *G* transformants was dramatically lower than that of *G-AtBAG2* transformants (57.6 ± 4.73% and 80.9 ± 3.97%, respectively, at 30 min; 43.3 ± 4.22% and 73.6 ± 5.37%, respectively, at 60 min). To confirm these results, we performed the TB exclusion assay [23]; in this assay, only dead cells are expected to stain blue as they cannot exclude the dye. No TB-positive *G*- and *G-AtBAG2*-transformed cells were observed at 27 °C after 60 min (Figure 2B). However, after a 60 min incubation at 55 °C, more than 50% of the *G*-transformed cells appeared to be dead (intense blue staining), while the *G-AtBAG2* transformants were alive (mild blue staining) (Figure 2B). Taken together, our results consistently demonstrated that *G-AtBAG2* overexpression resulted in a remarkable increase in the thermotolerance of yeast cells.

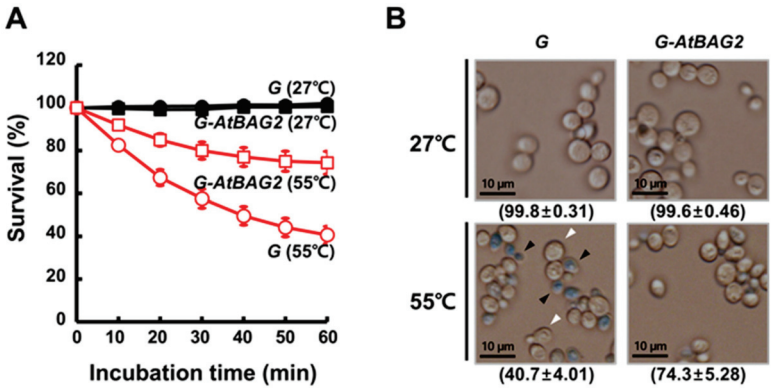


**Figure 2.** Ectopic expression of *AtBAG2* enhances the thermotolerance of yeast cells. (**A**) Effect of heat stress on yeast cell viability. Yeast cells transformed with pYES2-G-AtBAG2 (*G-AtBAG2*) or pYES2-G (*G*) were grown in YPD media supplemented with 2% galactose (Gal). The transformed cells ($5 \times 10^7$ cells/mL) were incubated at 27 °C or 55 °C and sampled at the indicated time points to count the number of viable cells. The cell survival rate (%) of each transformant was calculated as the ratio of the viable cell count at a given time point to the viable cell count at the 0 min time point. Data represent the mean ± SD of at least three independent experiments. (**B**) TB exclusion assay. Samples of *G-AtBAG2* and *G* transformants incubated at 55 °C for 1 h in (**A**) were visualized by fluorescence microscopy after staining with TB. White and black arrowheads indicate TB-negative and -positive cells, respectively. Scale bars, 10 μm. Data represent the mean ± SD of at least three independent experiments.

### *3.3. AtBAG2 Functions as a Molecular Chaperone*

Since *AtBAG2* conferred thermotolerance in both Arabidopsis and yeast, we explored the mechanism of action of the AtBAG2 protein by examining whether it exhibits molecular chaperone activity. To study the molecular chaperone activity of AtBAG2, we examined its surface hydrophobicity using bis-ANS, which binds to hydrophobic patches on proteins and fluoresces at an emission maximum of ~470 nm [24], and explored its ability to prevent the heat-induced aggregation of CS and MDH or dithiothreitol (DTT)-induced aggregation of insulin by measuring light scattering at 340 nm. The hydrophobicity of GST-fused AtBAG2 (G-AtBAG2) and its ability to prevent protein aggregation were compared with those of GST-fused Ypt1p (G-Ypt1p), a previously characterized molecular chaperone [19] (positive control), and GST alone (G; negative control). The fluorescence signal of bis-ANS-bound G-AtBAG2 was greater than that of G-Ypt1p (Figure 3A), suggesting that the chaperone activity of G-AtBAG2 is higher than that of G-Ypt1p. High surface hydrophobicity is a typical characteristic of molecular chaperones [25]. Based on these results, we investigated the chaperone activity of G-AtBAG2, along with those of G-Ypt1p (positive control) and G (negative control). In light scattering experiments, when CS (2 μM), a substrate protein [19,20], was heated alone or in the presence of 5-fold molar excess (8.35 μM) of the G protein, light scattering increased rapidly; however, when 2 μM CS was heated in the presence of 5-fold molar excess of G-AtBAG2 and G-Ypt1p, its aggregation was successively inhibited, confirming that G-AtBAG2 and G-Ypt1p function as molecular chaperones (Figure 3B). Interestingly, the aggregation prevention activity of G-AtBAG2 was greater than that of G-Ypt1p (Figure 3B). Furthermore, when MDH (1.67 μM) was heated alone or along with a two-fold molar excess (3.34 μM) of G, light scattering increased rapidly; however, when 1.67 μM MDH was heated in the presence of increasing amounts of G-AtBAG2 (0.5-, 1-, and 2-fold molar excess), its aggregation was inhibited in a concentration-dependent manner, confirming that G-AtBAG2 exhibits chaperone activity (Figure 3C). When G-AtBAG2 was present at two-fold molar excess (3.34 μM), approximately 90% of the MDH protein was protected against heat-induced aggregation during the 15 min incubation period (Figure 3C). G-AtBAG2 also inhibited the DTT-induced aggregation of insulin in a concentration-dependent manner (Figure 3D). These observations clearly demonstrate that AtBAG2 functions as a molecular chaperone.

### *3.4. BD Is Required for the Molecular Chaperone Activity of AtBAG2*

The analysis of the protein domain structure on the InterPro website (http://www.ebi.ac.uk/interpro/) revealed that the amino acid sequence of AtBAG2 contains one UBL domain and one BD. Therefore, we investigated the roles of both of these domains in the molecular chaperone function of AtBAG2. First, we generated GST fusion constructs of three serial deletion derivatives of AtBAG2 (G-AtBAG2-Nt, G-AtBAG2-M, and G-AtBAG2-Ct) (Figure 4A). Among the three AtBAG2 deletion variants, G-AtBAG2-Nt contains the UBL domain, G-AtBAG2-M contains the BD, and G-AtBAG2-Ct contains no unique domain. The recombinant GST fusion proteins were then produced in *E. coli* and purified, and their hydrophobicities were compared with that of G-AtBAG2 using bis-ANS. Upon binding to bis-ANS, G-AtBAG2-M showed high fluorescence, as with G-AtBAG2, whereas G-AtBAG2-Nt and G-AtBAG2-Ct showed very low fluorescence (Figure 4B). We then compared the chaperone activities of G-AtBAG2-Nt, G-AtBAG2-M, and G-AtBAG2-Ct with that of G-AtBAG2 by measuring their ability to prevent the heat-induced denaturation of the substrate protein MDH. Light scattering increased rapidly when MDH (1.67 μM) was heated alone or with a 5-fold molar excess (8.35 μM) of G-AtBAG2-Nt and G-AtBAG2-Ct; however, when MDH (1.67 μM) was heated in the presence of a five-fold molar excess of G-AtBAG2 and G-AtBAG2-M, its aggregation was successively prevented (Figure 4C), confirming the molecular chaperone activity of G-AtBAG2-M and G-AtBAG2. Based on this information, we compared the effects of the overexpression of *G-AtBAG2*, *G-AtBAG2-Nt*, *G-AtBAG2-M*, and *G-AtBAG2-Ct* on the thermotolerance of yeast cells. The *G-AtBAG2-Nt*, *G-AtBAG2-M*, and *G-AtBAG2-Ct* genes were cloned under the control of the *GAL1* promoter

to conditionally overexpress the recombinant proteins, and the constructs were named as *pYES2-G-AtBAG2-Nt*, *pYES2-G-AtBAG2-M*, and *pYES2-G-AtBAG2-Ct*, respectively. These three constructs as well as *pYES2-G-AtBAG2* and *pYES2-G* were separately transformed into the W303 cells. A spot assay was then performed using the heat-shocked *G*, *G-AtBAG2*, *G-AtBAG2-Nt*, *G-AtBAG2-M*, and *G-AtBAG2-Ct* transformants grown in media supplemented with 2% galactose (Gal) and sampled at the mid-exponential stage. The heat-shocked yeast cells overexpressing *G*, *G-AtBAG2-Nt*, and *G-AtBAG2-Ct* showed little difference in viability; by comparison, those overexpressing *G-AtBAG2* and *G-AtBAG2-M* showed significantly greater viability (Figure 4D). Taken together, these results suggest that the BD is critical for the molecular chaperone activity of AtBAG2, and overexpression of the BD-containing *AtBAG2* gene sequence significantly improves the viability of yeast cells under heat stress.

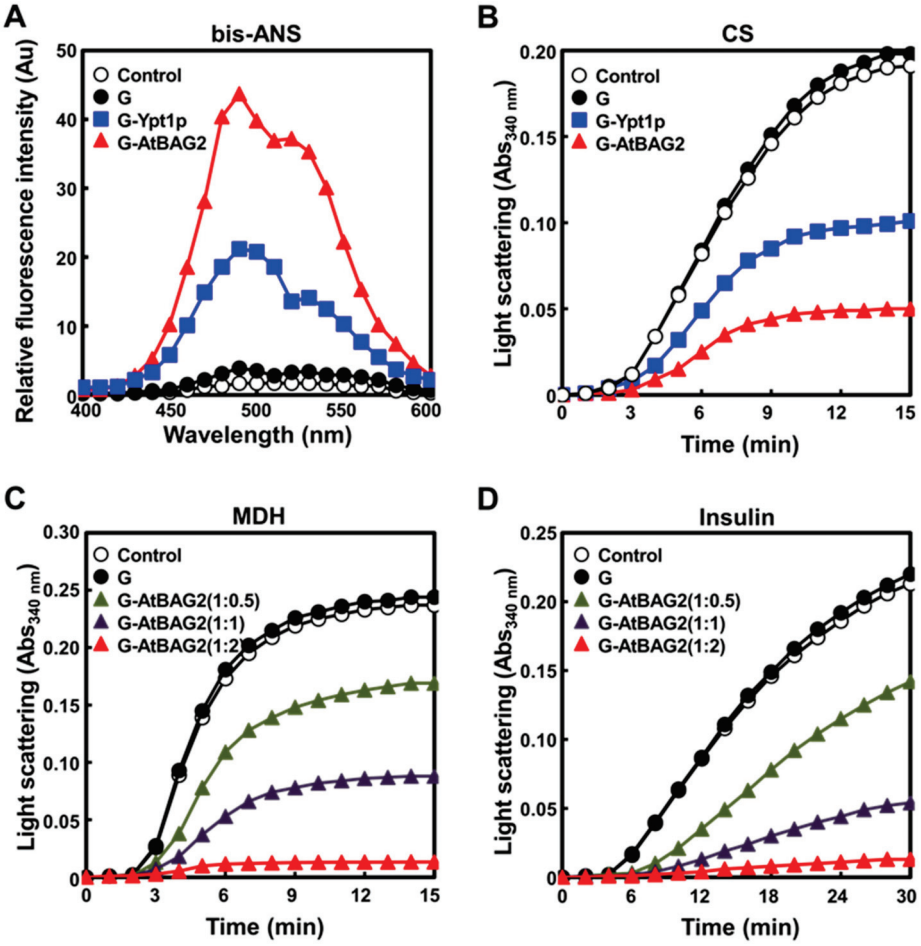


**Figure 3.** AtBAG2 acts as a molecular chaperone. (**A**) Results of bis-ANS-binding assay used to investigate the effect of heat shock on the hydrophobic domains of GST alone (G), GST-AtBAG2 fusion (G-AtBAG2), and G-Ypt1p fusion (G-Ypt1p; control). The fluorescence spectra of 10 μM bis-ANS (-○-) and 10 μM bis-ANS plus 30 μM G-AtBAG2 (-▲-), G-Ypt1p (-■-), or G (-●-) were measured at 380 nm (excitation wavelength) and 400–600 nm (emission wavelengths). (**B**–**D**) Molecular chaperone activity assay of AtBAG2 using CS (**B**), MDH (**C**), and insulin (**D**). Light scattering was monitored at 340 nm. In (**B**), 1 μM CS was incubated either alone (-○-) or with 2 μM G (-●-), G-Ypt1p (-■-), or G-AtBAG2 (-▲-) in 50 mM HEPES buffer (pH 7.0) in a spectrophotometer cell at 43 °C. In (**C**), 1.67 μM MDH was incubated alone (-○-), with 0.84 μM (-▲-), 1.67 μM (-▲-), or 3.34 μM (-▲-) G-AtBAG2, or with 3.34 μM G (-●-) in 50 mM HEPES buffer (pH 7.0) in a spectrophotometer cell at 45 °C. In (D), 1 μM insulin was incubated alone (-○-), with 0.5 μM (-▲-), 1 μM (-▲-), or 2 μM (-▲-) G-AtBAG2, or with 2 μM G (-●-) in 50 mM HEPES buffer (pH 8.0) containing 10 mM DTT in a spectrophotometer cell at 25 °C. Data represent the mean of at least three independent experiments.

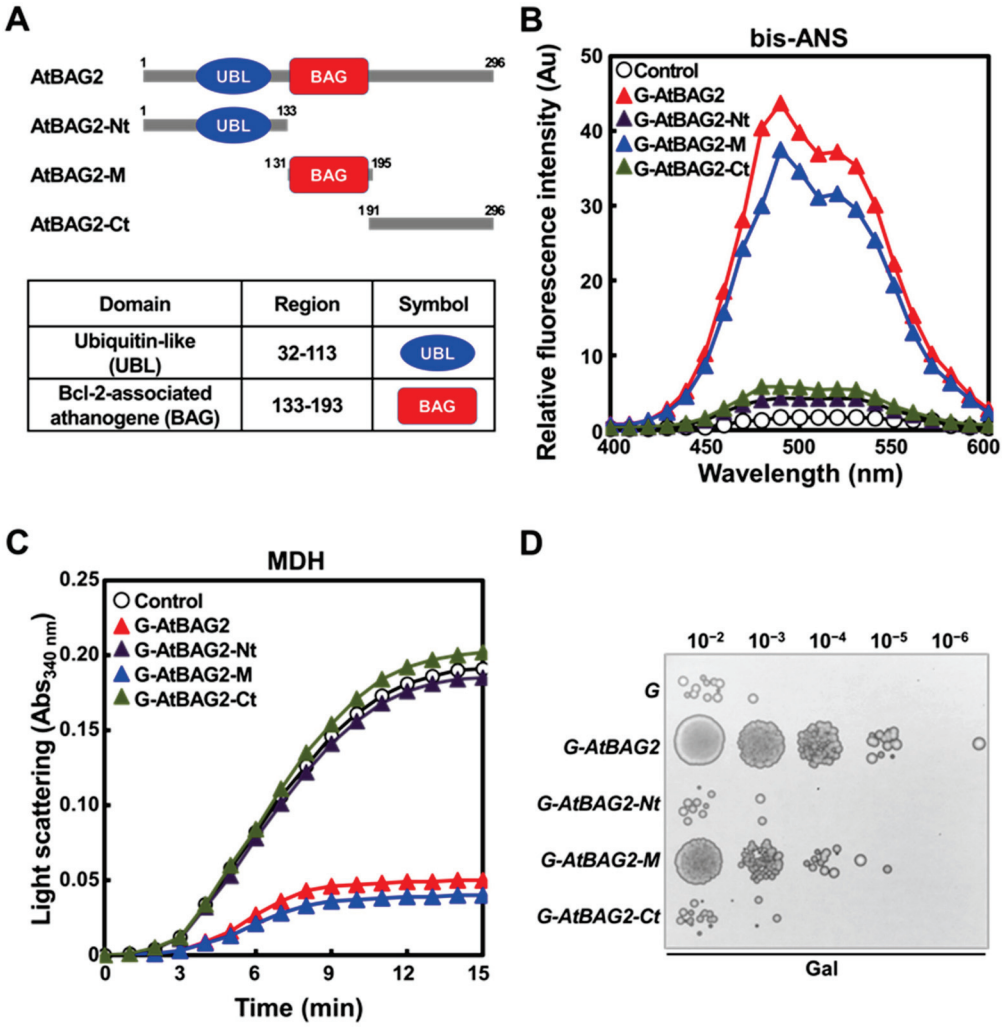


**Figure 4.** Identification of the functional domain responsible for the molecular chaperone activity of AtBAG2. (**A**) Schematic representation of AtBAG2 and its serial deletion variants (AtBAG2-Nt, -M, and -Ct). The circle and box indicate the UBL domain and BD, respectively. The amino acid positions of each deletion variant are indicated. G-AtBAG2 and G-AtBAG2-Nt, -M, and -Ct represent the four GST (G)-fusion constructs containing the indicated fragments of AtBAG2. (**B**) Results of bis-ANS-binding assays used to study the effect of heat shock on the hydrophobic domains of G-AtBAG2 and G-AtBAG2-Nt, -M, and -Ct. The samples used were 10 μM bis-ANS (-○-), 10 μM bis-ANS plus 30 μM G-AtBAG2 (-▲-), G-AtBAG2-Nt (-▲-), G-AtBAG2-M (-▲-), and G-AtBAG2-Ct (-▲-). (**C**) Molecular chaperone activity assay. Solutions containing either 1.67 μM MDH alone (-○-) or with 2.5 μM G-AtBAG2 (-▲-), G-AtBAG2-Nt (-▲-), G-AtBAG2-M (-▲-), and G-AtBAG2-Ct (-▲-) in 50 mM HEPES buffer (pH 7.0) were incubated in a spectrophotometer cell at 45 °C. (**D**) Yeast spot assay. Yeast cells transformed with pYES2-G-AtBAG2 (*G-AtBAG2*), pYES2-G-AtBAG2-Nt (*G-AtBAG2-Nt*), pYES2-G-AtBAG2-M (*G-AtBAG2-M*), pYES2-G-AtBAG2-Ct (*G-AtBAG2-Ct*), or pYES2-G (*G*) were grown in YPD media supplemented with 2% Gal. The transformed cells ($5 \times 10^7$ cells/mL) were incubated at 55 °C for 1 h. Next, 6 μL aliquots of 10-fold serial dilutions of each cell suspension were spotted on YPD plates. The plates were incubated at 27 °C for 3 days and then photographed.

## 4. Discussion

The BAG family proteins, known to act as co-chaperones, share a well-conserved BD at their C-terminus, which facilitates their interaction with HSP70/HSC70 [1,5]. For example, the most representative BAG protein, human BAG1, functions as a nucleotide exchange factor (NEF) for the chaperone HSP70, and triggers the release of chaperone-bound proteins as a co-chaperone [26,27]. AtBAG1 also interacts with HSC70 via the BD [3], thus acting

as a cofactor of HSC70 for the proteasomal degradation of unimported plastid proteins. However, it is difficult to find literature on BAG protein activity. According to the results of this study, AtBAG2 performs its own biochemical function without associating with other proteins. In particular, AtBAG2 was found to function as a molecular chaperone that prevents the heat- or chemical-induced aggregation of substrate proteins. AtBAG2 exhibited superior molecular chaperone activity with CS, which has frequently been used as a general substrate in molecular chaperone studies compared with Ypt1 (Figure 3B), which was previously identified as a molecular chaperone. In addition, AtBAG2 prevented the aggregation of thermally denatured MDH and chemically denatured insulin proteins, and its activity increased in a concentration-dependent manner (Figure 3C,D). Molecular chaperones exhibit high surface hydrophobicity [19,28], and AtBAG2 was no exception (Figure 3A). Based on these results, AtBAG2 was characterized as a novel molecular chaperone in this study.

To determine the region of AtBAG2 essential for its molecular chaperone activity, three deletion variants of the protein were generated, AtBAG2-Nt (UBL domain-containing N-terminal region), AtBAG2-Ct (domain-less C-terminal region), and AtBAG2-M (BD-containing middle region), and the activity of each variant protein was tested. Among the three derivative proteins, only AtBAG2-M exhibited molecular chaperone activity (Figure 4C), suggesting that the BD is responsible for the molecular chaperone function of AtBAG2. This suggests the possibility that the BD can act as a functionally active site in molecular chaperones, proposing new research directions and the applicability of BAG proteins in the future. In humans, the first and second helices of BAG1 BD are important for its binding to the serine/threonine kinase Raf-1 [29], while the second and third helices are known to mediate its binding to the ATPase domain of Hsc70/Hsp70 [30]. In the future, it will be interesting to find out which helices and amino acid residues in the BD of AtBAG2 are essential for the high hydrophobicity and molecular chaperone activity of the protein.

The maintenance of intracellular protein homeostasis is one of the most basic processes required for survival. Under stress conditions, intracellular proteins are often damaged and lose function, and molecular chaperones play a major role in maintaining life by preserving protein structure and function. For example, in humans, small heat shock proteins (sHsps), such as Hsp27, maintain cytoplasmic protein function and protect cells under stress conditions [31]. Ypt1p, a small GTPase in yeast, is generally responsible for intracellular trafficking; however, upon exposure to heat shock, Ypt1p undergoes structural changes which amplify its molecular chaperone function to protect cells [19]. In Arabidopsis, AtPP5 usually participates in cell signal transduction as a phosphatase; however, under thermal stress, it protects plants by acting as a molecular chaperone and preventing protein aggregation [32]. AtP3B, a ribosomal protein, exhibits high molecular chaperone activity, and its gene expression increases at high temperatures to protect Arabidopsis plants from heat-induced damage [28]. Similarly, AtBAG2 was found to exhibit excellent molecular chaperone activity in this study (Figure 3). Moreover, the *atbag2* mutant seedlings were sensitive to heat shock (Figure 1), whereas yeast cells overexpressing the *AtBAG2* gene showed enhanced survival under heat stress conditions (Figure 2), indicating that AtBAG2 enhances thermotolerance. Unfortunately, *AtBAG2* complementation lines in the *atbag2* mutant background could not be obtained, and therefore phenotypically characterized, in this study. Given that AtBAG2 enhances the tolerance to multiple abiotic stresses by regulating the expression of downstream genes involved in abscisic acid (ABA) response and stress-induced reactive oxygen species (ROS) production [13], revealing the relationship between its stress response mechanism and molecular chaperone activity is a research task that has yet to be accomplished. Our results, which identified and characterized AtBAG2 as a novel molecular chaperone, are expected to expand the research horizon for key factors such as HSP70 that regulate essential cellular functions by associating with BAG proteins.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/life13030687/s1, Figure S1: The original image for the Figure 1B (top); Figure S2: The original image for the Figure 1B (bottom); Figure S3: The original image for the Figure 1C (top); Figure S4: The original image for the Figure 1C (bottom).

**Author Contributions:** Conceptualization, C.H.K. and C.O.L.; data curation, C.H.K. and J.H.L.; formal analysis, C.H.K. and J.H.L.; funding acquisition, C.H.K., J.C.H. and C.O.L.; investigation, C.H.K., J.H.L., C.Y.K. and S.I.L.; methodology, C.H.K., Y.-J.K. and S.I.L.; project administration, C.H.K., J.C.H. and C.O.L.; resources, C.Y.K., J.C.H., Y.-J.K.; software, C.H.K. and C.Y.K.; supervision, C.O.L.; validation, C.H.K. and C.Y.K.; visualization, C.H.K.; writing—original draft, C.H.K. and C.O.L.; writing—review and editing, C.H.K. and C.O.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by a grant from the BioGreen21 Agri-Tech Innovation Program (Project PJ015862), Rural Development Administration, and by grants from the Basic Science Research Program funded by the Ministry of Education (grant numbers, 2022R1I1A1A01066231 and 2020R1A6A1A03044344) through the National Research Foundation of Korea (NRF), Republic of Korea.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are contained within the article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Drought Tolerance Assessment of Okra (*Abelmoschus esculentus* [L.] Moench) Accessions Based on Leaf Gas Exchange and Chlorophyll Fluorescence

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**Abstract:** Physiological and complementary phenotypic traits are essential in the selection of drought-adapted crop genotypes. Understanding the physiological response of diverse okra genotypes under drought stress conditions is critical to the selection of drought-tolerant accessions for production or breeding. The objective of this study was to assess the levels of drought tolerance in preliminarily selected okra accessions based on leaf gas exchange and chlorophyll fluorescence to determine best-performing genotypes for drought-tolerance breeding. Twenty-six genetically diverse okra accessions were screened under non-stressed (NS) and drought-stressed (DS) conditions under a controlled glasshouse environment using a 13 × 2 alpha lattice design in three replicates, in two growing seasons. Data were subjected to statistical analyses using various procedures. A significant genotype × water condition interaction effect was recorded for transpiration rate (T), net $CO_2$ assimilation (A), intrinsic water use efficiency (WUE*i*), instantaneous water use efficiency (WUE*ins*), minimum fluorescence ($Fo'$), maximum fluorescence ($Fm'$), maximum quantum efficiency of photosystem II photochemistry ($Fv'/Fm'$), the effective quantum efficiency of *PSII* photochemistry ($\phi PSII$), photochemical quenching ($qP$), nonphotochemical quenching ($qN$) and relative measure of electron transport to oxygen molecules (ETR/A). The results suggested variable drought tolerance of the studied okra accessions for selection. Seven principal components (PCs) contributing to 82% of the total variation for assessed physiological traits were identified under DS conditions. Leaf gas exchange parameters, T, A and WUE*i*, and chlorophyll fluorescence parameters such as the $\phi PSII$, $Fv'/Fm'$, $qP$, $qN$, ETR and ETR/A had high loading scores and correlated with WUE*i*, the $\phi PSII$, $qP$ and ETR under DS conditions. The study found that optimal gas exchange and photoprotection enhance drought adaptation in the assessed okra genotypes and tested water regimes. Using the physiological variables, the study identified drought-tolerant accessions, namely LS05, LS06, LS07 and LS08 based on high A, T, $Fm'$, $Fv'/Fm'$ and ETR, and LS10, LS11, LS18 and LS23 based on high *AES*, *Ci*, *Ci/Ca*, *WUEi*, *WUEins*, $\phi PSII$ and AES. The selected genotypes are high-yielding (≥5 g/plant) under drought stress conditions and will complement phenotypic data and guide breeding for water-limited agro-ecologies.

**Keywords:** abiotic stress; chlorophyll fluorescence; drought tolerance; leaf gas exchange; physiological traits

**Citation:** Mkhabela, S.S.; Shimelis, H.; Gerrano, A.S.; Mashilo, J. Drought Tolerance Assessment of Okra (*Abelmoschus esculentus* [L.] Moench) Accessions Based on Leaf Gas Exchange and Chlorophyll Fluorescence. *Life* **2023**, *13*, 682. https://doi.org/10.3390/life13030682

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 17 January 2023
Revised: 27 February 2023
Accepted: 28 February 2023
Published: 2 March 2023



## 1. Introduction

Okra (*Abelmoschus esculentus* [L.] Moench), belonging to the Malvaceae family, is an important crop mainly cultivated as fruits, vegetables and seed oil. It is extensively grown in tropic and subtropic regions [1] and arid and semi-arid regions with limited and erratic

rainfall conditions [2]. The tender and immature pods of okra are consumed as cooked vegetables [3]. The pods are rich in protein content (25 %) and amino acids, notably lysine and tryptophan [4], fat, fibre, vitamins (A, C and K), vital mineral elements such as calcium, potassium, sodium, magnesium, iron, zinc and manganese [5], and soluble sugars such as sucrose (110.4 g/100 g FW), fructose (34.8 g/100 g FW and glucose (30.9 g/100 g FW [6]. In addition, minor quantities of organic acids, including citric, oxalic and malic acid, are present in the succulent pods [6]. The mature and dry seeds are a vital source of edible oils. The seed oil content ranges from 20–40%, consisting of the following major fatty acids: linoleic, palmitic, oleic, diacylglycerols and triacylglycerols acids [7].

Continental Asia accounts for a total annual okra production of 6 million tons from 592,375 million hectares of cultivated land, whereas Africa is the second major producer, with 3 million tons per annum from approximately 1.9 million ha of cultivated land [8]. Commercial and small-scale farmers produce okra. In sub-Saharan Africa (SSA), the crop is mainly grown in marginal conditions characterised by low and erratic rainfall, with minimal agricultural inputs and production technologies. In SSA, okra is mainly cultivated under rainfed conditions, and these agro-ecologies face moderate to severe droughts during the growing season [9]. Drought stress significantly reduces growth, biomass and yield [10]. Drought alone accounts for yield losses ranging between 30 and 100% in okra, primarily when the stress occurs during the flowering and pod-filling stages [3]. Breeding okra cultivars with drought adaptation is the major objective in improvement programs. Physiological and complementary phenotypic traits are critical in the selection of drought-adapted crop genotypes.

Phenotyping of plants using gas exchange and chlorophyll fluorescence traits has been reported as a preferred approach for selecting drought-tolerant okra accessions [11]. Some gaseous exchange traits used to assess drought tolerance include photosynthesis rate, stomatal conductance, chlorophyll content and transpiration rate. Further, chlorophyll fluorescence parameters (e.g., minimum fluorescence, maximum fluorescence, effective quantum efficiency of *PSII* photochemistry, photochemical quenching and non-photochemical quenching) have been used in phenotyping for drought tolerance [11–13]. Drought stress affects okra growth and productivity, disrupting physiological functions and the photosynthetic rate, resulting in yield losses [11,13,14]. Mkhabela et al. [3] reported that okra yield loss under drought stress could be significantly minimised by breeding drought-tolerant ideotypes with intrinsic water use efficiency. Hence, understanding the physiological response of diverse okra genotypes under drought stress conditions is essential for the selection of drought-tolerant accessions for production or breeding.

There has been limited progress in the breeding of okra for drought tolerance. This could be due to limited accessions identified as good drought and heat tolerance sources and insect pests and disease resistance [15]. Some unique accessions, including Sabz Pari [16], NHAe 47-4 [17], Pusa Sawari, Iraq P, Hala [1] and Xianzhi [18], were identified as useful sources of genes for enhancing drought tolerance under water-limited conditions. Compared to the highest genetic diversity reported in the cultivated okra [19], the identified accessions with tolerance to drought are relatively few. Therefore, there is a need for concerted research and development in okra to develop market-led and improved varieties for water-limited conditions.

In South Africa, okra is an important but under-researched and under-utilised crop. It is grown under rainfed conditions using local and unimproved accessions with poor adaptation and low yield potential. Genetically unique okra accessions could be sourced from different geographical regions to enhance okra pre-breeding programs [3]. Morphological traits associated with drought tolerance in okra include the number of pods per plant, fresh pod length, number of seeds per pod, hundred seed weight, number of branches per plant, plant height and total pod production [1,19]. Reportedly, a higher number of branches, pod length and number of pods per plant, plant height between 150 and 170 cm and pod weight have a direct influence on pod yield [19]. Drought tolerance assessment of okra accessions using the combination of morphological and physiological

traits could increase the efficiency of identifying and selecting drought-tolerant accessions for cultivar development under dry environments. Therefore, this study aimed to assess the levels of drought tolerance in preliminarily selected okra accessions based on leaf gas exchange and chlorophyll fluorescence to determine best-performing genotypes for drought-tolerance breeding.

## 2. Materials and Methods

### *2.1. Plant Materials and Study Site*

Twenty-five genetically distinct okra accessions were used for the study. The accessions were sourced from the Agricultural Research Council, Vegetable, Industrial and Medicinal Plants (ARC-VIMP) gene bank, and one local variety was included. The accessions were previously studied for their morphological responses to drought stress under field and glasshouse environments [3]. Detailed information on their geographical origin and drought resistance index are presented in Table 1. The experiment was conducted under glasshouse conditions at the Controlled Environment Facility (CEF) of the University of KwaZulu-Natal during the 2020/2021 growing seasons. The first experiment was conducted from September 2020 to December 2020, and the second from February 2021 to May 2021. The accessions were evaluated under non-stressed (NS) and drought-stressed (DS) conditions in the glasshouse environment. Drought tolerance index was calculated as DTI = (Ys/Yn)/(Ms/Mn), where Ys and Yn are the genotype yields under stress and non-stress, and Ms and Mn are the mean yields of the accessions under stressed and non-stressed conditions, respectively [20].

**Table 1.** Accession code, accession number, database, geographical origin, drought tolerance index and stem colour of the okra accessions evaluated in the study.

| Accession Code | Accession Number | Database Name | Geographical Origin | DTI | Stem Colour |
|---|---|---|---|---|---|
| LS01 | VI033775 | ARC/South Africa | Malaysia | 0.02 | Red |
| LS02 | VI033797 | ARC/South Africa | Malaysia | 1.16 | Green |
| LS03 | VI056457 | ARC/South Africa | Yugoslavia | 1.46 | Red |
| LS04 | VI039651 | ARC/South Africa | Bangladesh | 0.67 | Green |
| LS05 | VI046561 | ARC/South Africa | Thailand | 1.80 | Red |
| LS06 | VI047672 | ARC/South Africa | Bangladesh | 1.00 | Green |
| LS07 | VI050150 | ARC/South Africa | Taiwan | 0.13 | Green |
| LS08 | VI050957 | ARC/South Africa | Zambia | 0.04 | Green |
| LS09 | VI050960 | ARC/South Africa | Zambia | 0.31 | Green |
| LS10 | VI055110 | ARC/South Africa | Malaysia | 0.15 | Red |
| LS11 | VI055119 | ARC/South Africa | Myanmar | 0.73 | Red |
| LS12 | VI055219 | ARC/South Africa | Malaysia | 0.99 | Red |
| LS13 | VI055220 | ARC/South Africa | Malaysia | 4.67 | Green |
| LS14 | VI055421 | ARC/South Africa | Viet Nam | 1.02 | Green |
| LS15 | VI056069 | ARC/South Africa | Cambodia | 0.14 | Red |
| LS16 | VI056079 | ARC/South Africa | Cambodia | 3.15 | Green |
| LS17 | VI056081 | ARC/South Africa | Cambodia | 0.53 | Red |
| LS18 | VI056449 | ARC/South Africa | United States of America | 0.43 | Red |
| LS19 | VI060131 | ARC/South Africa | Mali | 0.00 | Green |
| LS20 | VI060313 | ARC/South Africa | Tanzania | 6.49 | Green |
| LS21 | VI060679 | ARC/South Africa | India | 0.61 | Green |

**Table 1.** *Cont.*

| Accession Code | Accession Number | Database Name | Geographical Origin | DTI | Stem Colour |
|---|---|---|---|---|---|
| LS22 | VI060803 | ARC/South Africa | Turkey | 8.64 | Green |
| LS23 | VI060817 | ARC/South Africa | Brazil | 0.45 | Green |
| LS24 | VI060822 | ARC/South Africa | Nigeria | 0.31 | Green |
| LS25 | VI060823 | ARC/South Africa | Nigeria | 0.00 | Green |
| LS26 | Clemson Spineless | ARC/South Africa | South Africa | 0.23 | Green |

ARC = Agricultural Research Council, DTI = drought tolerance index.

### *2.2. Experimental Design and Crop Establishment*

Five seeds were initially planted in 5 L capacity plastic pots filled with composted pine bark growing media. Later, two plants were established per pot for each genotype. The day and night temperatures in the greenhouse (GH) were 30 °C and 20 °C, respectively, and the relative humidity ranged between 45 and 55% during the study. Inorganic fertilizers consisting of nitrogen (N), phosphorus (P) and potassium (K) were applied at a rate of 120, 30 and 30 kg $ha^{-1}$, based on soil fertility recommendations using urea (46-0-0), phosphorus pentoxide ($P_2O_5$) and potassium oxide ($P_2O$), respectively.

The trials were established using a 13 × 2 alpha lattice design under drought-stressed and non-stressed conditions with three replications. Drought stress was imposed at 50% flowering until physiological maturity by withholding irrigation until the soil water content reached 30% field capacity for plants under DS. The duration of stress was seven days before sampling. Plants under NS conditions were irrigated regularly to maintain soil moisture content at field capacity until physiological maturity. To determine pod yield, plants reached maturity, and pods were harvested sequentially at the soft, most digestible and immature stage. Tensiometers, moisture monitors (Spectrum Technologies, Inc, Chicago, IL, USA), were used to detect soil moisture levels at the root zone. Agronomic performance of the test genotypes was reported in Mkhabela et al. [19].

### *2.3. Data Collection*

Gas exchange and chlorophyll fluorescence parameters were measured using an LI-6400 XT Portable Photosynthesis system (Licor Bioscience, Inc. Lincoln, NE, USA) integrated with an infrared gas analyser (IGRA) attached to a leaf chamber fluorometer (LCF) (640040B, 2 $cm^2$ leaf area, Licor Bioscience, Inc, Lincoln, NE, USA). External leaf $CO_2$ concentration ($C_a$) and artificial saturating photosynthetic active radiation (PAR) were set at 400 µmol $mol^{-1}$ and 1000 µmol $m^{-2}$ $s^{-1}$, respectively. Water flow rate and relative humidity were maintained at 500 µmol and 43%, respectively. The leaf-to-air vapour pressure deficit in the cuvette was maintained at 1.7 kPa to avoid stomatal closure due to low air humidity. Gas exchange and chlorophyll fluorescence measurements were taken on the third half fully formed leaf inside the sensor head. Under both NS and DS conditions, measurements were taken from five plants of each accession.

The following gas exchange parameters were determined: stomatal conductance (gs), net $CO_2$ assimilation rate (A), transpiration rate (T), intercellular $CO_2$ concentration ($C_i$) and the ratio of intercellular and ambient $CO_2$ ($C_i/C_a$) concentrations. The ratio of net $CO_2$ assimilation rate to intercellular $CO_2$ concentration ($A/C_i$) was computed according to Kitao et al. [21]. The ratio of A and gs was used to compute intrinsic water use efficiency [22] and the ratio of A and T was used to calculate instantaneous water use efficiency) [23].

To estimate chlorophyll fluorescence variables, a saturation flash intensity of 1300 µmol $m^{-2}$ $s^{-1}$ was applied. The following parameters were recorded. The minimum ($Fo'$) and maximum fluorescence ($F_m'$) of light-adapted leaves under natural glasshouse conditions. The steady-state fluorescence ($F_s$) was also determined in light-adapted photosynthesis. Equation (1) was used to de-

termine the variable fluorescence in light-adapted leaves, while Equation (2) calculated fluorescence changes [24].

$$Fv' = Fm' F_0' \quad (1)$$

$$\Delta F = Fm' - Fs \quad (2)$$

Additional chlorophyll fluorescence parameters were estimated according to Evans [25], $Fv'/Fm'$, the maximum quantum efficiency of photosystem II photochemistry, the effective quantum efficiency of photosystem II photochemistry ($\phi PSII$), photochemical quenching ($qP$), non-photochemical quenching ($qN$) and electron transport rate (ETR). The ratio of ETR and A was used to calculate a relative measure of electron transport to oxygen molecules. The alternative electron sink (AES) was calculated as the ratio of photosystem II effective quantum efficiency to net $CO_2$ assimilation (A) [26]. Chlorophyll fluorescence was measured using a pulse-amplitude modulated (PAM) fluorometer, which applies a short pulse of light to the sample and measures the resulting fluorescence emitted by the chlorophyll. This measurement provided information on the photosynthetic efficiency and health of the crop. Gas exchange and chlorophyll fluorescence parameters were measured on fully expanded leaves. At the end of the second experiment, yield per plant (YPP) was determined by harvesting fresh pods when 50% of the pods were 3–5 cm long by hand every third day.

### *2.4. Statistical Analysis*

Data were subjected to analysis of variance using Genstat 20th edition (VSN International, Hempstead, UK). The mean data for the two seasons were combined for analysis. Means were separated using Fisher's protected least significant difference (LSD) test at the 5% significance level. Pearson's correlation coefficients were calculated using IBM SPSS Statistics 25.0 (SPSS Inc., Chicago, IL, USA) to determine the magnitude of the relationship among physiological traits. Principal component analysis (PCA) based on a correlation matrix was used to identify influential traits under NS and DS conditions using R Studio version 4.0, ggplot2 (R Core Team, 2018). Biplots were built using XLSTAT to determine relationships among the accessions and response variables (physiological traits). Principal component biplot diagrams were used to identify drought-tolerant and drought-susceptible okra accessions using XLSTAT. ClustVis (https://biit.cs.ut.ee/clustvis_large (accessed on 23 November 2022)) was used to visualise the heatmap analysis of physiological traits.

## 3. Results

### *3.1. Leaf Gas Exchange and Chlorophyll Fluorescence Parameters in Response to Drought*

The effects of genotype, water regime and interaction of genotype × water regime were significantly different for most evaluated traits of leaf gas exchange and chlorophyll fluorescence (Table 2). Drought stress significantly reduced gs, A and A/C*i* among the evaluated accessions (Tables 3 and 4). Accessions LS02, LS09, LS10, LS17, LS19 and LS26 recorded *gs* values of >0.3 mmol $m^{-2}$ $s^{-1}$ under NS conditions. Under DS, accessions LS04, LS11, LS13 and LS20 recorded *gs* values <0.1 µmol $m^{-2}$ $s^{-1}$. Regarding T, accessions LS03, LS13, LS15, LS19, LS23 and LS24 recorded values $\geq$ 7.01 mmol $H_2O$ $m^{-2}$ $s^{-1}$ under NS conditions, while, under DS conditions, genotypes LS01, LS03, LS04, LS08, LS09, LS11, LS12, LS14, LS19 and LS22 recorded T values $\leq$ 1.00 mmol $H_2O$ $m^{-1}$ $s^{-1}$. Under NS conditions, A values of $\geq$ 30 µmol $CO_2$ $m^{-2}$ $s^{-1}$ were observed from accessions LS08, LS10 and LS21, while values $\leq$ 20 µmol $CO_2$ $m^{-2}$ $s^{-1}$ were recorded for accessions LS03 and LS06.

**Table 2.** Analysis of variance indicating mean squares and significant tests of leaf gas exchange and chlorophyll fluorescence parameters of 26 okra genotypes evaluated under non-stress and drought-stress conditions averaged across two seasons.

| Source of Variation | d.f. | Leaf Gas Exchange Parameters | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *gs* | *T* | *A* | *Ci* | *A*/$C_i$ | $C_i/C_a$ | $WUE_i$ | $WUE_{ins}$ |
| Replications | 1 | 0.07 * | 3.19 ns | 34.76 ns | 806,541 * | 0.03 ns | 7.30 * | 324,534 * | 3675 ns |
| Incomplete Blocks | 1 | 0.01 ns | 1.92 ns | 54.88 ns | 15,807 ns | 0.05 ns | 1.48 ns | 4185 ns | 685 ns |
| Genotype (G) | 25 | 0.13 ** | 13.45 ** | 65.50 ns | 165,972 ns | 0.06 ns | 1.13 ns | 140,060 * | 652,347 ** |
| Water Regime (WR) | 1 | 0.38 ** | 75.62 ** | 448.16 ** | 1,830,530 * | 0.04 ns | 20.94 ** | 3,444,897 ** | 20,180,480 ** |
| G × WR | 25 | 0.01 ns | 14.46 ** | 30.41 * | 100,917 ns | 0.06 ns | 1.03 ns | 140,099 * | 644,150 ** |
| Residual | 50 | 0.11 | 4.33 | 31.35 | 174,990 | 0.07 | 1 | 56,471 | 205,739 |

| Source of Variation | d.f. | Chlorophyll Fluorescence Parameters | | | | | | | | | YPP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $F_O'$ | $F_m'$ | $F_v'/F_m'$ | *ϕPSII* | *qP* | *qN* | ETR | ETR/A | AES | |
| Replications | 1 | 8892 ns | 32,989 ns | 0.22 * | 0.08 ns | 0.11 ns | 0.25 ns | 3.72 ns | 586,500 ns | 2518 ns | 1186.7 * |
| Incomplete Blocks | 1 | 8535 ns | 468,996 * | 0.02 ns | 0.06 ns | 0.24 * | 1.69 * | 1.40 ns | 164,477 ns | 15,882 ns | 127.6 ns |
| Genotype (G) | 25 | 28,927 ** | 292,297 * | 0.17 * | 0.17 ** | 0.25 ** | 1.94 ** | 2.86 * | 356,680 ns | 14,198 ns | 1023.2 * |
| Water Regime (WR) | 1 | 844,279 ** | 20,220,415 ** | 0.66 ** | 1.35 ** | 1.20 ** | 8.68 ** | 4.16 ns | 101,913 ** | 424,290 ** | 6913.0 ** |
| G × W | 25 | 19,264 * | 472,144 ** | 0.05 * | 0.16 ** | 0.20 ** | 1.69 ** | 1.86 ns | 301,433 * | 12,027 ns | 194.9 * |
| Residual | 50 | 9080 | 115,681 | 0.08 | 0.03 | 0.14 | 0.38 | 1.2 | 156,859 | 11,175 | 183.9 |

d.f.: degree of freedom, *gs*: stomatal conductance, *T*: transpiration rate, *A*: net $CO_2$ assimilation, *Ci*: intercellular $CO_2$ concentration, *A*/*Ci*: $CO_2$ assimilation rate/intercellular $CO_2$ concentration, *Ci*/*Ca*: ratio of intercellular and atmospheric $CO_2$, $WUE_i$: intrinsic water use efficiency, $WUE_{ins}$: instantaneous water use efficiency, $F_0'$: minimum fluorescence, $Fm'$: maximum fluorescence, $Fv'/Fm'$: maximum quantum efficiency of photosystem II photochemistry, *ϕPSII*: the effective quantum efficiency of PSII photochemistry, *qP*: photochemical quenching, *qN*: non-photochemical quenching, ETR: electron transport rate, ETR/A: relative measure of electron transport to oxygen molecules, AES: alternative electron sinks, YPP: yield per plant, * and ** denote significance at 5 and 1% probability levels, respectively, ns: non-significant.

**Table 3.** Means of leaf gas exchange and chlorophyll fluorescence parameters of okra accessions under non-stressed conditions.

| Genotype | Leaf Gas Exchange Parameters | | | | | | | | | Chlorophyll Fluorescence Parameters | | | | | | | YPP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *gs* | T | A | *Ci* | A/*Ci* | *Ci*/*Ca* | WUE*i* | WUE*ins* | $Fo'$ | $Fm'$ | $Fv'/Fm'$ | *ϕPSII* | *qP* | *qN* | ETR | ETR/A | AES | |
| LS01 | 0.19 | 1.52 | 21.85 | 1.33 | 0.12 | 1.33 | 171.30 | 17.77 | 388.4 | 828.1 | 0.75 | 0.33 | 0.14 | 1.24 | 22,026 | 1129.9 | 56.59 | 7.02 |
| LS02 | 0.3 | 1.06 | 27.91 | 0.74 | 0.15 | 0.74 | 162.40 | 26.39 | 302.9 | 787.7 | 0.41 | 0.19 | 0.19 | 2.23 | 30,068 | 1082.6 | 26.09 | 7.83 |
| LS03 | 0.26 | 7.01 | 16.24 | 0.84 | 0.14 | 0.84 | 62.00 | 2.32 | 311.6 | 871.8 | 0.57 | 0.30 | 0.35 | 2.78 | 13,186 | 801.6 | 17.98 | 8.79 |
| LS04 | 0.24 | 2.52 | 24.93 | 0.78 | 0.17 | 0.78 | 102.20 | 10.58 | 168.8 | 860 | 0.28 | 0.29 | 0.48 | 0.75 | 21,020 | 807.40 | 20.85 | 6.09 |
| LS05 | 0.20 | 2.01 | 21.05 | 1.37 | 0.07 | 1.37 | 164.90 | 10.45 | 186.8 | 421.4 | 0.61 | 0.04 | 0.02 | 1.23 | 2815 | 137.10 | 33.70 | 7.33 |
| LS06 | 0.23 | 6.56 | 26.95 | 0.68 | 0.15 | 0.68 | 195.90 | 4.18 | 179.7 | 252.3 | 0.37 | 0.37 | 0.25 | 0.74 | 8148 | 306.70 | 18.36 | 8.00 |
| LS07 | 0.22 | 1.56 | 29.82 | 0.62 | 0.18 | 0.62 | 136.60 | 28.44 | 166.3 | 678 | 0.44 | 0.24 | 0.15 | 0.68 | 24,945 | 739.40 | 18.34 | 2.92 |
| LS08 | 0.29 | 1.52 | 30.01 | 0.75 | 0.11 | 0.75 | 109.70 | 20.87 | 444.4 | 552.3 | 0.40 | 0.24 | 0.16 | 0.76 | 37,921 | 1226.80 | 26.13 | 2.63 |
| LS09 | 0.39 | 4.02 | 21.02 | 1.65 | 0.14 | 1.65 | 55.10 | 11.61 | 227.4 | 822 | 0.64 | 0.40 | 0.14 | 1.24 | 23,498 | 1113.60 | 26.52 | 7.17 |
| LS10 | 0.34 | 1.26 | 35.54 | 1.66 | 0.15 | 1.66 | 108 | 28.26 | 434 | 811.10 | 0.80 | 0.30 | 0.33 | 1.23 | 52,313 | 1464.40 | 18.63 | 6.88 |
| LS11 | 0.27 | 1.01 | 28.85 | 0.78 | 0.11 | 0.78 | 107.40 | 28.44 | 207.10 | 139.6 | 0.53 | 0.37 | 0.14 | 2.31 | 23,806 | 776.30 | 14.05 | 9.23 |
| LS12 | 0.29 | 5.52 | 23.9 | 0.59 | 0.16 | 0.59 | 80.40 | 5.90 | 263.6 | 769 | 0.35 | 0.29 | 0.83 | 1.22 | 24,860 | 1047.40 | 37.65 | 7.68 |
| LS13 | 0.28 | 9.02 | 25.05 | 0.78 | 0.18 | 0.78 | 92.80 | 2.78 | 193.7 | 864.70 | 0.51 | 0.24 | 0.48 | 0.77 | 9184 | 378.90 | 15.30 | 6.13 |
| LS14 | 0.19 | 2.02 | 29.08 | 1.90 | 0.17 | 1.90 | 173 | 14.42 | 391.3 | 845.40 | 0.44 | 0.28 | 0.13 | 2.76 | 26,106 | 852.30 | 15.70 | 8.56 |
| LS15 | 0.50 | 7.56 | 29.91 | 0.66 | 0.13 | 0.66 | 61.30 | 4.39 | 370.7 | 472.90 | 0.93 | 0.37 | 0.26 | 1.76 | 37,168 | 1223.10 | 30.08 | 4.82 |
| LS16 | 0.14 | 5.51 | 16.5 | 0.83 | 0.06 | 0.83 | 303.10 | 10.20 | 174.8 | 824 | 0.13 | 0.03 | 0.16 | 0.75 | 26,910 | 1553.60 | 24.12 | 0.01 |
| LS17 | 0.53 | 2.51 | 22.08 | 0.71 | 0.12 | 0.71 | 41.40 | 12.74 | 289.8 | 794.50 | 0.74 | 0.31 | 0.36 | 1.24 | 21,657 | 961.70 | 33.09 | 6.00 |
| LS18 | 0.22 | 6.41 | 27.08 | 0.69 | 0.18 | 0.69 | 142.80 | 4.43 | 355.9 | 903.20 | 0.45 | 0.43 | 0.15 | 1.77 | 18,416 | 690.20 | 22.91 | 6.10 |
| LS19 | 0.32 | 8.02 | 28.84 | 0.61 | 0.18 | 0.61 | 88.70 | 3.60 | 260.5 | 692.70 | 0.36 | 0.40 | 0.12 | 1.69 | 26,298 | 918.80 | 18.44 | 11.55 |

**Table 3.** *Cont.*

| Genotype | Leaf Gas Exchange Parameters | | | | | | | | Chlorophyll Fluorescence Parameters | | | | | | | | YPP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *gs* | T | A | *Ci* | A/*Ci* | *Ci*/*Ca* | WUE*i* | WUE*ins* | Fo′ | Fm′ | *Fv′/Fm′* | *ϕPSII* | *qP* | *qN* | ETR | ETR/A | AES | |
| LS20 | 0.14 | 1.22 | 21.77 | 1.00 | 0.12 | 1.00 | 163.30 | 57.12 | 79.6 | 909.80 | 0.59 | 0.00 | 0.10 | 2.80 | 6732 | 323.60 | 12.72 | 8.08 |
| LS21 | 0.17 | 1.26 | 38.69 | 2.27 | 0.13 | 2.27 | 238.50 | 30.68 | 146.1 | 790.50 | 0.51 | 0.33 | 2.33 | 0.73 | 59,155 | 1528.80 | 61.12 | 9.58 |
| LS22 | 0.24 | 1.11 | 22.39 | 0.98 | 0.10 | 0.98 | 98 | 55.81 | 321 | 229.10 | 0.48 | 0.17 | 0.33 | 1.78 | 17,721 | 763.20 | 12.77 | 11.44 |
| LS23 | 0.16 | 7.51 | 21.38 | 1.73 | 0.18 | 1.73 | 168.70 | 3.25 | 124.2 | 155.40 | 0.49 | 0.27 | 0.2 | 0.82 | 20,206 | 939.50 | 32.53 | 8.00 |
| LS24 | 0.27 | 9.26 | 27.77 | 1.34 | 0.10 | 1.34 | 102.60 | 3.07 | 229.5 | 500.20 | 0.50 | 0.25 | 0.10 | 0.80 | 35,469 | 1381.90 | 30.68 | 13.25 |
| LS25 | 0.28 | 1.12 | 23.3 | 0.76 | 0.1 | 0.76 | 86.4 | 26.53 | 111.6 | 927.5 | 0.33 | 0.36 | 0.39 | 0.78 | 28,312 | 1215.7 | 59.69 | 7.04 |
| LS26 | 0.33 | 3.57 | 23.68 | 0.69 | 0.11 | 0.69 | 73.1 | 7.91 | 200.6 | 495.2 | 0.57 | 0.09 | 0.13 | 1.25 | 28,203 | 1191.7 | 17.33 | 5.24 |
| Mean | 0.27 | 3.91 | 25.6 | 1.03 | 0.14 | 1.03 | 126.52 | 16.62 | 251.17 | 661.48 | 0.51 | 0.26 | 0.32 | 1.39 | 24,852 | 944.47 | 26.98 | 7.2 |
| *p*-value | * | * | * | ns | ns | * | * | * | ns | ** | * | ** | ** | ** | ns | ns | ns | ** |
| SED | 0.09 | 2.23 | 5.5 | 201 | 0.44 | 0.51 | 52.13 | 19.36 | 122 | 191.1 | 0.16 | 0.08 | 0.23 | 0.43 | 13,977 | 459.9 | 15.5 | 4.35 |
| LSD (5%) | 0.18 | 4.59 | 11.35 | 415 | 0.09 | 1.05 | 107.4 | 29.87 | 251.3 | 393.6 | 0.33 | 0.16 | 0.47 | 0.89 | 28,786 | 947 | 32.06 | 5.55 |
| CV (%) | 32.2 | 55.97 | 25.52 | 51.74 | 32.79 | 39.54 | 41.2 | 48.47 | 48.58 | 28.89 | 31.66 | 30.25 | 71.11 | 31.04 | 56.24 | 48.69 | 47.7 | 33.78 |

*gs*: stomatal conductance (mmol m $^{-2}$ s$^{-1}$), T: transpiration rate (mmol $H_20$ m $^{-1}$ s$^{-1}$), A: net $CO_2$ assimilation (µmol $CO_2$ m$^{-1}$ s$^{-1}$), A/Ci: $CO_2$ assimilation rate/intercellular $CO_2$ concentration (µmol.mol $^{-1}$), Ci: intercellular $CO_2$ concentration (µmol.mol $^{-1}$), Ci/Ca: ratio of intercellular and atmospheric $CO_2$, WUEi: intrinsic water use efficiency ((µmol $(CO_2)m^{-2}$), WUEins: instantaneous water use efficiency (µmol.mol$^{-1}$), $F_0$′: minimum fluorescence, Fm′: maximum fluorescence, *Fv′/Fm′*: maximum quantum efficiency of photosystem II photochemistry (ratio), *ϕPSII*: the effective quantum efficiency of *PSII* photochemistry, *qP*: photochemical quenching, *qN*: non-photochemical quenching, ETR: electron transport rate (µmol e$^{-1}$ m$^{-2}$ s$^{-1)}$, ETR/A: relative measure of electron transport to oxygen molecules (µmol e µmol$^{-1}$ $CO_2$), AES: alternative electron sinks, SED: standard deviation, YPP: yield per plant (g/plant), LSD: least significant difference, CV: coefficient of variation, * and ** denote significance at 5 and 1% probability levels, respectively, ns: non-significant.

**Table 4.** Means of leaf gas exchange and chlorophyll fluorescence parameters of okra accessions under drought-stressed conditions.

| Genotype | Leaf Gas Exchange Parameters | | | | | | | | Chlorophyll Fluorescence Parameters | | | | | | | | YPP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *gs* | T | A | *Ci* | A/*Ci* | *Ci*/*Ca* | WUE*i* | WUE*ins* | *Fo′* | *Fm′* | *Fv′/Fm′* | *ϕPSII* | *qP* | *qN* | ETR | ETR/A | AES | |
| LS01 | 0.16 | 0.01 | 24.6 | 425.1 | 0.12 | 1.11 | 847.90 | 1881.90 | 442.20 | 1826 | 0.36 | 0.06 | 0.11 | 3.75 | 27,140 | 1111 | 263 | 3.92 |
| LS02 | 0.31 | 1.01 | 29.03 | 316.1 | 0.23 | 0.80 | 193.30 | 1212.90 | 420.80 | 1733 | 0.40 | 0.05 | 0.11 | 3.72 | 19,975 | 682 | 116.60 | 2.58 |
| LS03 | 0.16 | 0.01 | 11.11 | 216.9 | 0.14 | 3.55 | 72.60 | 1196.30 | 465.60 | 1775 | 0.33 | 0.05 | 0.13 | 0.72 | 19,196 | 1782 | 98.00 | 2.50 |
| LS04 | 0.09 | 0.01 | 19.9 | 1064 | 0.07 | 1.97 | 225.80 | 2164.70 | 489.80 | 282 | 0.24 | 0.04 | 0.21 | 2.80 | 16,373 | 791 | 66.80 | 4.19 |
| LS05 | 0.13 | 4.51 | 15.11 | 763.3 | 0.15 | 0.66 | 178.50 | 372.80 | 443.50 | 1890 | 0.41 | 0.00 | 0.05 | 1.78 | 10,688 | 683 | 562.80 | 6.17 |
| LS06 | 0.12 | 2.51 | 15.27 | 920.7 | 0.05 | 2.86 | 312.20 | 696.50 | 54.80 | 1775 | 0.36 | 0.03 | 0.09 | 2.74 | 13,797 | 898 | 113.70 | 5.05 |
| LS07 | 0.17 | 4.51 | 16.03 | 728.6 | 0.13 | 0.83 | 198.90 | 312.60 | 104.80 | 1746 | 0.49 | 0.04 | 0.06 | 0.71 | 18,534 | 1156 | 104.20 | 7.92 |
| LS08 | 0.10 | 0.01 | 16.82 | 225.8 | 0.15 | 1.08 | 252.70 | 1354.60 | 509.10 | 1774 | 0.34 | 0.06 | 0.13 | 1.72 | 34,541 | 2044 | 86.00 | 6.58 |
| LS09 | 0.29 | 0.01 | 24.22 | 881.6 | 0.05 | 2.79 | 98.60 | 1933.30 | 483.00 | 1598 | 0.38 | 0.10 | 0.11 | 0.66 | 39,986 | 1902 | 199.90 | 4.60 |
| LS10 | 0.27 | 6.12 | 30.16 | 671.2 | 0.13 | 3.07 | 565.20 | 522.10 | 449.5 | 2867 | 0.37 | 0.04 | 0.03 | 3.7 | 16,452 | 551 | 263.50 | 14.00 |
| LS11 | 0.03 | 0.01 | 20.65 | 1205.9 | 0.12 | 1.83 | 923.40 | 1986.30 | 506.8 | 1809 | 0.24 | 0.05 | 0.12 | 3.74 | 20,909 | 1007 | 70.40 | 6.76 |
| LS12 | 0.21 | 0.01 | 22.17 | 221.40 | 0.14 | 0.57 | 155.90 | 1918.90 | 461.2 | 344 | 0.42 | 0.05 | 0.34 | 1.75 | 23,095 | 1057 | 111.50 | 2.85 |
| LS13 | 0.02 | 2.01 | 25.32 | 1058 | 0.05 | 2.74 | 1438.8 | 734.60 | 505.7 | 640 | 0.32 | 0.05 | 0.10 | 2.67 | 23,537 | 871 | 112.80 | 2.00 |
| LS14 | 0.17 | 0.51 | 24.54 | 290.40 | 0.10 | 1.25 | 311.4 | 761.80 | 519.20 | 963 | 0.34 | 0.06 | 0.16 | 0.69 | 24,880 | 1041 | 114.40 | 4.48 |
| LS15 | 0.34 | 1.01 | 22.62 | 598.30 | 0.13 | 2.06 | 565.6 | 995.90 | 497.90 | 1805 | 0.35 | 0.06 | 0.16 | 1.74 | 29,353 | 1316 | 170.70 | 4.71 |
| LS16 | 0.15 | 2.01 | 17.14 | 234.40 | 0.15 | 1.09 | 122.9 | 330.50 | 539 | 1533 | 0.37 | 0.02 | 0.09 | 1.32 | 8937 | 516 | 67.60 | 2.63 |
| LS17 | 0.28 | 9.01 | 26.83 | 959.40 | 0.10 | 2.49 | 901.1 | 1048 | 429.90 | 806 | 0.38 | 0.09 | 0.19 | 2.71 | 41,445 | 1542 | 89.20 | 3.69 |
| LS18 | 0.25 | 3.67 | 23.51 | 1167.80 | 0.09 | 1.71 | 696.1 | 601.70 | 478.40 | 1709 | 0.33 | 0.04 | 0.19 | 3.69 | 19,453 | 842 | 311.90 | 5.42 |
| LS19 | 0.14 | 0.01 | 16.48 | 641.60 | 0.04 | 1.65 | 764.1 | 1392.50 | 373.80 | 1803 | 0.47 | 0.05 | 0.2 | 1.75 | 20,599 | 1228 | 91.50 | 0.50 |
| LS20 | 0.02 | 3.51 | 24.42 | 909 | 0.05 | 2.36 | 1256.1 | 1039.60 | 449.40 | 1714 | 0.26 | 0.05 | 0.11 | 2.7 | 19,890 | 849 | 163.10 | 0.75 |
| LS21 | 0.16 | 0.62 | 24.17 | 211.60 | 0.28 | 0.54 | 836.5 | 97.70 | 498.80 | 762 | 0.39 | 0.06 | 0.12 | 0.72 | 25,724 | 1072 | 82.20 | 4.17 |
| LS22 | 0.10 | 0.01 | 21.33 | 718.40 | 0.08 | 1.85 | 476.4 | 2161 | 307 | 1826 | 0.33 | 0.03 | 0.05 | 0.8 | 8129 | 381 | 64.90 | 1.75 |
| LS23 | 0.10 | 4.51 | 24.23 | 234.80 | 0.12 | 3.60 | 511.8 | 375.60 | 505.90 | 1651 | 0.23 | 0.05 | 0.09 | 0.67 | 10,832 | 485 | 154.50 | 5.88 |
| LS24 | 0.26 | 3.66 | 24.96 | 863.40 | 0.10 | 3.23 | 297.5 | 257.80 | 518.20 | 1848 | 0.26 | 0.04 | 0.13 | 2.25 | 18,797 | 772 | 102.9 | 4.17 |
| LS25 | 0.11 | 4.01 | 19.88 | 697.70 | 0.06 | 2.30 | 366.5 | 706.30 | 364.20 | 1774 | 0.30 | 0.05 | 0.00 | 0.78 | 21,796 | 1140 | 360.60 | 0.01 |
| LS26 | 0.10 | 4.01 | 17.15 | 815.40 | 0.06 | 2.09 | 184.6 | 1379.50 | 397.10 | 1874 | 0.34 | 0.04 | 0.26 | 0.79 | 8071 | 466 | 80.00 | 4.00 |
| Mean | 0.16 | 2.20 | 21.45 | 644 | 0.11 | 1.93 | 490.55 | 1055.21 | 431.37 | 1543.35 | 0.35 | 0.05 | 0.13 | 1.96 | 20,851.1 | 1007.12 | 154.72 | 4.31 |
| *p*-value | * | ** | * | ns | ns | * | * | ** | ** | ** | ns | * | ns | ** | * | ** | ns | * |
| SED | 0.07 | 1.91 | 4.5 | 503 | 0.08 | 1.21 | 317.1 | 641.5 | 57.65 | 442.6 | 0.1 | 0.02 | 0.09 | 0.61 | 6837 | 304.6 | 148.1 | 2.25 |

**Table 4.** *Cont.*

| Genotype | Leaf Gas Exchange Parameters | | | | | | | | Chlorophyll Fluorescence Parameters | | | | | | | | YPP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *gs* | T | A | *Ci* | A/*Ci* | *Ci*/*Ca* | WUE*i* | WUE*ins* | *Fo′* | *Fm′* | *Fv′*/*Fm′* | *ϕPSII* | *qP* | *qN* | ETR | ETR/A | AES | |
| LSD (5%) | 0.14 | 3.92 | 9.3 | 1036 | 0.18 | 2.5 | 653.1 | 1321 | 118.7 | 911.6 | 0.21 | 0.04 | 0.19 | 1.23 | 14081 | 627.3 | 305 | 4.21 |
| CV (%) | 42.44 | 46.4 | 20.98 | 76.77 | 74.92 | 62.98 | 64.65 | 71.47 | 13.36 | 28.68 | 28.96 | 39.83 | 69.85 | 30.87 | 32.79 | 30.25 | 95.71 | 25.76 |

*gs*: stomatal conductance (mmol m $^{-2}$ s$^{-1}$), T: transpiration rate (mmol $H_20$ m $^{-1}$ s$^{-1}$), A: net $CO_2$ assimilation (µmol $CO_2$ m$^{-1}$ s$^{-1}$), A/*Ci*: $CO_2$ assimilation rate/intercellular $CO_2$ concentration (µmol·mol $^{-1}$), *Ci*: intercellular $CO_2$ concentration (µmol·mol $^{-1}$), *Ci*/*Ca*: ratio of intercellular and atmospheric $CO_2$, WUE*i*: intrinsic water use efficiency (µmol ($CO_2$)m$^{-2}$), WUE*ins*: instantaneous water use efficiency (µmol·mol$^{-1}$), $F_0'$: minimum fluorescence, *Fm′*: maximum fluorescence, *Fv′*/*Fm′*: maximum quantum efficiency of photosystem II photochemistry (ratio), *ϕPSII*: the effective quantum efficiency of *PSII* photochemistry, *qP*: photochemical quenching, *qN*: non-photochemical quenching, ETR: electron transport rate (µmol e$^{-1}$ m$^{-2}$ s$^{-1}$), ETR/A: relative measure of electron transport to oxygen molecules (µmol e µmol$^{-1}$ $CO_2$), AES: alternative electron sinks, YPP: yield per plant (g/plant), SED: standard deviation, LSD: least significant difference, CV: coefficient of variation, * and ** denote significance at 5 and 1% probability levels, respectively, ns: non-significant.

Non-significant ($p > 0.05$) differences were observed among accessions under NS and DS conditions for *Ci*. Okra genotypes LS02 and LS21 exhibited high $A/C_i$ values of 0.23 and 0.28 µmol. mol $^{-1}$, respectively, under DS conditions compared to other accessions. Significant ($p < 0.05$) differences were observed in $C_{i/}C_a$ values among accessions under both NS and DS conditions. Intrinsic water use efficiency and instantaneous water use efficiency were increased by drought stress (Table 4). Accessions LS13 and LS20 had the highest $WUE_i$ under drought-stress conditions, with 1438.80 and 1256.10 µmol $CO_2$ m$^{-2}$, respectively. The highest $WUE_{ins}$ values under drought stress were recorded for accessions LS04 (2164.70 µmol·mol$^{-1}$) and LS22 (2161.00 µmolmol$^{-1}$).

The effect of drought stress on chlorophyll fluorescence parameters among the tested okra accessions are highlighted in Table 2. Chlorophyll fluorescence parameters indicated significant differences for genotype, water regime and genotype x water regime interaction, showing that the evaluated genotypes responded differently under non-stress and drought-stress conditions. Non-significant differences were observed for $Fo'$ under non-stress, while significant ($p < 0.001$) differences were recorded under drought-stress conditions (Tables 3 and 4). Genotypic variability ($p < 0.001$) with respect to $F_m'$ was observed under non-stress and drought-stress conditions. Drought stress decreased *Fv′*/*Fm′*, from 0.51 under non-stressed to 0.35 under drought-stressed conditions. The *ϕPSII* varied significantly among the tested genotypes under non-stress and drought-stress conditions. LS07, LS12 and LS19 revealed considerably higher values for $\phi PSII \geq 0.40$ compared to other genotypes under non-stress conditions.

Photochemical quenching was significantly reduced from 0.32 to 0.13 by drought stress among the evaluated genotypes, of which LS04, LS12 and LS13 had the highest values of $qP > 0.40$. A variable genotypic response was observed with respect to *qN* under non-stress and drought-stress conditions. The mean for *qN* was higher under drought-stress (1.96) than non-stress conditions (1.39). The *qN* values ranged from 0.68 to 2.80 under non-stress (Table 3) and from 0.66 to 3.75 under drought-stress conditions (Table 4). LS02, LS03 and LS11 revealed *qN* values $\geq 2$ under non-stress conditions. Genotypes LS01, LS02, LS10, LS11 and LS18 showed *qN* values $\geq 3$ under drought-stress conditions. Non-significant differences were observed for ETR under non-stress conditions, while genotypic variation was observed for ETR under drought-stress conditions. LS08, LS09 and LS17 revealed the highest ETR value of $\geq$34,541 µmol e$^{-1}$ m$^{-1}$ s$^{-1}$, whereas LS16, LS22 and LS26 showed the lowest ETR $\leq$ 8071 under DS conditions. Drought stress significantly increased ETR/A (Table 4). The highest ETR/A ($\geq$1542 µmol e µmol-1 $CO_2$) was recorded from LS03, LS08, LS09 and LS17 under drought-stress conditions. Drought stress significantly increased AES (154.72) compared to NS (26.98). AES ranged from 12.77 to 61.12 under non-stress and from 64.90 to 562.80 under drought-stress conditions. Yield per plant was significantly reduced, from 7.20 g/plant to 4.31 g/plant, by drought stress among the evaluated genotypes. Accessions LS11, LS19, LS21, LS22 and LS24 had the highest yield (>9 g/plant) under NS conditions, whereas LS05, LS06, LS07, LS08, LS10, LS11, LS18 and LS23 exhibited the highest yield (>5 g/plant) under DS conditions.

### *3.2. Correlation between Leaf Gas Exchange and Chlorophyll Fluorescence Parameters under Non-Stressed and Drought-Stressed Conditions*

Pearson correlation coefficients showing relationships among leaf gas exchange and chlorophyll fluorescence parameters among the tested okra accessions under NS and DS conditions are presented in Table 5. Under NS conditions, Ci/Ca was highly and significantly correlated with Ci ($r$ = 1, $p < 0.001$), WUE*i* with gs ($r = -0.75$, $p < 0.001$), WUE*ins* with T (r = −0.75, $p < 0.001$) and *ϕPSII* with A/Ci ($r$ = 0.61, $p < 0.001$). In addition, *qP* was positively and significantly correlated with A ($r$ = 0.55, $p < 0.05$), C*i* ($r$ = 0.48, $p < 0.05$) and C*i*/C*a* ($r$ = 0.48, $p < 0.05$). ETR was positively and highly significantly correlated with A ($r$ = 0.71, $p < 0.001$) and *qP* ($r$ = 0.52, $p < 0.001$). Positive and high significant correlation was observed between ERT/A and ETR ($r$ = 0.86, $p < 0.001$) and AES and q*P* ($r$ = 0.52, $p < 0.001$), while a negative and highly significant association was observed between YPP and A ($r = -0.69$, $p < 0.001$). A significant positive correlation was observed between YPP and ETR/A (r = 0.49, $p < 0.05$), YPP and Ci ($r$ = 0.34, $p < 0.05$) and YPP and C*i*/C*a* (r = 0.45, $p < 0.05$), while a negative significant correlation was observed between YPP and qN ($r = -0.45$, $p < 0.05$) under NS conditions.

**Table 5.** Correlation coefficients for gas exchange and chlorophyll fluorescence parameters under non-stressed (bottom diagonal) and drought-stressed (top diagonal) conditions.

| Traits | *gs* | T | A | $C_i$ | A/$C_i$ | $C_i$/$C_a$ | $WUE_i$ | $WUE_{ins}$ | $F_O'$ | $F_m'$ | $F_v'/F_m'$ | *ϕPSII* | *qP* | *qN* | ETR | ETR/A | AES | YPP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *gs* | 1.00 | 0.17 ns | 0.57 * | −0.16 ns | 0.31 ns | −0.18 ns | −0.33 ns | −0.13 ns | 0.19 ns | 0.23 ns | 0.47 * | 0.54 ** | 0.14 ns | 0.11 ns | 0.45 * | 0.21 ns | 0.18 ns | 0.25 ns |
| T | 0.13 ns | 1.00 | 0.23 ns | 0.30 ns | −0.12 ns | 0.30 ns | 0.14 ns | −0.55 ** | −0.22 ns | 0.28 ns | 0.27 ns | −0.07 ns | −0.21 ns | 0.11 ns | −0.16 ns | 0.24 ns | 0.45 * | 0.31 ns |
| A | 0.14 ns | −0.19 ns | 1.00 | 0.12 ns | 0.15 ns | 0.09 ns | 0.48 * | −0.34 ns | 0.46 ns | −0.18 ns | 0.37 ns | 0.42 * | −0.03 ns | 0.43 * | 0.45 * | −0.26 ns | 0.02 ns | 0.18 ns |
| *Ci* | −0.31 ns | −0.29 ns | 0.30 ns | 1.00 | −0.61 ** | 0.31 ns | 0.38 ns | 0.26 ns | −0.26 ns | 0.03 ns | 0.16 ns | −0.03 ns | −0.03 ns | 0.43 * | 0.04 ns | 0.14 ns | 0.14 ns | 0.66 ** |
| A/*Ci* | 0.04 ns | 0.29 ns | 0.35 ns | −0.02 ns | 1.00 | −0.57 ** | −0.28 ns | −0.29 ns | 0.23 ns | −0.16 ns | 0.03 ns | −0.06 ns | −0.07 ns | 0.01 ns | 0.15 ns | 0.01 ns | 0.03 ns | 0.24 ns |
| *Ci*/*Ca* | −0.31 ns | −0.29 ns | 0.38 ns | 1.00 ** | −0.02 ns | 1.00 | 0.13 ns | 0.07 ns | 0.22 ns | 0.36 ns | 0.28 ns | −0.19 ns | −0.19 ns | 0.01 ns | 0.03 ns | 0.03 ns | −0.19 ns | 0.67 ** |
| WUE*i* | −0.75 ** | −0.14 ns | 0.16 ns | 0.45 ns | −0.20 ns | 0.35 ns | 1.00 | −0.17 ns | 0.24 ns | −0.12 ns | 0.33 ns | 0.22 ns | −0.07 ns | 0.43 * | 0.23 ns | 0.68 ** | −0.04 ns | 0.48 * |
| WUE*ins* | −0.24 ns | −0.74 ** | 0.16 ns | 0.13 ns | −0.35 ns | 0.13 ns | 0.14 ns | 1.00 | 0.15 ns | −0.29 ns | 0.03 ns | 0.30 ns | 0.39 * | 0.16 ns | 0.29 ns | 0.23 ns | 0.27 ns | −0.23 ns |
| *Fo'* | 0.36 ns | −0.06 ns | 0.39 ns | 0.01 ns | 0.12 ns | 0.01 ns | −0.35 ns | −0.21 ns | 1.00 | −0.22 ns | 0.14 ns | 0.24 ns | 0.24 ns | 0.18 ns | 0.24 ns | 0.18 ns | 0.03 ns | 0.83 ** |
| *Fm'* | 0.01 ns | −0.12 ns | −0.15 ns | 0.02 ns | 0.26 ns | 0.02 ns | −0.01 ns | −0.08 ns | 0.13 ns | 1.00 | 0.33 ns | −0.27 ns | 0.55 ** | 0.13 ns | −0.27 ns | −0.12 ns | 0.25 ns | 0.40 * |
| *Fv'/Fm'* | 0.21 ns | −0.17 ns | 0.193 ns | −0.12 ns | 0.23 ns | −0.12 ns | 0.03 ns | −0.18 ns | 00.21 ns | 0.27 ns | 1.00 | 0.46 * | 00.08 ns | 0.197 ns | 0.53 ** | 0.19 ns | 0.28 ns | 0.36 ns |
| *ϕPSII* | 0.38 ns | 0.36 ns | 0.42 ns | 0.02 ns | 0.51 ** | 0.02 ns | −0.36 ns | −0.44 ns | 0.30 ns | 0.04 ns | 0.05 ns | 1.00 | 0.23 ns | 0.16 ns | 0.87 ** | 0.67 ** | 0.29 ns | −0.09 ns |
| *qP* | −0.12 ns | −0.24 ns | 0.55 * | 0.48 * | 0.08 ns | 0.48 * | 0.23 ns | 0.13 ns | −0.19 ns | 0.29 ns | 0.13 ns | 0.29 ns | 1.00 | 0.15 ns | 0.28 ns | 0.13 ns | 0.48 * | −0.21 ns |
| *qN* | −0.01 ns | −0.25 ns | −0.13 ns | −0.27 ns | 0.17 ns | −0.27 ns | −0.14 ns | 0.18 ns | 0.35 ns | 0.10 ns | −0.12 ns | −0.17 ns | −0.25 ns | 1.00 | 0.08 ns | −0.17 ns | 0.13 ns | 0.25 ns |
| ETR | 0.22 ns | −0.19 ns | 0.71 * | 0.38 ns | −0.25 ns | 0.48 * | 0.06 ns | 0.11 ns | 0.31 ns | 0.12 ns | 0.15 ns | 0.37 ns | 0.52 ** | −0.24 ns | 1.00 | 0.82 ** | 0.07 ns | −0.03 ns |
| ETR/A | 0.22 ns | −0.16 ns | 0.38 ns | 0.25 ns | −0.22 ns | 0.25 ns | 0.16 ns | −0.15 ns | 0.37 ns | 0.23 ns | 0.27 ns | 0.27 ns | 0.31 ns | −0.37 ns | 0.86 ** | 1.00 | 0.29 ns | −0.60 ** |
| AES | −0.03 ns | −0.20 ns | 0.10 ns | 0.33 ns | −0.33 ns | 0.33 ns | 0.27 ns | −0.14 ns | −0.10 ns | 0.21 ns | 0.12 ns | 0.38 ns | 0.52 ** | −0.45 * | 0.37 ns | 0.49 * | 1.00 | 0.19 ns |
| YPP | −0.29 ns | −0.26 ns | −0.69 ** | 0.45* | 00.32 ns | 0.45* | 0.37 ns | 0.35 ns | 0.35 ns | −0.35 ns | −0.04 ns | −0.29 ns | −0.23 ns | 0.22 ns | 0.16 ns | 0.11 ns | 0.16 ns | 1.00 |

*gs*: stomatal conductance, T: transpiration rate, A: net $CO_2$ assimilation, A/*Ci*: $CO_2$ assimilation rate/intercellular $CO_2$ concentration, *Ci*: intercellular $CO_2$ concentration, *Ci*/*Ca*: ratio of intercellular and atmospheric $CO_2$, WUE*i*: intrinsic water use efficiency, WUE*ins*: instantaneous water use efficiency, $F_0'$: minimum fluorescence, *Fm'*: maximum fluorescence, *Fv'*/*Fm'*: maximum quantum efficiency of photosystem II photochemistry (ratio), *ϕPSII*: the effective quantum efficiency of *PSII* photochemistry, *qP*: photochemical quenching, *qN*: non-photochemical quenching, ETR: electron transport rate, ETR/A: relative measure of electron transport to oxygen molecules, AES: alternative electron sinks, YPP: pod yield per plant. * and ** denote significance at 5 and 1% probability levels, respectively, ns: non-significant.

Under DS conditions, a significant positive correlation was detected between A and gs ($r$ = 0.57, $p < 0.05$), while A/C*i* was negatively and highly significantly correlated with C*i* ($r = -0.61$, $p < 0.001$). A highly significant negative association was observed between

C*i*/C*a* and A/Ci ($r = -0.57$, $p < 0.001$). WUE*i* was positively and significantly correlated with A ($r = 0.48$, $p < 0.05$), while WUE*ins* was negatively and highly significantly correlated with T ($r = -0.55$, $p < 0.001$). $Fv'/Fm'$ was positively correlated with gs ($r = 0.47$, $p < 0.05$). $\phi PSII$ was positively and highly significantly correlated with gs ($r = 0.54$, $p < 0.001$), while significantly associated with A ($r = 0.42$, $p < 0.05$) and $Fv'/Fm'$ ($r = 0.46$, $p < 0.05$). *qP* was positively correlated with WUEins ($r = 0.39$, $p < 0.05$) and highly significantly correlated with $Fm'$ (r = 0.55, $p < 0.001$). Positive correlations were observed between *qN* and A ($r = 0.48$, $p < 0.05$) and C*i* ($r = 0.48$, $p < 0.05$) and WUE*i* ($r = 0.43$, $p < 0.05$). ETR was positively correlated with gs ($r = 0.45$, $p < 0.05$), A ($r = 0.45$, $p < 0.05$), $Fv'/Fm'$ ($r = 0.53$, $p < 0.001$) and $\phi PSII$ (r = 0.82, $p < 0.001$). Relative measure of electron transport to oxygen molecules was positively and significantly correlated with WUE*i* ($r = 0.68$, $p < 0.001$) and ETR ($r = 0.82$, $p < 0.001$), while AES was positively correlated with T ($r = 0.45$, $p < 0.05$) and *qP* ($r = 0.48$, $p < 0.05$). YPP was highly positively correlated with C*i* ($r = 0.66$, $p < 0.001$), $Fo'$ ($r = 0.83$, $p < 0.001$) and Ci/Ca ($r = 0.67$, $p < 0.001$), while significantly associated with WUE*i* ($r = 0.48$, $p < 0.05$) and Fm′ ($r = 0.40$, $p < 0.05$) and negatively correlated with ETR/A ($r = -0.60$, $p < 0.001$) under DS conditions.

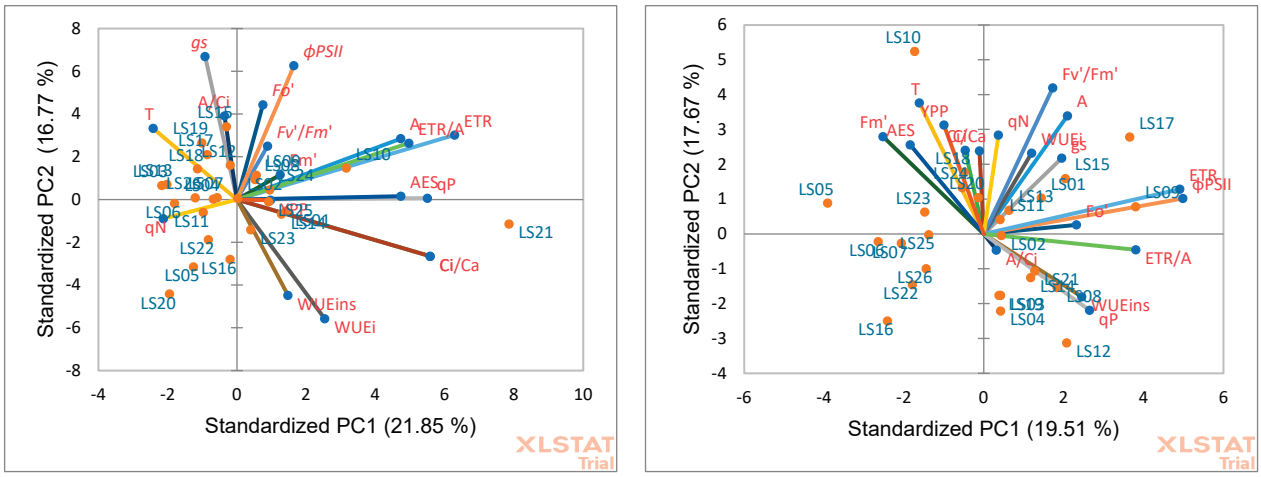
### *3.3. Principal Component Analysis (PCA) for Leaf Gas Exchange and Chlorophyll Fluorescence Traits*

Values of PCA, eigenvalues, percent, and cumulative explained variances are summarised in Table 5. Under NS conditions, seven principal components exhibited eigenvalues > 1 and accounted for 81% of total phenotypic variation. Net $CO_2$ assimilation, $C_i$, $C_i/C_a$, *qP*, ETR, ETR /A, AES and YPP were positively correlated with PC1, which accounted for 22% of the total variation. PC2 was positively correlated with gs, $Fo'$ and $\phi PSII$, whereas $WUE_i$ and $WU_{Eins}$ were negatively correlated with PC2, which accounted for 17% of the total variation. Transpiration rate was negatively correlated with PC3, whereas $WUE_{ins}$, *qP* and YPP were positively correlated with PC3, which contributed 11.42% of total variation. A/C*i* positively correlated with PC4 accounted for 10.67% of total variation. PC5 was positively correlated with $Fm'$ and $F_v'/F_m'$, contributing 8% of total variation, whereas PC6 was positively correlated with Fm′, contributing 7% of total variation.

Similarly, under DS conditions, seven PCs with eigenvalues > 1 were detected, which contributed 80% of the total phenotypic variability. Yield per plant was negatively correlated with PC1, whereas $\phi PSII$, ETR and ETR/A were positively correlated with PC1, which accounted for 20% of total variation. Transpiration rate, net $CO_2$ assimilation, $C_i$, $WUE_i$, $F_v'/F_m'$, *qN* and YPP were positively associated with PC2, accounting for 18% of the total variation. Stomatal conductance and A/Ci were positively correlated with PC3, whereas Ci and $WUE_{ins}$ negatively associated with PC3 contributed 14% of the total variation. Net $CO_2$ assimilation and *qN* were positively correlated with PC4, whereas ETR/A was negatively correlated with PC4, accounting for 10% of total variation. Instantaneous water use efficiency was positively correlated with PC5, which accounted for 7% of total variation, whereas stomatal conductance and photochemical quenching were positively correlated with PC6, which contributed 6% of total variation.

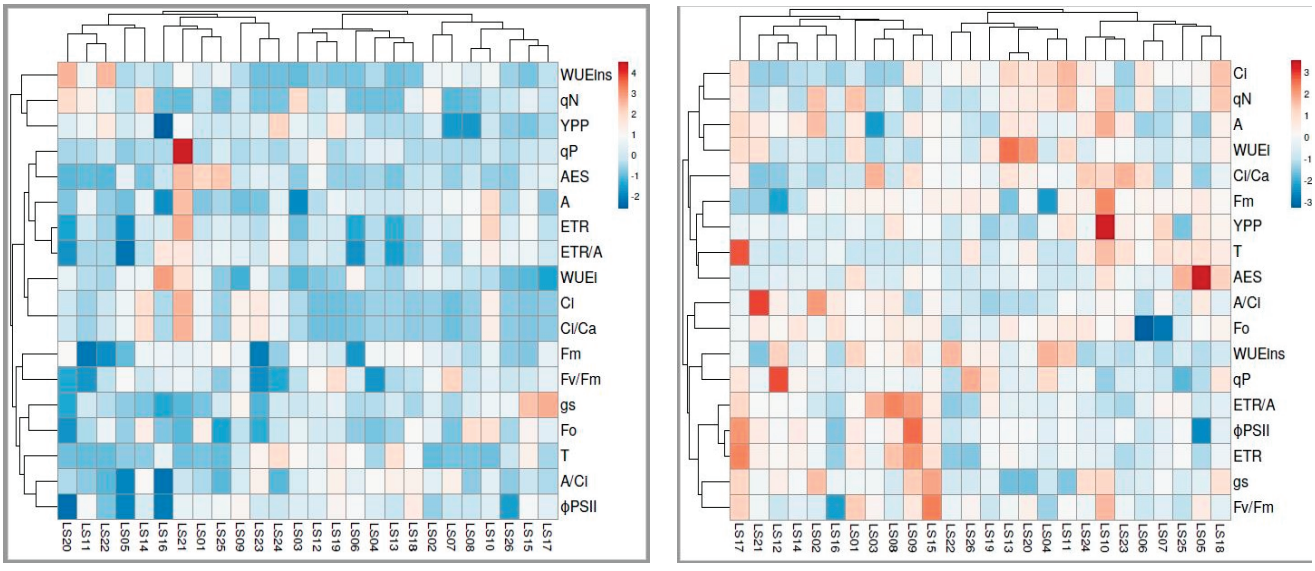
Principal component biplots based on PCA analysis were used to indicate the relationships among okra accessions for leaf gas exchange and chlorophyll fluorescence parameters under NS (Figure 1A) and DS (Figure 2B) conditions. Traits presented by parallel vectors or those close to each other revealed a strong positive association, and those located nearly opposite (at 180°) showed a highly negative association, while the vectors toward sides expressed a weak relationship. Under NS conditions, accessions LS06, LS11, LS22, LS05 and LS20 were grouped based on high *qN*. Accessions LS19, LS17 and LS18 were grouped together based on high gs, T and A/Ci. LS02, LS10 and LS24 were grouped based on high $\phi PSII$, $Fo'$, $F_v'/F_m'$, A, ETR, ETR/A, AES and *qP*. Accessions LS25, LS01, LS23 and LS16 were grouped together based on high $C_i/C_a$, $WUE_i$ and $WUE_{ins}$. Under DS conditions, accessions LS10, LS24, LS25, LS05 and LS06 were clustered together based on high $Fm'$, AES, T, $C_i$ and YPP. LS13, LS15, LS17 and LS09 were grouped together based on high $C_i/Ca$,

$F_v'/F_m'$, $WUE_i$ and *ϕPSII*. Accessions LS02, LS19, LS21, LS08 and LS12 were grouped based on high *Fo′*, ETR/A, $WUE_{ins}$ and *qP*.




(**A**) (**B**)

**Figure 1.** Principal component (PC) biplot of PC1 vs. PC2 depicting the relationships among physiological traits among 26 okra accessions evaluated under non-stressed (**A**) and drought-stressed (**B**) conditions.




(**A**) (**B**)

**Figure 2.** Heatmap showing the relationship among physiological traits among 26 okra accessions evaluated under non-stressed (**A**) and drought-stressed (**B**) conditions.

### *3.4. Heatmap Analysis for Leaf Gas Exchange and Chlorophyll Fluorescence Traits*

A heatmap based on leaf gas exchange and chlorophyll fluorescence traits under NS and DS conditions was constructed using a hierarchical clustering method to discern the relationship of 26 okra accessions based on Jaccard's coefficient (Figure 2). Under NS (Figure 2A) conditions, physiological traits were grouped into four main clusters. The first cluster consists of two subclusters, dominated by eight accessions, including LS19, LS12, LS06, LS18, LS13, LS07 and LS02, which were grouped based on high negative correlations with WUE*ins*, *qN* and YPP. The second subcluster consisted of accessions LS22, LS11, LS1, LS08, LS20 and LS14, which were negatively correlated with A/Ci and T. LS25, LS01, LS21, LS16, LS24 and LS23 dominated the fourth subcluster under NS conditions and positively

correlated with *qP*. Under DS, physiological traits were grouped into three main clusters and six subclusters. The first cluster is dominated by accessions LS19, LS09, LS03, LS15, LS14 and LS17, based on their positive correlations with ETR and ETR/A. LS26, LS22, LS04, LS20, LS13 and LS11 dominated the second cluster under DS conditions, with positive correlations with WUE*i*, WUE*ins*, *qN* and YPP. AES was positively correlated with LS25 and LS05 in the third cluster under DS conditions.

## 4. Discussion

Okra is one of the most important commercial vegetable crops grown for its fresh fruits and dry seeds. Drought is the major impediment to okra production in dry regions. To adapt to drought stress, plants have undergone many biochemical, molecular, and physiological changes. These changes increase the plants' tolerance to drought stress. Drought stress influences plant performance by reducing gas exchange and altering chlorophyll fluorescence formation. Gas exchange and chlorophyll fluorescence confer drought tolerance in okra [11,27]. Plants alter gene expression, disrupting the production of photosynthetic pigments and regulating stomatal function to adapt to and tolerate stress conditions [27]. Developing new strategies for maintaining high yield under drought-stress conditions is one of the major challenges in the current crop production system.

In this study, various physiological drought responses were assessed in okra accessions. Reductions in okra's stomatal conductance and transpiration rates have been associated with water conservation that allows plants to tolerate drought stress and the loss of physiological functions [9]. Stomatal closure leads to a reduction in $CO_2$ assimilation and minimises the rate of water loss through transpiration. This role of drought-induced stomatal closure limits $CO_2$ uptake by the leaves and possibly leads to increased susceptibility to photodamage [11]. Similar findings were reported for okra accessions under water shortages [11,27]. These physiological changes increase the plants' resistance to drought stress, enabling the crop to survive in environments with limited water availability.

Drought tolerance should be considered as a comprehensive evaluation of carbon assimilation during global climate change challenges [28]. In the current study, okra accessions exhibited a reduction in net $CO_2$ assimilation under drought-stressed conditions (Table 4). The decrease in net $CO_2$ assimilation during water-stressed conditions might be reversible initially. However, drought in the pod-filling stage might cause irreversible damage to the photosynthetic pathway, thereby affecting carbon assimilation [29]. Further, utilisation of assimilates is relevant in addition to the photosynthetic performance of leaves. The evaluated okra accessions revealed high water use efficiency under drought-stressed conditions (Table 4). Enhancing water use efficiency to sustain okra production under water-limited conditions remains the most important task for water management. Hence, specific responses for enhancing water use efficiency could be achieved with more precise data on crop stress detection [11]. Drought-tolerant accessions exhibited high $WUE_i$ and $WUE_{ins}$ compared to drought-susceptible accessions (Table 2). This indicates that the evaluated accessions use water efficiently, attributed to drought escape mechanisms such as the transpiration rate. Drought-tolerant accessions use water efficiently, maintain tissue water status, reduce water loss and produce stable yield during water shortages [30].

Chlorophyll fluorescence is a non-invasive measurement detecting the authenticity of photosystem II [31]. Chlorophyll fluorescence parameters, including photosystem II photochemistry, minimum fluorescence, maximum fluorescence, photochemical quenching and electron transport rate are useful for detecting drought-stress severity, genetic variation and determining damage to *PSII* [32]. $F_v'/F_m'$ is considered the most important parameter of chlorophyll fluorescence, widely used to evaluate drought-stress response. In this study, a reduced $F_v'/F_m'$ value was recorded under drought-stress conditions, corroborating with results reported by Ahmed and El-Sayed, [27]. According to Paknejad et al. [33], reduced $F_v'/F_m'$ under drought-stress conditions indicates the presence of a protective mechanism of light absorption in response to water shortages. Hence, the $F_v'/F_m'$ parameter can be applied to determine the potential efficiency of *PSII*.

In the present study, drought-tolerant okra accessions showed an efficient photosynthetic affinity compared to sensitive accessions. Photosystem *II* is highly drought tolerant. However, under drought-stress conditions, photosynthetic electron transport through *PSII* is inhibited [24]. The decrease in *PSII* might be due to the photo-protective increase in thermal energy dissipation induced by the excess of absorbed light [34]. However, there are contradictory reports on the direct effect of *PSII* functionality under drought-stress conditions. A study reported that, under mild water stress, *PSII* is not affected [35], while another study reported that, under drought-stress conditions, damage occurs to both photosystem I and photosystem II [36]. The current study found that *PSII* was significantly affected by drought stress. Under drought-stress conditions, the *PSII* thermal energy dissipation was strongly limited due to damage to *PSII* structure and functionality. A decrease in photochemical quenching was observed in the studied okra accessions under drought-stress conditions. Similar results were reported by Ashraf et al. [37] in the study of gas exchange characteristics and water relations in some elite okra cultivars under water-deficit conditions. The decrease in $qP$ is attributable to either a decrease in the rate of consumption of reductants and ATP produced from non-cyclic electron transport relative to the rate of excitation of open *PSII* reaction centres or damage to *PSII* reaction centres [24].

Positive correlations were observed between non-photochemical quenching and intrinsic water use efficiency under drought-stress conditions, indicating a protective mechanism by the plants against reactive oxygen species that harm antenna pigments and closing reactions in the photosystem. Drought stress also affects the electron transport rate (ETR) and alternative electron sink (AES) [38]. An increase in alternative electron sink was observed among the studied okra accessions under drought-stress conditions. Drought-tolerant accessions indicated higher AES values. An increase in AES was reported as an indicator of drought stress [39]. Alternative electron sink is the second most important mechanism after photosynthesis used to remove electrons, which occurs at high rates in the leaves under drought stress conditions [40].

## 5. Conclusions

Drought is one of the most important factors affecting physiological traits and yield in crop plants, including okra. In the present study, it was observed that drought stress affected physiological processes such as reduced stomatal conductance, transpiration rate, net carbon dioxide assimilation, maximum quantum efficiency, effective quantum efficiency of *PSII* photochemistry, photochemical quenching and electron transport rate among the studied okra accessions. These physiological traits could be useful for drought-tolerance breeding in okra. Principal component analysis-based biplots allowed the identification of drought-tolerant accessions such as LS05, LS06, LS07 and LS08 based on high A, T, $Fm'$, $Fv'/Fm'$ and ETR, and LS10, LS11, LS18 and LS23 based on high *AES*, *Ci*, *Ci/Ca*,*WUEi*, *WUEins*, $\phi PSII$ and AES. The selected genotypes are high yielding ($\geq$5 g/plant) under drought-stress conditions. These accessions are suitable candidates for parental genotypes for drought-tolerance breeding in okra to enhance water use efficiency under water-limited conditions.

**Author Contributions:** Conceptualisation, S.S.M., H.S. and A.S.G.; methodology, S.S.M.; validation, S.S.M., H.S., A.S.G. and J.M.; formal analysis, S.S.M.; investigation, S.S.M.; resources, H.S. and A.S.G.; data curation, S.S.M.; writing—original draft preparation, S.S.M.; writing—review and editing, S.S.M., H.S., A.S.G. and J.M.; supervision, H.S. and A.S.G.; project administration, S.S.M., H.S. and A.S.G.; funding acquisition, H.S. and A.S.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Research Foundation, South Africa, grant number MND200618533646, the Agricultural Research Council through the PDP program, grant number 10013464 and the Moses Kotane Institute, grant number 215041664.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The University of KwaZulu-Natal is acknowledged for the support of the project, as well as the Agricultural Research Council through the Professional Development Programme for providing plant material, funding and research support. The National Research Foundation, South Africa and Moses Kotane Institute are acknowledged for funding this study.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Testing the Single and Combined Effect of Kaolin and Spinosad against *Bactrocera oleae* and Its Natural Antagonist Insects in an Organic Olive Grove

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**Abstract:** The presence and infestation level of *Bactrocera oleae* was monitored in an organic olive orchard divided into differently treated parcels with kaolin (K), spinosad (S) and with kaolin and spinosad (K + S) in alternate rows. The treatments did not seem to affect olive fruit fly population dynamics, while statistically significant protective effects were recorded against total and harmful infestation, but not against the active one. Eventually, neither kaolin nor spinosad were shown to have a particular detrimental effect against naturally occurring *B. oleae* parasitoids.

**Keywords:** *Bactrocera oleae*; spinosad; kaolin; organic oliviculture

**Citation:** Vizzarri, V.; Lombardo, L.; Novellis, C.; Rizzo, P.; Pellegrino, M.; Cruceli, G.; Godino, G.; Zaffina, F.; Ienco, A. Testing the Single and Combined Effect of Kaolin and Spinosad against *Bactrocera oleae* and Its Natural Antagonist Insects in an Organic Olive Grove. *Life* **2023**, *13*, 607. https://doi.org/10.3390/life13030607

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 29 December 2022
Revised: 10 February 2023
Accepted: 17 February 2023
Published: 22 February 2023



## 1. Introduction

Olive fly (*Bactrocera oleae*, Rossi) is the most important pest of olive in the Mediterranean Basin [1]. The direct damage is caused by larval feeding on fruits, determining quantitative (premature fruit drop and pulp destruction by grub) and qualitative (increase in acidity and peroxide levels and decrease of polar phenols) losses, resulting in relevant production shrinkages reaching up to 80% for oil and 100% for table olives in particularly susceptible cultivars [1–4]. It is a polyvoltine monophagous species, feeding exclusively on fruits of the genus Olea, whereas its main host plant is *O. europaea* subsp. *europaea* (cultivated and var. *sylvestris*). As such, the evolution and distribution of *B. oleae* is closely linked to the millennial history of this species following its domestication and diffusion [5]. Accordingly, Theophrastus (IV–III century BC) described (*Historia plantarum*, IV, XIV) a worm (σκώληξ) that insinuates into the pulp up to the stone of the olive drupes. The same observation (*vermiculationem*) was reported in the I century AD by Pliny the Elder (*Naturalis Historia*, XVII, XXXVII, 230). In spite of this, the first written reference to the responsibility of a fly for these worms generated in olives is due to Sieuve in 1769 [6], inasmuch as he believed that oviposition occurred on the trunk with the larvae subsequently reaching the drupes. Only a few years later (1773), Grimaldi [7] reported the observations of Calabrian olive growers according to whom the fly laid its eggs directly in the drupe. The correct identification of the pest led to the development of the first forms of control, including anticipated harvest and the employment of mixtures based on cobalt, potassium arsenite or tar [8]. With the advent of synthetic insecticides, the management of olive fly was mainly based on the use of organophosphates (OPs) in cover and bait sprays (e.g., dimethoate and fenthion), while more recently there has been an increase in the use of pyrethroids [1]. Nevertheless, the development of mechanisms of resistance, as well as the evidence of negative effects on the agricultural useful entomofauna and on human health due to the presence of residues in the oil, raised several concerns, resulting in the ban of several OPs worldwide [9,10]. Paradigmatic in this sense for olive growing is the case of the cytotropic insecticide dimethoate, which was abundantly and predominantly used for decades against *B. oleae*, and whose employment was revoked in the EU in 2020 due to

failure to renew the European approval pursuant to regulation (EU) 2019/1090, as it was not possible to exclude its genotoxic potential (as well as the one of its main metabolite, methoate which is an *in vivo* mutagenic agent). The Authority also concluded that there is a dimethoate-correlated high risk for mammals and non-target arthropods (including honey bees). The selection of alternative control methods with little or no environmental impact is therefore becoming a pressing challenge for oliviculture. The pursuit of effective systems to contrast the olive fly is particularly important in the organic sector. In Italy, the organic surface planted with olive trees is equivalent to 242,708 ha, approximately 22% of the total, divided by over 37,000 farms (certified or in conversion phase [11]). However, the production of organic oil in the Italian market covers only slightly less than 10% of the total, corresponding to approximately 28,000 Mg. This gap can be attributed not only to the reduced presence of oil mills equipped for biological production, but also to the general lower production of organic surfaces (actually due to the reduced alternatives on the market that are allowed to be used for olive pathogen control) [12,13], and to the fact that only the commercial categories of virgin (VOO) and extra virgin (EVOO) olive oils can be labeled as organic [14], so that if *B. oleae* is not effectively countered, the risk of organoleptic defects and chemical changes in oil composition is high. In this regard, natural-origin and biological insecticides are now acquiring more and more interest because of their generally more environmental-friendly action and of their allowed use in organic farming (an exception is represented by rotenone, widely used also against olive fly, that in spite of its natural origin, has been excluded from the list of products allowed in organic farming in Europe, according to Annex I of EC Directive 91/414/2008 because of its high toxicity towards fish and bees). Among the naturalytes, spinosad, an environmentally safe molecule with an attractive and insecticidal action, obtained from the bacterium *Saccharopolyspora spinosa* (Mertz &Yao) through fermentation, has proven its effectiveness in dozens of crops to control several insect pests, including *B. oleae* [1,15–19]. It is a mixture of spinosyns A and D, altering the nicotinic and gamma-aminobutyric acid receptor functions, acting mainly by ingestion. Nevertheless, in an integrated pest management context, the need for the combination of several methods in order to reduce the amount of product applied and to prevent the onset of phenomena such as pesticide resistance and toxicity for non-target organisms is now clear. In this particular case, an interesting approach may rely on the integrated use of non-insecticidal control methods for the target pest to reduce selection pressures on a resistant population. Particle film technology (PFT) is a relatively new method for controlling arthropod and disease pests of food crops that is allowed in organic farming. When crops are dusted or sprayed with hydrophobic particles, a protective barrier against both plant pathogens and plant-feeding arthropods is created. In this regard, kaolin is a white, nonabrasive, fine-grained and inert aluminosilicate mineral that is purified and sized so that it easily disperses in water and creates a mineral barrier on plants that prevents oviposition and insect feeding and severely reduces insect movements by the attachment of particles to their bodies [20,21]. Previous studies have shown that hydrophobic formulations of kaolin-based particle films can effectively protect olive trees from *B. oleae*, *Prays oleae* (Bernard) and *Saissetia oleae* (Olivier) [22,23]. With particular reference to *B. oleae*, kaolin proved to have the same protective efficacy as dimethoate in a Tunisian [24] and a Portuguese [18] study, and to provide a higher degree of protection than copper at high infestation levels [25], whereas copper-based compounds are allowed to be used, with some limitations-, in organic farming and commonly used in integrated and conventional pest management. Despite its deterrent (insects move away after contact) action, kaolin is not included among the antiparasitic substances, but was admitted as a biostimulant in organic legislation.

In light of the above, the main objective of this study was to evaluate the effect, either alone or in combination, of kaolin and spinosad against *B. oleae* and its main naturally occurring parasitoids in an experimental organic olive grove.

## 2. Materials and Methods

### *2.1. Experimental Design*

The study was carried out during the 2019–2020 period in a CREA OFA/ARSAC (Regional Company for the development of Calabrian agriculture) experimental field located in Mirto (39.615974, 16.7771879), Calabria Region, Italy, managed according to the provisions contained in EU Regulation 848/2018 on the production of organic food and feed. The 0.75 ha olive grove was made up of plants of the cultivar Carolea at 4 × 4 m spacing. Carolea is one of the most important and widespread Calabrian varieties, whereas Calabria is the second major olive producing region in Italy [26]. Carolea is a highly susceptible cultivar to the olive fruit fly e.g., [27,28], and thus the monocultivar condition in a single environment allowed us to limit the influence of external factors affecting *B. oleae* infestation. This aspect is particularly important considering the vast Italian olive germplasm including over 800 accessions [29], with different degrees of susceptibility to olive fly, e.g., [27,30,31].

The orchard was split into five parcels consisting of two control plots interspersed by three blocks, differently treated with kaolin (K), spinosad (S) and with kaolin and spinosad (K + S) in alternate rows, respectively, as shown in Figure 1. An untreated buffer zone of two rows of olive plants divided each pair of contiguous parcels. A spinosad-based protein bait with specific attractive substances (commercial product: Spintor™ Fly; Dow Agro-Sciences LLC, Indianapolis, IN, USA) was administered at a dose of 1 L of product/ha and diluted in 4 L of water, every 6–10 days from 8 August to 11 November 2019 and from 27 July to 23 October of 2020. It was sprayed on all the perimeter plants and to alternate plants in the internal rows, in a canopy portion free from drupes and facing south.

**Figure 1.** Subdivision of the experimental field according to the treatment. Highlighted in green: control; purple: kaolin; yellow: spinosad; grey: buffer zones. The red spots indicate the positioning of the chromotropic adhesive traps.

Kaolin was administered at a dose of 5 kg/ha with three treatments (early August, early September and early October in 2020 and end of July, mid-August, and mid-October in 2020) defined on the basis of the rainy events that occurred in the two years, and in accordance with [18,32].

Temperature data were recorded by a meteorological station situated a few hundred meters away from the experimental olive orchard but within the ARSAC property.

Active (the presence of eggs and the first and second instar larvae), harmful (third instar larvae, pupae and abandoned tunnels) and total infestation (all the alive or dead preimaginal stages, parasitized and abandoned tunnels) were determined by examination samples of 100 olive drupes per treatment (C, K, S and K + S) and per sampling date under a binocular microscope, randomly collected every 7 to 10 days from August to November of each year. The effectiveness of the two products and their combined action was assessed in terms of active, harmful, and total infestation in the treated blocks compared to the control parcels.

The presence and population trend of *B. oleae* adults were monitored during the study period every 8–10 days through the placement of 14 chromotropic adhesive traps throughout the five plots so as to have a global coverage of the orchard (Figure 1).The traps consisted of yellow plexiglass panels measuring 15 × 20 cm wrapped with a transparent sticky film that was replaced at each monitoring (8–10 days). They were positioned in the medium-low part of the canopy and hooked to a branch on the most exposed side of the tree to make them more visible to the insects.

Male and female captures were discriminated by treatment in order to evaluate them for a possible repulsive effect.

### *2.2. Identification and Population Dynamic Monitoring of Naturally Occurring B. oleae Egg/Larval Parasitoids*

The presence of the main natural antagonists of the olive fly during the experimental trial was monitored through different approaches: (i) adult captures through the chromotropic adhesive traps positioned for monitoring olive fly population dynamics; (ii) evaluation of the level of ectoparasitism in actively infested drupes; and (iii) evaluation of the level of endoparasitism through the collection of *B. oleae* puparia from infested drupes maintained in Petri dishes at laboratory conditions (24 ± 1 °C, 60 ± 10% RH) until the eventual parasitoids emergence from olive fly puparia. Captured/reared adult parasitoids were stored at −20 °C and then observed under the stereomicroscope for taxonomic identification by examining their morphological characteristics.

The percentage of ecto- and endoparasitism was calculated as follows:

$$\% \text{ ectoparasitism} = (\text{number of parasitoids feeding on } \textit{B. oleae} \text{ eggs-larvae found in infested drupes/number of } \textit{B. oleae}\text{-infested drupes}) \times 100 \quad (1)$$

$$\% \text{ endoparasitism} = (\text{number of parasitoids emerging from } \textit{B. oleae} \text{ puparia/number of } \textit{B. oleae} \text{ puparia}) \times 100 \quad (2)$$

### *2.3. Statistical Analysis*

The effects of treatments on the total male and female captures and fruit damages in the two years were initially tested through a factorial ANOVA with interaction effects performing the "aov" function in the "stats" package. Since only the factor "treatment" was significant, we proceeded to the analysis with a one-way ANOVA with the same package and function. Normality and homoscedasticity of residuals were tested using a Shapiro–Wilk test (function "Shapiro.test" in package "dplyr") and Bartlett's test (function "bartlett.test" in package "stats"). After residue analysis, data were log(x + 1) transformed in order to avoid heterogeneity of variances. Statistical significance was considered at $p \leq 0.01$ and $p \leq 0.05$.

A post hoc multiple comparison of means at confidence levels of 99 and 95% was made using a Tukey's test (function "TukeyHSD" in package "stats"). All analyses were done using R 3.4.1 software [33].

## 3. Results and Discussion

### *3.1. B. oleae Population Dynamics*

The overall (males plus females) trend of *B. oleae* presence in the organic olive orchard during the July to November and July to end of October periods in 2019 and 2020, respectively, is presented in Figure 2. Temperature, and in particular maximum temperature,

seemed to represent the principal factor driving the increase and decrease of individuals in 2019, with maximum temperatures above 35 °C causing a drastic decline in the total number of olive fruit flies. A 35 °C threshold as a lethal temperature has been previously highlighted in a laboratory-scale study [34], even if in open-field conditions other variables, primarily air relative humidity, intervene in determining the survival/mortality rate. Accordingly, a negative correlation (Pearson's r = −0.84) was found between maximum temperatures and *B. oleae* catches. A similar observation was also valid for 2020 until August, whereupon the drop in temperatures did not support an increase in the population of *B. oleae* for the whole month of September (Figure 2). Since this anomalous trend was uniformly found in the five parcels and considering that the orchard is located in a highly olive-vocated area, we are led to hypothesize that the reason for the zeroing of the number of catches, accompanied by an equally low rate of active infestation, might be sought in the use of insecticides in response to the peak recorded on 12 August 2020 in the adjacent orchards, having had an impact on the total population of the olive fly.

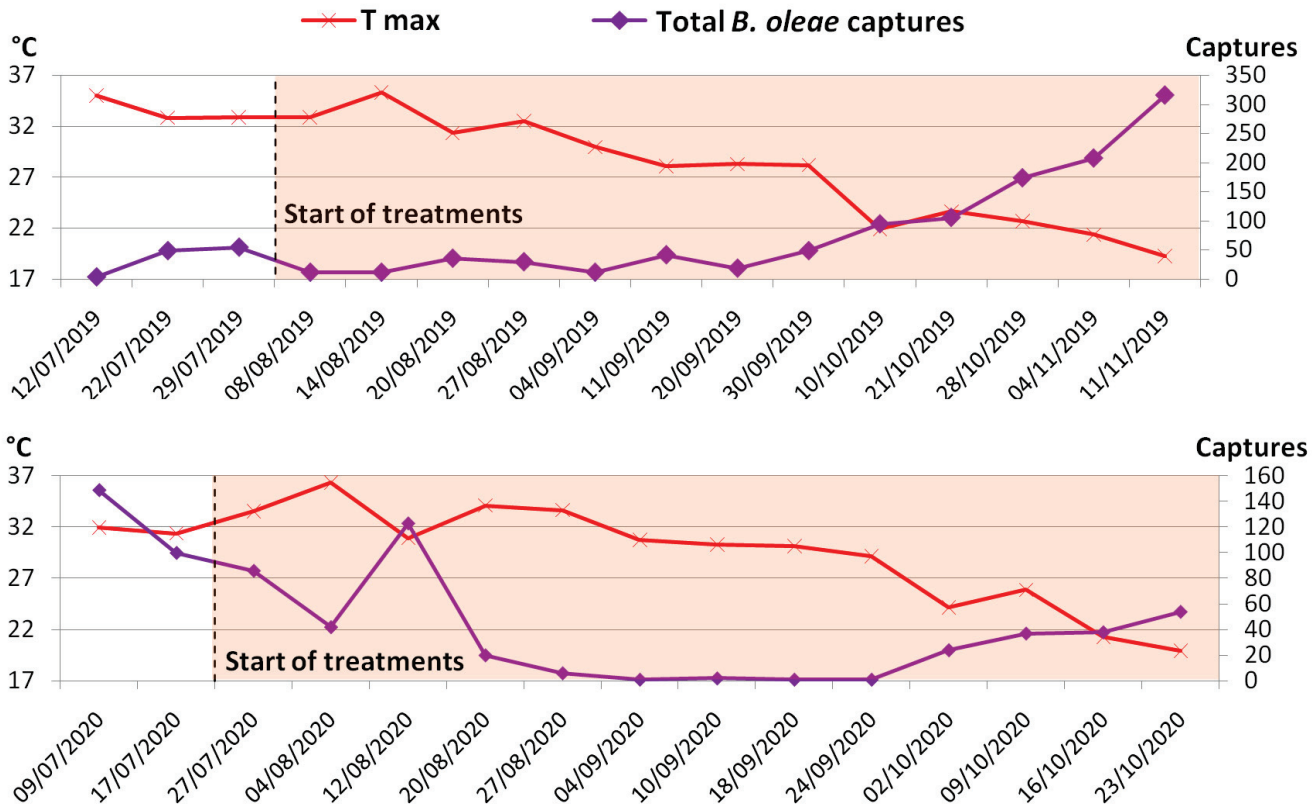


**Figure 2.** Trend of maximum temperatures and total captures of individuals of *B. oleae* in the 14 sticky traps scattered in the olive grove recorded during the two-year trial.

The higher number of catches found at the beginning of July 2020 compared to the same period of 2019 was likely due to the rains that occurred during the month of June 2020 and which are the basis of the different population trends observed in the two years. In this sense, the highest values were recorded in November in 2019 with 524 catches and in July in 2020 with 335 catches. The resulting highest values per single trap were recorded on 11 November 2019 (56) and on 9 July 2020 (61).

Regarding sex ratio, a balanced distribution was observed via adult captures through chromotropic adhesive traps (Figure 3) in both years, while, as easily predictable, a strong correlation (Pearson's r = 0.96 and 0.80 for 2019 and 2020, respectively) was found between female captures and active infestation, namely representing the most "recent" portion of the infestation, including the presence of eggs and the first and second instar larvae inside the drupes' pulp. Regarding the positioning of the traps, in the untreated period, a statistically

significant difference ($p$ = 0.046) in *B. oleae* captures was only found between the parcels subsequently sprayed with kaolin and spinosad (with higher values in the former) in the two years, with a consequent general uniform spread of this dipterous.

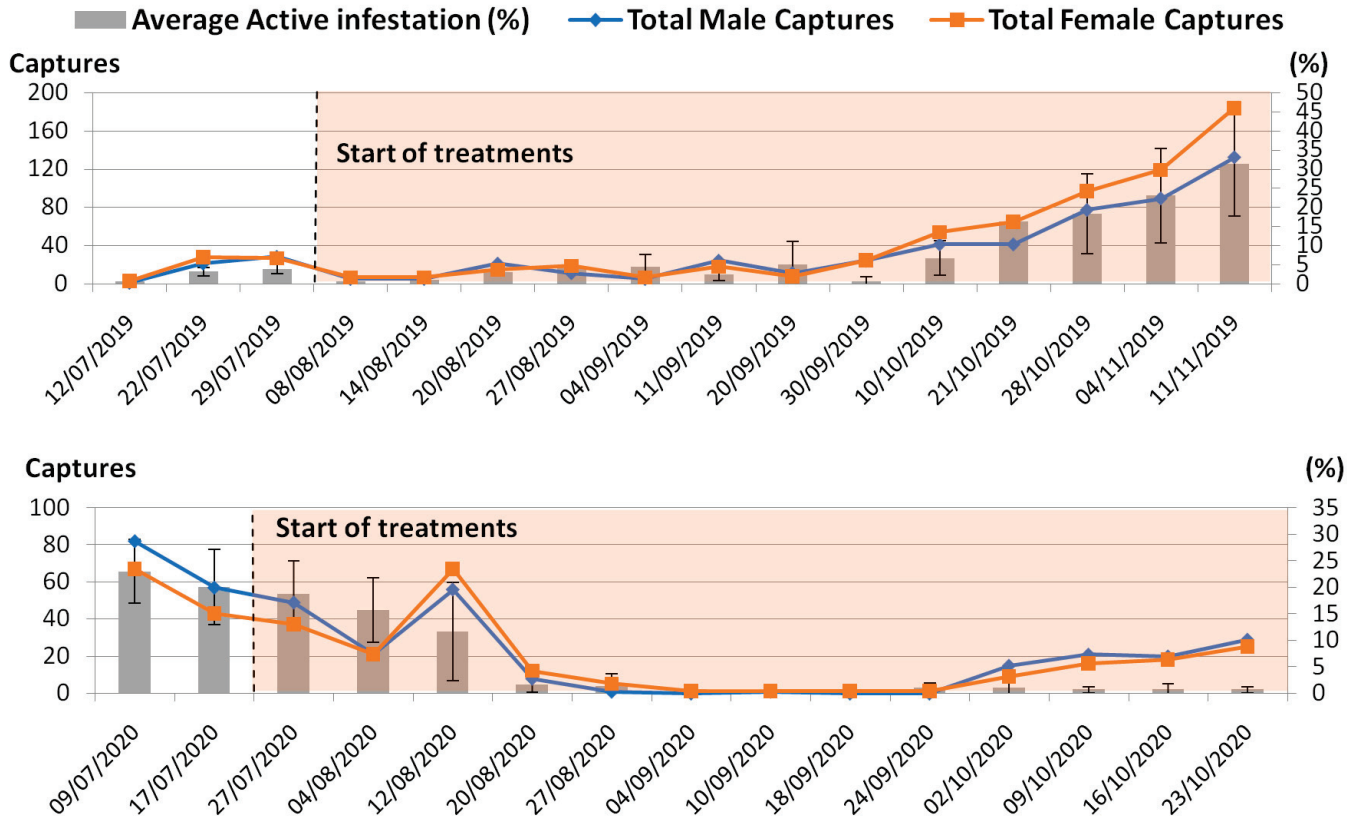


**Figure 3.** Trend of average active infestation (with standard deviation bars) and total captures of male and female individuals of *B. oleae* during the two-year trial, regardless of treatment.

### *3.2. Effect of Treatment on Drupes' Infestation Level and B. oleae Captures*

The total effect of treatments on total, active and harmful infestation over the two years (2019 and 2020) is shown in Figure 4.

The comparison of the two years shows that 2019 had higher levels of total infestation than 2020 ($p$-value = 0.006) regardless of treatment, but no significant differences were found among single treatments by considering interaction effects. Total infestation was statistically significantly higher in the control parcels in both years compared to the treated ones. No difference emerged from the comparison of the effect given by the three treatments with each other (Figure 4a). Active infestation was not overall significantly affected by treatment either in 2019 ($p$-value = 0.691) and in 2020 ($p$-value = 0.869), with tendentially higher values in the untreated plot (C, Figure 4b), while a Tukey's test showed that either in 2019 and in 2020 the three treatments (K, S and K + S) were significantly different from the control for harmful infestation, presenting lower values with a comparable protective effect in a highly susceptible cultivar such as Carolea (Figure 4c). Eventually, the treatment did not seem to influence the number of sterile punctures.

Regarding treatment, in general kaolin showed the best results, with lower infestation levels in absolute value; which was even better considering that the highest numbers of fly captures before treatments were recorded in this parcel. This finding is in accordance with the work by Rizzo and collaborators [35] recording a higher efficacy of kaolin against *B. oleae* compared to a spinosad-based bait in three Sicilian organic olive orchards planted with the medium-high susceptible cvs Cerasuola and Nocellara del Belice, without however testing their combined use. Similarly, field experiments on the cv Carolea demonstrated a greater effectiveness of kaolin compared to rotenone [36], copper and copper mixed to

kaolin [37]. These studies also revealed the neutral effect of kaolin on the chemical and sensory characteristics of the extracted oils. Conversely, kaolin (as well as copper) treatment was demonstrated to affect the sensorial attributes of table olives and the microbial population during fermentation [38].

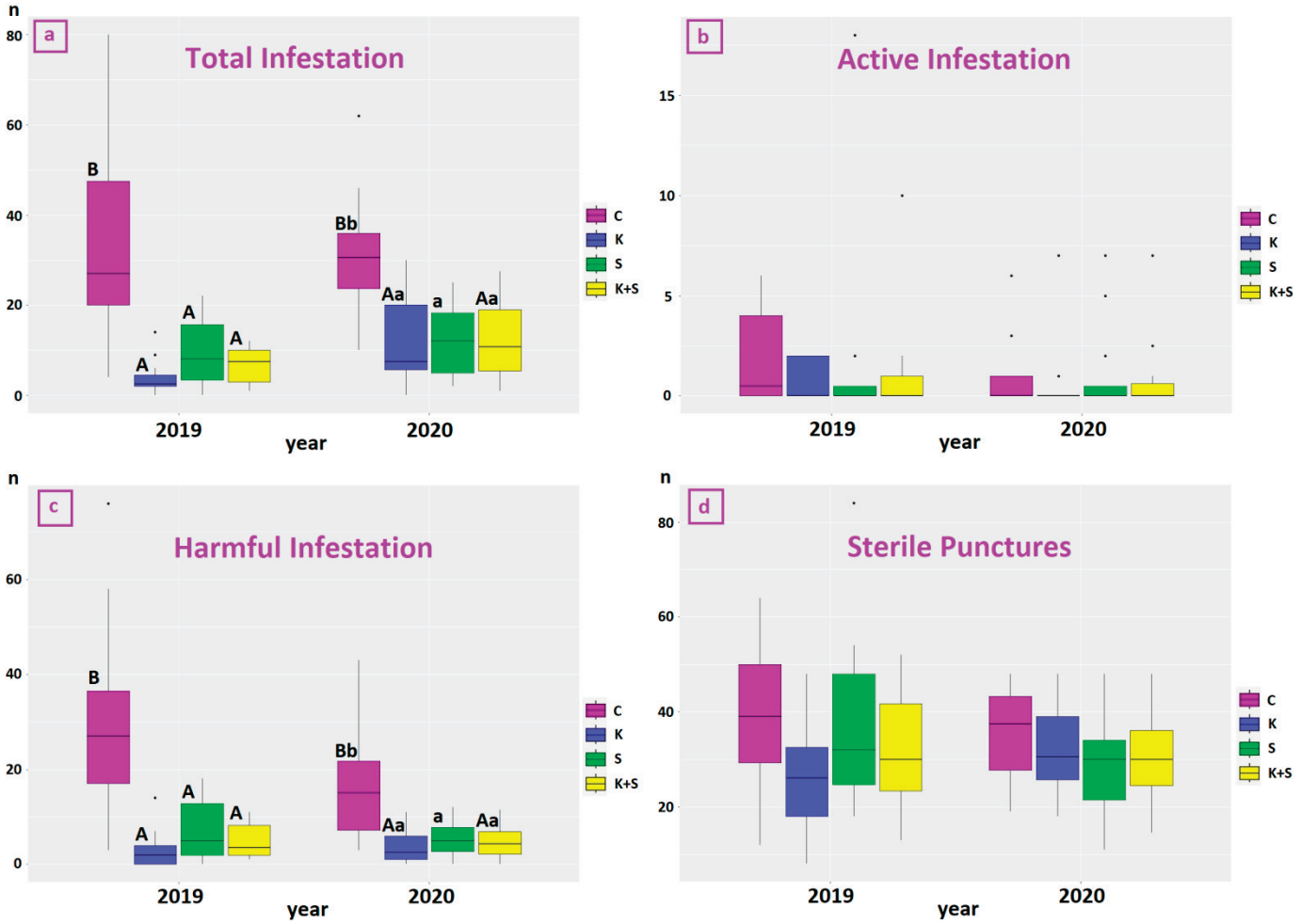


**Figure 4.** Box plots showing the level of (**a**) total, (**b**) active and (**c**) harmful infestation and (**d**) the number of sterile punctures over the two year (2019 and 2020) experiment. Control is shown in purple, kaolin in blue, spinosad in green, and synergic in yellow. Different uppercase letters indicate statistical significance at 99% level; different lowercase letters indicate statistical significance at 95% through post-hoc multiple means comparison by Tukey's honestly significant difference (HSD) test. Years have been analyzed separately.

Nevertheless, in the present study, K, S and K + S have proven to be good alternatives (and also interchangeable), with similarly effective results, especially considering that we are led to believe that the actual effectiveness of the treatments is somehow masked by the limited presence of the olive fly detected in several lapses of time in the biennium of the study. This evidence is particularly important for olive growers in the definition of olive fruit fly management systems aimed at avoiding the generation of selection pressures, at limiting prolonged effects on (even beneficial) non-target organisms and, no less important, at cost reduction through a rationalization in the use and dosage of control methods. In fact, in orchard experiments, the onset of resistance to spinosad in olive fly [39], as well as toxicity for non-target insects [40–42] has been described, whereas the drawbacks related to kaolin employment concern the onset of resistance [38], leaching by rainfall (and so the need for repeated applications during the olive season), and the negative effects on abundance and the diversity of non-target arthropods [23,43,44] and the community of

natural enemies [25]. However, this negative effect could be somewhat mitigated through the sustainable management of the olive grove (such as organic management) that has clear positive effects on some soil quality parameters including microbial and pedofaunal communities abundance and diversity [45–47].

As for the male and female catches, no significant differences between treatments were highlighted ($p$-value > 0.05) in either year, indicating no particular deterrent effect on *B. oleae* flight of either kaolin or spinosad. In the overlapping period of experimentation of the two years (August 8–October 23), the average total catches for single traps did not show major differences, ranging from 31.66 ± 36.12 (C) to 15.333 ± 15.41 (K) to 30.16± 27.12 (S) to 22.75 ± 15.87 (K + S) in 2019, and from 15 ± 4.6 (C) to 18.83 ± 21.03 (K) to 25.33 ± 27.50 (S) to 22.08 ± 7.66 (K + S) in 2020.

### *3.3. Identification and Monitoring of Naturally Occurring Parasitoids of B. oleae*

The morphological examination of captured and/or reared adults allowed for the verification of the presence of five natural antagonists of the olive fly. Specifically, three ectoparasitoids (Figure 5): *Eurytoma martellii* Domenichini (Hymenoptera: Chalcidoidea, Eurytomidae), *Prolasioptera berlesiana* Paoli (Diptera: Cecidomyiidae) and *Eupelmus urozonus* Dalman (Hymenoptera: Chalcidoidea, Eupelmidae), and two endoparasitoids (Figure 6): *Psyttalia concolor* Szépligeti (Hymenoptera: Braconidae), and *Baryscapus sylvestrii* Viggiani& Bernardo (Hymenoptera: Chalcidoidea, Eulophidae), were identified. As regards the latter, it was during this trial that this insect was first observed in Calabria [48].

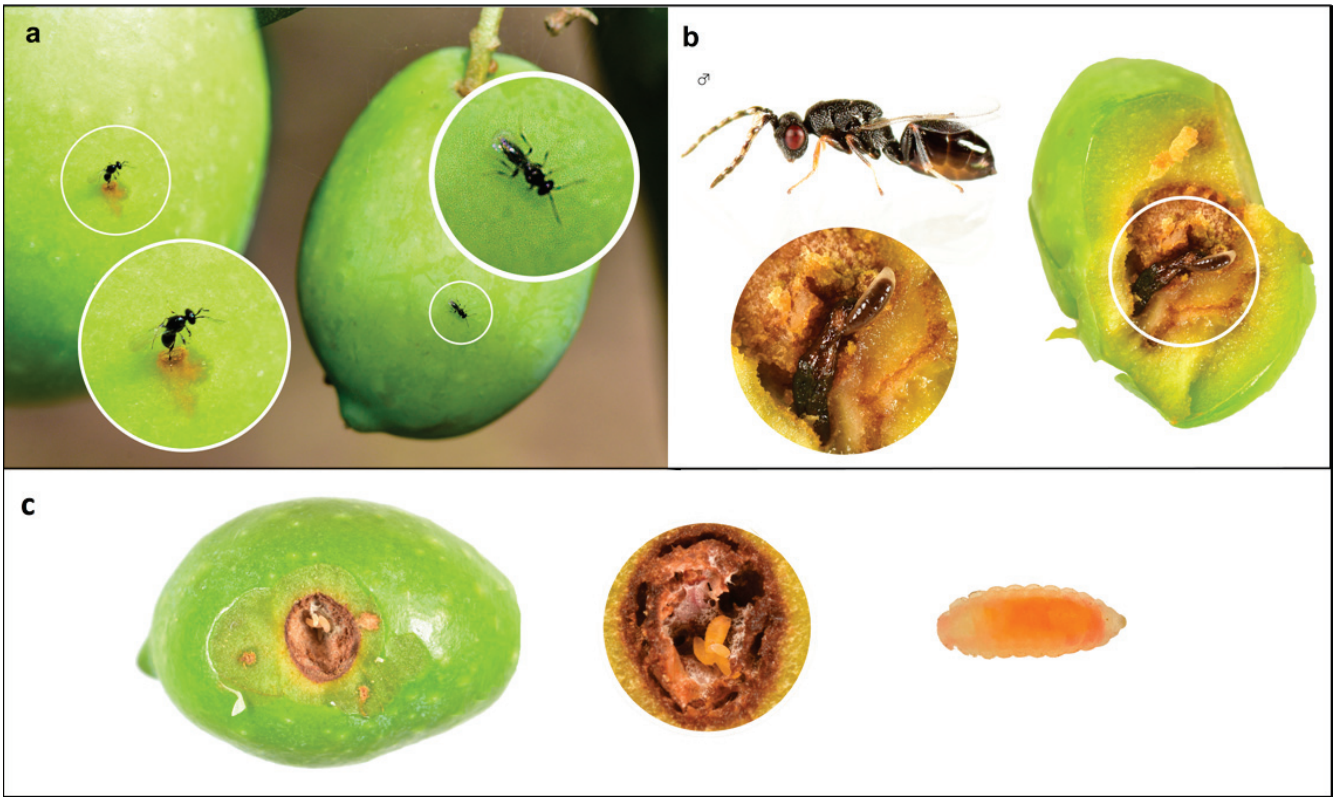


**Figure 5.** Images of the three different naturally occurring ectoparasitoids found in the experimental organic olive grove. (**a**): Female adults of *Eupelmus urozonus* in the act of egg-laying in a oviposition puncture of *B. oleae*. (**b**): Larva of *Eurytoma martellii* feeding on a third instar larva of *B. oleae* and a captured female adult. (**c**): Larvae of *Prolasioptera berlesiana* feeding on the mycelium of the symbiotic fungus *Botryosphaeria dothidea* within the olive fruit mesocarp.

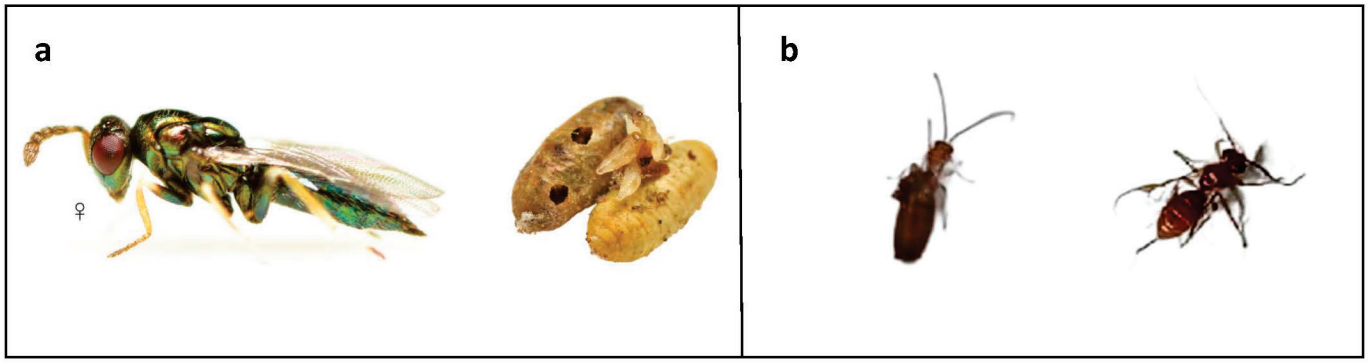


**Figure 6.** Images of the two different naturally occurring endoparasitoids found in *B. oleae* puparia from infested drupes of Carolea cv. (**a**): Pupae of *Baryscapus sylvestrii* emerged from pupae of *B. oleae*. (**b**): Adult of *Psyttalia concolor* emerging/emerged from *B. oleae* puparium.

The number of adult captures and the level of endo- and ectoparasitism in infested drupes turned out to be quite homogeneous in the differently managed parcels (Table 1) without statistically significant differences among the treatments. Nonetheless, as this was largely predictable, indigenous parasitoids did not seem to significantly impact the population size of *B. oleae*. However, the mass introduction of natural antagonists could be part of an integrated control strategy, also allowed under the organic legislation, although it needs to be said that a significant limitation in this regard is represented by a generally inefficient parasitoids' mass rearing [49]. The higher number of insects emerging from puparia collected in the control plot only depends on the higher number of puparia found in the drupes of this parcel. This study suggests a harmless effect of kaolin and spinosad against the indigenous *B. oleae* antagonists. Therefore, kaolin's deterrent effect is likely only limited once the fly has laid its eggs inside the drupes. These results confirm the neutral effect of kaolin on *P. concolor,* as demonstrated in controlled environments [50,51], as well as in the open field [24]. Laboratory tests highlighted the non-harmful nature of kaolin against the *B. oleae* parasitoids *P. concolor* and *Chrysoperla carnea,* albeit with some effect on their fecundity, and other beneficial insects of the olive grove agroecosystem [52], as well as against the natural enemies' communities [53].

Regarding spinosad, some evidence of moderately harmful or harmful effects on parasitoids have been reported [54–56]; however, for *B. oleae* antagonists, these have been described in laboratory experiments but not in field ones [57]. Notwithstanding this, it cannot be excluded that the lower values on the number of parasitoids found in the spinosad-treated plot could be attributed to its residual toxicity, which lasted from three to ten days after treatment [50].

Lastly, a separate discussion is required for *Prolasioptera berlesiana* female that, with oviposition, also inoculates its symbiotic fungus *Botryosphaeria dothidea* (anamorph of *Fusicoccum aesculi*) inside the drupe via *B. oleae* puncture, causing necrotic spots and depressions on the fruit [58]. This primarily visual damage known as fruit rot can cause significant production losses, especially for table olives, but can simultaneously favor the onset of other diseases [59]. Although *P. berlesiana* is not the exclusive source of inoculum of the fungus [60] (and even the fungi *Neofusicoccum australe* and *N. vitifusiforme* have been associated to olive fruit rot symptoms [61]), a more in-depth analysis of the actual economic and ecological benefits must be carried out before the employment of this dipterous in the biological control of *B. oleae.* In this study, symptoms of fruit rot were evident in 3.77, 2.33, 2.41 and 2.28% of the drupes collected in C, in K, S and K + S, respectively, implying significant losses for a double aptitude (for oil and table olives) cultivar such as Carolea.

**Table 1.** Number of total (captured + reared) individuals collected and percentage of endo- and ectoparasitism for the five naturally occurring *B. oleae* parasitoids found in the olive orchard.

| Parasitoid | Treatment | Total Individuals | | % of Endoparasitism | | % of Ectoparasitism | |
|---|---|---|---|---|---|---|---|
| | | 2019 | 2020 | 2019 | 2020 | 2019 | 2020 |
| *Eurytoma martellii* | C | 5 | 7 | 2.02 | 2.23 | | |
| | K | 1 | 4 | 2.08 | 3.17 | | |
| | S | 1 | 3 | 1.67 | 2.46 | | |
| | K + S | 2 | 4 | 1.85 | 2.31 | | |
| *Prolasioptera berlesiana* | C | 9 | 11 | 3.03 | 3.36 | | |
| | K | 4 | 5 | 4.17 | 3.17 | | |
| | S | 3 | 6 | 3.33 | 3.28 | | |
| | K + S | 3 | 6 | 3.70 | 3.84 | | |
| *Eupelmus urozonus* | C | 4 | 6 | 1.51 | 1.86 | | |
| | K | 1 | 3 | 2.08 | 2.38 | | |
| | S | 1 | 4 | 1.67 | 2.46 | | |
| | K + S | 1 | 3 | 1.85 | 1.54 | | |
| *Psyttalia concolor* | C | 12 | 6 | | | 5.78 | 3.48 |
| | K | 3 | 1 | | | 6.89 | 3.84 |
| | S | 1 | 1 | | | 2.78 | 0 |
| | K + S | 2 | 1 | | | 2.63 | 3.12 |
| *Baryscapus sylvestrii* | C | 8 | 6 | | | 4.04 | 4.65 |
| | K | 1 | 3 | | | 3.44 | 3.84 |
| | S | 2 | 2 | | | 2.77 | 2.5 |
| | K + S | 2 | 1 | | | 5.26 | 3.12 |

## 4. Conclusions

*Bactrocera oleae* is responsible for remarkable quantitative (and qualitative) yearly losses in the olive orchards worldwide. Thus, the adoption of low environmental impact containment strategies is a key point in the organic sector, made even more difficult by the limited choice of products permitted by the current legislation. In this sense, in this two year trial, kaolin and spinosad have been found to be effective in limiting total and harmful fly infestation, even in combination, thus reducing application costs and preventing phenomena such as the onset of resistance and prolonged harmful exposures for non-target organisms. Moreover, these treatments did not appear to have adverse effects on the indigenous natural antagonists of *B. oleae*, thus paving the way for the investigation of case-specific integrated pest management strategies. This information is even more interesting considering the need for alternatives to replace the withdrawn traditionally used pesticides in conventional oliviculture. Further experimental tests are needed, as the fight against *Bactrocera oleae* is a continuing one.

**Author Contributions:** Conceptualization, V.V.; Methodology, V.V., L.L., P.R. and A.I.; Software, A.I. and L.L.; validation, V.V., L.L. and A.I.; Formal Analysis, V.V., L.L., C.N., P.R., M.P., G.C., G.G., F.Z. and A.I.; Investigation, V.V., L.L., C.N., P.R., M.P., G.C., G.G., F.Z. and A.I.; Resources, V.V.; Data Curation, V.V., L.L. and A.I., Writing—Original Draft Preparation, V.V., L.L. and A.I.; Writing—Review and Editing, V.V., L.L. and A.I.; Visualization, V.V., L.L., P.R., G.G. and A.I.; Supervision, V.V.; Funding acquisition, V.V. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the Italian Ministry of Agricultural, Food and Forestry Policies in the context of the "Piano Olivicolo Oleario" (Interministerial Decree DI.OL n. 23774-06/09/2017).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data sharing not applicable.

**Conflicts of Interest:** The authors declare that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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# The Modification of Cell Wall Properties Is Involved in the Growth Inhibition of Rice Coleoptiles Induced by Lead Stress

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**Abstract:** Lead (Pb) is a widespread heavy metal pollutant that interferes with plant growth. In this study, we investigated the effects of Pb on the mechanical and chemical properties of cell walls and on the growth of coleoptiles of rice (*Oryza sativa* L.) seedlings grown in the air (on moistened filter paper) and underwater (submerged condition). Coleoptile growth of air-grown seedlings was reduced by 40% by the 3 mM Pb treatment, while that of water-grown ones was reduced by 50% by the 0.5 mM Pb. Although the effective concentration of Pb for growth inhibition of air-grown coleoptiles was much higher than that of water-grown ones, Pb treatment significantly decreased the mechanical extensibility of the cell wall in air- and water-grown coleoptiles, when it inhibited their growth. Among the chemical components of coleoptile cell walls, the amounts of cell wall polysaccharides per unit fresh weight and unit length of coleoptile, which represent the thickness of the cell wall, were significantly increased in response to the Pb treatment (3 mM and 0.5 mM Pb for air- and water-grown seedlings, respectively), while the levels of cell wall-bound diferulic acids (DFAs) and ferulic acids (FAs) slightly decreased. These results indicate that Pb treatment increased the thickness of the cell wall but not the phenolic acid-mediated cross-linking structures within the cell wall in air- and water-grown coleoptiles. The Pb-induced cell wall thickening probably causes the mechanical stiffening of the cell wall and thus decreases cell wall extensibility. Such modifications of cell wall properties may be associated with the inhibition of coleoptile growth. The results of this study provide a new finding that Pb-induced cell wall remodeling contributes to the regulation of plant growth under Pb stress conditions via the modification of the mechanical property of the cell wall.

**Keywords:** cell wall extensibility; cell wall polysaccharide; coleoptile; growth inhibition; lead (Pb); rice

**Citation:** Wakabayashi, K.; Soga, K.; Hoson, T.; Masuda, H. The Modification of Cell Wall Properties Is Involved in the Growth Inhibition of Rice Coleoptiles Induced by Lead Stress. *Life* **2023**, *13*, 471. https://doi.org/10.3390/life13020471

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 27 December 2022
Revised: 6 February 2023
Accepted: 6 February 2023
Published: 8 February 2023



## 1. Introduction

Lead (Pb) is one of the most abundant heavy metal contaminants in both terrestrial and aquatic environments. Pb is not biodegradable and accumulates in organisms. Pb has no biological function, but it is highly toxic to living organisms even at low concentrations and causes disease, such as neurotoxicity and kidney damage in animals [1]. In plants, a prominent Pb toxicity is the inhibition of organ growth [2–5]. The toxic effects of Pb on cellular functions have been extensively studied; for example, incorporated Pb stimulates the production of reactive oxygen species (ROS), elevates the levels of lipid hydroperoxide, and increases the activities of antioxidant enzymes, while it decreases the chlorophyll contents and thus declines the photosynthetic activity [2–10]. Alterations in metabolic and biochemical processes may cause physiological changes in plant development under Pb stress conditions. In addition to metabolic and biochemical alterations, it has been shown that Pb disturbed the microtubule organization in meristem cells and interfered with cell

division in roots [11,12]. Therefore, the suppression of cell division in root meristem may be associated with the inhibition of root growth under the Pb stress condition. In addition to the increment of cell number, the increase in cell volume is an important factor determining the growth rate of plant organs. Lane et al. [13] showed that Pb interfered with auxin-induced cell elongation in segments of wheat (*Triticum aestivum*) coleoptiles. They also showed that Pb treatment decreased the deformation ability of turgid coleoptile segments under constant inflection load. These results imply that Pb affected the mechanical properties of the cell walls when it inhibited auxin-induced growth of the segments. However, it has not been clarified whether Pb directly affects the cell wall mechanical properties in growing stem organs.

Plant cell walls surround each protoplast and provide protoplasts with mechanical rigidity. Furthermore, cell walls play an important role in the regulation of growth and morphogenesis in plants [14–16]. Cell wall extensibility, a parameter of cell wall mechanical property, represents the capacity of the cell wall to extend and thus the parameter is related to the elongation capacity of plant cells [15–17]. Cell walls of growing plant tissues are mainly composed of polysaccharides, such as cellulose and a variety of matrix polysaccharides. The quantities and chemical structures of cell wall polysaccharides are considered to be factors determining cell wall extensibility [14,15,17,18]. In addition to polysaccharides, the cell wall of gramineous (cereal) plants, such as rice (*Oryza sativa*), wheat, and maize (*Zea mays*), contain a significant amount of phenolic acid monomers, such as ferulic acid (FA), which are ester-bound to matrix polysaccharides [19,20]. Some FA residues undergo a coupling reaction to produce diferulic acid (DFA), which forms cross-links between matrix polysaccharides [21,22]. The formation of the cross-linkages by phenolic acids makes the cell wall mechanically rigid [20].

Plant cell walls play an efficient barrier to the entry of heavy metals into the protoplast [23]. Heavy metals, including Pb, increased the thickness of root cell walls in several plant species, such as *Vicia faba*, *Oryza sativa*, and *Allium cepa*, and protonemata cells of *Funaria hygrometrica* [24–27]. The thickening of the cell wall is associated with a decrease in cell wall extensibility [15,17,28]. An increase in cell wall thickness in seedling stems is accompanied by an increase in cell wall constituents, especially polysaccharides [17,28]. It is expected that Pb increases levels of cell wall constituents, such as polysaccharides and cell wall-bound DFA and FA in coleoptiles, which may promote the cell wall thickening and the formation of cross-linkages within the cell wall and thereby decrease cell wall extensibility. In the present study, we investigated the above possibility using air- and water-grown rice coleoptiles. The present results revealed a key role of the plant cell wall in the regulation of organ growth under heavy metal stress conditions.

## 2. Materials and Methods

### *2.1. Plant Materials and Growth Conditions*

Caryopses of rice (*Oryza sativa* L. cv. Koshihikari) were sterilized in ca. 1% (*v*/*v*) sodium hypochlorite solution for 1 h and then soaked in deionized water for two days at 25 °C in the dark. Germinated caryopses were grown for four days in the dark at 25 °C under two different cultural conditions: on moistened filter paper (air-grown) and underwater (water-grown). For the cultivation in air, germinated caryopses were placed on one layer of filter paper in a cylindrical polycarbonate box (15 cm in diameter and 8 cm in height) which contained 30 mL of 2 mM MES-KOH buffer (pH 6.0) containing different concentrations (0, 0.1, 0.3, 1.0, and 3.0 mM) of $PbCl_2$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cultivation underwater was as follows: germinated caryopses were submerged in a test solution (ca. 7 cm in depth) in a polycarbonate cylinder (4 cm in diameter and 11 cm in height). Each cylinder contained 80 mL of 2 mM MES-KOH buffer (pH 6.0) containing different concentrations (0, 0.25, 0.5, and 1.0 mM) of $PbCl_2$. For the transplant experiment, water-grown seedlings that had been grown for 2 days in the dark at 25 °C in 2 mM MES-KOH buffer (pH 6.0) were immediately transferred to the same buffer containing 0 and 0.5 mM $PbCl_2$ and grown for a further 2 days in the

same conditions. On the days after planting, the lengths of coleoptiles and roots were measured with a commercially obtainable ruler. After the measurement, coleoptiles were excised. Since the coleoptile excised from air-grown seedlings contained a primary leaf inside, a vertical slit was made at the basal portion of the coleoptile and then the primary leaf was removed using forceps. After the removal of the primary leaf, the fresh weight of the coleoptile was measured using an electric balance. In contrast, the primary leaf inside the coleoptile scarcely grew under submerged conditions. So, the coleoptile excised from water-grown seedlings was used readily for the measurement of fresh weight. All manipulations were performed under dim green light (ca. 0.09 μmol $m^{-2}$ $s^{-1}$ at handling level). The growth experiment was repeated at least three times. The amounts of cell wall constituents, the cellular osmotic concentration, and the Pb content were determined using three samples obtained from three independent experiments. The measurement of the cell wall mechanical properties was repeated twice using samples obtained from two independent experiments.

### *2.2. Assay of Pb Content*

Shoots consisted of coleoptile and the inner primary leaf and roots were used for the assay of Pb content. Seedlings were grown in the air for 4 days in the presence (1 mM and 3 mM) or absence of $PbCl_2$, as described in the above section. After the cultivation, seedlings were washed several times with deionized water, and then shoots and roots were excised. Their fresh weights were measured using an electric balance. Shoots and roots excised from the control and 1 mM Pb-treated seedlings were put in Teflon vessels and immediately oven-dried at 60 °C for 2 days. The dried samples were digested completely with $HNO_3/HClO_4$ (2:1, *v*/*v*) solution at 140 °C for 24 h. After the acidic solution was evaporated completely, the digested samples were dissolved in 0.1 N $HNO_3$ and analyzed for Pb content using inductive coupled plasma–mass spectrometry (ICP-MS) (SPQ 9700; Hitachi High-Tech Science Corp., Tokyo, Japan). For the measurement of Pb content in cytoplasmic fluid, shoots excised from 1 mM and 3 mM Pb-treated seedlings and roots from 1 mM Pb-treated ones were boiled for 10 min in 10 mL of 80% ethanol. The ethanol extract was dried in Teflon vessels. Dried samples were digested with $HNO_3/HclO_4$ and then dissolved in 0.1 N $HNO_3$, as described above. The Pb content in the cytoplasmic fluid of 3 mM Pb-treated roots could not be analyzed because roots of air-grown seedlings hardly grew at this concentration.

### *2.3. Measurement of the Osmotic Concentration of Cell Sap*

The extraction and collection of cell sap were carried out according to the method of Ooume et al. [29]. The coleoptiles obtained from air-grown and water-grown seedlings were put in a plastic mini-column and then immediately frozen with liquid nitrogen. The cell sap was collected from frozen–thawed coleoptiles by centrifugation at 1500× *g* for 10 min at 4 °C. The osmotic concentration of the collected cell sap was measured with a vapor pressure osmometer (Model 5500C; Wescor, Logan, UT, USA).

### *2.4. Measurement of the Mechanical Properties of the Cell Wall*

The coleoptiles prepared from air-grown and water-grown seedlings were immediately boiled for 10 min in 80% ethanol and then stored in fresh 80% ethanol. Before the measurement of cell wall mechanical properties, ethanol-fixed samples were rehydrated for several hours. Cell wall extensibility was measured with a tensile tester (RTM-25; Toyo Baldwin Co., Tokyo, Japan) [30]. The subapical region (1–2 mm below the tip) of air-grown coleoptile was fixed between two clamps 2 mm apart, and stretched by lowering the bottom clamp at a speed of 20 mm/min to produce a stress of 10 g. In the case of water-grown plants, a segment 10 mm in length was excised from the tip of the coleoptile. The segment was fixed between two clamps 2 mm apart and stretched at the same speed to produce a stress of 4 g. Cell wall extensibility (μm/g) was determined by measuring the rate of the

increase in stress just before it reached the maximum stress (4 g and 10 g for water-grown and air-grown coleoptiles, respectively).

### *2.5. Fractionation of Cell Wall Constituents*

Cell wall materials were prepared and fractionated according to the method of Wakabayashi et al. [31]. Briefly, cell wall preparation was treated with 1 M NaOH to extract ester-linked phenolic acids. Then, the residual material was extracted with 17.5% NaOH containing 0.02% $NaBH_4$. The fraction extracted with 17.5% NaOH was neutralized with acetic acid. After the extraction of cell wall-bound phenolic acids from the 1 M NaOH solution as described below, the remaining solution was combined with the 17.5% NaOH extracts, and designated as the matrix polysaccharide fraction. The alkali-insoluble fraction was designated as the cellulose fraction. The cellulose fraction was dissolved with 72% sulfuric acid. The total sugar content in each fraction was determined by the phenol-sulfuric acid method [32] and expressed as glucose equivalents.

### *2.6. Determination of Cell Wall-Bound Phenolic Acids*

Analysis of cell wall-bound phenolic acids was carried out according to the method of Wakabayashi et al. [31]. Ester-linked phenolic acids liberated from the cell wall with 1 M NaOH (see above) were recovered into ethyl acetate by acidification. The liberated phenolic acids were analyzed using an HPLC system equipped with a reversed-phase column and a photodiode array detector with a gradient elution of methanol. FA and *p*-coumaric acid (*p*-CA) were identified and quantified using authentic *trans*-FA and *trans*-*p*-CA (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The peaks of DFA isomers were identified and quantified using response factors [33].

### *2.7. Statistical Analysis*

For each measurement, the means and the standard errors of the means (SE) were calculated. The significance of differences among the treatments with different Pb concentrations was analyzed using Tukey's HSD test ($p < 0.05$). The significance of differences between the control and single Pb treatment was analyzed using Student's *t*-test ($p < 0.05$).

## 3. Results

### *3.1. Effects of Pb on Air-Grown Seedlings*

When germinated rice caryopses were grown for 4 days on moistened filter paper, the lengths of primary roots and coleoptiles reached about 33 mm and 10 mm, respectively, in the absence of Pb (Figure 1). Root growth was significantly inhibited by the 0.3 mM Pb treatment and the inhibitory effect significantly increased in a concentration-dependent manner. Roots scarcely elongated at 3 mM Pb. In contrast, coleoptile growth was not inhibited by Pb up to 1 mM, but was significantly inhibited at 3 mM (Figure 1). Growth of the first leaf inside the coleoptile synchronized with that of the coleoptile until the start of leaf emergence. Pb also inhibited the growth of first leaves at 3 mM, but lower concentrations did not affect leaf growth; the lengths of first leaves on 4 days were $8.4 \pm 0.3$, $8.4 \pm 0.2$, $8.3 \pm 0.3$, $8.1 \pm 0.2$, and $4.8 \pm 0.3$ mm (each $n = 18$–$20$) for 0, 0.1, 0.3, 1, and 3 mM Pb, respectively. These results suggest that the inhibitory effect of Pb on the growth of aboveground organs in air-grown rice seedlings was much smaller than that of roots.

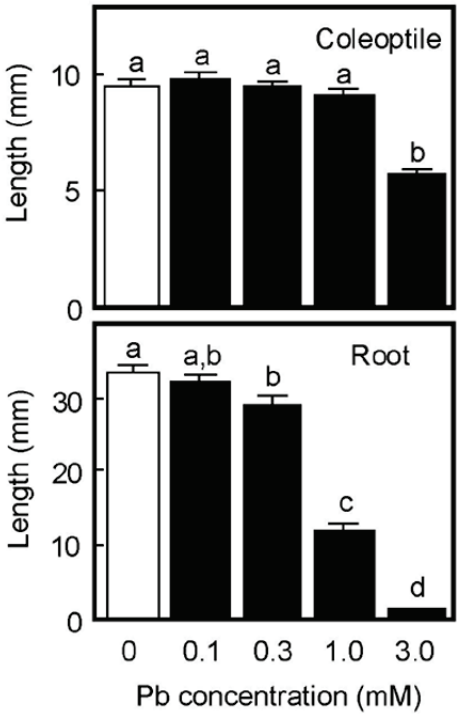


**Figure 1.** Effects of Pb on the growth of coleoptiles and roots of air-grown rice seedlings. Germinated caryopses were planted on filter paper containing a 2 mM MES-KOH buffer (pH 6.0) with or without different concentrations of Pb and then grown for 4 days in the dark. Data are means ± SE ($n$ = 18–20). Different letters above the bars represent statistically significant differences (Tukey's HSD test, $p < 0.05$).

Next, the accumulation of Pb in shoots consisting of coleoptile and first leaf and in roots of air-grown seedlings was analyzed by ICP-MS. When germinated caryopses were grown for 4 days on moistened filter paper containing a buffer solution with or without 1 mM Pb, root growth was substantially inhibited by 1 mM Pb, but shoot growth was not, as shown in Figure 1. The Pb contents in the 1 mM Pb-treated shoot and root were 119 ± 25 and 1025 ± 86 ng/organ, respectively ($n$ = 3), while the contents in the control seedlings were negligible (Pb contents in the control shoot and root were 0.1 and 0.4 ng/organ, respectively). We further examined the Pb accumulation in the cytoplasmic fluid of shoots and roots that had been grown for 4 days in the presence of 1 mM and 3 mM Pb. The Pb contents in the cytoplasmic fluid of the 1 mM and 3 mM Pb-treated shoots were 0.33 ± 0.07 and 3.10 ± 0.29 μg/g fresh weight (FW), respectively, while that of the 1 mM Pb-treated roots was 4.29 ± 0.18 μg/g FW ($n$ = 3, respectively). The Pb content in 3 mM Pb-treated roots could not be analyzed because roots hardly grew at this concentration (Figure 1). The calculated concentrations of Pb in the cytoplasmic fluid were 1.6, 15, and 21 μM for the 1 mM Pb-treated, the 3 mM Pb-treated shoots, and the 1 mM Pb-treated roots, respectively.

Cell wall extensibility and the osmotic concentration of air-grown coleoptiles are shown in Figure 2. Treatment with Pb at a concentration of 1 mM did not affect either cell wall extensibility or the cellular osmotic concentration (Figure 2A,B), similar to the effect on coleoptile growth. The Pb treatment at 3 mM significantly decreased cell wall extensibility and increased the cellular osmotic concentration, when it inhibited coleoptile growth (Figure 1).

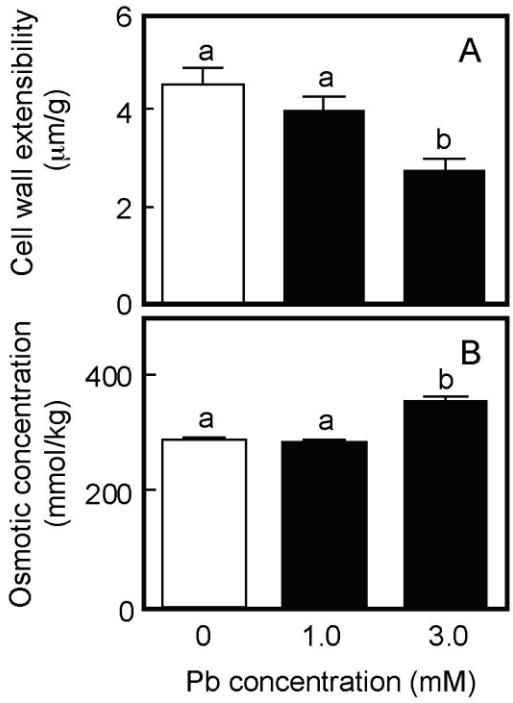


**Figure 2.** Effects of Pb on cell wall extensibility (**A**) and the cellular osmotic concentration (**B**) in coleoptiles of air-grown rice seedlings. Growth conditions are shown in Figure 1. (**A**) The cell wall extensibility of the upper region of coleoptiles was measured with a tensile tester. Data are means ± SE ($n$ = 16–18). (**B**) The osmotic concentration of the cell sap obtained from coleoptiles was measured with a vapor pressure osmometer. Data are means ± SE ($n$ = 3). Different letters above the bars represent statistically significant differences (Tukey's HSD test, $p < 0.05$).

The chemical properties of cell walls are considered to be factors determining the mechanical properties of the cell wall. We next analyzed the amounts of cell wall polysaccharides and cell wall-bound phenolic acids in air-grown coleoptiles. Cell wall polysaccharides were fractionated into two fractions, the matrix polysaccharides and cellulose. The amount of matrix polysaccharides was almost equivalent to that of cellulose in coleoptiles grown for 4 days in the air (Figure 3A). On the basis of unit length and unit fresh weight of coleoptile, Pb treatment at 3 mM significantly increased the amounts of both matrix polysaccharides and cellulose (Figure 3A).

Cell walls of gramineous plants contain phenolic acid monomers, such as FA and *p*-coumaric acid (*p*-CA). Our previous study showed that the cell walls of dark-grown rice shoots contained three predominant DFA isomers: 5-5, 8-*O*-4, and 8-5 DFA [34]. On the basis of unit matrix polysaccharide content, Pb treatment at 3 mM significantly decreased the amounts of both phenolic acid monomers, although amounts of *p*-CA were substantially lower than those of FA (Figure 3B). Furthermore, among DFA isomers, the amounts of 8-*O*-4 and 8-5 DFAs in Pb–treated coleoptiles were significantly lower than those in control ones (Figure 3B).

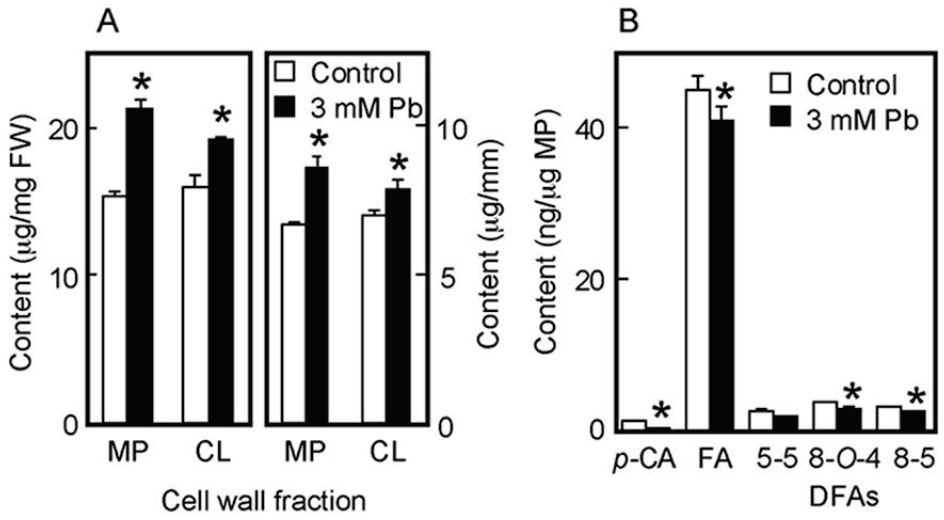


**Figure 3.** Effects of Pb on the amounts of cell wall polysaccharides (**A**) and cell wall-bound phenolic acids (**B**) in air-grown rice coleoptiles. Coleoptiles were grown for 4 days in the presence or absence of 3 mM Pb. (**A**) The sugar content in each cell wall fraction was determined by the phenol-sulfuric acid method. Amounts of cell wall polysaccharides were expressed on the basis of unit length and unit fresh weight (FW) of the coleoptile. MP, matrix polysaccharides; CL, cellulose. (**B**) Phenolic acids were analyzed by the HPLC and their amounts were expressed on the basis of unit matrix polysaccharide (MP) content. *p*-CA, *p*-coumaric acid; FA, ferulic acid; DFAs, diferulic acids. Data are means ± SE ($n$ = 3). * Mean values were significantly different between the control and Pb treatment (Student's $t$-test, $p < 0.05$).

### *3.2. Effects of Pb on Water-Grown Seedlings*

Rice is a semiaquatic plant and its coleoptile grows faster underwater than in air [35,36]. In contrast, the root formation and the growth of the primary leaf inside the coleoptile are strongly suppressed when caryopses are germinated underwater [35]. As shown in Figure 4A, the root formation was strongly suppressed under submerged conditions, even in the absence of Pb. When germinated caryopses were grown for 4 days underwater, the length of the control coleoptiles reached approximately 35 mm (Figure 4B). In contrast to the air-grown seedlings, the growth of water-grown coleoptiles was significantly inhibited by the treatment with 0.25 mM Pb and the inhibitory effect increased with increasing Pb concentration. Coleoptile growth was reduced by about 50% by the 0.5 mM Pb treatment (Figure 4B).

The effects of Pb treatment at 0.5 mM on cell wall extensibility and the cellular osmotic concentration of water-grown coleoptiles are shown in Figure 4C,D. The Pb treatment significantly decreased cell wall extensibility, but did not affect the cellular osmotic concentration. Furthermore, when seedlings grown under submerged conditions for 2 days without Pb were transferred to the Pb-containing medium, Pb at 0.5 mM significantly inhibited the coleoptile growth afterward and it also significantly lowered cell wall extensibility (Table 1).

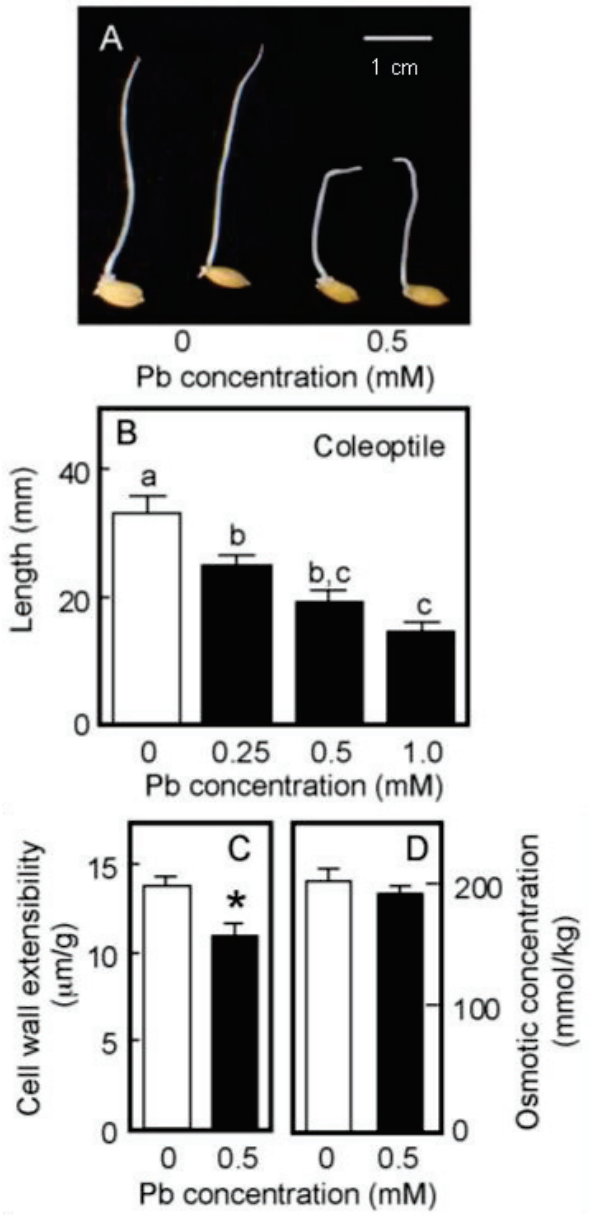


**Figure 4.** Effects of Pb on the growth of coleoptiles (**A**,**B**), the cell wall extensibility (**C**), and the cellular osmotic concentration (**D**) in coleoptiles of water-grown rice seedlings. Germinated caryopses were submerged in a 2 mM MES-KOH buffer (pH 6.0) with or without different concentrations of Pb and then grown for 4 days in the dark. (**A**) Photograph showing seedlings grown for 4 days under submerged conditions. Scale bar = 1 cm. (**B**) The length of water-grown coleoptiles. Data are means ± SE ($n$ = 18). Different letters above the bars represent statistically significant differences (Tukey's HSD test, $p < 0.05$). (**C**) The cell wall extensibility of the upper region of coleoptiles grown for 4 days with or without 0.5 mM Pb was measured. Data are means ± SE ($n$ = 16–18). * Mean values were significantly different between the control and Pb treatment (Student's $t$-test, $p < 0.05$). (**D**) The osmotic concentration of the cell sap obtained from coleoptiles that had been grown for 4 days with or without 0.5 mM Pb was measured. Data are means ± SE ($n$ = 3).

**Table 1.** Effects of Pb on the growth and cell wall extensibility of water-grown rice coleoptiles. Germinated caryopses were grown for 2 days in a 2 mM MES-KOH buffer (pH 6.0) (Initial), and then the seedlings were immediately transferred to the same buffer with or without 0.5 mM Pb. The transferred seedlings were grown for a further 2 days (the control and Pb treatment). The cell wall extensibility of the upper region of the coleoptile was measured. Data are means ± SE ($n$ = 15–18). * Mean values were significantly different between the control and Pb treatment (Student's $t$-test, $p < 0.05$).

| Treatment | Coleoptile Length (mm) | Cell Wall Extensibility (μm/g) |
|---|---|---|
| Initial | 14.8 ± 0.9 | 11.2 ± 0.4 |
| Control | 38.6 ± 2.2 | 14.6 ± 0.8 |
| Pb treatment | 26.8 ± 2.6 * | 10.3 ± 1.1 * |

The amount of cell wall polysaccharides and cell wall-bound phenolic acids in water-grown coleoptiles (Figure 5A,B) were smaller than those in air-grown ones (Figure 3A,B). On the basis of unit length and unit fresh weight of the coleoptile, Pb treatment at 0.5 mM significantly increased the amounts of matrix polysaccharides and cellulose (Figure 5A). The amounts per unit matrix polysaccharide content of phenolic acid monomers and dimers were lower in Pb-treated coleoptiles than in control ones, particularly the amounts of *p*-CA and 8-*O*-4 DFA in Pb-treated coleoptiles, which were significantly lower than those in control ones (Figure 5B). The effects of Pb on the amounts of cell wall constituents were similar in air- and water-grown coleoptiles.

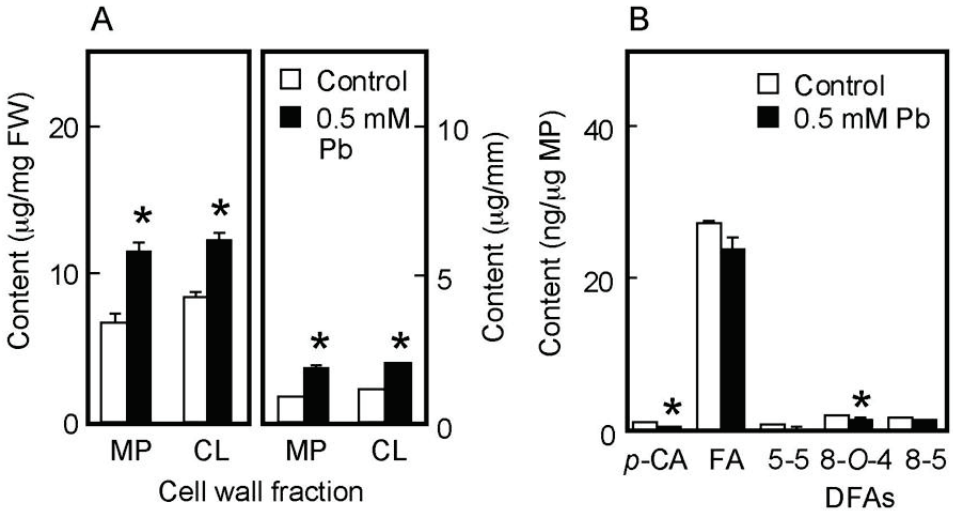


**Figure 5.** Effects of Pb on the amounts of cell wall polysaccharides (**A**) and cell wall-bound phenolic acids (**B**) in water-grown rice coleoptiles. Coleoptiles were grown under submerged conditions for 4 days in the presence or absence of 0.5 mM Pb. (**A**) The sugar content in each cell wall fraction was determined by the phenol-sulfuric acid method. Amounts of cell wall polysaccharides were expressed on the basis of unit length and unit fresh weight (FW) of the coleoptile. MP, matrix polysaccharides; CL, cellulose. (**B**) Phenolic acids were analyzed by the HPLC and their amounts were expressed on the basis of unit matrix polysaccharide (MP) content. *p*-CA, *p*-coumaric acid; FA, ferulic acid; DFAs, diferulic acids. Data are means ± SE ($n$ = 3). * Mean values were significantly different between the control and Pb treatment (Student's $t$-test, $p < 0.05$).

## 4. Discussion

Plant cell expansion is caused by the influx of water into the cell and the osmotic concentration of the cell sap provides the driving force for water uptake. In this context, an increase in osmotic concentration of the cell sap is expected to promote the growth rate, while a decrease slows it down. The cellular osmotic concentration, along with cell wall extensibility, is thought to be involved in the regulation of plant growth. In the present

results, Pb had no negative effect on the osmotic concentration in air- and water-grown coleoptiles (Figures 2B and 4D), suggesting that the cellular osmotic concentration is not related to the growth inhibition induced by Pb. In contrast, the Pb treatment decreased cell wall extensibility of both air- and water-grown coleoptiles, when it inhibited their growth (Figures 1, 2A and 4B,C, Table 1). It is suggested by these results that the decrease in the ability of the cell wall to extend is associated with the inhibition of coleoptile growth in response to the Pb exposure. The relationship between the growth inhibition of stem organs and the decrease in cell wall extensibility has been extensively examined in studies of environmental stimuli and plant hormones on stem growth [17,30,37–39].

The quantitative changes in cell wall constituents underlie the modification of mechanical properties of the cell wall [15,17]. The amounts of cell wall polysaccharides per unit fresh weight and per unit length of the stem show the proportion and the cross-sectional mass of the cell wall in the stem organ, respectively, and thus those values are thought to represent the thickness of the cell wall [28]. On these bases, the amounts of matrix polysaccharides and cellulose in Pb-treated coleoptiles were higher than those in control ones in both cultivation conditions (Figures 3A and 5A), indicating that the cell wall thickness of Pb-treated coleoptiles was greater than that of control ones. The increase in the thickness of the cell walls results in a decrease in the cell wall extensibility of stem organs [28,30,37,40]. Therefore, Pb-induced cell wall thickening may be primarily involved in the decrease in cell wall extensibility in rice coleoptiles.

In addition to cell wall polysaccharides, the increases in the amounts of DFA and FA were associated with a decrease in the ability of the cell wall to extend in gramineous shoots [20,34,41,42]. The present results, however, showed that the levels of cell wall-bound DFAs, FA, and *p*-CA in Pb-treated coleoptiles were lower than those in control ones (Figures 3B and 5B), suggesting that Pb decreased the concentration of DFA-mediated cross-linkages within cell wall architecture. These results suggest that cell wall-bound phenolic acids were not involved in the Pb-mediated decrease in cell wall extensibility. As for the effect of Pb on cell wall-bound phenolic acids, the Pb treatment only slightly affected the ratio of the total amount of three DFA isomers to the amount of FA, which was 0.20 and 0.19 for the control and Pb-treated coleoptiles grown in air and 0.15 and 0.14 for the control and Pb-treated ones grown underwater, respectively (calculated using data in Figures 3B and 5B). These results suggest that Pb scarcely affects the coupling step of FA to produce DFA. Therefore, the decreases in DFA levels in Pb-treated coleoptiles may be attributed to the reduced FA level. The Pb treatment decreased the amounts of both FA and *p*-CA that are synthesized via the phenylpropanoid pathway [43]. Therefore, Pb may affect the reactions in the pathway and/or the feruoylation and coumaroylation of matrix polysaccharides, such as arabinoxylans [20,44,45].

Plant cell walls are able to bind metal cations and a large number of heavy metals incorporated into plants were localized in the cell walls [2,46,47]. Therefore, plant cell walls function not only as a barrier limiting the penetration of heavy metals but also as a sink for the accumulation of heavy metals [23]. Plant cell walls serve to sequester heavy metals from the cytoplasm, as do phytochelatins and metallothioneins, proteins that bind heavy metals. Because the cell wall can accumulate and immobilize a significant amount of heavy metals, Pb-induced cell wall thickening is thought to enhance the defense mechanism against the Pb stress [23]. The present study showed that Pb induced the cell wall thickening in coleoptiles and that the thickening caused a decrease in cell wall extensibility. Therefore, Pb-induced cell wall thickening may contribute not only to the defense strategy against Pb stress but also to the growth regulation of the aboveground organ by modifying the cell wall's mechanical properties.

At present, the mechanism by which Pb promotes cell wall thickening in coleoptiles is not clarified. Pb treatment stimulated the production of ROS in plant cells [7,8,48]. Although ROS have toxic effects on cellular functions, they act as signaling molecules in stress-induced cellular responses in plants. Among ROS, hydrogen peroxide is thought to be involved in the structural modification processes of the cell wall in response to abiotic

stresses [49]. The application of hydrogen peroxide affected plant growth responses, such as root gravitropism [50]. Therefore, it is conceivable that hydrogen peroxide signaling may be involved in Pb-induced cell wall thickening in rice coleoptiles. In addition, Pb may affect the autolytic activity of the cell wall. Plant cell walls contain various kinds of enzymes that are involved in cell wall remodeling [51,52]. Since Pb is able to bind to acidic sugar residues of matrix polysaccharides [23,46], it is likely that Pb accumulated within the cell walls may interfere with the action and activity of enzymes involved in the degradation of cell wall polysaccharides. This possibility remains to be clarified in a future study.

Pb strongly inhibits root growth. So, it has been believed that the inhibitory effect of Pb on the growth of plant aboveground organs is attributed to the inhibition of root development [2]. The present results showed that Pb inhibited the coleoptile growth of water-grown seedlings, which did not develop roots (Figure 4A), suggesting that Pb directly inhibits the growth of aboveground organs. In the case of rice caryopses, the cell division ceases about 60 h after sowing and the coleoptile growth afterward is mainly due to cell elongation [36]. Therefore, the results of the transplant experiment (Table 1) suggest that Pb inhibited the cell elongation process of coleoptiles by reducing cell wall extensibility. In air-grown seedlings, the effective concentration of Pb for the inhibition of coleoptile growth was much higher than that of root growth (Figure 1). However, the dose–response of Pb for the inhibition of the growth of water-grown coleoptiles was similar to that of air-grown roots (Figure 6). Furthermore, when concentrations of Pb in the cytoplasmic fluid were elevated to the order of ten μM, severe growth inhibition was observed in shoots and roots of air-grown seedlings. It is suggested by these results that there are no apparent differences in the organ susceptibility to Pb between aboveground organs and roots in rice seedlings.

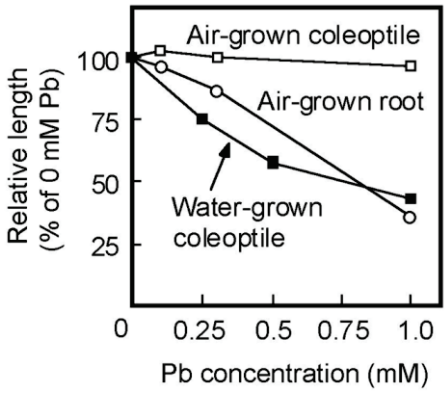


**Figure 6.** Dose–response curves of Pb on the growth of coleoptiles of air- and water-grown seedlings and of roots of air-grown seedlings. Lengths of coleoptiles and roots are shown as a percentage of the control (0 mM Pb) value. Values were calculated using the data in Figures 1 and 4B. Mean values are shown.

## 5. Conclusions

Pb treatment increases the thickness of the cell walls of rice coleoptiles irrespective of cultivation conditions, which may decrease cell wall extensibility. The decrease in cell wall extensibility is associated with the inhibition of coleoptile growth. It is conceivable that the growth inhibition of stem organs by other heavy metals also involves cell wall remodeling similar to that of Pb. Finally, water-grown rice seedlings may serve as a good experimental system to investigate how the cell walls in the aboveground organs of plants resist the penetration of heavy metals into the cells.

**Author Contributions:** K.W., K.S. and T.H. designed the research; K.W., K.S. and H.M. performed experiments; K.W., K.S., T.H. and H.M. analyzed data; K.W. and T.H. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available upon request from the corresponding author.

**Acknowledgments:** We thank Kazuya Izumoto, Yui Kagotani, Masashi Ueda, and Saki Terazawa of our laboratory for their efforts and cooperation during the course of the present study. We also thank Tomomi Shimonaka of Osaka Metropolitan University for excellent technical assistance in ICP-MS analysis.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Growth Stage-, Organ- and Time-Dependent Salt Tolerance of Halophyte *Tripolium pannonicum* (Jacq.) Dobrocz.

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**Abstract:** *Tripolium pannonicum* (Jacq.) Dobrocz. is a member of the diverse group of halophytes with the potential for the desalination and reclamation of degraded land. The adaptive processes of *T. pannonicum* to salinity habitats are still not well recognized. Therefore, we evaluated the effect of NaCl (0, 200, 400, and 800 mM) on: (1) two plant growth stages, (2) the activity of antioxidant enzymes and concentration of $H_2O_2$ and the proline in roots, stems, and leaves, and (3) the effect of long- and short-term salt stress on physiological responses. Germination, pot experiments, and a biochemical analysis were performed. The effective *T. pannonicum*'s seed germination was achieved in the control. We demonstrated that halophyte's organs do not simply tolerate high-salt conditions. The activities of APX, POD, and catalase observed at 400 mM and 800 mM NaCl were varied between organs and revealed the following pattern: root > leaves > stem. Proline was preferentially accumulated in leaves that were more salt-tolerant than other organs. Salt stress enhanced the activity of antioxidant enzymes and concentrations of salinity stress indicators in a time-dependent manner. Our study has indicated that salt tolerance is a complex mechanism that depends on the growth phase, organs, and duration of salinity exposure. The results have potential for further proteomic and metabolomic analyses of adaptive salt tolerance processes.

**Keywords:** salinity; halophytes; *Tripolium pannonicum*; proline; hydrogen peroxide; antioxidant enzymes

**Citation:** Ludwiczak, A.; Ciarkowska, A.; Rajabi Dehnavi, A.; Cárdenas-Pérez, S.; Piernik, A. Growth Stage-, Organ- and Time-Dependent Salt Tolerance of Halophyte *Tripolium pannonicum* (Jacq.) Dobrocz. *Life* **2023**, *13*, 462. https://doi.org/10.3390/life13020462

Academic Editors: Hakim Manghwar, Wajid Zaman and Balazs Barna

Received: 4 January 2023
Revised: 26 January 2023
Accepted: 3 February 2023
Published: 7 February 2023



## 1. Introduction

Soil salinity is a dynamic and globally spreading issue for over one hundred countries [1]. Salinity affects almost all stages of plant development and causes osmotic stress and ionic and nutrient imbalance [1,2]. Halophytes are one of the most suitable models of salt stress tolerance mechanisms, due to their salt resistance [3]. The newest reports indicated that halophytes have considerable potential for the restoration of salt contaminated lands and potential for the phytosanitation and phytoremediation of the soil [4].

The adaptiveness of *T. pannonicum* to salinity habitats are poorly studied and 'adaptive plant strategies' are not explained. Therefore, the experimental model in our studies is the sea aster *Tripolium pannonicum* (Jacq.) Dobrocz. (formerly *Aster tripolium* L.) from the *Asteraceae* family, which grows in the salt marshes and coastal areas of temperate zones and in non-tidal saline areas [5]. The plant is also part of highly specialized habitats: Atlantic salt meadows (1330) and inland salt meadows (1340) listed on the Habitats Directive-Natura 2000 and the European Red List of Habitats [6]. As a plant from the coastal areas, *T. pannonicum* is periodically flooded with sea water while in inland habitats are surrounded by outflows of salty groundwater and are classified as coastal wetland-specific species [7].

This species tolerates long-term flooding, making it more successful than other halophytes that cannot survive long-term flooding [8]. *T. pannonicum* is present also in saline, moist, and nitrogen-rich anthropogenic habitats, e.g., the surroundings of the soda factory in Inowrocław (Poland) with the highest maximum salinity of 140 $dS \cdot m^{-1}$. However, in natural habitats, such as the nature reserve in Ciechocinek (Poland), salinity can be lower (1.58–38.5 $S \cdot m^{-1}$) [9]. Piernik identified that the ecological growth optima of *T. pannonicum* correspond with very strong saline soils [9]. The variability of the parental habitat's salinity can be the basis of the extreme environmental adaptation of this plant. In addition, the 'adaptive plant strategies' may differ depending on the phase of growth, the plant's organs, and the length of time of exposure to the stress factors and even between and within species [10]. Different halophytes can express different salt stress adaptation strategies that are essential both in the context of their protection/restoration and of the future saline agriculture development [11]. Enhancing ROS and/or osmolyte production and antioxidant defence mechanism improvement are the most documented examples [11,12]. Therefore, studies on the not well-examined endangered halophyte *T. pannonicum* can help to better understand the plant's response to salinity stress and plant-environment relations, especially in the context of extreme climate change and habitat disturbances [13].

The initial growth, occurring at the germination and seedling stages, can influence a plant's capacity to capture resources in later growth when competition for light and soil nutrients becomes more intense [14,15]. Therefore, successful germination and seedling development are crucial steps in the effective growth of a new plant. The salt tolerance of the germination differs between halophytes, therefore it is essential to evaluate the salinity effect on the not well-studied halophyte species. A common effect of abiotic and biotic stressors is an excessive production of the reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$) causing cellular oxidative stress and damage of the crucial macromolecules [3,16,17] and osmolytes (such as proline or glycine betaine) [18–20]. The role of these compounds in *T. pannonicum*'s adaptation and tolerance of salinity is not well investigated and the patterns of correlation between these compounds are not documented yet. There is a lack of knowledge of whether the activities of antioxidant enzymes are correlated with the concentration of salinity stress indicators during *T. pannonicum's* adaptation to salinity. There is no evidence as to which organs (roots, stem, or leaves) are most affected by different NaCl concentrations. It is also not clear how the activity of antioxidant enzymes and salinity stress indicators (proline and $H_2O_2$) are correlated between organs and related to each other. The studies by Ievinsh et al. indicated a high dispersion of leaf water content, $Na^+$ vs. $K^+$ concentration in water from leaf tissue, and high sodium accumulation with low potassium levels in the leaves of the sea aster but without a dipper biochemical analysis that is crucial for understanding the salinity tolerance and adaptation [7].

Few studies have actually shown plant responses to different durations of salinization in short- and long-term periods. The researchers usually focus only on one type of response to salt stress, short- or long-term [21,22]. Therefore, in our study we want to explain how the short- and long-term NaCl stress act on the activity of antioxidant enzymes and on the concentration of salinity stress indicators. Examining the plant stress response on a wide time scale will allow us to fully understand the adaptation of *T. pannonicum* to extreme and variable soil salinity in the habitat. From the perspective of autecological studies on halophyte adaptation to salinity, the long-term stress effect seems to be even more significant than the short time salinity effect.

The main goal of our research was to determine at which levels (plant growth, organ, time) salinity can modify the stress response of *T. pannonicum*. We performed this autecological study to evaluate the following hypotheses: (1) even though *T. pannonicum* is a halophyte, salinity significantly affects germination and late growth, (2) salinity significantly affects the activity of antioxidant enzymes (APX, POD, and CAT) and salinity stress indicators (hydrogen peroxide and proline) in the root, stem and leaves of *T. pannonicum*, (3) the duration of salinity exposure modifies plant physiological responses. We hope that

the results will provide a novel view to understanding the interactions of individual species with the extreme environment and to recognize the salinity tolerance of this plant.

## 2. Materials and Methods

### *2.1. Seed Collection*

The seeds were collected in November 2019 from the anthropogenic inland saline habitat near a soda factory in the town of Inowrocław (52°48′ N, 18°15′ E). This site represents an industrial saline area in Poland, with salinity associated with waste from the soda production. In this area, the $EC_e$ value reached even 140 $dSm^{-1}$ (which corresponds to 1400 mM NaCl) [23]. Experiments were conducted at the Nicolaus Copernicus University in Torun, Poland in 2020. The permission to work with the seeds of a protected plant was provided by the regional director of the Environmental Protection in Bydgoszcz (WOP.6400.17.2020JC).

### *2.2. Germination Experiment*

Prior to the germination experiment, cold stratification was performed (4 °C, 30 days). Then, the seeds were sown on Petri dishes (Ø 7 cm) containing Whatman No. 2 filter paper (three replicates of 35 seeds in each salinity variant). We watered the seeds with four variants of a solution: control (0 mM NaCl), 200, 400, and 800 mM NaCl. NaCl concentrations were selected according to those observed in the field studies [23]. Piernik et al. [23] indicated the minimum (ca. 2 dS/m), maximum (ca. 100 dS/m), and optimum (ca. 30 dS/m) salinity for *T. pannonicum* growth. These correspond, respectively, to 20, 1000, and 300 mM NaCl. However, we did not obtain seedlings in the germination experiment at 1000 mM NaCl. Therefore, we established finally an upper limit of 800 mM NaCl. The Petri dishes with seeds were put into the growth chamber with day/night (25 °C), a humidity of 50–60%, a photon flux density of 1000 mmol $m^{-2}$ $s^{-1}$, and a photoperiod of 16/8 h (light/dark) (LED lights with white, full-spectrum light) [24]. The number of germinated seeds was determined daily (in the same part of the day) until the end of the 14th day after sowing.

The germination parameters were calculated based on the International Seed Testing Association (ISTA) method [25].

Germination percentage (GP):

$$GP = (n/N) \times 100, \quad (1)$$

where n is the number of normally germinated seeds and N is the number of all seeds sown.

Germination index (GI):

$$GI = \sum(G_t/T_t), \quad (2)$$

where $G_t$ is the number of seeds germinated on day t, and $T_t$ is the number of days.

Mean germination time (MGT):

$$MGT = \sum(T_i \times N_i)/\sum N_i, \quad (3)$$

where $N_i$ is the number of newly germinated seeds at time $T_i$.

Germination energy (GE) was assessed on the fourth day by counting the number of typical seedlings according to the ISTA (2006) standard [25].

### *2.3. Pot Experiments*

Following 14 days of germination, the seeds were transferred into individual pots (height: 5.3 cm, diameter: 5.5 cm) with a mixture of vermiculite and sand (1:1) as a substrate. Each pot was saturated to full capacity by solutions of 0, 200, 400, and 800 mM NaCl (ca 35 mL of solution for 1 pot with the substrate) to reflect the salinity of the soil in the field. For individual variants of salinity, we prepared six pots (total 6 pots × 4 variants of NaCl concentration). The pots were located on individual trays filled with 210 mL NaCl solution

(35 mL × 6 pots). Because in the first step we saturated the pots to the full capacity with NaCl solution, the NaCl was still present in the "soil solution" and the concentration of NaCl in this medium did not change during the experiment [24,26,27]. Seedlings were irrigated for three months with 210 mL of Hoagland's solution added to each tray every two days [22]. Following three months of plant development, the growth parameters and biochemical parameters were estimated per triplicate for each NaCl concentration (plants were randomly selected). We evaluated the salinity effect on six growth parameters: shoot length (SL), root length (RL), total fresh mass ($FM_T$), shoot fresh mass ($FM_S$), root fresh mass ($FM_R$), and number of leaves in the rosette ($No.L_R$); and on biochemical parameters: the activity of antioxidant enzymes (APX and POD) and salinity stress indicators (hydrogen peroxide and proline) in the roots, stems and leaves.

The phenotype photos after treatment with different salt concentrations were performed with a Sony digital camera and processed according to Cárdenas-Pérez et al. [24].

### *2.4. Long- and Short-Term Effects of Salinity*

The three-month-old plants (growing without NaCl application) were stressed with 800 mM NaCl and the leaves were harvested after 1 h, 3 h, and 5 h (short-term salinity stress), and after 24 h, 48 h, 5 days, and 7 days (long-term salt stress) of NaCl addition. Then, the antioxidant enzyme activities (POD and APX) and salinity stress indicators (hydrogen peroxide and proline) were assessed.

### *2.5. Biochemical Analysis*

For the determination of the activity of antioxidant enzymes (POD and APX), the leaves, roots, and stems were homogenized in 50 mM potassium phosphate buffer pH 7.0, including 0.1 mM EDTA on ice in a mortar. Then, the homogenate was centrifuged at 15,000× *g* for 15 min at 4 °C. The obtained supernatant was used for the determination of the antioxidant enzyme activities and protein content. The peroxidase activity (POD) was examined according to Maehly and Chance [28]. The enzymatic reaction was initiated by adding 100 µL of supernatant to the mixture of 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, and 40 mM $H_2O_2$. Changes in the absorbance of the reaction solution at 470 nm were read every minute. One unit of the enzyme activity was defined as the amount of enzyme causing a 0.001 change in absorbance per minute. The enzyme activity was presented as $U \cdot mg^{-1}$. The protein concentration was determined by the Bradford method [29]. The absorbance of the protein solution was measured at 595 nm with bovine serum albumin (BSA) as a standard. The ascorbate peroxidase activity (APX) was followed by the method of [30]. The assay mixture contained 0.1 mL of supernatant with enzyme, 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM $H_2O_2$, and 1 mL of potassium phosphate buffer (pH 7.0). The decrease in the absorbance of ascorbate at 290 nm was measured and one unit of the enzyme activity was defined as the amount of enzyme causing a 0.001 change in absorbance per minute. The activity of APX was expressed as $U \cdot mg^{-1}$.

Hydrogen peroxide in plant organs was examined according to Sergiev et al. [31] with modifications described by Velikova et al. [32]. Plant tissues (500 mg) were homogenized in an ice bath with 5 mL 0.1% TCA. Then, the homogenate was centrifuged at 12,000× *g* for 15 min (4 °C), and 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The solution was incubated in the dark for one hour and the absorbance of the supernatant was read at 390 nm. The final $H_2O_2$ concentration was expressed as µM.

The proline level was assessed according to the methodology of Abrahám et al. [33] with a small modification. Fresh plant material (500 mg) was homogenized on ice in a mortar with 3% aqueous sulfosalicylic acid solution (5 µL of solution per one mg of plant material). The homogenate was centrifuged (18,000× *g*, 10 min, 4 °C), and the supernatant was collected. The reaction mixture was composed of 2 mL of glacial acetic acid, 2 mL of acidic ninhydrin reagent, and 2 mL of supernatant. An acidic ninhydrin reagent was prepared according to Bates et al. [34]. The reaction mixture was shaken and incubated at

100 °C for 30 min. The reaction was inhibited by placing the samples on ice. To extract the chromophore, 4 mL of toluene was added and quantified spectrophotometrically at 520 nm. Proline concentrations were presented in μg/mL.

### *2.6. Detection of Catalase Activity by Non-Denaturing Polyacrylamide Gel Electrophoresis (PAGE) in Roots, Shoots, and Leaves*

Leaves, roots, and stems were homogenized in 50 mM potassium phosphate buffer pH 7.0, including 0.1 mM EDTA on ice in a mortar. Then, the homogenate was centrifuged at 15,000× *g* for 15 min at 4 °C, and 10 μg of protein per sample was loaded on 6% resolving gel solution [35]. The electrophoresis was run at 15 mA at 4 °C for 2 h. Following electrophoresis, the gel was soaked in distilled water for 5 min at room temperature. Then, the gel was incubated with 100 mL of a solution with 4 mM $H_2O_2$ (10 min, RT) and washed with 100 mL of distilled water. Following this step, the gel was moved to 100 mL of a solution (1% (*w*/*v*) ferric chloride and 1% (*w*/*v*) potassium ferricyanide). When the gel turned dark green, the ferric chloride/potassium ferricyanide solution was removed and washed with distilled water. Bands of catalase activity were marked as clear bands and their intensity corresponded with the activity of CAT [36].

### *2.7. Statistical Analysis*

One-way ANOVA with Tukey's post hoc comparison was applied to estimate the significance of the differences in (1) germination and growth parameters, (2) the activity of antioxidant enzymes and concentrations of proline in the roots, stem, and leaves, and (3) the salinity stress duration. A principal component analysis (PCA) was used to determine the correlation pattern between traits, and then Pearson's correlation coefficients were used for the correlation assessment. The statistical significance between treatments was agreed at the $p < 0.05$ level. For calculations, Statistica version 8.0 [37] and Canoco 5.0 [38] packages were used.

## 3. Results

### *3.1. Effect of Salinity on Two Growth Stages*

Salinity affected similarly on the germination and late-growth parameters of *T. pannonicum*. The differences between treatments were significant in all measured parameters except $FM_R$ (Tables 1 and S1). Salinity significantly reduced GP, GI, and GE and increased MGT. Germination was highest at 0 mM NaCl, and lowest at 800 mM NaCl (Table 1). The MGT was highest at 800 mM NaCl, but in the control, 200 mM, and 400 mM NaCl, we observed no statistically significant differences. Salinity also significantly reduced the parameters of late growth: SL, RL, $FM_T$, and $FM_S$. The stem was longest in the control (31.3 cm), and the roots were longest in the control and 200 mM NaCl (ca 8.3 cm). $FM_T$ was highest in the control and 200 mM NaCl (ca 24 g) and lowest at 400 and 800 mM NaCl (ca 20 g). We observed no significant difference in $FM_S$ and $No.L_R$ between the control and 200 mM and between 400 mM and 800 mM NaCl (Table 1). $FM_S$ was lowest at 400 and 800 mM NaCl (18 g and 16.7 g, respectively). $FM_R$ was not significantly affected by salinity.

**Table 1.** Effect of NaCl on the germination and growth parameters of *T. pannonicum*.

| Trait | Control | 200 mM | 400 mM | 800 mM | ANOVA |
|---|---|---|---|---|---|
| | | NaCl | | | |
| Germination parameters | | | | | |
| GP | 61.7 $^{a}$ ± 0.88 | 50.3 $^{b}$ ± 0.33 | 3.67 $^{c}$ ± 0.33 | 1.33 $^{d}$ ± 0.67 | $p < 0.0001$ |
| GI | 6.65 $^{a}$ ± 0.18 | 5.85 $^{b}$ ± 0.03 | 0.42 $^{c}$ ± 0.056 | 0.053 $^{d}$ ± 0.01 | $p < 0.0001$ |
| MGT | 4.89 $^{b}$ ± 0.045 | 4.49 $^{b}$ ± 0.04 | 5.66 $^{b}$ ± 0.29 | 7.33 $^{a}$ ± 0.17 | $p < 0.0001$ |
| Plant morphology |   | | | | |
| Growth parameters | | | | | |
| SL (cm)RL (cm) | 31.3 $^{a}$ ± 0.67<br>8.27 $^{a}$ ± 0.15 | 28.5 $^{b}$ ± 0.29<br>8.30 $^{a}$ ± 0.12 | 21.0 $^{c}$ ± 0.58<br>6.83 $^{b}$ ± 0.18 | 16.3 $^{d}$ ± 0.21<br>5.07 $^{c}$ ± 0.09 | $p < 0.0001$<br>$p < 0.0001$ |
| $FM_T$ (g) | 24.4 $^{a}$ ± 0.64 | 23.4 $^{a}$ ± 0.37 | 20.9 $^{b}$ ± 0.26 | 20.04 $^{b}$ ± 0.13 | $p = 0.0003$ |
| $FM_S$ (g) | 21.3 $^{a}$ ± 0.50 | 21.1 $^{a}$ ± 0.43 | 18 $^{b}$ ± 0.35 | 16.7 $^{b}$ ± 0.35 | $p = 0.0002$ |
| $FM_R$ (g) | 2.83 $^{ab}$ ± 0.43 | 2.40 $^{b}$ ± 0.12 | 3.29 $^{ab}$ ± 0.30 | 3.66 $^{a}$ ± 0.23 | $p = 0.1077$ |
| $No.L_R$ | 18 $^{a}$ | 17 $^{a}$ | 16 $^{b}$ | 15 $^{b}$ | $p < 0.0001$ |

Average values ($n = 3$) with standard error (SE) are given. GP = germination percentage; GI = germination index; MGT = mean germination time; GE = germination energy; SL = shoot length; RL = root length; $FM_T$ = total fresh mass; $FM_S$ = shoot fresh mass; $FM_R$ = root fresh mass; $No.L_R$ = number of leaves in a rosette. Differences between groups based on Tukey's range test are marked by different letters and are significantly different at $p \leq 0.05$.

### *3.2. Effect of Salinity on Organ Stress Responses*

Salinity significantly affected the activity of antioxidant enzymes (APX and POD) and concentrations of salinity stress indicators (hydrogen peroxide and proline) in the roots, stems, and leaves of *T. pannonicum* (Table S2). The main effects of salinity on the organs, and the interaction effects between them, were significant for all measured parameters (Table 2). In all organs, salinity increased APX and POD activity. The activity of APX and POD was highest at 800 mM NaCl in all analyzed plant organs (Figure 1). In addition, the zymogram analysis indicated that CAT enzyme activity was also highest at 800 mM NaCl (Figure 2).

**Table 2.** Analysis of variance (mean squares) for the activity of antioxidant enzymes and salinity stress indicators in two organs (O) of *T. pannonicum* and four salt concentrations (S).

| Trait | Sources of Variations | | | |
|---|---|---|---|---|
| | S | O | SxO | Error |
| **df** | **2** | **4** | **8** | **24** |
| APX | 10,691 ** | 11,643 ** | 1824 ** | 1.082 |
| POD | 15.44 ** | 52.15 ** | 3.56 ** | 0.096 |
| $H_2O_2$ | 97.73 ** | 221.34 ** | 26.14 ** | 0.409 |
| P | 13,145.01 ** | 3869.41 ** | 437.55 ** | 31.615 |

Average values ($n = 3$) with errors within group variance are given. APX = ascorbate peroxidase activity; POD = peroxidase activity; $H_2O_2$ = $H_2O_2$ concentration; P = proline concentration; df = degrees of freedom; ** = $p \leq 0.01$.

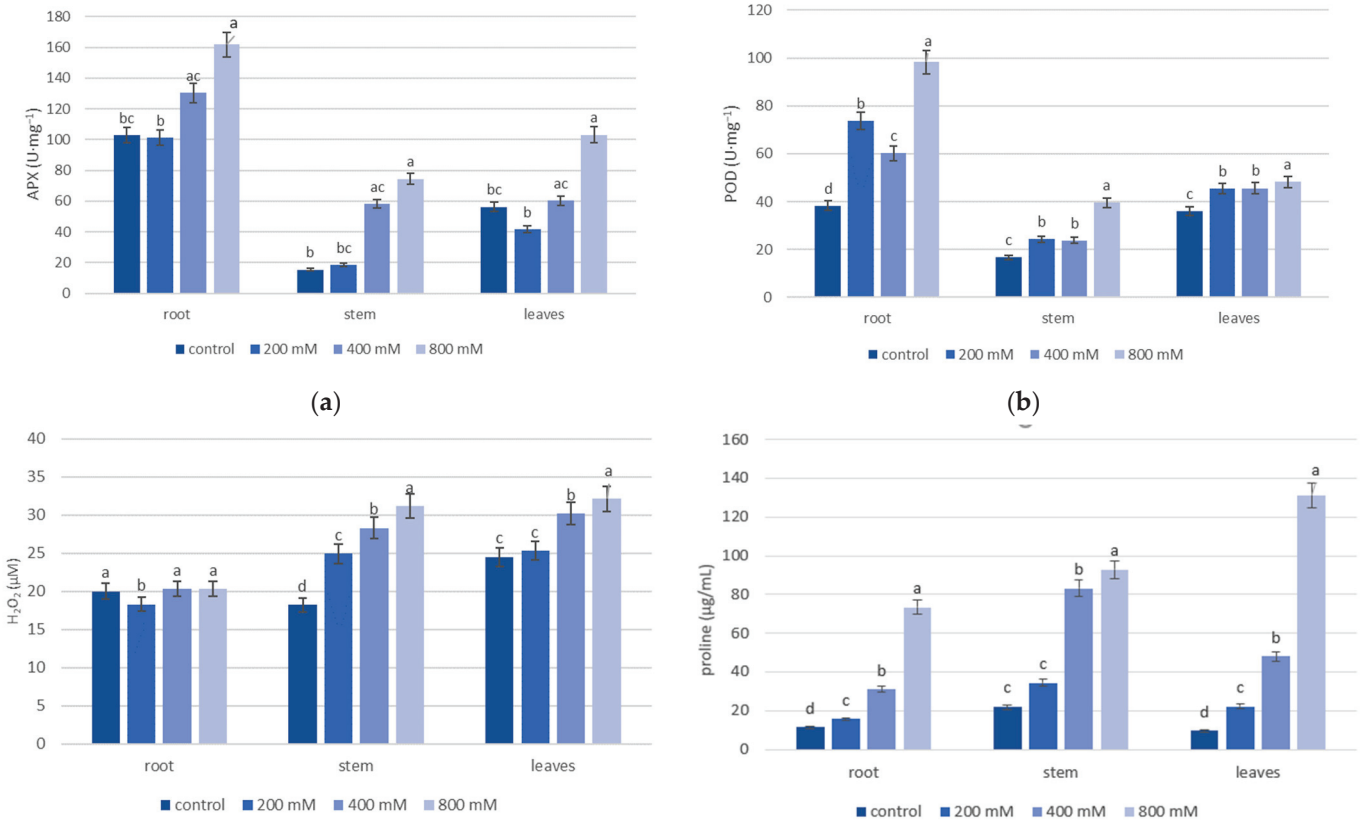


**Figure 1.** Effect of NaCl on the activity of antioxidant enzymes—(**a**) APX and (**b**) POD, and salinity stress indicators—(**c**) $H_2O_2$ and (**d**) proline in the roots, stems, and leaves of *T. pannonicum*. Differences between groups based on Tukey's range test are marked by different letters and are significantly different at $p \leq 0.05$. One unit of the enzyme activity was defined as the amount of enzyme causing a 0.001 change in absorbance per minute. Average values with standard error are given $n = 3$.

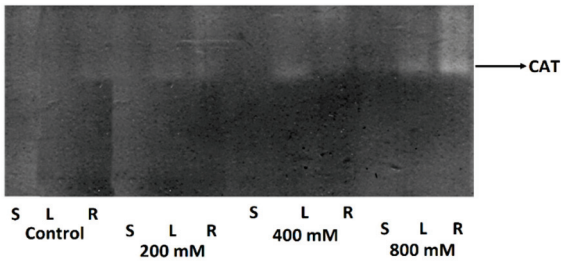


**Figure 2.** Zymogram of CAT enzyme activity in the roots (R), stem (S), and leaves (L) after 0 (control), 200, 400, and 800 mM NaCl treatment. Bands of catalase activity were marked as clear bands and their intensity corresponded with the activity of CAT (shown by the arrow) between organs.

Of all organs, the activity of APX and POD was lowest for the stem in the control (Figure 1a,b). The lowest activity of APX in the root and leaves was observed at 200 mM NaCl. The activity of POD for all organs was lowest in the control. We observed no significant difference in POD activity in the leaves in 200 and 400 mM NaCl (Figure 1b). The highest intensity of the bands in the CAT zymogram was observed for the roots after the 800 mM NaCl treatment. In addition, we detected one isoform of catalase in all NaCl treatments (Figure 2). The highest concentrations of $H_2O_2$ and proline were observed at 800 mM NaCl in all analyzed organs (Figure 1c,d). The lowest concentration of $H_2O_2$ between all organs was noticed for the stem in the control (18.2 µM). The lowest $H_2O_2$ concentration in the root and leaves was observed in 200 mM NaCl (Figure 1c). The

concentration of $H_2O_2$ in the root seems to be independent of the increasing salinity because no significant difference was observed between NaCl treatments. The amount of proline for all organs was lowest in the control (11.4 µg/mL for the root, 21.8 µg/mL for the stem, and 9.49 µg/mL for the leaves). For all organs, proline concentration increased with the growing salinity and was greatest in the leaves (Figure 1d).

### *3.3. Effect of the Duration of Salinity on the T. pannonicum Stress Responses*

Based on the above result of our experiment, we investigated the effect of long- and short-term salt exposure on the enzyme activity, and the concentration of hydrogen peroxide and proline in the leaves as the main organ affected by salinity and the organ responsible for salt extrusion by the shedding of rosette old leaves saturated by salt [39]. Because the most significant effect of the salinity was obtained for 800 mM NaCl, we selected this concentration of salt for further analysis.

The duration of salinity exposure significantly affected the activity of antioxidant enzymes and salinity stress indicator concentrations (Table S3). We observed that salinity acts on analyzed parameters over different time scales (Figure 3). The activities of both APX and POD enzymes were similar for each analyzed timeframe, however, significant differences in the APX activity, between 5 h and 48 h, were observed (Figure 3a). The highest activity of APX and POD was observed 1 h after NaCl application (short-term salt stress) (26.2 U·mg$^{-1}$ for APX and 26.7 U·mg$^{-1}$ for POD) and 48 h (long-term salt stress) (32.3 U·mg$^{-1}$ for APX and 27.6 U·mg$^{-1}$ for POD). The highest concentrations of $H_2O_2$ and proline were at 48 h and in 5 days after NaCl application, respectively (Figure 3b,c). There were no significant differences in the salinity stress indicator concentrations in short-term salt stress (1 h, 3 h, and 5 h), nor in long-term salt stress (5 days and 7 days).

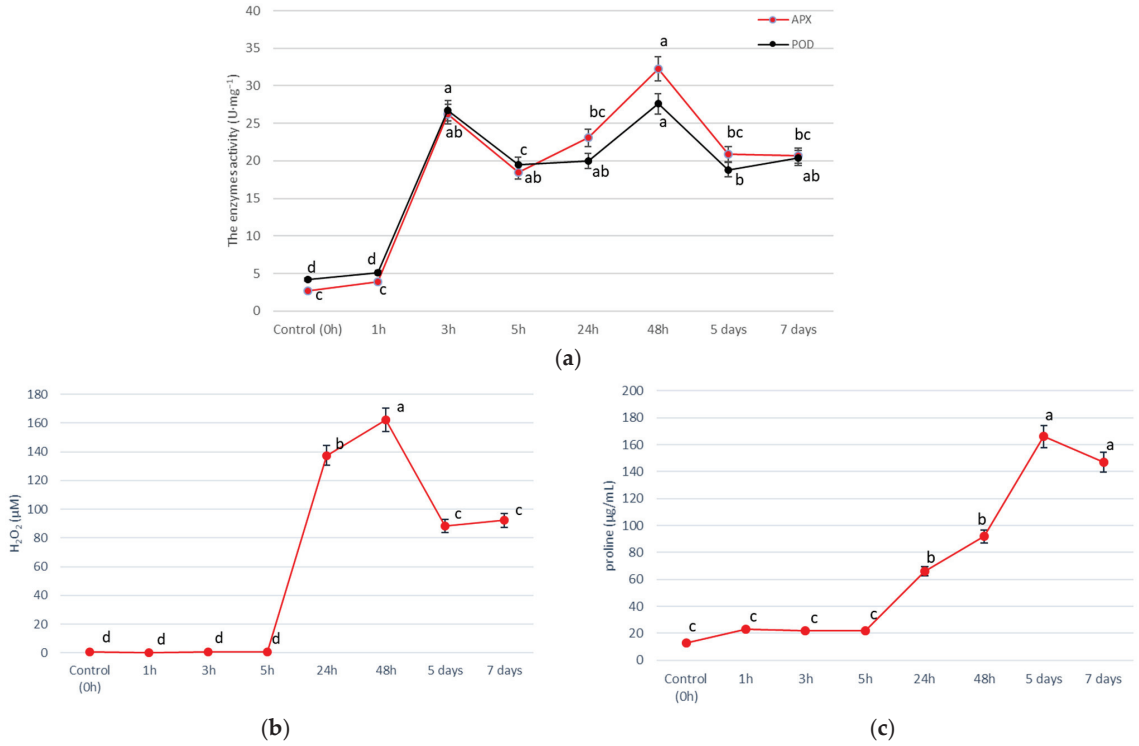


**Figure 3.** Effect of the duration of salinity exposure on the activity of antioxidant enzymes—(**a**) APX and POD, and salinity stress indicators—(**b**) $H_2O_2$, and (**c**) proline, in the leaves of *T. pannonicum*. Differences between groups based on Tukey's range test are marked by different letters and are significantly different at $p \leq 0.05$. One unit of the enzyme activity was defined as the amount of enzyme causing a 0.001 change in absorbance per minute. Average values with standard error are given $n = 3$.

### *3.4. Patterns of Correlation between Growth Stage, Organs, and Duration of Salinity Exposure*

All of the variables (germination and growth parameters, antioxidant enzymes, and salinity stress indicators in organs) were evaluated in the NaCl concentration, while biochemical parameters were also evaluated in the time scale using PCA. The response to salinity was dependent on the growth stage, organs, and duration of salinity stress, where the first ordination axis represents the salinity gradient (Figure 4a–c).

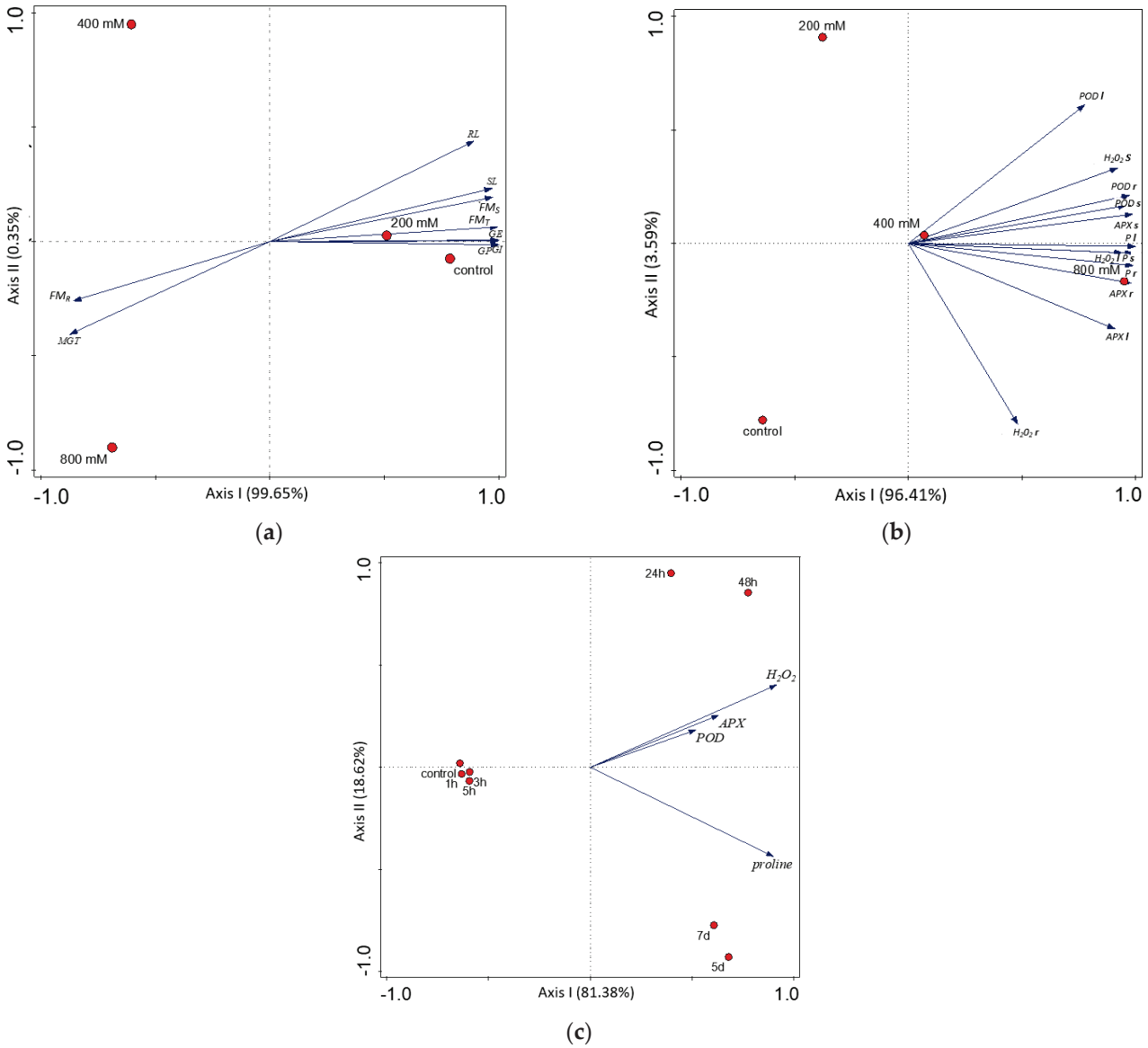


**Figure 4.** Result of the PCA analysis between: (**a**) salinity treatments and germination and growth parameters; (**b**) salinity treatments and stress responses in different organs; (**c**) duration of salinity exposure and stress responses in the leaves. Abbreviations as in Tables 1 and S2. The concentrations of NaCl (**a**,**b**) and duration of NaCl stress (**c**) are marked by red points.

We noticed a strong and statistically significant positive correlation between POD in the roots and the concentration of $H_2O_2$ in the stem (Pearson's r = 0.864) and a very strong positive correlation between APX in the roots and the concentration of $H_2O_2$ in the leaves (Pearson's r = 0.961). The concentration of proline was also correlated significantly with the activity of antioxidant enzymes, especially with APX activity. A very strong positive correlation was observed for the proline concentration in the stems and APX activity in the roots (Pearson's r = 0.990) and for the proline concentration in the leaves and APX activity

in the roots (Pearson's r = 0.985). The concentration of salinity stress indicators ($H_2O_2$ and proline) was positively correlated between organs. The strongest significant correlation was found for the concentration of proline in the stem and $H_2O_2$ in the leaves (Pearson's r = 0.981) (Figure 4b, Table S5). The activity of antioxidant enzymes and concentration of salinity stress indicators were strongly correlated with the type of salinity response early up to 5 h and late from 24 h, represented by the first PCA axis explaining ca 81% of the traits' variance (Figure 4c). The differentiation in the late response, represented by the second PCA axis, explained ca 18%. All analyzed parameters were positively correlated with the duration of salinity stress (Figure 4c, Table S6). The activities of APX and POD were significantly and very strongly correlated with each other (Pearson's r = 0.984). The positive correlation was high between APX and $H_2O_2$ (Pearson's r = 0.666), and moderate between POD and $H_2O_2$ (r = 0.532). The correlation between two salinity stress indicators (proline and $H_2O_2$) was also significant and moderate (r = 0.385; Table S6).

## 4. Discussion

### *4.1. Salinity Affects Germination and Late Growth of T. pannonicum*

Germination is a crucial part of the plants' growth, but more so for halophytes, and salt tolerance usually varied between halophytes [40]. To survive in a saline habitat, halophytes required successful seed germination [41]. Most parameters of germination and growth were affected by salinity Tables 1 and S2). These results are in line with our previous studies on glycophytic species, e.g., fodder beet [42], sorghum [26], maize, millet, and oat [2]. In our study, the effective *T. pannonicum* seed germination was achieved in the control (0 mM NaCl) although the model plant is an obligatory halophyte. The negative effect of salinity was also visible in the reduction of the germination energy (Table 1), because reducing the osmotic potential of the solution inhibits the imbibition of water by the seeds. MGT parameter (determining the time for the seed to germinate) was almost the same in the control, 200 mM and 400 mM NaCl (Table 1) which can be an example of a *T. pannonicum*'s seed strategy for efficient germination even under high soil salinity. Salinity had a significant effect on germination time (Table 1), as in a study of *P. sativum* and *L. sativus* [14]. The higher NaCl concentrations lengthen the germination time until the seeds develop a tolerance and start to germinate. Faster and early germination under lower salinity confers an ecological advantage upon halophyte seedlings [40].

Our study has shown that salinity strongly reduces the growth parameters of three-month-old plants, and maximum growth is obtained under non-saline and low-saline conditions (Table 1) as in a study by Geissler et al., 2009 [43], not all plant growth parameters were affected by salinity at the same level. The best growth parameters were observed in the control and 200 mM NaCl, which indicates the optimal concentration of NaCl for growth success. In addition, the total biomass of *T. pannonicum* was similar in 400 mM and 800 mM NaCl, however lower than in the control and 200 mM NaCl, indicating effective adaptation to higher levels of salinity. High variability of salinity in the *T. pannonicum* wet habitats promotes a broad spectrum of NaCl tolerance which was observed also by Ievinsh et al. [7]. They found *T. pannonicum* also in habitats with low salinity [7]. In addition, Karlsons et al. 2008 [44] demonstrated a higher decrease in the roots and leaves biomass of this species at 400 mM NaCl, compared with our studies. However, the adaptations to environmental stress can evolve within populations of the same species [45] and can be genetically established within a population as the result of local adaptation.

### *4.2. Response to Salinity Depends on the Organs of T. pannonicum*

The result of our study demonstrated that the halophyte's organs do not simply tolerate high-salt conditions (Tables 2 and S2). All biochemical parameters (activity of APX and POD, concentration of $H_2O_2$ and proline) in the analyzed organs were affected by salinity. The higher salt tolerance of numerous halophytes is related to proper ROS homeostasis by the activation of their antioxidant systems under salt stress [22]. The highest activities of APX, POD, and CAT were observed at 800 mM NaCl in all analyzed plant

organs and were greatest for the roots (Figures 1a,b and 2). The activities of APX and POD observed at 400 mM and 800 mM NaCl were varied between organs and revealed the following activity pattern: root > leaves > stem (Figure 1). Increased antioxidant activity has been observed in several salt-tolerant plants, indicating that antioxidants are an important factor in the salt stress response [46]. Enzymatic oxidative stress defenses to high salt concentration are more generally in the roots (such as CAT or APX activation), while root-specific antioxidant enzymes (i.e., SOD, and MDHAR) have also been found [47].

As shown in our study, the highest concentration of salinity stress indicators ($H_2O_2$ and proline) was observed at 800 mM NaCl in all analyzed organs (Figure 1c,d). Salt stress also generated oxidative stress in halophytes of the genus *Juncus* [48]. The highest efficiency of the antioxidant system in the root was the reduced $H_2O_2$ concentration in this organ which indicated that ROS scavenging mechanism is most effective in the roots, compared to the stems and leaves. An interesting observation is that proline was preferentially accumulated in the leaves (Figure 1d), however, in increasing NaCl concentrations, we noticed proline accumulation in all organs. Our results indicated that, in the context of proline accumulation, leaves are more salt-tolerant than other organs. Organ-specific accumulation of proline in the leaves of lupine and halophyte *K. virginica* was also observed by Rady et al., 2016 [18] and Wang et al., 2015 [49] and $H_2O_2$ in the leaves of rice [50].

### *4.3. Response to Salinity Depends on the Duration of Salinity Stress*

Studies of the effects of salinity on halophytes on a wide time scale (in short-time and long timescale) are significant to fully understand the adaptation strategy and plant interaction with the environment. The response of *T. pannonicum* to salinity stress was two stages to enable more efficient and comprehensive adaptiveness (Figure 3a–c). The $H_2O_2$ concentration peaked at 48 h (long-term salt stress), which corresponds with the increased activity of APX and POD, the key enzymes responsible for $H_2O_2$ scavenging during salinity stress in plants. A significant result is that the activity of both investigated peroxidases is synergistic (Figure 3a). It seems reasonable to cope with NaCl stress "with redoubled strength". It is also evidence of an early- and late-cellular stress response, which was for the first time demonstrated for *T. pannonicum*. Similar onset/reinforcement of antioxidant systems were also observed for groundnut [12] and soybean [51] under salt stress. For halophyte *S. aralocaspica*, adaptation to the external salinity changes for a period of 24–48 h has been reported [52]. Hernández et al., 2000 [53] noticed that the induction of antioxidant defence is at least one component of the tolerance mechanism of *Pisum sativum* L. to long-term salt stress. According to Fraire-Velazquez and Emmanuel [17], the observed initial, fast response to salinity stress is temporary and thus separated clearly from the pathological consequences of exposure to the same level of salinity over a longer period, which is catastrophic for non-adapted plants.

The highest concentrations of $H_2O_2$ (162.1 μM) and proline (166.08 μg/mL) were recorded at 48 h and 5 days after salinity treatment, respectively. Huang et al., 2013 [20] observed the same dependencies in *H. tuberosus* during the initial 72-h period. Compared with enzyme activity, the increase in the concentration of salinity stress indicators was a part of the late stress response (Figure 3b,c) therefore these compounds may be more responsible for the long-term adaptation process of the plant to the extreme environment than antioxidant enzymes, as in the study of Naliwajski and Skłodowska 2021 [54]. The action of $H_2O_2$ and proline is also synergistic to intensify the defense mechanisms against NaCl stress. Studies by Huang et al. 2013 [20] demonstrated that the gene expression of key enzymes in the proline biosynthetic pathway changed significantly in roots of *H. tuberosus* after 4 h treatment, which may be responsible for the increase in the concentration of proline observed after 5 h of salinity treatment in our study (Figure 3b).

### *4.4. Salt-Tolerance and Salt-Adaptation of T. pannonicum*

We indicated that germination success determines the future plant biomass ($FM_T$) and biomass of shoots ($FM_S$) (Table S4). Bayuelo-Jiménez et al., 2002 [15] observed that faster

germination allowed emerging seedlings to obtain a higher biomass. The strong negative correlation observed in our study for RL and MGT (Table S4) corresponds with the findings by Robin et al., 2016 [55]. Salinity induced a reduction in the surface area of the root and changes in the main root in wheat [55], which may explain the extended mean germination time of *T. pannonicum* seeds under salinity. Although *T. pannonicum* is a halophyte, saline conditions at the germination stage will be defined as the further adaptation success of this plant. Low soil salinity under the first step of plant growth allowed this plant to obtain a better biomass and overcome interspecific competition in the environment. A PCA analysis indicated a correlation between the activity of antioxidant enzymes and proline concentrations in the investigated organs under NaCl stress, which can be essential for *T. pannonicum* adaptation (Figure 4b, Table S5). The early cellular stress response observed after 1 h and 5 h of NaCl exposure (Figures 3a–c and 4c) can help the plant to restore its performance more quickly and is essential to stress neutralization and further plant survival [17]. Then, the changes in enzyme activities affecting the concentrations of salinity stress indicators ($H_2O_2$ and proline) were part of the late cellular stress response observed 24 h after salinity stress (Figure 3a–c). The first line of defense against ROS caused by oxidative stress comprises antioxidant enzymes, so they are activated more quickly than the production/accumulation of proline [56]. The growth response to salinity stress can be divided into two steps: a fast reaction to the increase of the external osmotic pressure, and a slower response as a result of the accumulation of $Na^+$ in plant organs, which was documented by Munns and Tester 2008 [57]. The results of our studies strongly indicate that the levels of stress indicators and the activity of antioxidant enzymes contribute to the tolerance and adaptation of *T. pannonicum* to salinity stress.

The high salt tolerance was noticed for the germination and late growth of *T. pannonicum* results from its natural growth in very stressful and variable habitats. *T. pannonicum* is part of inland and coastal plant communities [8,9,23]. The ecological habitats of sea aster are extremely unfavorable: inland saline meadows flooded in the spring after snowmelt and dried in the summer, or coastal seashores with sea tides. This species in anthropogenic areas can be also exposed to salty waste from the soda or potassium industry [58]. These difficult growth conditions increase the adaptability of *T. pannonicum.* Investigated salt-adaptation traits, such as a high total biomass under salinity, the high and organ-specific activities of APX, POD, and CAT, and the highest efficiency of the antioxidant system in the root with a leaves-specific accumulation of proline, are examples of plant answers for the high variability of the habitats where they grow.

## 5. Conclusions

*T. pannonicum* is a member of a diverse group of halophytes that seems to be a promising cash crop to desalinize and reclaim degraded land. However, some basic physiological, biochemical, and molecular mechanisms in the adaptive processes of *T. pannonicum,* such as salt tolerance, are still not well recognized. Our study and recent genetic and omic experiments have indicated that salt tolerance is a complex mechanism that depends on the growth phase, organs, and duration of salinity exposure. The antioxidant system of *T. pannonicum* was very active at 800 mM NaCl and APX. POD, and CAT activity were greatest for the roots. The demonstrated different responses of the organs to NaCl application have significant potential to further proteomic and metabolomic analyses of halophyte adaptions. The time-dependent regulation of adaptive processes involved in the tolerance of high and extended salinity, but also shorter episodes of salinity, also requires a deeper explanation. A better understanding of the adaptabilities of *T. pannonicum* to high salinity could be helpful in the restoration of the fragmented aster population and studies of these plants' application as energy crops for cultivation on saline lands or as cash crop vegetables.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/life13020462/s1, Table S1: Analysis of variance (mean squares) for the germination and the growth parameters of *T. pannonicum* in four salt concentrations, Table S2: Analysis of variance (mean squares) for the activity of antioxidant enzymes (APX and POD) and salinity stress indicators

($H_2O_2$ and proline) of *T. pannonicum* in four salt concentrations, Table S3: Analysis of variance (mean squares) for the activity of antioxidant enzymes (APX and POD) and salinity stress indicators ($H_2O_2$ and proline) of *T. pannonicum* in eight different times of measurements, Table S4: Correlation coefficient matrix between germination and growth parameters under NaCl stress, Table S5: Correlation coefficient matrix between antioxidant enzymes and salinity stress indicators under NaCl stress, Table S6: Correlation coefficient matrix between antioxidant enzymes and salinity stress indicators under the different times of the duration of salinity stress.

**Author Contributions:** Conducted experiments and data interpretation, wrote the manuscript, A.L.; supported biochemical analysis, A.C.; supported statistical analysis, A.R.D.; supported time-dependent experiments, S.C.-P.; supported data interpretation, A.P.; reviewed & edited the manuscript, A.C., A.R.D., S.C.-P. and A.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by funds for the science from Nicolaus Copernicus University in Toruń, Poland, and IDUB Emerging Field Ecology & Biodiversity.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets generated during the current study are available from the corresponding author upon reasonable request.

**Acknowledgments:** Thanks are extended to Katarzyna Roszek (Department of Biochemistry, NCU) for technical and equipment support.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Examination of Different Sporidium Numbers of *Ustilago maydis* Infection on Two Hungarian Sweet Corn Hybrids' Characteristics at Vegetative and Generative Stages

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**Abstract:** Corn smut is one of the major diseases in corn production. The cob infection causes high economic and quality loss. This research investigated the effects of three different concentrations of corn smut infection (2500, 5000, and 10,000 sporidia/mL) on two Hungarian sweet corn hybrids (Desszert 73 and Noa). Plants were infected at the vegetative (V4–V5) and the generative (V7) stages. The effects of the corn smut infection were evaluated at 7 and 14 days after the pathogen infection (DAPI) at vegetative and at 21 DAPI at generative stages. The photosynthetic pigments (relative chlorophyll, chlorophyll-*a* and *b*, and carotenoids), malondialdehyde (MDA), and proline concentration, activities of the antioxidant enzymes [ascorbate peroxidase (APX), guaiacol peroxidase (POX), and superoxide dismutase (SOD)], morphological characteristics (plant height, stem and cob diameter, cob length, cob and kernel weights), mineral contents (Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P, Pb, S, Sr, and Zn), and quality parameters (dry matter, fiber, fat, ash, nitrogen, and protein) were measured. At both sampling times (7 and 14 DAPI) in both hybrids, the corn smut infection reduced the photosynthetic pigments (relative chlorophyll, chlorophylls-*a*, and *b*, and carotenoids) irrespective of the spore concentration. Under the same conditions, the MDA and proline contents, as well as the activities of APX, POX, and SOD increased at both sampling times. The negative effects of the corn smut infection were also observed at the generative stage. Only the 10,000 sporidia/mL of corn smut caused symptoms (tumor growth) on the cobs of both hybrids at 21 DAPI. Similarly, this treatment impacted adversely the cob characteristics (reduced cob length, kernel weight, and 100 grains fresh and dry weight) for both hybrids. In addition, crude fat and protein content, Mg, and Mn concentration of grains also decreased in both hybrids while the concentration of Al and Ca increased. Based on these results, the sweet corn hybrids were more susceptible to corn smut at the vegetative stage than at the generative stage.

**Keywords:** antioxidant enzymes; chlorophylls; corn smut; fungus infection; maize; MDA; proline; quality

**Citation:** Szőke, L.; Moloi, M.J.; Kaczur, D.; Radócz, L.; Tóth, B. Examination of Different Sporidium Numbers of *Ustilago maydis* Infection on Two Hungarian Sweet Corn Hybrids' Characteristics at Vegetative and Generative Stages. *Life* **2023**, *13*, 433. https://doi.org/10.3390/life13020433

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 5 January 2023
Revised: 30 January 2023
Accepted: 31 January 2023
Published: 3 February 2023



## 1. Introduction

Successful crop production depends on many factors, such as crop type [1], variety [2], and environmental factors [3], including climatic conditions [4], soil [5], and water [6]. Biotic and abiotic stresses also affect yield [7]. Direct protection against abiotic factors such as drought [8], salinity [9], extreme cold or heat [10], heavy metal stress [11], and water deficiency or excess [12] is achieved through resistance. However, biotic stresses are also important [13]. The use of pesticides protects against diseases, pests, and weeds (which cause great economic losses) [14,15]. Their use is disadvantageous because it contributes to environmental pollution [16]. In addition, resistance breeding is widely used to reduce the use

of pesticides. Plants have a specific defense system that is activated after infection [17]. The cuticle is the plants' first defense against fungal invasion [18]. The structure of the cuticle can be different in different plant species [19] and its effectiveness against pathogens is also different [20]. When the fungal invasion is successful, plants produce secondary metabolites to protect themselves [21]. Phenolic terpenes and nitrogen/sulfur-containing compounds are synthesized in plants [22]. The roles of phenolic compounds against pathogens are well studied [23]. For example, benzaldehyde (against *Botrytis cinerea*, [24]), protocatechuic acid (against *Colletotrichum circinans*, [25]), salicylic acid (against *Eutypa lata*, [26]), vanillic acid (against *Phytophthora infestans*, [27]), chlorogenic acid (against *Fusarium osysporum*, [28]), naringin (against *Penicillium digitatum*, [29]), flavones (against *Aspergillus*, [30]), oleuropein (against *Phytophthora*, [31]), Nobiletin (against *Phoma tracheophyta*, [32]), Geinstein (*Monilinia fructicola*, [33]), and Hordatin A (against *Helminthosporium sativum*, [34]). Other important molecules in the plant-pathogen interaction are the reactive oxygen species (ROS) [35]. After infection, plant cells produce ROS such as hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), superoxide anions ($O_2^-$), and hydroxyl radicals ($^-OH$) [36]. ROS can be important molecules for signal transduction (at low concentration) or toxic (at high concentration) for plants [37]. One of the most detrimental effects of ROS is the induction of lipid peroxidation in the cell membrane [38]. ROS are mainly produced in peroxisomes [39], mitochondria [40], and chloroplasts [41]), which can interfere with metabolic processes. To eliminate ROS, plants use antioxidant enzymes [42]. Ascorbate peroxidase (APX) degrades $H_2O_2$ to water using ascorbic acid as a substrate. Several APX isoforms in plants are distributed in different cellular compartments such as chloroplasts, mitochondria, peroxisomes, and cytosol [43]. Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion radical ($O_2^-$) to water and $H_2O_2$. Their classification is based on their subcellular location and bound metal cofactor (Cu/Zn, Mn, Fe, and Ni) [44]. Guaiacol peroxidase (POX) is essential for lignin biosynthesis and neutralizes $H_2O_2$ [45]. Corn smut is a major corn pathogen capable of infecting corn at vegetative and generative stages, leading to serious yield losses [46], especially in sweet corn, which has a great economic impact [47]. Corn smut is a biotrophic pathogen, which causes galls on all aerial parts of its host plants but does not cause the death of cells. To evaluate the impact of a biotrophic pathogen on its host plants, many physiological, morphological, biochemical, and quality parameters give an understanding of the impacts of pathogen infection. Biotrophic pathogens live and complete their life cycle in the host plants. To survive, they derive nutrients from the host plant, leading to reduced growth [48]. Corn smut infection has a significant impact on the leaf chlorophyll content, which can be measured using different methods. During corn smut infection, chlorosis would appear 3–5 days after infection, which is an indication of chlorophyll loss [49]. Therefore, measurements of chlorophyll content are valuable. Infection may also cause oxidative stress in plants [50,51]. The oxidative burst in host plants may activate the antioxidative mechanisms. Szőke et al. [52] showed that the corn smut infection increased the activities of antioxidant enzymes (SOD, APX, and POX) and the malondialdehyde (MDA) content in the infected fodder and sweet corn hybrids.

Corn smut also has a significant impact on sweet corn yield. Clough et al. [53] found a strong correlation between the intensity of the corn smut infection and kernel characteristics. They stated that when the gall size was bigger, fresh weight, length, diameter, and kernel depth were smaller. According to Pál-Fám et al. [54], corn smut infection significantly decreased the ear, grain and cob weights of the fodder corn, causing significant economic damage. Moreover, they stated that the corn smut-infected cob had lower dry matter, fiber, and ash contents. Keszthelyi et al. [55] also stated that the corn smut infection reduced the dry matter, protein, fat, fiber, and ash contents of the fodder corn.

The effects of different diseases on the nutrient content of the host plant have also been reported. A high concentration of the corn smut infection increased the amounts of Fe and Zn in the shoots and roots of infected plants [56]. The *Candidatus phytoplasma* L. asiaticus infection decreased the N and P contents of citrus species [57]. The Fusarium-infected tomato plants had a lower Cu content compared to uninfected control plants [58]. Mineral

nutrients have an important function in the interaction between plants and pathogens [59]. The element contents of crop plants may differ depending on the host plant and the type of plant pathogen [60].

Szőke et al. [52] showed that infection of sweet corn at the vegetative stage with a high amount of corn smut sporidia (10,000 sporidia/mL), under controlled conditions in the greenhouse negatively impacted the photosynthesis pigments and growth parameters. They suggested a follow-up study to establish the effects of corn smut at lower loadings. During the experiment, the two infection periods were simulated that are the most typical in corn cultivation. The first peak of infection mostly affects young corn plants, which are most characteristic during the period of development of mechanical damage caused by the frit fly or mechanical inter-row weeding cultivation. Another such period is the stage of the emergence of young corn cob initiation. The different sporidia concentrations are a good representation of the level of infection pressure, which may be related to the cultivation variants. The physiological changes that occur as a result of the infection are a good representation of the defense reactions and physiological changes of the individual corn plants. These changes in plant physiology are not only correlated with the amount of infectious sporidia material but also strongly depend on the type of crop (for example, sweet or fodder corn) and its phyto-phenological state of development. Therefore, the current research examined the effects of different concentrations (2500, 5000, and 10,000 sporidia/mL) of corn smut inoculum, on different morphological (plant height and stem diameter) and biochemical parameters (chlorophyll, protein, and MDA contents; the activities of SOD, APX, and POX) at the vegetative (V4–V5) and generative (V7) stages. The corn smut attacks the embryonic tissue when the tissue is already in the differentiation phase and smut is not able to infect during this stage, meaning that there will be no tumor formation.

The goal for including different infection times, which was not established by Szőke et al. [52], was to examine if there are any other roles of corn smut infection besides tumor formation (e.g., effects on quality and quantity). In addition, monosporidial inoculation does not cause tumor formation but has negative impacts on plant growth and several other physiological processes. At the V7 stage, measurements focused on cob parameters like cob length and diameter, kernel weight, 100 grains fresh and dry weight, element content of grains, and the quality characteristics (dry matter, fiber, fat, ash, nitrogen, and protein). The first goal of this study was to examine the tumor formation at the V4–V5 phenological stage because the first hypothesis was that there is no tumor formation at low (2500 sporidia/mL) inoculation, The second hypothesis of this research was that the corn smut infection negatively affects the morphological, physiological, biochemical, and quality parameters, as well as the element content and other quality characteristics of grains irrespective of the lower dosage. The third hypothesis was that the 10,000 sporidia/mL has more negative impacts on the measured parameters relative to the 2500 and 5000 sporidia/mL treatments. Furthermore, the goal was to examine which phenological stage (V4–5 or V7) is more susceptible to the corn smut infection.

## 2. Materials and Methods

### *2.1. Experimental Conditions*

The experiment was conducted under field conditions in the Demonstration Garden of the Institute of Plant Protection (47°33′07.7″ N 21°36′00.3″ E). The maize plants [Desszert 73 (Topcorn Ltd., Budapest, Hungary) and Noa (Rédei Kertimag Ltd., Réde, Hungary) sweet corn hybrids] were sown on 29 April 2021. The temperature was ideal for the growth of the maize and the corn smut proliferation because it was between 10 °C and 25 °C during the experiment (Figure 1).

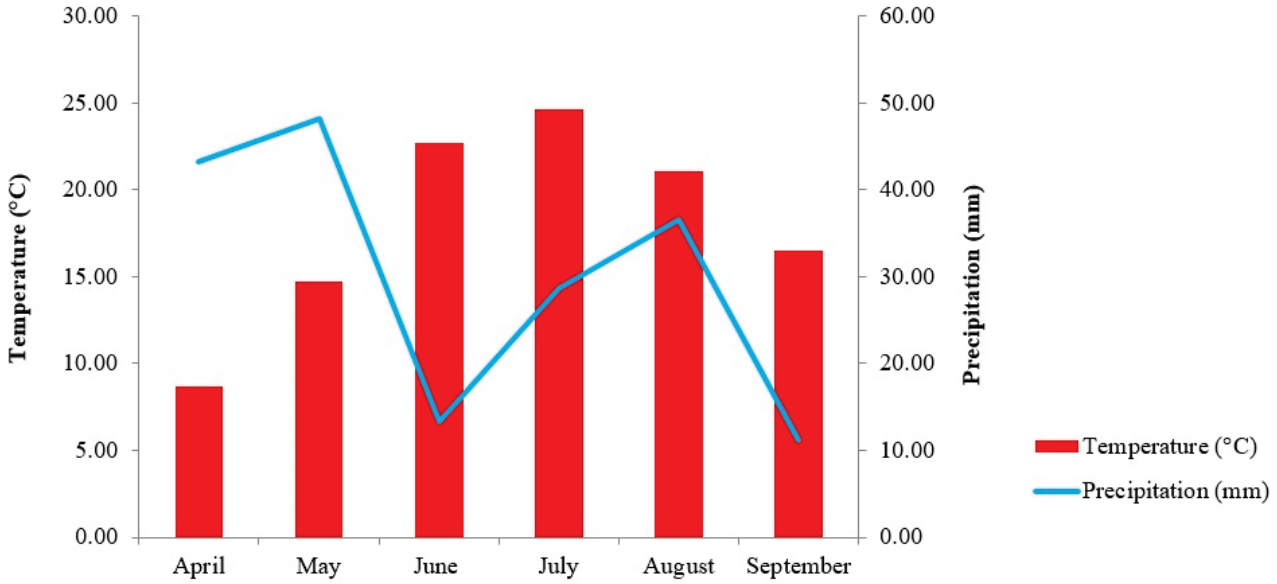


**Figure 1.** Temperature (°C) and precipitation (mm) during the experimental period. The data originates from the Institute of Plant Protection, the University of Debrecen's meteorological station (47°33′07.7″ N 21°36′00.3″ E).

A randomized complete block design was used as the experiment design. The size of the plots was 3 × 5 (vertical × horizontal) meters, and the soil disinfectant Bomber (tefluthrin) was sprayed into the rows in one pass with the sowing. A total of 4 corn rows/plots were sown with 25 plants per row. Plants were sown 5–6 cm deep, with 75 cm row spacing, and 20 cm seedling spacing. Plant irrigation was continuous, due to a drought watering took place every 2–3 days with 40–50 milliliters of water. Weed control was carried out by Principal Plus (nicosulfuron + dicamba + rimsulfuron) and insecticide treatment was carried out with Karate Zeon 5 CS (lambda-cyhalothrin). Fungicide was not used during the experiment.

The inoculum was created from the infected cobs using the method by Szőke and Tóth [61] under laboratory conditions at the laboratory of the Institute of Plant Protection, at the University of Debrecen. The infected corn cobs were collected from fields, and then the teliospores were sprayed on the corn smut-specific substrate. Then the pure culture was created. Next, the pathogen was replicated in the liquid-specific substrate and the dilution series (one -, ten -, one hundred -, one thousand -, ten thousand -, one hundred thousand-, and one million-fold) were made. Twenty-two strains were created and the first, seventh, and twelfth strains were compatible with each other. For the infection, the monosporidial strains were propagated on a liquid medium, grown for 48 h, and then mixed into a ratio of 1:1 before the inoculation. The cell numbers were set up to 2500, 5000, and 10,000 sporidia/$cm^3$ in the Burker chamber. The infection was carried out at the vegetative stage (4–5 leaf stage, 3 June 2021) and at the generative stage (at the beginning of cob development, 29 June 2021). The biochemical and morphological parameters were measured at 7 and 14 days after the pathogen infection (DAPI)—because the symptoms appeared 7 days after the pathogen infection, the plants perished more at 14 days than after the pathogen infection—and the mineral contents and quality parameters were determined at 21 days after the pathogen infection (DAPI). The tumors surfaced on cobs at 21 days after the pathogen infection (DAPI).

### *2.2. Amount of Photosynthetic Pigments*

The relative chlorophyll content was measured in the fourth leaf of sweet corn leaves with a SPAD-502+ Chlorophyll Meter (Minolta, Japan). The amount of the photosynthetic pigments was determined from the fourth leaves after the relative chlorophyll content mea-

surement by the method of Moran and Porath [62], which was reformed by Wellburn [63]. Fifty mg of fresh leaf sample was collected from the fourth leaf and dissolved in 5 mL N, N-dimethylformamide at 4 °C for 72 h. The absorbance of the extract was measured spectrophotometrically and adjusted to wavelengths of 470, 647, and 664 nm. (Nicolet Evolution 300 UV-Vis Spectrometer; Thermo Fisher Scientific, Waltham, MA, USA).

### *2.3. MDA Content Determination*

The MDA concentration was determined fluorometrically from the fifth leaf [64]. The leaf sample (0.1 g) was pounded and homogenized with 1 mL 0.25% (*w*/*v*) thiobarbituric acid (TBA) and 10% (*w*/*v*) trichloroacetic acid (TCA). After the sample was centrifuged at 10,800× *g* for 25 min at 4 °C, 0.3 mL, the supernatant was transferred into the Eppendorf tube which contains 0.7 mL 5% (*w*/*v*) TBA and 20% (*w*/*v*) TCA. After the vortexing, the mixture was heated in a thermoshaker (Bioshan TS-100) at 95 °C for 30 min before being immediately cooled on ice. The absorbance was taken at 532 and 600 nm (Nicolet Evolution 300 UV-VIS Spectrometer) and the concentration of MDA was determined with the use of 155 $mM^{-1}$ $cm^{-1}$ as extinction coefficient.

### *2.4. Activities of Antioxidant Enzymes*

The leaf samples were prepared using the method described by Pukacka and Ratahczak [65], and the activities of ascorbate peroxidase (APX) and guaiacol peroxidase (POX) were measured. Leaf samples (0.2 g) were frozen in liquid nitrogen and ground in a 1 mL 50 mM potassium phosphate buffer (pH 7.0) containing 2% (*w*/*v*) PVP, 1 mM ascorbate, 0.1% (*v*/*v*) Triton X-100, and 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 15,000× *g* for 20 min at 4 °C. The supernatant was collected and kept on ice until further processing. The activities of antioxidant enzymes were determined from the fifth leaves of the hybrids.

The APX activity was measured by the decrease in optical density due to ascorbic acid [66]. The final volume of the assay was 1 mL (550 µL of 50 mM potassium phosphate buffer (pH 7.0), 200 µL $H_2O_2$ (0.1 mM), 150 µL sodium ascorbate (0.5 mM), 50 µL EDTA (0.1 mM EDTA) and 50 µL sample extract). The ascorbate oxidation (decreased absorbance) was measured at 290 nm for 5 min at 20 °C compared to a blank that contained a phosphate buffer in place of the enzyme extract and the APX activity was calculated with the use of 2.8 $mM^{-1}$ $cm^{-1}$ as the extinction coefficient.

Zeislin and Ben-Zaken's [67] method was used for the determination of POX activity. A mixture of 50 µL 0.2 M $H_2O_2$, 100 µL 50 mM guaiacol, 340 µL purified water, 490 µL 80 mM phosphate buffer (pH 5.5), and 20 µL enzyme extract was created. The produced concentration of tetraguaiacol was used to calculate POX activity. The reaction compound's absorbance was measured at 470 nm for 3 min at 30 °C. In the blank, the sample extract was replaced with a 50 mM phosphate buffer. The extinction coefficient of 26.6 $mM^{-1}$ $cm^{-1}$ was utilized to estimate tetraguaiacol concentration.

The photochemical reduction of nitro blue tetrazolium chloride (NBT) was used to evaluate SOD activity [68]. The final volume of the assay was 4 mL containing 50 mM phosphate buffer (0.1 mM EDTA, 1% PVPP (*w*/*v*), 1 mM phenylmethylsulfonyl fluoride (PMSF) at pH 7.8). The homogenates were centrifuged at 10,000× *g* for 15 min. Twenty-five µL of plant extract, 25 µL of NBT (9 mM), 25 µL of riboflavin (0.25 mM), 250 L of methionine (0.16 M), and 2.675 mL of phosphate buffer (pH 7.8, 50 mM) were mixed and held at room temperature for 15 min. One SOD unit was defined as 50% inhibition of NBT. The absorbance was read at 560 nm. The blank contained 2.7 mL of phosphate buffer without plant extract.

### *2.5. Determination of Proline*

Ninhydrin and acetic acid were used for proline concentration measurement [69]. Fresh wheat leaves (0.1 g) were homogenized with liquid nitrogen before being mixed with 2 mL 70% (*v*/*v*) ethanol. The 1 mL reaction mix (1% ninhydrin in 60% (*v*/*v*) acetic acid)

was added to 500 µL of ethanolic extract, incubated at 95 °C for 20 min, cooled on ice, and centrifuged at 12,000× *g* for 1 min. The absorbance was read at 520 nm. Solutions of pure proline were used to calibrate the assays. The proline concentration was measured from the fifth leaves of the hybrids.

### *2.6. Measurement of Morphological Parameters*

A sliding caliper was used to measure the diameter of the stem between the second and third nodes.

The plant's height was measured from the peat surface to the tip of the youngest leaf.

The cob length was measured with a tape measure from the beginning of the cob to the end of the cob. The cob diameter was measured with a caliper before the corn shelling. After the shelling, 100 grains were counted, the kernel's weight was weighed fresh and after drying, the dry weight was subtracted from the fresh weight. Drying took place at 65 °C for 3 days in an exsiccator. After the sampling, the cob mass was weighed on an analytical scale; in the case of infected plants, the infected cob mass was first weighed, then after the tumors were removed, the tumors were weighed, and the tumor's weight was subtracted from the cob's weight.

### *2.7. Measurement of the Quality Parameters of Grains*

The quality parameters of the grains were determined by the method of Csapó et al. [70]. Nitrogen and protein content was determined by the method of Kjeldahl. The 5 g corn grain samples were digested at 420 °C in sulphuric acid ($H_2SO_4$) for 30 min. The end of digestion was indicated by the discoloration of the solution. Potassium sulfate ($K_2SO_4$) was then added, and ammonium sulfate ($NH_4$) was formed. The volatile ammonia formed was removed from the solution by steam distillation, and the distillate was collected in 0.1 M hydrochloric acid (HCl). It was then titrated with 0.2 M NaOH until the color changed to red.

The nitrogen and protein contents were calculated by these formulae:

$$\text{nitrogen\%} = \frac{(\text{S} - \text{L}) \times 0.0028016 \times 100}{\text{b}} \quad (1)$$

S = amount of 0.1 M $H_2SO_4$ (mL) in the volumetric flask.
L = amount of 0.2 M NaOH (mL) consumed for the back titration of sulphuric acid.
B = Sample weight (g).
0.0028016 = nitrogen content which is corresponding to 1 mL of 0.1 M sulphuric acid quantity (g).

$$\text{protein\%} = \text{nitrogen\%} \times 6.25 \quad (2)$$

6.25 = conversion factor.

To determine the ash content, 5 g of corn grain was measured and placed in the ashing crucible (which was measured before taking the plant sample) and placed in the drying oven (with a temperature of 550 °C) for 3 h. Then it was cooled in the desiccator and then weighed. The ash content was calculated with this formula:

$$\text{ash\%} = \frac{m1 - m2}{m0} \times 100 \quad (3)$$

$m_0$ = the plant sample weight (g).
$m_1$ = the plant sample and ashing crucible weight (g).
$m_2$ = the ashing crucible weight (g)

To determine the fiber content, 2 g cob grain was weighed, and 150 mL distilled water and 50 mL 0.51 M sulphuric acid were added and boiled for 30 min. Then 50 mL of cold distilled water was added, allowed to cool to room temperature, then filtered through a

silk sieve. The residue was washed with warm water to make it an acid-free solution, then returned to the beaker. After 50 mL potassium hydroxide was added and it was boiled for 30 min and washed again with 50 mL of cold distilled water. The residue was filtered on filter paper (which was dried at 105 °C for one hour and the weight was measured before the filtering) (B). Then the filter paper with residue was washed with acetone and dried at 105 °C for between five and eight hours. Then it was cooled in a desiccator and the weight was measured (A). The A-B is the fiber that contains ash. Then the fiber ash was weighed, first weighed the heated porcelain dish (D) and placed the filter paper containing the fiber in it. Then, it was cremated in a muffle furnace at 550 °C for three hours. It was taken to the desiccator and the weight was measured (C). The fiber content is calculated by the following formula:

$$\text{fiber\%} = \frac{a - b}{m} \times 100 \quad (4)$$

*a*: the fiber which contains ash weight (g), A–B.
*b*: the ash weight (g), C–D.
*m*: the sample weight (g).

To calculate the dry matter of the kernels, 5 g grain was measured and put in the porcelain dish, which was taken into the drying oven set to 105 °C for 4 h, and weighed. After the plant samples were transferred into the porcelain dish and it was put into the drying oven set to 105 °C for 24 h and weighed. The dry matter content is calculated by the following formula:

$$\text{dry matter\%} = \text{A} - \text{B} * 100 \quad (5)$$

A: Dried porcelain dish weight (g) with plant samples.
B: Dried porcelain dish weight (g) without plant samples.

To determine crude fat content, 5 g of grain was measured in a fat-free extraction tube and the tube was sealed with fat-free absorbent cotton. The tube containing the sample to be analyzed was placed in the middle part of the extraction apparatus and then connected to the flask that contained 4–5 pieces coarse gravel, pre-weighed ($m^2$), and filled with n-hexane or petroleum ether filled to 3/4 full. After installing the cooler, the samples were extracted for 6 h by heating them so that the sample would only contact fresh solvent (at least ten times per hour). After six hours of extraction, the tube with the sample was removed and the solvent in the flask was distilled into the middle part while being continuously removed from there. The flasks with the fat and solvent residues were placed in the drying oven at a temperature of 98 °C for one hour, then cooled in an exsiccator and weighed. The raw fat content is calculated by the following formula:

$$\text{crude fat\%} = \frac{m1 - m2}{m0} \times 100 \quad (6)$$

$m_0$: the sample weight (g).
$m_1$: the flask, pumice stone, and dried sample weight (g).
$m_2$: the flask and pumice stone weight (g).

### *2.8. Measurement of Mineral Elements Concentration*

The collected corn kernels were dried at 30 °C for four days, then 1 g sample was measured and ground. Fifteen ml $HNO_3$ (65% *v*/*v*) was added to 1 g of the sample and incubated overnight at room temperature. The materials were then pre-digested for 30 min at 60 °C. Finally, 5 mL $H_2O_2$ (30 m/m%) was added for 270 min while the solution was boiled at 120 °C. The solutions were prepared to a volume of 50 mL, homogenized, and filtered using Filtrak 388 filter paper. Analytical grade $HNO_3$ (65%) solution (manufactured by Merck) was utilized for solution preparation. Merck solutions, as well as analytically pure compounds, were used in the manufacturing of basic solutions.

An OPTIMA 3300 DV type ICP-OES was used for the analytical definition [71].

### *2.9. Method of Statistical Analysis*

The IBM SPSS Statistics 25 software (Armonk, NY, USA) was used for statistical analysis. The normality of data was determined by Kolmogorov—Smirnov and Shapiro—Wilk tests [72]. One-way and three-way ANOVA [73] were used for the analysis, and the means were compared by the Tukey-HSD test [74] at a significance level of 5% ($p \leq 0.05$) because of the normal distribution of data. The number of replicates was five per treatment per investigated parameter. The statistical difference was labeled by small characters (a, b, c, d, e, and f) in the text. For cluster analysis, the samples were standardized with the descriptive group. Thus, we obtained the Z values needed for the cluster analysis. Then the K-means cluster analysis was performed based on the treatments with the Z values and one-way ANOVA was carried out for the evaluation of the significant differences among the created clusters. The Pearson correlation also was performed for linear correlation that measures the strength and direction of the relationship between two variables.

## 3. Results

Three-way ANOVA was performed to evaluate the significance of the independent variables [hybrid (H), treatment (T) as inoculum concentration, and sampling time (S)] separately and their interactions. The hybrid impact was significant for Chl-b, MDA, APX, POX, plant height, and stem diameter. Treatment impacts were significant for measured characteristics except for chlorophyll-b and carotenoids. Sampling time also had substantial effects on all physiological parameters (SPAD, MDA, APX, POX, SOD, proline, plant height, and stem diameter), except Chl-*a*, -*b*, and carotenoids. There were significant interactions between the hybrid and the treatment for Car, MDA, APX, SOD, and plant height. The interaction between hybrid and sampling time was significant for Chl-*a*, MDA, POX, plant height, and stem diameter. Significant interactions were observed between treatment and sampling time for MDA, APX, POX, SOD, proline, and plant height. The three-way interaction (hybrid x treatment x sampling time) only had a significant impact on Chl-*a*, -*b*, Car, APX, proline, plant height, and stem diameter (Table 1).

**Table 1.** Combined analysis of variance for measured parameters of two sweet corn cultivars with three treatments over two sampling times.

| | Hybrid (H) | Treatment (T) | Sampling Time (S) | HxT | HxS | TxS | HxTxS |
|---|---|---|---|---|---|---|---|
| SPAD | 2.693 | 732.922 * | 84.791 * | 9.333 | 4.18 | 58.41 | 23.831 |
| Chl-*a* | 3.158 | 2.158 * | 1.589 | 16.52 | 11.150 * | 7.119 | 7.1331 * |
| Chl-*b* | 4.335 * | 2.778 | 4.598 | 12.891 | 14.516 | 11.010 | 8.987 * |
| Car | 5.112 | 1.889 | 5.115 | 20.150 * | 12.260 | 9.116 | 7.668 * |
| MDA | 799.322 * | 4257.701 * | 14,799.288 * | 3119.895 * | 2648.025 * | 2204.624 * | 232.441 |
| APX | 0.219 * | 1.497 * | 0.116 * | 0.031 * | 0.007 | 0.054 * | 0.020 * |
| POX | 0.020 * | 0.536 * | 0.265 * | 0.001 | 0.028 * | 0.031 * | 0.011 |
| SOD | 0.597 | 28.101 * | 3.135 * | 0.930 * | 0.037 | 0.967 * | 0.084 |
| Proline | 0.013 | 0.001 * | 0.078 * | 0.003 | 0.001 | 0.001 * | 0.001 * |
| Height | 78,375.168 * | 87,860.924 * | 8455.813 * | 913.530 * | 1391.294 * | 10,121.012 * | 2547.550 * |
| Diameter | 374.978 * | 165.483 * | 107.648 * | 1.041 | 107.648 * | 0.919 | 0.919 |

The data were evaluated by three-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by * $p \leq 0.05$. SPAD = relative chlorophyll content, Chl-*a*: chlorophyll-*a*, Chl-*b*: chlorophyll-*b*, Car: carotenoids, MDA: malondialdehyde, APX: ascorbate peroxidase, POX: guaiacol peroxidase, SOD: superoxide dismutase, Height: plants height, Diameter: stem diameter.

The applied fungal treatments (2500, 5000, and 10,000 sporidia/mL) significantly reduced the SPAD-unit ($p < 0.05$) (40%, 52%, and 57%, respectively) in the Desszert 73 and Noa hybrid (37, 53, and 42%, respectively) at 7 DAPI. Similarly, these fungal treatments substantially ($p < 0.05$) reduced relative chlorophyll contents (61%, 69%, and 65% in Desszert 73; and 110%, 122%, and 113% in Noa hybrid) at 14 DAPI (Figure 2).

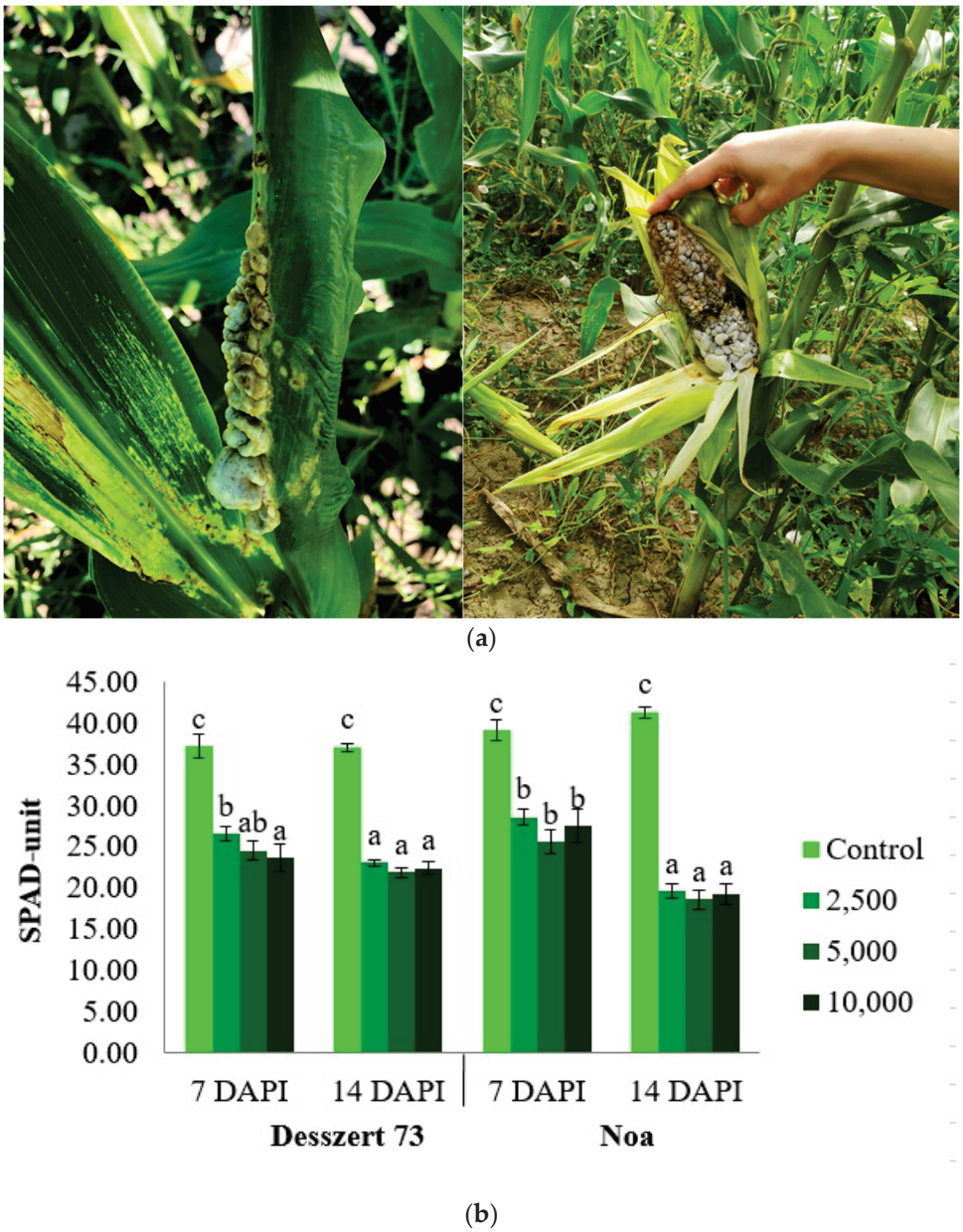


**Figure 2.** (**a**) Corn smut infected leaves and cob at 10,000 sporidia/mL on Desszert 73 hybrid. (**b**) The relative chlorophyll content (SPAD-unit) of the four leaves of corn smut infected Desszert 73 and Noa hybrids at 7 and 14 DAPI (mean ± SD, n = 25). The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b, and c). DAPI: days after the pathogen infection.

Since the relative chlorophyll content is an index, the amounts of photosynthetic pigments (chlorophyll-*a*, chlorophyll-*b*, and carotenoids) were also measured. The increased spore concentration (2500, 5000, and 10,000 sporidia/mL) had significant effects on chlorophyll-*a* in both hybrids at both sampling times. Chlorophyll-*a* content of Desszert 73 and Noa significantly decreased ($p < 0.05$) when infected by the three corn smut concentrations (87%, 127%, and 146% in Desszert 73, and 79%, 75%, and 106% in Noa) at 7 DAPI compared to the control plants. At the second sampling time (14 DAPI), the reductions were 254%, 286%, and 316% for Desszert 73 and 127%, 160%, and 167% for Noa, respectively. Noa had the most significant reduction when the highest sporidium treatment was applied (means for at 7 and 14 DAPI at 10,000 sporidia/mL followed by different letters) (Figure 3A).

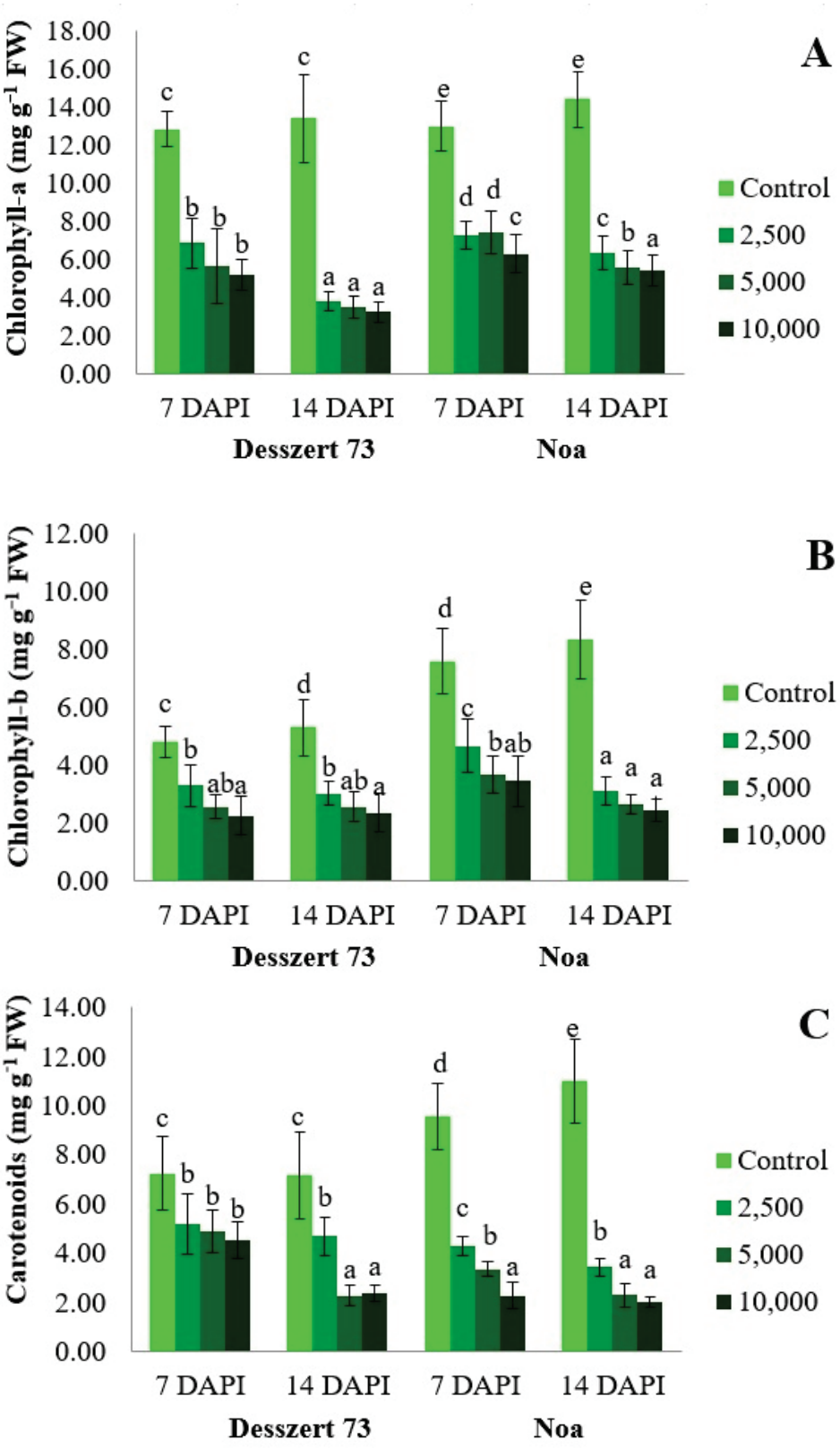


**Figure 3.** (**A**) The effects of corn smut infection on chlorophyll-*a* (mg g$^{-1}$ FW) (mean ± SD, n = 5), (**B**) The effects of corn smut infection on chlorophyll-*b* (mg g$^{-1}$ FW) (mean ± SD, n = 5), (**C**) The effects of corn smut infection on carotenoids (mg g$^{-1}$ FW) (mean ± SD, n = 5) of the fourth leaves of Desszert 73 and Noa hybrids at 7 and 14 DAPI. The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b, c, d, and e). DAPI: days after the pathogen infection, FW: fresh weight.

Corn smut infection also had negative effects on the amounts of chlorophyll-*b* in the Desszert 73 and Noa hybrids ($p < 0.05$). Increased concentration of sporidia diminished chlorophyll-*b* by 46%, 89%, and 116% in Desszert 73 and 63%, 107%, and 121% in Noa compared to the control plants at 7 DAPI. Furthermore, the reduction was 76%, 108%, and 127% in Desszert 73 and 170%, 214%, and 244% in Noa hybrids at 14 DAPI (Figure 3B).

The carotenoid content was reduced at 7 DAPI by 40% ($p = 0.016$) and 123% ($p = 0.006$) in the Desszert 73 and Noa hybrids treated 5000 sporidia/mL; 49% ($p = 0.023$) and 185% ($p = 0.006$) in Desszert 73 and Noa hybrids treated with 10,000 sporidia/mL. Similarly, at 14 DAPI, carotenoid content was also significantly reduced under 2500, 5000, and 10,000 sporidia/mL in Desszert 73 [53% ($p = 0.013$), 222% ($p = 0.005$), and 216% ($p= 0.004$)] and Noa [380% ($p = 0.000$), 203% ($p = 0.030$), and 450% ($p = 0.000$] (Figure 3C).

The respective corn smut treatments significantly increased ($p \leq 0.001$) the MDA content of Desszert 73 and Noa hybrids' leaves (140%, 181%, 224% and 194%, 215%, and 280% reduction) at 7 DAPI. The MDA content was significantly ($p \leq 0.001$) increased at 14 DAPI in Desszert 73 and Noa infected with the three concentrations of corn smut (147%, 191%, and 260% increase; 205%, 242%, and 282% increase) in the infected plants due to the different concentration of the inoculum at 14 DAPI (Figure 4).

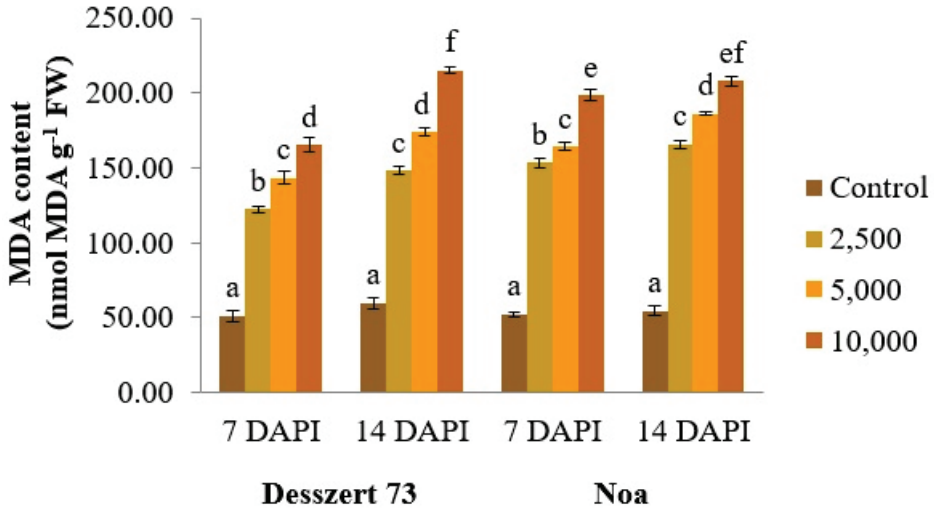


**Figure 4.** The MDA content (nmol MDA $g^{-1}$ FW) of the fifth leaves of corn smut infected Desszert 73 and Noa hybrids at 7 and 14 DAPI (mean ± SD, n = 5). The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b, c, d, e, and f). DAPI: days after the pathogen infection. FW: fresh weight, MDA: malondialdehyde.

The APX activity in the leaves of Desszert 73 and Noa hybrids significantly ($p \leq 0.001$) increased with infection intensity (100%, 132%, 147%, and 67%, 96%, and 119%, respectively, significant differences under 2500, 5000, and 10,000 sporidia/mL) for Desszert and Noa hybrids at 7 DAPI. Similarly, all applied treatments increased APX activity in Desszert 73 and Noa hybrids (124%, 157%, 186%, 115%, 188%, and 192%, respectively) compared to the control plants ($p \leq 0.000$) at 14 DAPI (Figure 5).

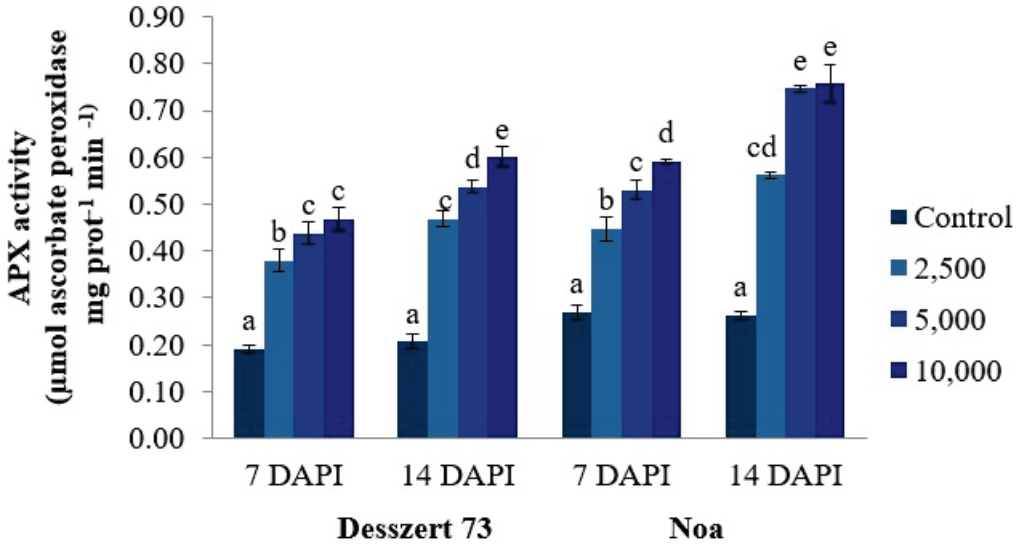


**Figure 5.** The effect of corn smut infection on the APX activity (µmol ascorbate peroxidase $min^{-1}$ mg $prot^{-1}$) of the fifth leaves of Desszert 73 and Noa hybrids at 7 and 14 DAPI (mean ± SD, n = 5). The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b, c, d, and e). DAPI: days after the pathogen infection.

The POX activity in the leaves of the infected two hybrids was also elevated due to the difference in sporidia of corn smut at both sampling dates. The POX activity increased substantially ($p \leq 0.001$), at 7 DAPI, for corn smut infected (2500, 5000, and 10,000 sporidia/mL) Desszert 73 (118%, 159%, and 212%) and Noa (76%, 81%, and 110%). At the second sampling (14 DAPI), POX increased by 207%, 287%, and 333% ($p \leq 0.001$) in Desszert 73 and 83%, 178%, and 243% ($p \leq 0.001$) in the Noa hybrid (Figure 6). The increased concentration of corn smut (2500, 5000, and 10,000) induced higher SOD activity in Desszert 73 (1.82; 2.09 and 2.40 times higher; $p \leq 0.001$) and Noa (1.75; 2.51 and 3.08 times higher; $p \leq 0.001$) hybrids at 7 DAPI. At 14 DAPI, this activity was still significantly ($p \leq 0.001$) high 2.89; 3.84, and 4.02 times higher in Desszert 73; 1.49; 3.05, and 3.63 times higher in Noa (Figure 7).

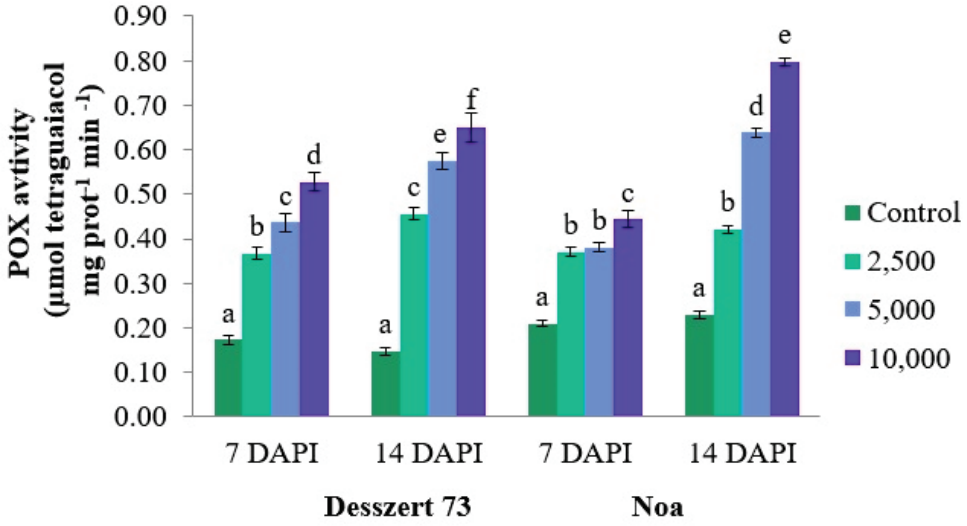


**Figure 6.** The effect of corn smut infection on the POX activity (µmol tetraguaiacol $min^{-1}$ mg $prot^{-1}$) of the fifth leaves of Desszert 73 and Noa hybrids at 7 and 14 DAPI (mean ± SD, n = 5). The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b, c, d, e, and f). DAPI: days after the pathogen infection.

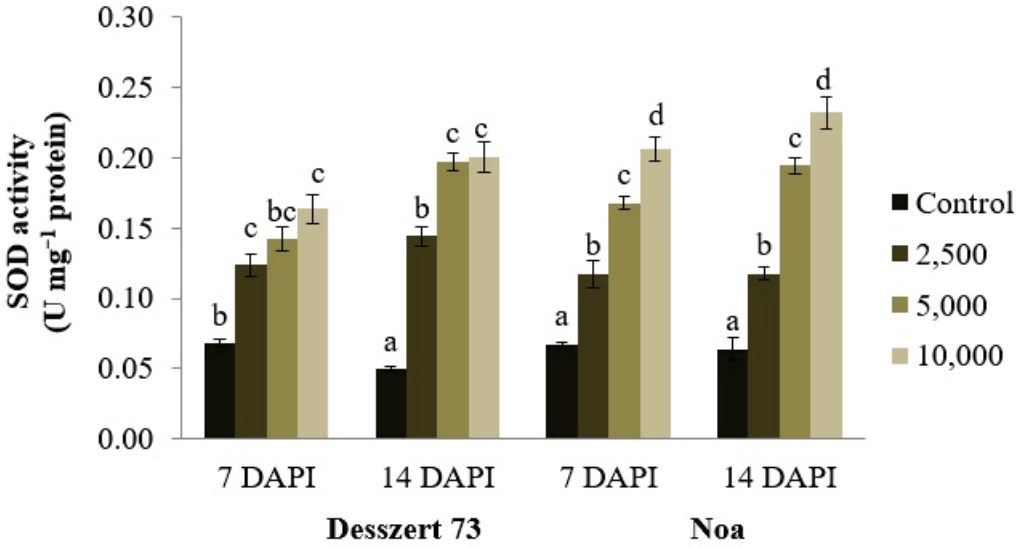


**Figure 7.** The effect of corn smut infection on the SOD activity (U mg$^{-1}$ protein) of the fifth leaves of Desszert 73 and Noa hybrids at 7 and 14 DAPI (mean ± SD, n = 5). The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b, c, and d). DAPI: days after the pathogen infection.

Corn smut infection did not have a constant effect on the proline concentration in the leaves of sweet corn hybrids. At the first sampling time (7 DAPI), there were no significant differences in proline across different sporidia at Desszert 73. This, however, increased significantly at 5000 sporidia/mL at 14 DAPI in Desszert 73. Proline was significantly lower in Noa infected with 5000 and 10,000 sporidia/mL (15% and 18%, $p \leq 0.050$). At 14 DAPI, proline content was not significantly affected by the different sporidia in Noa (Figure 8).

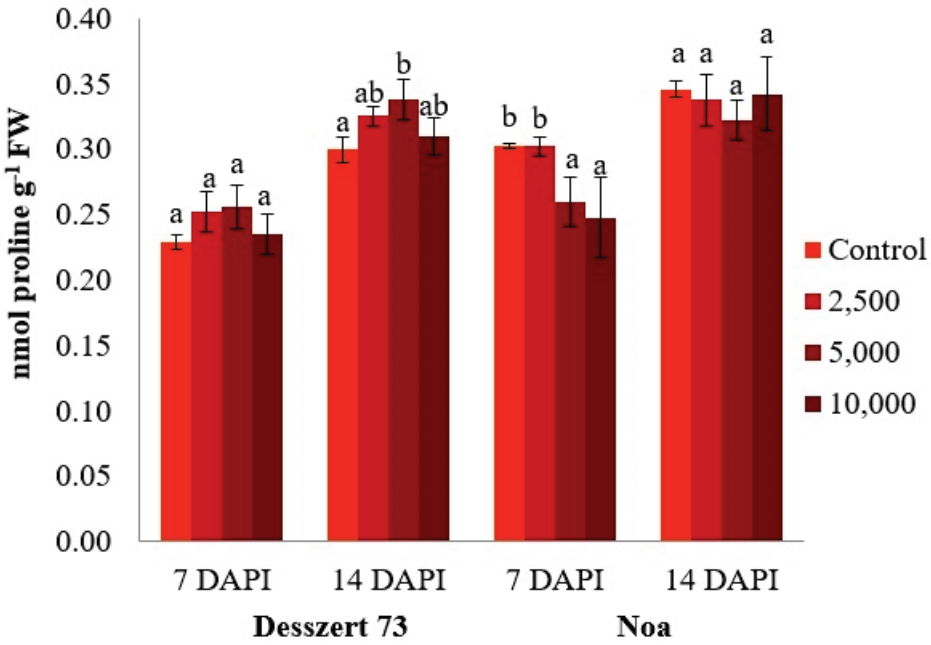


**Figure 8.** The proline concentration (nmol proline g$^{-1}$ FW) of the fifth leaves of corn smut infected Desszert 73 and Noa hybrids at 7 and 14 DAPI (mean ± SD, n = 5). The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, and b). DAPI: days after the pathogen infection, FW: fresh weight.

The K-mean cluster analysis created three groups (cluster 1, cluster 2, and cluster 3). It compares the clusters (groups) to an average. Where the values are positive the cluster is above the average, where the values are negative the cluster is below the average. For chlorophyll-*a*, the first and second groups were above average, and the third group was below average. For chlorophyll-*b*, groups one and three are below average, and group two is above average. For carotenoids, the first group (cluster) is above average, the second and third are below average. For MDA, APX, and POD, the first and second clusters are below average, and the third is above average. At SOD, the first and third groups are below average, and the second is above average. For proline, the first group is above average, and the second and third are below average (Figure 9).

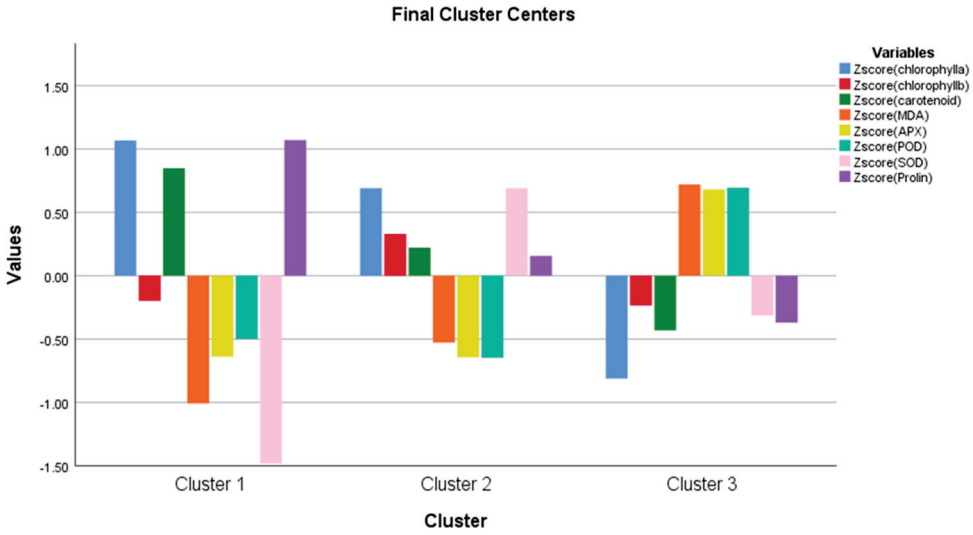


**Figure 9.** K-mean cluster analysis for chlorophylls (chlorophyll-*a*, and *b*), carotenoids, malondialdehyde (MDA), ascorbate peroxidase (APX), guaiacol peroxidase (POD), superoxide dismutase (SOD), and proline.

The one-way ANOVA analysis shows that there is a significant difference among the clusters presented in Figure 9 (Table 2).

**Table 2.** One-way ANOVA analysis for the three clusters created with K-mean clusters for chlorophylls (chlorophyll-*a*, and *b*), carotenoids, malondialdehyde (MDA), ascorbate peroxidase (APX), guaiacol peroxidase (POD), superoxide dismutase (SOD), and proline, df: degree of freedom.

| | Cluster | | Error | | F | Sig. |
|---|---|---|---|---|---|---|
| | Mean Square | df | Mean Square | df | | |
| Zscore (chlorophyll*a*) | 24.716 | 2 | 0.394 | 75 | 62.765 | 0.000 |
| Zscore (chlorophyll*b*) | 2.939 | 2 | 0.948 | 75 | 3.099 | 0.051 |
| Zscore (carotenoid) | 7.162 | 2 | 0.814 | 75 | 8.797 | 0.000 |
| Zscore (MDA) | 18.332 | 2 | 0.525 | 75 | 34.932 | 0.000 |
| Zscore (APX) | 17.036 | 2 | 0.598 | 75 | 28.475 | 0.000 |
| Zscore (POD) | 16.822 | 2 | 0.577 | 75 | 29.141 | 0.000 |
| Zscore (SOD) | 18.260 | 2 | 0.546 | 75 | 33.471 | 0.000 |
| Zscore (Proline) | 7.567 | 2 | 0.844 | 75 | 8.970 | 0.000 |

Chlorophyll-*a* had a significant positive correlation with carotenoids and proline, and a negative correlation with MDA, APX, and POX. Chlorophyll-*b* was negatively correlated with APX and POX. Carotenoids were negatively correlated with MDA. MDA was negatively correlated with SOD and proline and strongly positively correlated with APX and POX. There was a strong positive correlation between APX and POX. In addition, there was a significant negative correlation between POX and SOD (Table 3).

**Table 3.** Correlations based on the average values for chlorophyll-*a*, and *b* (Chl-*a*, Chl-*b*), carotenoids (Car), malondialdehyde (MDA), ascorbate peroxidase (APX), guaiacol peroxidase (POD), superoxide dismutase (SOD), and proline for all treatments combined.

| | **Chl-*a*** | **Chl-*b*** | **Car** | **MDA** | **APX** | **POX** | **SOD** | **Proline** |
|---|---|---|---|---|---|---|---|---|
| Chl-*a* | 1 | ns | 0.673 ** | −0.679 ** | −0.479 ** | −0.421 ** | ns | 0.412 ** |
| Chl-*b* | ns | 1 | ns | ns | −0.291 ** | −0.291 ** | ns | ns |
| Car | 0.673 ** | ns | 1 | −0.382 ** | ns | ns | ns | ns |
| MDA | −0.679 ** | ns | −0.382 ** | 1 | 0.562 ** | 0.424 ** | −0.226 * | −0.691 ** |
| APX | −0.479 ** | −0.291 ** | ns | 0.562 ** | 1 | 0.802 ** | −0.180 | −0.243 * |
| POX | −0.421 ** | −0.291 ** | ns | 0.424 ** | 0.802 ** | 1 | −0.246 * | ns |
| SOD | ns | ns | ns | −0.226 * | ns | −0.246 * | 1 | ns |
| Proline | 0.412 ** | ns | ns | −0.691 ** | −0.243 * | ns | ns | 1 |

* correlation is significant at the 0.05 level (2-tailed) ($p$ = 5%), ** correlation is significant at the 0.01 level (2-tailed) ($p$ = 1%), ns: not significant.

The effects of the corn smut infection on the host plants' morphological parameters (plant height and stem diameter) were also investigated. Plant height was reduced in both hybrids by all treatments for corn smut infection. Desszert 73 plant height was reduced by 21%, 31%, and 24%, and that of Noa was reduced by 21%, 23%, and 29% due to the 2500, 5000, and 10,000 sporidia number of corn smut infection at 7 DAPI. Similarly, in the second sampling (14 DAPI), the corn smut treatments (in their increasing order) decreased plant height of Desszert 73 (45%, 60%, and 67%) and Noa (23%, 37%, and 38%) (Table 4).

**Table 4.** Plant height (mm) and stem diameter (mm) of corn smut infected Desszert 73 and Noa hybrids at 7 and 14 DAPI (mean ± SD, n = 5).

| | **Plant Height** | | | | **Stem Diameter** | | | |
|---|---|---|---|---|---|---|---|---|
| | Desszert 73 | | Noa | | Desszert 73 | | Noa | |
| | 7 DAPI | 14 DAPI | 7 DAPI | 14 DAPI | 7 DAPI | 14 DAPI | 7 DAPI | 14 DAPI |
| Control | 453 ± 17.08 b | 640 ± 18.26 c | 544 ± 15.17 b | 647 ± 5.77 c | 22.92 ± 1.44 a | 27.36 ± 0.77 b | 25.34 ± 0.85 a | 28.82 ± 0.94 b |
| 2500 | 375 ± 19.15 ab | 445 ± 19.15 b | 450 ± 10 a | 528 ± 12.58 b | 26.26 ± 0.70 b | 31.84 ± 0.81 c | 27.88 ± 0.84 ab | 33.66 ± 1.46 c |
| 5000 | 345 ± 12.91 a | 403 ± 15.28 ab | 444 ± 23.02 a | 473 ± 20.82 a | 28.42 ± 0.75 b | 31.94 ± 1.11 c | 28.18 ± 0.95 b | 34.92 ± 0.93 c |
| 10,000 | 365 ± 12.91 a | 383 ± 9.57 a | 422 ± 13.04 a | 470 ± 15.81 a | 28.2 ± 0.70 b | 33.94 ± 1.09 c | 29.58 ± 0.68 b | 35.72 ± 0.91 c |

The data were evaluated by One-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b, and c). DAPI: days after the pathogen infection.

As a result of the increasing corn smut infection (2500, 5000, and 10,000 sporidia/mL), both hybrids had increased stem diameter (15%, 24%, and 23% increase for Desszert 73; 10%, 11%, and 17%, increase for Noa). Stem diameter for Desszert 73 and Noa also increased significantly with sporidium treatment (control means represented by different letters) (Table 3).

Figure 10 shows that the plant height's first and second clusters are below the average, while the third is above average. Regarding the stem diameter, the first and third clusters are below the average, and the second is above the average. Based on the one-way ANOVA analysis, there is a significant difference among the three clusters (data are not shown). Furthermore, there is no significant correlation between plant height and stem diameter according to the Pearson correlation (results are not shown).

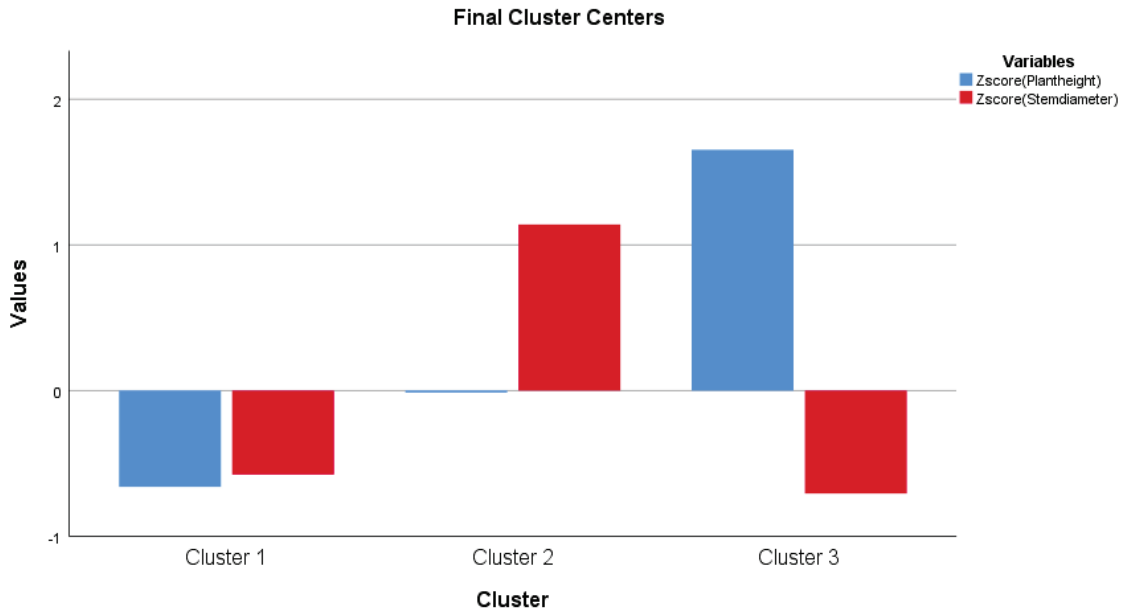


**Figure 10.** K-mean cluster analysis for plant height and stem diameter.

The negative effects of corn smut infection were also observed at the generative stage. Only the 10,000 sporidia/mL of corn smut caused symptoms (tumor growth) on the cobs of both hybrids at 21 DAPI. This infection significantly reduced the cob length in Desszert 73 and Noa (19% and 30%, $p \leq 0.050$) at 21 DAPI. The cob diameter was not affected by the corn smut infection. The 10,000 sporidia/mL infected plants had significantly lower kernel weights (41% reduction in Desszert 73, and 18% reduction in Noa hybrid; $p \leq 0.050$) at 21 DAPI. The 100 grains fresh weight was significantly reduced in the Desszert 73 and Noa hybrids' infected plants (by 21% and 17% compared to the control plants; $p \leq 0.050$) by the corn smut infection (10,000 sporidia/mL) at 21 DAPI. The 100 grains' dry weight was also reduced by 51% and 59% ($p \leq 0.050$) in both hybrids (Table 5).

**Table 5.** The effect of the corn smut infection on cob length (cm), cob diameter (cm), kernel weight (g), 100 grains fresh weight (g), and 100 grains dry weight (g) (mean ± SD, n = 5) of Desszert 73 and Noa hybrids at 21 DAPI.

| | **Desszert 73** | | **Noa** | |
|---|---|---|---|---|
| | **Control** | **Infected** | **Control** | **Infected** |
| Cob length (cm) | 21.34 ±1.31 b | 17.32 ± 3.33 a | 23.59 ± 0.19 b | 16.51 ± 0.99 a |
| Cob diameter (cm) | 4.40 ± 0.45 b | 3.62 ± 0.41 a | 4.68 ± 0.16 a | 3.72 ± 0.18 a |
| Kernel weight (g) | 275.81 ± 32.07 b | 161.42 ± 29.44 a | 311.80 ± 24.43 b | 259.30 ± 12.22 a |
| 100 grains fresh weight (g) | 42.69 ± 1.31 b | 34.42 ± 3.30 a | 45.46 ± 4.01 b | 37.92 ± 1.04 a |
| 100 grains dry weight (g) | 9.04 ± 1.15 b | 4.39 ± 0.72 a | 7.99 ± 0.27 b | 3.29 ± 0.15 a |

The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, b).

The cluster analysis shows that the 100 grains' fresh weight is above the average, while every other kernel parameters are below the average in the first cluster. While the cob length is above the average in the second, and all of the kernel parameters are above the average in the third cluster (Figure 11). In addition, the one-way ANOVA analysis shows that there is a significant difference among the clusters presented in Figure 11 (Table 6).

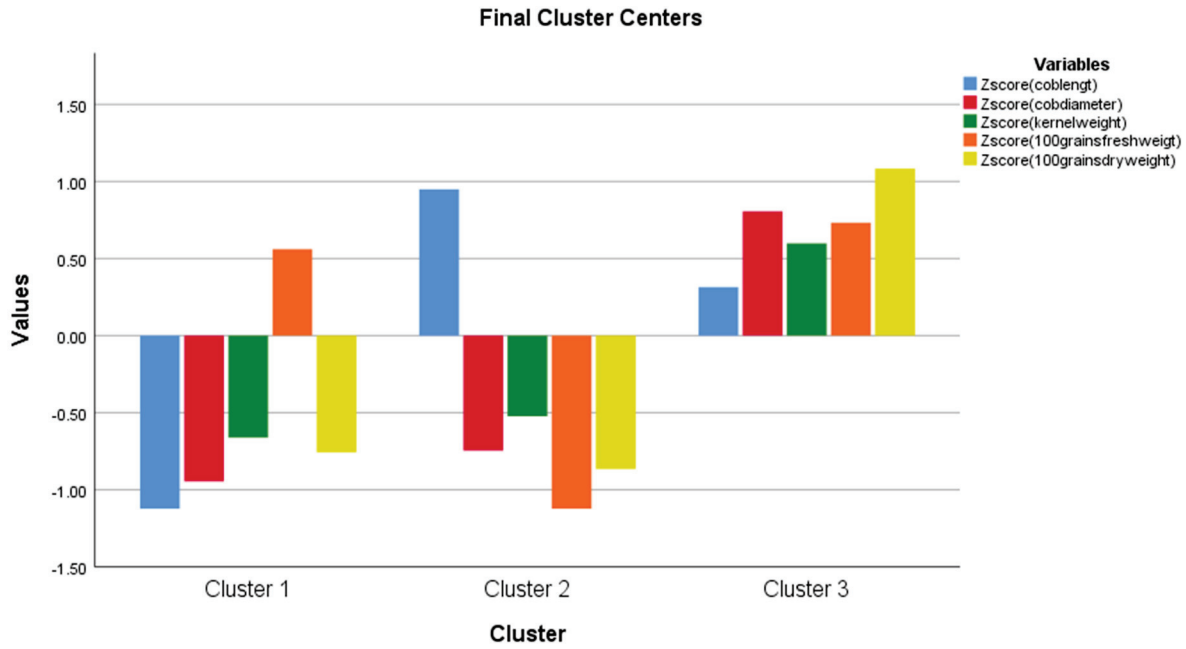


**Figure 11.** K-mean cluster analysis for cob length, cob diameter, kernel weight, 100 grains fresh and dry weight.

**Table 6.** One-way ANOVA analysis for the three clusters created with K-mean clusters for cob length, cob diameter, kernel weight, 100 grains fresh and dry weight.

| | Cluster Mean Square | df | Error Mean Square | df | F | Sig. |
|---|---|---|---|---|---|---|
| Zscore (coblenght) | 2.865 | 2 | 0.610 | 7 | 4.697 | 0.051 |
| Zscore (cobdiameter) | 3.170 | 2 | 0.150 | 7 | 21.077 | 0.001 |
| Zscore (kernelweight) | 1.650 | 2 | 0.233 | 7 | 7.067 | 0.021 |
| Zscore (stograinweigt) | 3.900 | 2 | 0.685 | 7 | 5.693 | 0.034 |
| Zscore (stograinsusa) | 4.395 | 2 | 0.032 | 7 | 136.657 | 0.000 |

The Desszert 73 and Noa corn smut-infected kernel had lower dry matter (10% and 5%), crude fat (9% and 11%), and protein (13% and 15%) ($p \leq 0.050$) contents compared to the control, non-infected. The infection did not affect crude fiber, crude ash, and nitrogen contents (Figure 12).

The K-mean cluster analysis created three groups (cluster 1, cluster 2, and cluster 3). The dry matter, crude fiber, crude fat, and protein are below the average at the first cluster, while these are above the average at the third cluster. For nitrogen, the first and third clusters are above the average, and the second is below the average (Figure 13). There are significant differences among the clusters based on one-way ANOVA (results are not shown).

Infection reduced the Mg and Mn content in both hybrids kernel compared to the control (15% and 25% in Desszert 73 and 8% and 15% in Noa hybrid, respectively; $p \leq 0.050$). The concentration of Al, Ca, and S were significantly increased by 182% ($p \leq 0.000$), 12% ($p = 0.038$), and 7% ($p = 0.045$) in Desszert 73 and by 105% ($p = 0.002$), 8% ($p = 0.045$), and 14% ($p = 0.041$), in the Noa hybrid, respectively, due to the corn smut infection. The infected kernel of Desszert 73 had lower level of B, P, and Zn by 11%, 12%, and 6% ($p \leq 0.050$), respectively, compared to the control, non-infected. Corn smut infection significantly decreased K concentration (5% reduction; $p \leq 0.050$) but increased the Na concentration (13% increase; $p \leq 0.050$) in the Noa hybrid's kernel (Table 7).

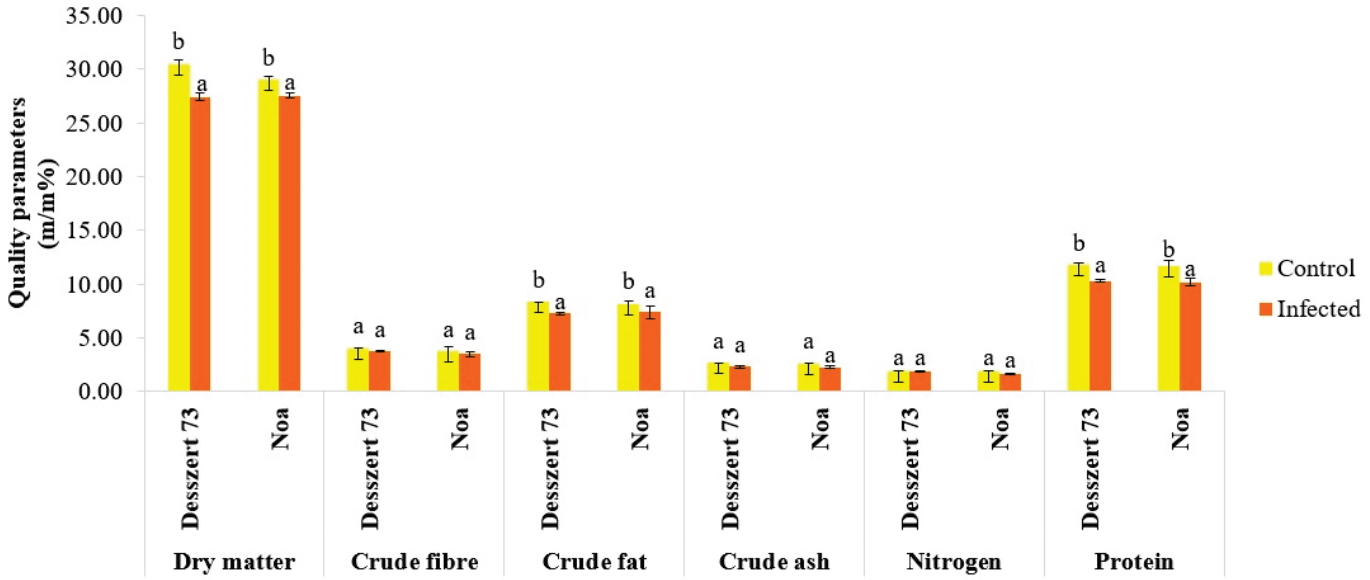


**Figure 12.** The effects of corn smut infection on the quality parameters of the kernel (*m/m*%) of Desszert 73 and Noa hybrids at 21 DAPI (mean ± SD, n = 5). The data were evaluated by one-way ANOVA followed by the Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, and b). DAPI: days after the pathogen infection.

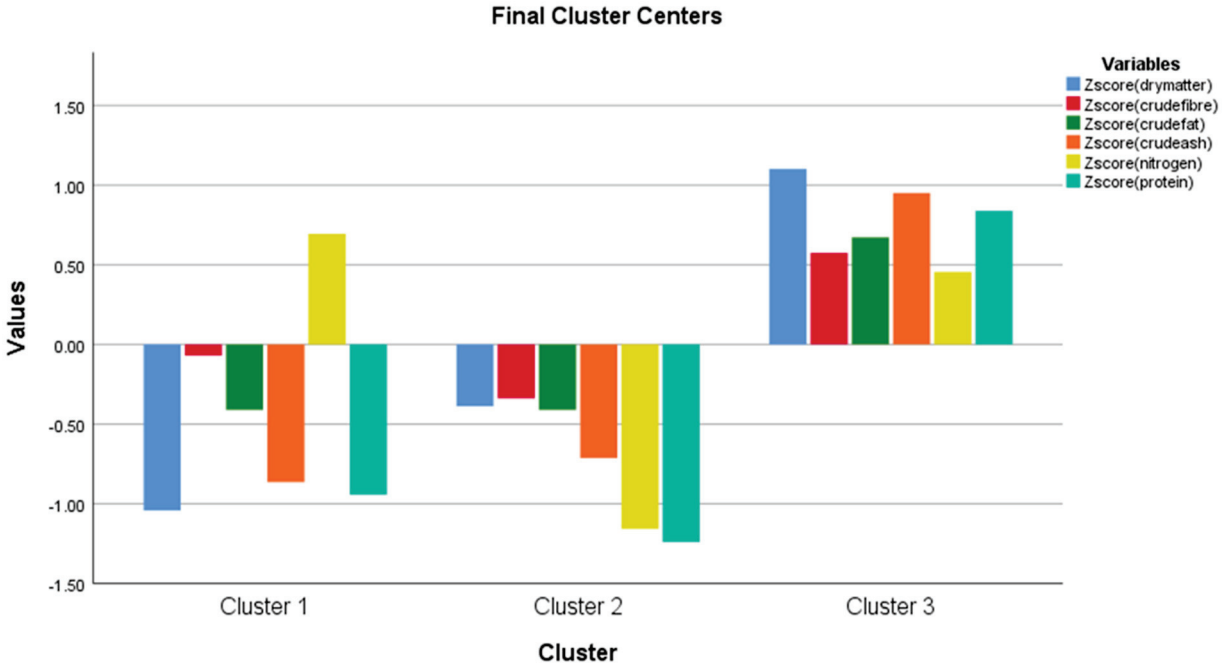


**Figure 13.** K-mean cluster analysis for the quality parameters of kernel (dry matter, crude fiber, crude fat, crude ash, nitrogen, and protein (m/m%) of Desszert 73 and Noa hybrids at 21 DAPI.

**Table 7.** The effect of the corn smut infection on elements concentration (mg/kg DW) of Desszert 73 and Noa hybrids' kernel at 21 DAPI (mean ± SD, n = 5).

| | Desszert 73 | | Noa | |
|---|---|---|---|---|
| | **Control** | **Infected Plants** | **Control** | **Infected Plants** |
| Al | 0.38 ± 0.20 a | 1.07 ± 0.33 b | 0.81 ± 0.08 a | 1.66 ± 0.42 b |
| B | 3.21 ± 0.02 b | 2.88 ± 0.13 a | 3.38 ± 0.14 a | 3.00 ± 0.21 a |
| Ca | 67.31 ± 2.64 a | 75.48 ± 4.80 b | 67.16 ± 1.62 a | 72.60 ± 4.61 b |
| Cr | 0.11 ± 0.003 a | 0.11 ± 0.008 a | 0.12 ± 0.01 a | 0.14 ± 0.005 a |
| Cu | 2.85 ± 0.15 a | 2.55 ± 0.21 a | 2.34 ± 0.09 a | 2.46 ± 0.14 a |
| Fe | 12.34 ± 0.26 a | 12.12 ± 0.51 a | 12.59 ± 0.48 a | 13.37 ± 0.75a |
| K | 6132.94 ± 77.68 a | 6093.44 ± 113.27 a | 5966.32 ± 202.62 b | 5716.64 ± 148.06 a |
| Mg | 789.62 ± 16.81 b | 686.91 ± 7.66 a | 746.868 ± 12.12 b | 690.20 ± 10.74 a |
| Mn | 9.41 ± 0.40 b | 7.52 ± 0.52 a | 8.45 ± 0.40 b | 7.34 ± 0.53 a |
| Na | 21.35 ± 1.35 a | 22.65 ± 0.84 a | 21.73 ± 1.03 a | 28.05 ± 1.96 b |
| P | 2998.66 ± 151.78 b | 2687.77 ± 63.30 a | 2777.86 ± 62.22 a | 2677.32 ± 50.32 a |
| Pb | 0.11 ± 0.003 a | 0.18 ± 0.006 b | 0.13 ± 0.023 a | 0.13 ± 0.007 a |
| S | 870.61 ± 42.84 a | 930.92 ± 54.18 b | 824.195 ± 18.56 a | 940.99 ± 64.51 b |
| Sr | 0.62 ± 0.09 a | 0.79 ± 0.04 a | 0.56 ± 0.03 a | 0.47 ± 0.11 a |
| Zn | 28.38 ± 0.25 b | 26.86 ± 1.94 a | 24.35 ± 0.90 a | 23.71 ± 1.54 a |

The data were evaluated by one-way ANOVA followed by Tukey-HSD test at 0.05 to determine significant differences indicated by different letters (a, and b). Al: Aluminum, B: Boron, Ca: Calcium, Cr: Chromium, Cu: Copper, Fe: Iron, K: Potassium, Mg: Magnesium, Mn: Manganese, Na: Sodium, P: Phosphorus, Pb: Lead, S: Sulfur, Sr: Strontium, and Zn: Zinc. DAPI: days after the infection, DW: dry weight.

In addition, K-mean cluster analysis shows that for Al, Ca, Cu, K, S, and Sr, the first and second clusters are below the average, while the third is above the average. For B, Mg, Mn, P, Pb, and Zn, the first cluster is below the average, the second and third are above the average. For Cr and Fe, the first and third clusters are below the average, and the second is above the average (Figure 14). Based on one-way ANOVA analysis, there are significant differences among the clusters for Al, Ca, Cr, K, Na, Pb, S, and Sr (results are not shown).

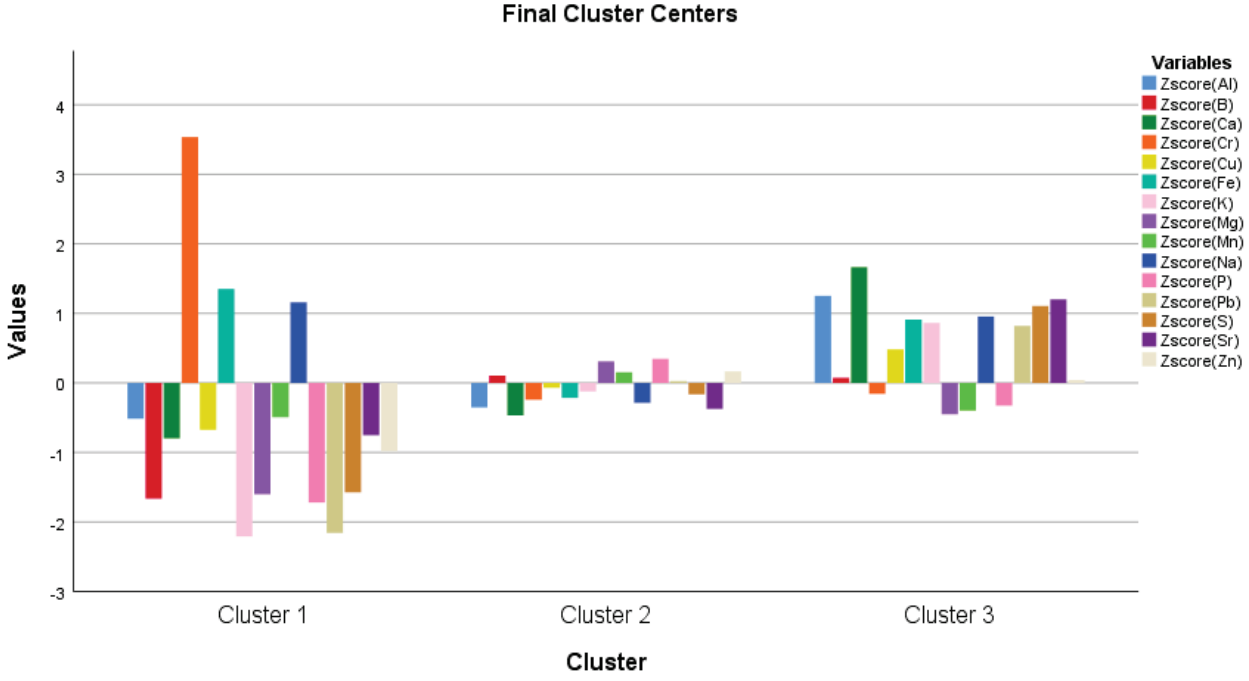


**Figure 14.** K-mean cluster analysis for on the kernel's elements concentration (Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, P, Pb, S, Sr, and Zn) of Desszert 73 and Noa hybrids at 21 DAPI.

## 4. Discussion

The effects of different corn smut inoculum treatments (2500, 5000, and 10,000 sporidia/mL) on the biochemical (amounts of photosynthetic pigments, MDA content and proline concentration, activities of APX, POX, and SOD enzymes), morphological (plant height and stem diameter), mineral contents, and quality parameters were determined. The first goal of this study was to examine the tumor formation at the V4–V5 phenological stage because the first hypothesis was that there is no tumor formation at low (2500 sporidia/mL) inoculation. Based on the outcome of the inoculation, tumor formation occurred at lower (2500 and 5000 sporidia/mL) and at high (10,000 sporidia/mL) corn smut sporidia numbers, too. The second hypothesis of this research was that the corn smut infection adversely affects the morphological, physiological, biochemical, and quality parameters, as well as the element content and other quality characteristics of grains irrespective of the lower dosage. The third hypothesis was that the 10,000 sporidia/mL has more negative impacts on the measured parameters relative to the 2500 and 5000 sporidia/mL treatments. The further goal was to examine which phenological stage (V4–5 or V7) was more susceptible to corn smut infection.

The first visible symptom of corn smut infection is the yellowing of the leaves which can be measured with the relative (SPAD Units) and absolute photosynthetic content (mg $g^{-1}$). The relative chlorophyll content (Figure 2) and the amount of photosynthetic pigments (Figure 3) of the infected plants were reduced in the fourth leaves in both hybrids at 7 and 14 DAPI. However, there were no significant differences among the treatments in the relative chlorophyll content for both hybrids, at 14 DAPI. These results do not confirm our second hypothesis because the 10,000 sporidia/mL treatment had no significant effects relative to 2500 and 5000 sporidia/mL treatments (Table 1). This effect was also observed in other studies. Frommer et al. [75] measured lower SPAD- units in sweet corn hybrids infected with corn smut. Szőke et al. [76] found that the 5000 and 10,000 sporidia/mL of corn smut reduced the relative chlorophyll content of the fodder corn hybrid. The reduction of photosynthetic pigments due to corn smut infection was also found in previous studies [77,78]. The corn smut disease causes chlorosis and necrosis on leaves, and this is the reason why the chlorophyll content was reduced [79].

Malondialdehyde content is an indicator of the presence of oxidative stress. Various biotic stress factors increase the MDA content in plants [80]. Meena et al. [81] found high MDA content in the *Alternaria alternata*-infected tomato plants. The *Plasmodiophora brassicae*-infected pakchoi plants had also high MDA content [82]. Chen et al. [83] stated that the *Puccinia striiformis* f. sp. *tritici* infection raised the concentration of MDA in the wheat leaves. In this study, the different treatments for corn smut infection also increased the concentration of MDA in the fifth leaves drastically in both hybrids (Figure 4). This effect proves that the corn smut infection was successful because of the increased MDA concentration as an indication of stress conditions. Secondly, this data confirms the second hypothesis that the corn smut infection had significant impacts on the physiological parameters of corn. The MDA content increased with the increasing corn smut sporidia. The highest MDA content was observed in both hybrids at both sampling times under 10,000 sporidia/mL inoculum. This result confirms the third hypothesis because there were significances among the 10,000 and 25,000 and 5000 sporidia/mL treatments. In addition, the MDA content was significantly influenced by the hybrids, treatment, sampling times, and the interactions of any two factors. The interactions of hybrid × treatment × sampling time did not have any significant impact on this parameter (Table 1). The hybrids did not tolerate the corn smut infection at the vegetative stage, and this was not dependent on the concentration of corn smut. The reason for this is that sweet corn hybrids are very susceptible to corn smut infection and the lower concentration infection can also cause severe damage to sweet corn.

The relationship between antioxidant enzyme activities and corn smut infection was also studied. Infection significantly increased the APX, POX, and SOD activities (Figures 5–7) for both hybrids' fifth leaves. Consistently with the second hypothesis, the activities of APX and POX were significantly affected by the hybrid (Noa had higher activities),

treatment (5000 and 10,000 sporidia/mL had higher activities relative to 2500), sampling time (14 DAPI had higher values), and the interaction of treatment and sampling time. The SOD activity was influenced by treatment, sampling time, and the interaction of hybrid and treatment, and treatment and sampling time (Table 1). However, there is no convincing evidence that the higher concentration of 10,000 sporidia/mL has more adverse impacts on antioxidant enzyme activities compared to the lower (2500 and 5000 sporidia/mL) sporidia numbers treatment. The activity of POX was significantly higher at all treatments at both hybrids and both sampling times at 10,000 sporidia/$cm^3$ treatments compared to the 2500 and 5000 sporidia/mL treatments. The APX activity was not significantly higher at 7 DAPI at Desszert 73 and at 14 DAPI at Noa when 10,000 sporidia/mL treatment was compared to the other two treatments. Significantly higher SOD activity was measured at Noa at both sampling times but not at Desszert 73. Similar studies also reported the effects of infection with various diseases on antioxidant enzyme activities. The Cucumber Green Mottle Mosaic Virus infection induced higher APX, POX, and SOD activities in cucumber plants [84]. The results of Kovács et al. [85] showed higher APX, POX, and SOD activities in European chestnut leaves infected with *Cryphonectria parasitica*. Barley plants infected with *Blumeria graminis* f. sp. *hordei* had higher APX and SOD activities compared with uninfected control plants [86]. The different pathogen infections produce different ROS and cause an oxidative burst [87]. Along with these progressions, plant antioxidant enzymes are activated after infection [88].

Proline content was also significantly elevated in the fifth leaf of Desszert 73 infected with 5000 sporidia/mL, at 14 DAPI. However, in Noa's fifth leaf, the proline content was significantly lower in the infected plants with 5000 and 10,000 sporidia/mL at 7 DAPI as compared to control, non-infected plants (Figure 8). Furthermore, proline concentration was significantly affected by treatment, sampling time, the interaction of treatment and sampling time, and hybrid × treatment × sampling time (Table 1). Based on the proline data, there are no significant differences between 10,000, 2500, and 5000 sporidia/mL treatments. This means corn smut sporidia number did not change significantly the proline content of the host plant. A biotic factor may initially lower the proline content in the plants [89]. In this study, the proline content in the infected plants was high under field conditions. Proline content is affected by other factors such as UV radiation [90], temperature [91], heavy metals [92], and so on. Furthermore, since the experiment was conducted under field conditions, these factors could not be controlled.

The applied treatments of corn smut infection also affected the plant height and stem diameter (Table 4) for both hybrids at both sampling times. The effect of the pathogen infection on the host plant height and stem diameter was also found in other studies. According to Szőke et al. [52], the corn smut infection decreased plant height and increased the stem diameter under greenhouse conditions at 7 DAPI. The tobacco mosaic virus decreased the plant height and stem diameter of pepper plants [93].

Adverse effects of the corn smut infection on the generative stage were also observed. Interestingly, only the 10,000 sporidia/mL caused symptoms on the infected cobs in both hybrids. Thus, the maize plants tolerated the lower concentrations (2500 and 5000 sporidia/mL) of the corn smut infection. This proves that the examined sweet corn hybrids are more susceptible to corn smut infection during the V4–5 stage than at the V7 stage. This is the first observation of these two (Desszert 73, and Noa) sweet corn hybrids' sensitivity comparison based on the phenological stage. The corn smut infection significantly diminished the dry matter, fat, and protein contents of the kernel, cob length and diameter, and kernel-100 grains of fresh and dry weights in both hybrids (Figure 12 and Table 5). These results are similar to some previous studies' results. According to Keszthelyi et al. [94], the corn smut infection decreased the protein, but not the fat content of a fodder corn hybrid's kernel. They stated that kernel and grain weights were also diminished by the corn smut infection. They also showed that the dry matter, protein, fat, ash, and nitrogen contents were decreased in other fodder corn hybrids by the corn smut infection [55].

Contrary to the second hypothesis of the study, the impacts of corn smut infection were different considering different elements. Magnesium and Mn had lower concentration while Al, Ca, and S had higher concentration in both hybrids' kernel at 21 DAPI. Boron, P, and Zn were reduced in Desszert 73, and K was reduced in the Noa hybrid's kernel (Table 7). Thus, the successful effect of the infection may depend on the cultivar. These differences proved that there is an effect of a hybrid on the case of grains' element contents as well. There are very few studies on the effects of plant pathogens on host plant element content. Shattuck et al. [95] showed that the Turnip mosaic virus did not alter mineral contents (Mg, Mn, Ca, Zn, B, and P) in rutabaga roots. Cesco et al. [96] reported that the *Plasmopara viticola*-infected plants had higher levels of the elements Ca, Fe, Mn, and Cu than control plants. Minerals can also influence the interaction between the pathogen and the host plant. The different mineral elements (K, Ca, Mn, Mg, and Zn) can increase the resistance of the host plant to different diseases [97].

The K-mean cluster analysis created three groups (cluster 1, cluster 2, and cluster 3) for measured parameters. The three clusters mean how different the three groups were from the average. The difference between the clusters was shown in the ANOVA table for a given parameter. The three groups were created based on the degree of freedom (df) in the cluster section for the created Z values, which is a recommendation of how many cluster groups should be created. This adds $+/-1$. Of course, we would have liked to create more cluster groups, we created them, however, the created a higher number of groups and did not show a significant difference among the clusters for any of the parameters, so the cluster analysis makes no sense. That is why we stayed at the recommended $2 +/- 1$ for the Z value, i.e., the three groups (Figures 9–11, 13 and 14).

## 5. Conclusions

The different concentrations of the corn smut infection had impacts on the physiological, morphological, quantity, and quality parameters of maize. In the vegetative stage, the hybrids did not tolerate the corn smut infection. Sweet corn is very susceptible to corn smut. Corn smut can cause severe damage regardless of the concentration of the pathogen. So, prevention is very important against corn smut. However, in the generative stage, only the concentration of 10,000 sporidia/mL of corn smut affected the content of corn cob elements and caused symptoms (tumor growth and galls). Thus, plants can better tolerate a lower concentration of infection at the generative stage. This proves that the examined sweet corn hybrids are more susceptible to corn smut infection during the V4–5 stage than at the V7 stage. This is the first observation of these two (Dessert 73, and Noa) sweet corn hybrids' sensitivity comparison based on the phenological stage. The results partly prove our hypothesis. Relative chlorophyll content (SPAD unit) and photosynthetic pigments (chlorophyll-*a*, chlorophyll-*b*, and carotenoids) content did not change significantly when 10,000 sporidia/mL were used compared to 25,000 and 5000 sporidia/mL. In addition, the sampling time also did not have any significant effect on these characteristics. Furthermore, MDA content and POX activity of the leaves were significantly higher at 10,000 sporidia/mL relative to 25,000 and 5000 sporidia/mL. This proves that photosynthesis and related parameters are less affected during corn smut infection than the defense system of the host plants. Only 10,000 sporidia/mL infected the corn cob during the generative stage, so we do not make any conclusion related to the impacts of sporidia concentration.

With appropriate indirect protection, i.e., by avoiding mechanical damage, using insecticide treatments, and proper care of the crop area, we can reduce the amount of pathogen inoculum (infectious material) concentration, because, with appropriate indirect protection, we reduce the optimal environmental conditions for the pathogen, as well as the optimal conditions for the development of the cultivated plant we create optimal conditions for the development of the cultivated plants. The experiment proved that resistant and less susceptible hybrids can tolerate lower concentrations of goiter infection (2500 and 5000 sporidia/mL) without pathogenic symptoms.

**Author Contributions:** Conceptualization and methodology B.T. and L.S.; Investigation L.S., D.K. and B.T.; Formal analysis B.T. and L.S.; Resources B.T. and L.R.; Supervision B.T.; Validation M.J.M.; Visualization L.S. and B.T.; Writing original draft B.T. and L.S.; Writing reviewing and editing M.J.M. All authors approved the published manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are contained within the article.

**Acknowledgments:** The authors thank László Tóth, Gábor Tarczali, Béla Szabó, and Mihály Sárvári for providing excellent technical assistance and materials for the experiment.

**Conflicts of Interest:** The authors declare that they have no conflict of interest.

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# Exploring the Potentiality of Native Actinobacteria to Combat the Chilli Fruit Rot Pathogens under Post-Harvest Pathosystem

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**Abstract:** Chilli is an universal spice cum solanaceous vegetable crop rich in vitamin A, vitamin C, capsaicin and capsanthin. Its cultivation is highly threatened by fruit rot disease which cause yield loss as high as 80–100% under congenial environment conditions. Currently actinobacteria are considered as eco-friendly alternatives to synthetic fungicides at pre and post-harvest pathosystems. Hence, this research work focuses on the exploitation of rhizospheric, phyllospheric and endophytic actinobacteria associated with chilli plants for their antagonistic activity against fruit rot pathogens *viz*., *Colletotrichum scovillei*, *Colletotrichum truncatum* and *Fusarium oxysporum*. In vitro bioassays revealed that the actinobacterial isolate AR26 was found to be the most potent antagonist with multifarious biocontrol mechanisms such as production of volatile, non-volatile, thermostable compounds, siderophores, extracellular lytic enzymes. 16S rRNA gene sequence confirmed that the isolate AR26 belongs to *Streptomyces tuirus*. The results of detached fruit assay revealed that application of liquid bio-formulation of *Stretomyces tuirus* @ 10 mL/L concentration completely inhibited the development of fruit rot symptoms in pepper fruits compared to methanol extracts. Hence, the present research work have a great scope for evaluating the biocontrol potential of native *S. tuirus* AR26 against chilli fruit rot disease under field condition as well against a broad spectrum of post-harvest plant pathogens.

**Keywords:** actinobacteria; *Streptomyces tuirus*; chilli fruit rot; *Colletotrichum scovillei*; *Colletotrichum truncatum*; *Fusarium oxysporum*; liquid bio-formulation

**Citation:** Renuka, R.; Prabakar, K.; Anandham, R.; Pugalendhi, L.; Rajendran, L.; Raguchander, T.; Karthikeyan, G. Exploring the Potentiality of Native Actinobacteria to Combat the Chilli Fruit Rot Pathogens under Post-Harvest Pathosystem. *Life* **2023**, *13*, 426. https://doi.org/10.3390/life13020426

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 28 November 2022
Revised: 13 January 2023
Accepted: 18 January 2023
Published: 2 February 2023



## 1. Introduction

Chilli (*Capsicum annuum* L.) is one of the most economically important spices cum solanaceous vegetable crops and is grown throughout the world for its green and red ripe fruits. It is a universal spice crop of India and occupies a major share in the Indian economy. In addition to adding pungency, taste, aroma and colour to cuisines, chilli have been used for centuries as medicine with countless health benefits, with antioxidant, anti-mutagenic, anti-carcinogenic, anti-arthritic and anti-inflammatory properties. Chilli fruits are rich in capsaicin, an appetite stimulant, and capsanthin, a pigment that gives its distinctive flavour and colour. Green chilli fruits contain more vitamin C than citrus fruits, whereas red chilli fruits contain more vitamin A than carrots [1,2]. Despite its rich nutritional and economic value, its commercial production is greatly threatened by fruit rot disease caused by complex pathogens including different species of *Colletotrichum*, *Fusarium* and *Alternaria* [3–5]. These pathogens extensively damage the fruits and significantly

reduce the quality, yield, appearance and marketability of the fruits [6,7]. It is a highly destructive pre- and post-harvest disease which causes yield losses up to 100% under congenial environmental conditions [8–10].

Though this disease can be managed with the repeated application of fungicides, pre- and post-harvest application of synthetic fungicides has been curtailed due to the persistence of fungicides on the fruits, which pose a direct risk to consumers and the environment through food chain contamination [11–13]. The use of naturally occurring bioactive compounds, especially those derived from antagonistic microorganisms, have been explored as prospective alternatives to synthetic fungicides due to their reduced toxicity and impact on humans and the environment [14–16]. Biological control of chilli anthracnose using antagonistic microorganisms or their metabolites is not a new concept, but a sustainable and ecologically acceptable approach in the context of leaving no toxic residues on the produce, safer application methods and ease of delivery, with minimal reliance on chemicals [17,18].

Although different groups of microorganisms have been employed for disease management, several research findings over the past few decades have highlighted the biocontrol potential of actinobacteria against a wide range of plant pathogens [19–21] through various mechanisms, including fungal cell-wall lysis, antibiosis, competition for nutrients, induction of host systemic resistance, phytotoxin degradation, plant growth stimulation, nutrient assimilation, rhizosphere competence and mineral availability [22–28].

Several species of actinobacteria were reported to have strong antagonistic activity against various species of *Colletotrichum* infecting a variety of crops. Taechowisan et al. [29] reported that *Streptomyces* spp. SRM1 exhibited antagonistic activity against *Colletotrichum musae* causing anthracnose in banana. *Streptomyces violaceoruber* reduced the incidence of chilli anthracnose by inhibiting the spore germination and mycelial growth of *Colletotrichum capsici* [30]. *Streptomyces ambofacines* S2 extract completely inhibited the expression of anthracnose symptoms of *Colletotrichum gloeosporioides* in red pepper fruits [18]. Actinobacteria not only prevent post-harvest pathogenic infection but also prolong the shelf life of a variety of crops without upsetting the natural balance.

A diverse group of actinobacteria inhabit the rhizosphere, phyllosphere and endosphere region of the plant and a subset of these provide a wide range of services and benefits to the plant in terms of suppressing plant diseases, promoting plant growth, increasing crop yield and enhancing soil fertility [31–33]. Particularly, when employed to curtail the plant infections, native actinobacterial isolates are more adaptable to their regular niche, have a higher success rate and are more resilient to local environmental challenges than the introduced microbes [34,35]. Furthermore, the introduced microbes must be able to co-habit with the native microbiome in order to provide more benefits to the plants. Hence, precolonization of the host by well adapted native biocontrol agents may prevent the growth and survival of plant pathogens.

The present study was, therefore, undertaken with the following objectives: (1) to isolate native actinobacterial isolates associated with rhizosphere, phyllosphere and surface sterilized tissues of chilli plants; (2) to identify the efficient actinobacterial isolate having antifungal potential against fruit rot pathogens *Colletotrichum* spp., and *Fusarium* sp.; (3) to unravel the antifungal mechanisms of potential actinobacterial isolates against fruit rot pathogens under in vitro conditions; (4) to assess the in vivo antifungal efficacy of liquid formulation and soluble metabolites of potential actinobacterial isolates on chilli fruits.

## 2. Materials and Methods

### *2.1. Fruit Rot Pathogens*

Fruit rot fungal pathogens *viz*., *Colletotrichum scovillei*, *Colletotrichum truncatum* and *Fusarium oxysporum* were isolated from infected chilli fruits collected from various locations of Tamil Nadu, India. The infected portion of the fruits were cut into small pieces (5 mm) using a sterile blade and surface sterilized with 1% $NaOCl_4$ for 1–2 min followed by 70% ethanol for 30 s and rinsed thrice with sterile distilled water [36]. The surface

disinfected fruit pieces were placed onto sterile Potato Dextrose Agar (PDA) medium amended with streptomycin sulphate (0.03 g $L^{-1}$) and incubated at 28 ± 2 °C for 7 days. Pure cultures of the pathogens were obtained by single hyphal tip method. The stock cultures of the pathogens were maintained as pure cultures on PDA slants at 4 °C.

### *2.2. Antagonistic Actinobacteria*

Rhizosphere actinobacteria were isolated from the rhizosphere soil of healthy chilli plants as described by Anwar et al. [37]. The soil samples were taken from a depth of 10–20 cm and subjected to dry heat pre-treatment for 4 h at 45 °C [38] to diminish the fast growing and abundant soil bacteria that would hinder slow growing actinobacteria [39]. Ten grams (10 g) of pre-treated soil was suspended in 90 mL of sterile distilled water, shaken thoroughly for 1 h at 100 rpm in an orbital shaker and allowed to settle for an hour. Subsequently, samples were serially diluted up to $10^{-5}$ dilutions and 1 mL aliquot from $10^{-3}$–$10^{-5}$ dilutions were plated on sterile Starch Casein Agar (SCA) supplemented with 25 µg/mL nalidixic acid and 50 µg/mL nystatin as antibacterial and antifungal agents [40].

Phyllospheric actinobacteria were isolated from the leaf, stem, flower and fruits of healthy chilli plants [41]. Ten grams (10 g) of samples were preheated at 70 °C for 15 min and transferred to 90 mL of 0.85% saline buffer (NaCl) and kept in an orbital shaker at 250 rpm for 30 min at 28 ± 2 °C. The solution thus obtained was subjected to the standard serial dilution pour plate technique on Starch Casein Agar (SCA) supplemented with nalidixic acid (25 µg/mL) and nystatin (50 µg/mL).

The actinobacteria from the surface sterilized plant tissues were isolated as per the procedure described by Li et al. [42]. A five-step procedure was employed for the sterilization of the plant tissues: (i) the tissue segments were surface-sterilized in 0.1% sterile Tween 20 for 1 min, (ii) the samples were sterilized with 5% sodium hypochlorite for 4 min (leaf samples) or 6 min (stem and root samples), (iii) the samples were then rinsed in 2.5% (*w*/*v*) sodium thiosulfate for 10 min and washed three times with sterile distilled $H_2O$, followed by (iv) immersing in 70% (*v*/*v*) ethanol for 4 min (leaf samples) or 6 min (stem and root samples), and finally (v) the samples were washed with sterile distilled water for a minimum of three times. To validate the successful surface disinfection process, 0.2 mL of water from the final wash was spread onto the isolation medium and incubated at 28 ± 2 °C. One gram of surface-sterilized plant tissues was homogenized in a mortar and pestle with 1 mL of 0.9% saline buffer (*w*/*v*). One millilitre of the tissue suspension was serially diluted and $10^{-3}$–$10^{-5}$ dilutions were plated on Starch Casein Agar plates. The plates were incubated at 28 ± 2 °C for 7–10 days. Powdery, bright actinobacterial colonies were purified, suspended in 20% glycerol and stored at −80 °C as stock culture [43].

### *2.3. In Vitro Antifungal Bioassay*

#### 2.3.1. Primary Screening of Actinobacterial Isolates for the Antifungal Activity against Chilli Fruit Rot Pathogens

Fifty-two actinobacterial isolates were screened for their antifungal activity against chilli fruit rot pathogens by dual-culture assay [44]. The test isolates were streaked at one corner of the PDA plates (10 mm from the periphery of a 90 mm diameter Petri dish) and incubated at 28 ± 2 °C for 4 days. After incubation, the 5-day-old pathogen fungal disc was placed opposite to actinobacterial streak (10 mm away from the periphery). Petri dishes without actinobacterial isolates served as the control. All plates were incubated at 28 ± 2 °C for 7 days. All the isolates were tested in triplicate. After incubation, the zone of inhibition was measured and the per cent inhibition of mycelial growth was calculated. The zone of inhibition (ZI) was measured as the diameter of the halo zone (in cm) between the actinobacteria and pathogen colony as and when the pathogen in the control plate covered the entire plate. Per cent inhibition of mycelial growth (PIMG) was determined according to the formula: PIMG = (C − T)/C 100, where C and T are the mycelial growth of pathogenic fungus in the control plate and dual culture plate, respectively. The degree of antifungal activity of various actinobacterial isolates against the

tested pathogens were evaluated based on the zone of inhibition (ZI) (in cm) and per cent inhibition of mycelial growth (PIMG) [45]. Based on the zone of inhibition, the antagonistic activity of actinobacterial isolates were grouped into four categories according to Lee and Hwang [46] as: − no inhibition (ZI $\leq$ 0); + weak inhibition (ZI = 0.1–1.0 cm); ++ moderate inhibition (ZI = 1.01–2 cm); and +++ strong inhibition (ZI $\geq$ 2 cm).

#### 2.3.2. Secondary Screening for the Antifungal Activity of Actinobacterial Isolates

The antifungal activity of six actinobacterial isolates which exhibited the strongest inhibition against the tested pathogens by dual culture assay was further confirmed by paired culture antibiosis assay as per the protocol of Liotti et al. [47] with slight modification. An 8 mm mycelial disc of the pathogen was placed at the centre of a Petri dish containing PDA medium and the actinobacterial isolate was streaked at equidistance on both sides of the pathogen, about 10 mm from the periphery of Petri dish. A control plate was maintained without actinobacteria. The experiment was replicated thrice. After 7 days of incubation at 28 $\pm$ 2 °C, the percentage inhibition of mycelial growth (PIMG) of the pathogen was calculated as per the formula described above.

#### 2.3.3. In Vitro Screening of Actinobacterial Isolates for Production of Extracellular Lytic Enzymes and Siderophore

The actinobacterial isolates were assayed for their biocontrol traits *viz*., amylase, cellulase, chitinase and protease production by spot inoculating 10 µL of culture in starch agar medium [48], Carboxy Methyl Cellulose (CMC) agar medium [49], colloidal chitin agar medium [50] and skim milk agar medium [51], containing starch, cellulose, colloidal chitin and casein as the respective substrates. Siderophore production was assayed on Chrome Azurol Sulphonate (CAS) agar medium according to the methodology of Sadeghi et al. [52]. The plates were incubated for 5–7 days at 28 $\pm$ 2 °C. Three replications were maintained for each actinobacterial isolate.

The amylase activity of the actinobacteria was evaluated by flooding the plate with Lugol's iodine solution for 30 s. A clear hydrolysis zone around the colonies against the blue background indicated the hydrolysis of starch by the amylase enzyme [53]. Cellulase activity was determined by flooding the plates with 0.1% Congo red solution and counter staining with 1 M NaCl for 15–20 min. The formation of a clear zone around the colony due to the hydrolysis of cellulose indicated a positive result for the production of the cellulase enzyme by the actinobacterial isolates. The isolates positive for chitinolytic and proteolytic activity produced a clear halo zone around the colonies due to the hydrolysis of chitin and casein in the respective media. The formation of a yellow to orange halo around the actinobacterial colonies due to the removal of iron from CAS represented a positive result for the production of siderophore.

#### 2.3.4. Antifungal Activity of Volatile Organic Compounds

The four most active actinobacterial isolates that showed significant positive results for the production of cell wall-degrading enzymes and siderophore were subjected to additional assays on the production of volatile, non-volatile and thermostable compounds. The antifungal activity of volatile organic compounds (VOCs) produced by the actinobacterial isolates was tested against the fruit rot pathogens by the double-sealed plate method [54]. A 90 mm diameter Petri dish bottom containing 15 mL of ISP4 medium was streaked with a loopful of actinobacterial culture. An 8 mm diameter mycelial plug of the pathogen was inoculated in the centre of another Petri dish bottom containing 15 mL of potato dextrose agar medium. A Petri dish "sandwich" was made with the antagonist Petri dish placed over the pathogen plate in such a way that the pathogen plate was at the bottom and antagonist plate was on the top. The sandwiched Petri dish was sealed together with a parafilm without any gaps and incubated at 28 $\pm$ 2 °C for 7–10 days. A Petri dish containing ISP4 medium without the antagonist placed over the pathogen plate served as the control. The parafilm-sealed plates ensured no physical contact between the pathogen and

antagonist. The experiment was conducted with three replications for each isolate. The rate of inhibition (%) of mycelial growth was calculated as described previously.

#### 2.3.5. Antifungal Activity of Non-Volatile Metabolites

The antifungal activity of non-volatile metabolites in the cell-free culture filtrate of actinobacterial isolates was determined using the seeded agar method [55,56]. Actinobacterial isolates were cultured in a 250 mL conical flask containing 100 mL ISP4 broth and incubated in an orbital shaker at 150 rpm for 7 days at 28 ± 2 °C. Then the culture broth was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant obtained was filtered through a 0.22 µm nitrocellulose membrane filter to obtain cell-free culture filtrate and subjected to an antifungal assay. The filtrate was mixed with warm PDA (25%) and plated in a sterile Petri dish. Finally, an 8 mm mycelial disc of the pathogen was placed at the centre of the seeded PDA medium in the Petri dish. The pathogen growth on the Petri dish without the cell-free culture filtrate served as the control. The plates were incubated at 28 ± 2 °C until the mycelial disc in the control plate completely covers the plate. Three replicates were maintained for each isolate. Per cent inhibition (PI) of mycelial growth was calculated as described previously.

#### 2.3.6. Antifungal Activity of Thermostable Compounds

The actinobacterial isolates were cultured in 100 mL ISP4 broth in a 250 mL conical flask with constant agitation in an orbital shaker (150 rpm) for 7 days at 28 ± 2 °C. The actinobacterial cells were harvested by centrifugation at 10,000 rpm for 15 min. Twenty-five millilitres of the supernatant were transferred to a conical flask containing 75 mL PDA medium and sterilized at 121 °C for 20 min. The actinobacterial metabolite-amended sterile medium was plated into Petri dish and a 9 mm mycelial disc of the tested pathogen was placed at the centre of solidified medium. The pathogen growth on PDA medium without actinobacterial metabolite served as the control. The Petri dishes were incubated at room temperature for 7 days and the Per cent Inhibition (PI) of mycelial growth of the pathogen was assessed as per the formula described above.

#### 2.3.7. Assessment of In Vitro Antifungal Traits

Among the six isolates, the best isolate with the highest antagonistic potential was selected based on a bonitur scale as described by Passari et al. [57] and El-Sayed et al. [58]. In this scale, points were given for each in vitro antifungal trait and the maximum bonitur score is 24 points. The per cent inhibition of mycelial growth (PIMG) was evaluated as follows: if PIMG is 30–54.9% = 1 point; 55–74.9% = 2 points; 75–95% = 3 points. Lytic enzyme production was evaluated with 1 point and siderophore with 2 points each.

### *2.4. Scanning Electron Microscopy (SEM)*

The interaction of the actinobacterial isolate AR26 which exhibited strong antifungal activity against the pathogens in the dual culture plate was documented by Scanning Electron Microscope (SEM) (Model: FAI QUANTA 250, Czech Republic) at 15 KV [59]. Mycelial discs (5 mm) of the pathogen from the periphery of inhibition zone in the dual culture plate as well as in the control plate were cut with a sterile scalpel and transferred to perforated capsules and fixed in 1.5% glutaraldehyde in phosphate buffer for 4 h [60]. Then, the specimens were washed with 0.2 M sodium cacodylate buffer (pH 6.2) and dehydrated with an increasing concentration of ethanol washes from 0–100% at 10 min intervals (0%, 30%, 50%, 70%, 80%, 90% and 100%). Later the specimens were mounted on aluminium stubs using conductive double-sided carbon tape. The stubs were then lyophilized, and sputter coated with gold (5 nm thickness). Finally, any morphological changes of the pathogen mycelium in the dual culture plate as well as in the control plate were examined under scanning electron microscope.

### *2.5. Molecular Characterization of Actinobacterial Isolates*

The genomic DNA of the actinobacterial isolate was extracted from the spore masses using the Cetyl Trimethyl Ammonium Bromide (CTAB) method [61]. The 1.5 kb full length 16S rRNA gene of actinobacteria was amplified by Polymerase Chain reaction (PCR) with a forward primer 27F (5′ AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) [62]. The PCR amplification was performed with a 25 µL reaction mixture which contained 10 µL of master mix, 1 µL of bacterial genomic DNA at a concentration of 20 ng, 1 µL of each primer at a concentration of 10 pM and 12 µL of sterilized deionized water. The PCR amplification conditions included an initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 40 s and final extension at 72 °C for 10 min. The PCR amplified products were visualized on 1% agarose gel with a UV transilluminator and photographed using the gel documentation system and sequenced at Biokart India Pvt. Ltd., Bangalore, India. The sequence similarities were determined by BLAST analysis (Basic Local Alignment Search Tool) (BLAST, (https://www.ncbi.nlm.nih.gov) (accessed on 27 November 2022) and submitted in GenBank. The most homologous sequences showing the highest similarity were retrieved from the NCBI GenBank database and multiple sequences were aligned using the ClustalW algorithm. A phylogenetic tree was constructed with closely related nucleotide sequences using the Neighbour-Joining (NJ) method [63] using MEGA (Molecular Evolutionary Genetics Analysis) 11 software [64] with bootstrap values of 1000. Evolutionary distances were calculated using the maximum combined likelihood method and are given in units of the number of base substitutions per site.

### *2.6. Antifungal Bioassay of Liquid Formulation of Actinobacterial Isolate on Chilli Fruits*

The antifungal activity of actinobacterial isolate AR26 on green chilli fruits was determined as per the antifungal bioassay of Liottia et al. [47]. Fresh fruits of the chilli hybrid "Ganga" of uniform size and maturity without wounds, scars and rots on their surface were surface sterilized as described previously. The surface sterilized green chilli fruits were wounded to the depth of 1 mm with a sterile needle and subjected to following treatments. (i) healthy control: chilli fruits were inoculated with 20 µL of sterile distilled water, (ii) pathogen-inoculated control: chilli fruits were inoculated with 6 mm mycelial disc of pathogen culture, (iii) antagonist inoculated control: chilli fruits were inoculated with 20 µL of liquid formulation of *S. tuirus* AR26 at10 mL/L containing $9 \times 10^8$ CFU/mL, (iv) chilli fruits were first inoculated with 20 µL of liquid formulation of *S. tuirus* at 5 mL/L and after an hour of incubation, 6 mm mycelial disc of respective pathogens were placed over it, (v) chilli fruits were first inoculated with 20 µL of liquid formulation of *S. tuirus* at 10 mL/L and 6 mm mycelial disc of respective pathogens were placed over it.

Inoculated fruits of each treatment were placed in separate glass Petri dishes, sealed with parafilm and incubated at 28 ± 2 °C for 7 days. The experiment was conducted statistically as a completely randomized design (CRD) in three replicates of five fruits each. The progress of the symptom on the fruits was measured as the lesion diameter after seven days of incubation. The percentage of inhibition of fruit rot symptom and disease incidence was calculated as per the formula given below. Per cent disease reduction = $[(D - d) \times 100]/D$, where D is the lesion diameter in pathogen-inoculated control fruits, and d is the lesion diameter in actinobacteria and pathogen co-inoculated fruits.

$$\text{Disease incidence} = (\text{Number of diseased chili fruits}/\text{Total number of chili fruits}) \times 100.$$

### *2.7. Antifungal Bioassay of Soluble Metabolites of Actinobacterial Isolate on Chilli Fruits*

This assay was conducted to differentiate whether the antifungal activity was mediated by the presence of actinobacterial culture or by its metabolites. The soluble metabolites produced by the isolate AR26 in the dual culture plate in PDA medium were extracted from the zone of inhibition by excising the PDA medium from the inhibition zone using a sterile scalpel. Excised PDA medium was blended with HPLC-grade acetonitrile in a 1:4 ratio

(5 g agar in 20 mL of HPLC grade acetonitrile). The mixture was incubated overnight at 28 ± 2 °C in an orbital shaker at 150 rpm. The homogenised samples were subjected to 10 min centrifugation at 10,000 rpm, and then filtered through Whatman No.1 filter paper to separate the agar particles and supernatant. The supernatant was dried in a vacuum flash evaporator (Roteva Equitron Make). After discarding the eluent, the final product was diluted in 1 mL of HPLC-grade methanol [65]. The extract obtained was tested for its ability to control chilli fruit rot pathogens. The assay was performed as described above with four treatments, using 20 µL of methanol extract of *S. tuirus* AR26 for treatments and 20 µL of methanol alone for control.

### *2.8. Statistical Analysis*

The data was subjected to a single factor test of significance (ANOVA) using the analytical software SPSS version 16.0. Significant differences between the average values of each treatment ($p \leq 0.05$) were determined using critical difference.

## 3. Results

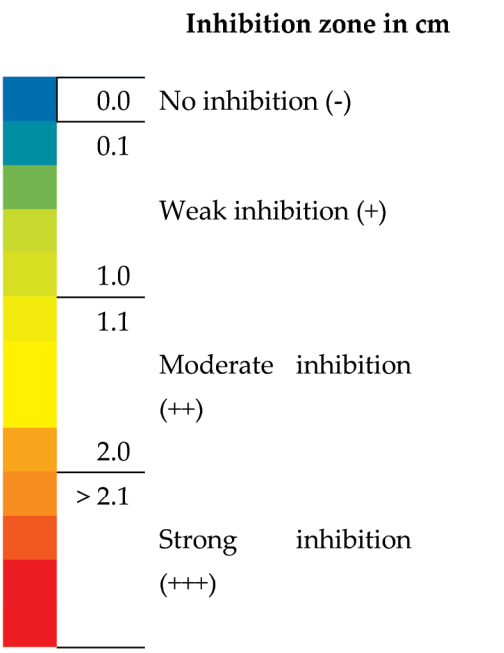
### *3.1. Primary Screening for Antifungal Activity of Actinobacterial Isolates*

In this study, 52 actinobacterial isolates obtained from rhizospheric (26), phyllospheric (16) and surface sterilized plant tissues (10) of chilli plants were screened for their antagonistic potential against chilli fruit rot pathogens *viz*., *C. scovillei*, *C. truncatum* and *F. oxysporum* by dual culture technique. About 19.2% of the rhizospheric isolates, 12.5% of phyllospheric isolates and 10.0% of the endophytic isolates exhibited strong antifungal activity against *C. scovillei*, whereas 15.4% of the rhizospheric isolates and 12.5% of the phyllospheric isolates and again 10.0 % of endophytic isolates showed antifungal activity against *C. truncatum*. With regard to *F. oxysporum*, 23.1% of rhizospheric, 12.5% of phyllospheric isolates and 10.0% of endophytic isolates showed strong antagonistic activity. Thirty-eight (73.07%) out of 52 isolates inhibited the mycelial growth of at least one out of the three pathogens with varying degrees of inhibitory action, ranging from 4.82% to 67.90% (weak to strong inhibition) (Supplementary Table S1 and Figure 1). Six isolates designated as AR1, AR10, AR26, AL5, AL7, and AFE2 strongly inhibited the growth of all three pathogens (Figure 2) with an inhibition zone (ZI) greater than 2 cm. Isolate AR26 was found to be significantly superior to other isolates, with the highest mycelial growth inhibition of 67.90%, 63.21%, and 60.37% and inhibition zones of 3.2 cm, 2.8 cm, and 2.7 cm respectively for *C. scovillei*, *C. truncatum*, and *F. oxysporum*, followed by the isolate AR10 (Figure 3).

### *3.2. Secondary Screening for the Antifungal Activity of Actinobacterial Isolates and Scanning Electron Microscopic Assay*

The six isolates (Figure 4) which showed the strongest antagonism against the three pathogens were further subjected to secondary screening by paired culture antibiosis to further confirm their antagonistic ability against *C. scovillei*, *C. truncatum* and *F. oxysporum*. The results of this assay indicated that all six isolates were capable of inhibiting the growth of *C. scovillei*, *C. truncatum* and *F. oxysporum*. The isolate AR26 was found to be significantly superior to other isolates in inhibiting mycelial growth of *C. scovillei* (59.63%), *C. truncatum* (61.18%) and *F. oxysporum* (63.58%), respectively followed by the isolate AR10 (Supplementary Figure S1). The isolate AFE2 recorded the lowest percentage of mycelial growth inhibition of the tested pathogens, relative to the other five isolates. Scanning Electron Microscopy (SEM) observations indicated a clear evidence for antifungal activity of isolate AR26 against *C. scovillei*, *C. truncatum* and *F. oxysporum*. The antifungal activity was observed as distinct morphological deformities in pathogen hyphae in the presence of antagonist and hyphae were found to be twisted and shrunk in *C. scovillei*, disintegrated in *C. truncatum*, aggregated into clusters in *F. oxysporum* with reduction in mycelial mat (Figure 5). In contrast, the hyphae in the control plate were dense, intact with regular structure.

| Isolate | *C. scovillei* | *C. truncatum* | *F. oxysporum* |
|---|---|---|---|
| AR1 | 2.4 | 2.3 | 2.1 |
| AR2 | 1.3 | 1.6 | 1.4 |
| AR3 | 1.1 | 0.5 | 0.4 |
| AR4 | 2.1 | 2.1 | 1.3 |
| AR5 | 1.3 | 1.3 | 0.9 |
| AR6 | 1.1 | 0.4 | 1.5 |
| AR7 | 0.5 | 1.2 | 1.7 |
| AR8 | 0.6 | 1.0 | 0.4 |
| AR9 | 1.5 | 1.2 | 0.8 |
| AR10 | 2.7 | 2.4 | 2.6 |
| AR11 | 2.0 | 1.1 | 1.1 |
| AR12 | 1.4 | 1.4 | 1.5 |
| AR13 | 1.2 | 0.0 | 1.5 |
| AR14 | 1.5 | 0.3 | 0.0 |
| AR15 | 1.6 | 1.2 | 0.9 |
| AR16 | 0.9 | 0.3 | 1.2 |
| AR17 | 0.0 | 0.0 | 0.0 |
| AR18 | 1.3 | 0.0 | 0.5 |
| AR19 | 0.0 | 0.0 | 0.0 |
| AR20 | 0.5 | 1.2 | 2.1 |
| AR21 | 0.8 | 0.4 | 1.1 |
| AR22 | 0.4 | 0.0 | 0.8 |
| AR23 | 1.5 | 1.2 | 0.1 |
| AR24 | 1.6 | 1.4 | 2.1 |
| AR25 | 2.7 | 1.4 | 2.2 |
| AR26 | 3.2 | 2.8 | 2.7 |
| AL1 | 0.8 | 0.6 | 1.7 |
| AL2 | 0.0 | 1.2 | 0.8 |
| AL3 | 0.0 | 0.0 | 0.0 |
| AL4 | 0.9 | 1.1 | 1.4 |
| AL5 | 2.3 | 2.1 | 2.4 |
| AL6 | 0.5 | 1.4 | 0.0 |
| AL7 | 2.3 | 2.2 | 2.3 |
| ASt1 | 0.0 | 2.0 | 0.7 |
| ASt2 | 0.4 | 0.1 | 0.8 |
| ASt3 | 1.7 | 1.2 | 1.0 |
| ASe1 | 1.6 | 1.5 | 1.2 |
| ASe2 | 0.8 | 1.3 | 1.1 |
| ASe3 | 0.0 | 0.8 | 1.0 |
| AF1 | 1.3 | 1.2 | 1.5 |
| AF2 | 1.1 | 1.2 | 0.8 |
| AF3 | 0.7 | 1.3 | 1.4 |
| ALE1 | 0.0 | 0.9 | 1.5 |
| ALE2 | 1.0 | 0.8 | 0.5 |
| ALE3 | 0.6 | 0.5 | 1.5 |
| ALE4 | 0.7 | 0.7 | 0.7 |
| AStE1 | 1.6 | 1.2 | 0.3 |
| ASeE2 | 0.2 | 1.3 | 1.3 |
| ASeE3 | 0.8 | 0.0 | 0.0 |
| AFE1 | 0.0 | 0.0 | 0.0 |
| AFE2 | 2.1 | 2.1 | 2.1 |
| AFE3 | 0.4 | 1.4 | 1.1 |
| Control | 0.0 | 0.0 | 0.0 |




**Degree of antifungal activity**

IZ = ≤ 0 cm – = no inhibition

IZ = 0.1–1.0 cm + = weak inhibition

IZ = 1.01-2 cm ++ = moderate inhibition

IZ = > 2 cm +++ = strong inhibition

**Figure 1.** Heat map showing the in vitro antagonism of actinobacterial isolates against chilli fruit rot pathogens as represented by zone of inhibition.

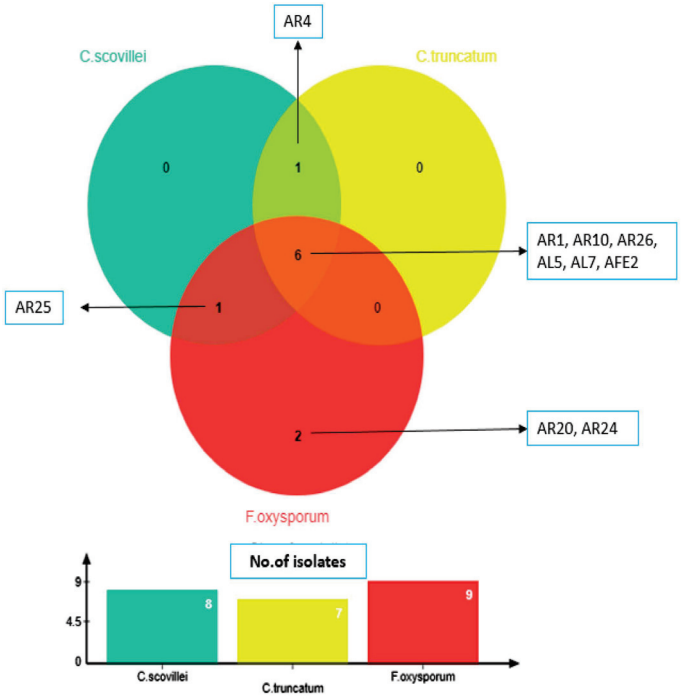



**Figure 2.** Venn diagram representing the actinobacterial isolates exhibiting strong antagonism against fruit rot pathogens.

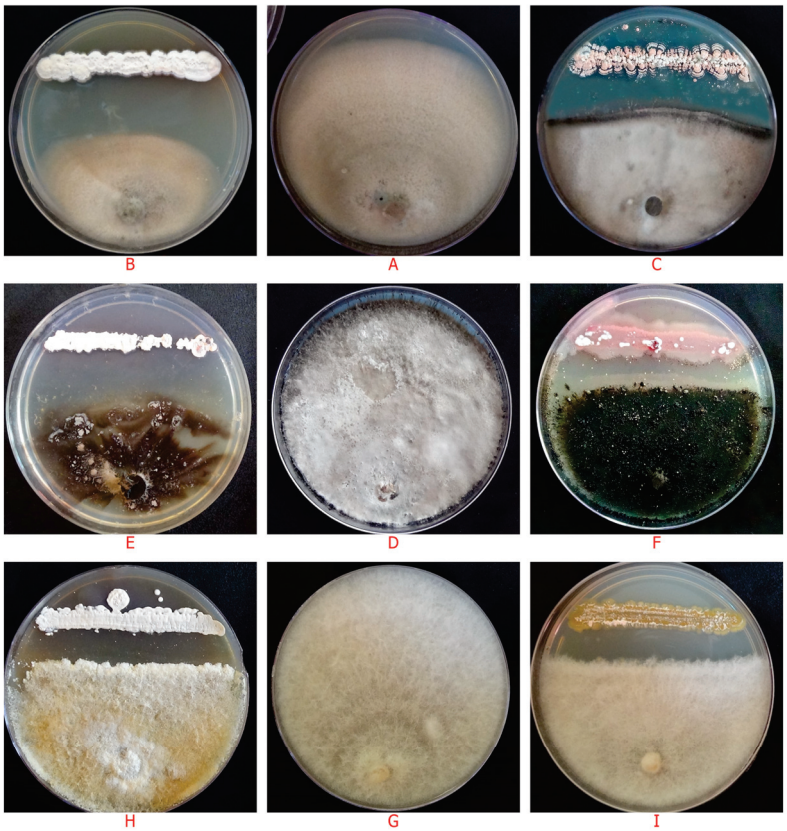



**Figure 3.** Antifungal activity of actinobacterial isolates against chilli fruit rot pathogens. (**A**) Control *C. scovillei* (**D**) Control *C. truncatum* (**G**) Control *F. oxysporum.* Antifungal activity of actinobacterial isolate AR26 against (**B**) *C. scovillei* (**E**) *C. truncatum* (**H**) *F. oxysporum*. Antifungal activity of actinobacterial isolate AR10 against (**C**) *C. scovillei* (**F**) *C. truncatum* (**I**) *F. oxysporum*.

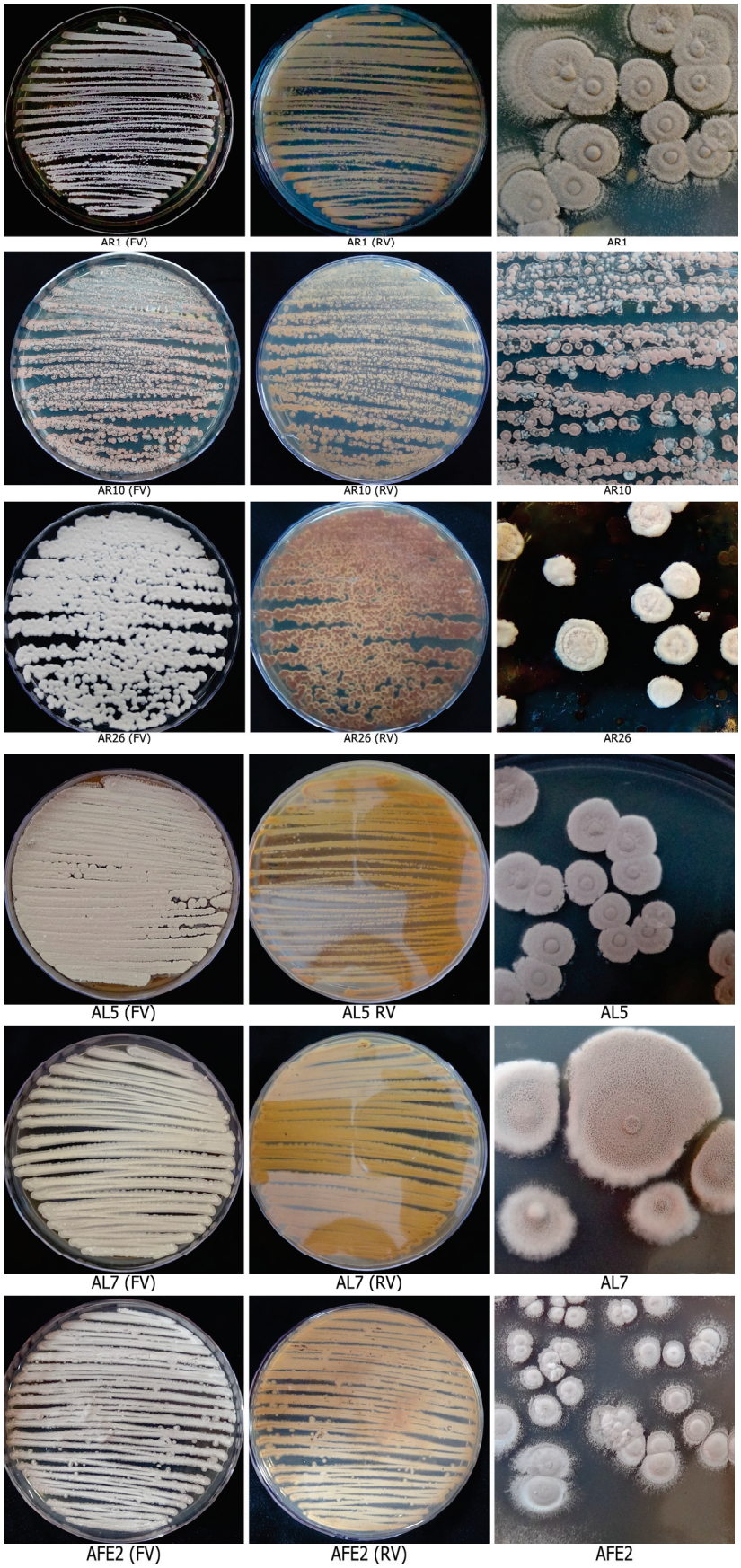



**Figure 4.** Actinobacterial isolates with potent antagonistic activity against fruit rot pathogens.

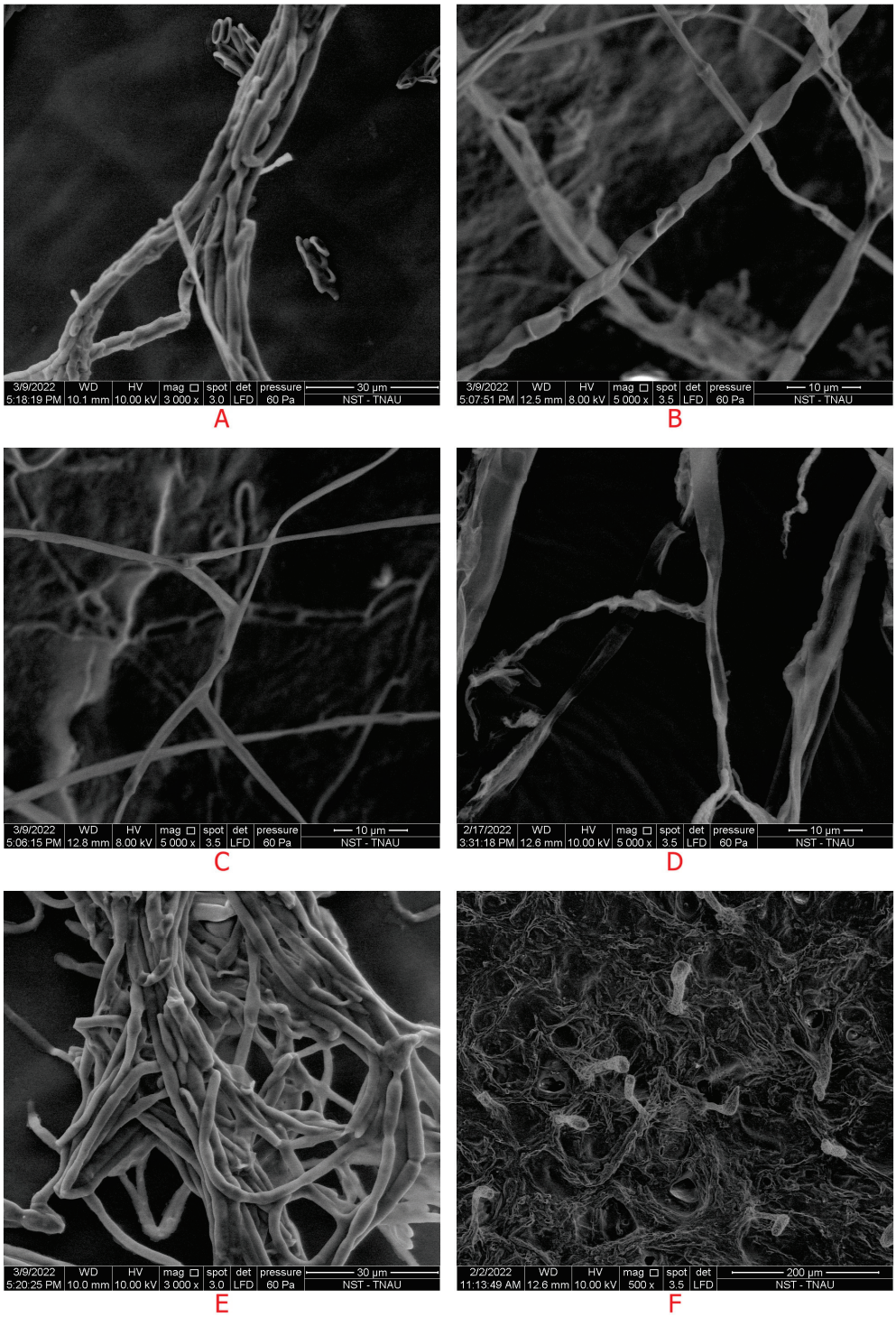



**Figure 5.** Scanning Electron Micrographs showing the interaction of antagonist. *S. tuirus* AR26 with *C. scovillei*, *C. truncatum* and *F. oxysporum*; (**A**) intact mycelium of *C. scovillei* in the absence of antagonist; (**B**) twisted and shrunken hyphae of *C. scovillei* in the presence of antagonist; (**C**) mycelium of *C. truncatum* in absence of antagonist; (**D**) distorted hyphae of *C. truncatum* in the presence of antagonist; (**E**) dense and intact mycelium of *F. oxysporum* in absence of antagonist; (**F**) aggregated hyphae of *F. oxysporum* in presence of antagonist.

### *3.3. Screening for the Production of Extracellular Lytic Enzymes and Siderophore by the Antagonists*

All six actinobacterial isolates were able to produce at least 4 out of 5 hydrolytic enzymes to different degrees. All the isolates tested positive for amylase and cellulase.

The isolates AR10, AR26 and AL7 produced siderophore and AR1, AR10, AR26 and AL7 recorded chitinase activity. The isolates AL5 and AFE2 produced protease while all other isolates tested negative for protease activity. The results revealed that the isolates AR10, AR26 and AL7 were positive for siderophore, amylase, cellulase, and chitinase. The isolate AR26 was the most potent antagonist to produce prominently siderophore, cellulase and chitinase (Supplementary Figures S2–S7).

### *3.4. Antifungal Activity of Volatile, Non-Volatile and Thermostable Compounds*

All of the four isolates AR10, AR26, AL5 and AL7 apparently produced volatile, non-volatile and thermostable compounds, and significant differences in the antifungal activity against the tested pathogens were observed among the isolates (Figure 6). The volatile compounds of isolates AR10, AR26, and AL5 were found to be more effective than non-volatile and thermostable compounds. The volatile organic compounds of isolate AR26 exhibited the maximum inhibitory effect against *C. scovillei* (77.04%), *C. truncatum* (72.63%) and *F. oxysporum* (69.53%). The thermostable compound of AR26 exhibited the strongest inhibitory action against *F. oxysporum*, followed by AR10.

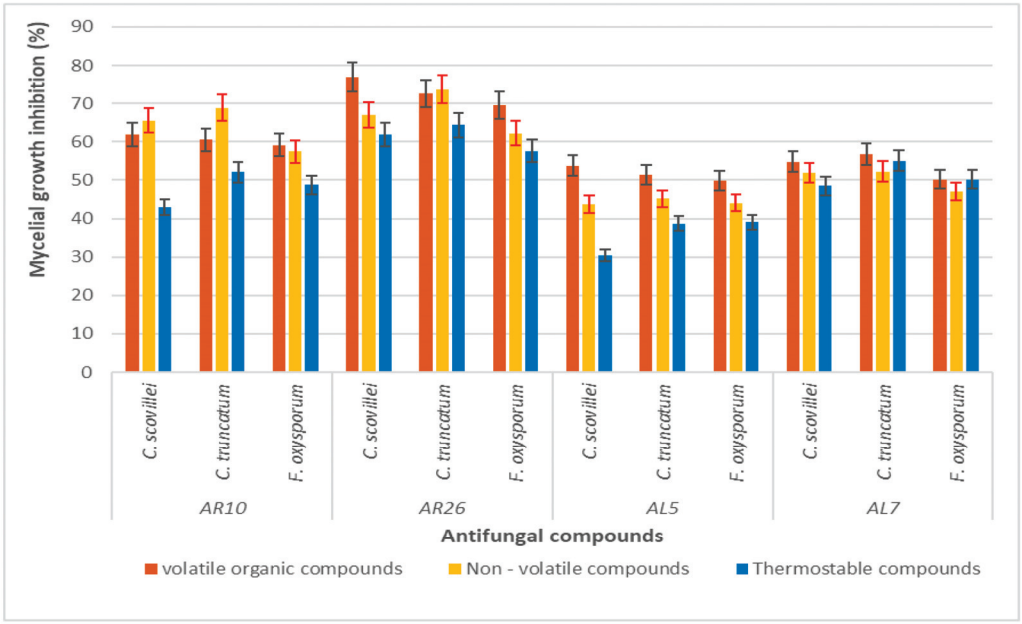



**Figure 6.** Antifungal activity of volatile, non-volatile and thermostable compounds. Error bars represent the standard deviation of the data set.

### *3.5. Assessment of In Vitro Antifungal Traits*

The results of the assessment for in vitro antifungal traits revealed that out of the six isolates screened, rhizospheric isolate AR26 showed the highest assessment value of 17 points followed by the isolate AR10 with 15 points. Hence, the actinobacterial isolate AR26 was selected as the most efficient antagonist for further studies (Table 1).

### *3.6. Molecular Confirmation of Actinobacterial Isolates*

The results of the 16S rRNA sequence analysis of the actinobacterial isolates revealed that five isolates were closely affiliated to the genus *Streptomyces.* Isolates AR1, AR10, AL5, AR26 and AL7 exhibited the highest similarity with *Streptomyces rochei*, *Streptomyces deccanensis*, *Streptomyces azureus*, *Streptomyces tuirus*, and *S. geysiriensis*, respectively (Table 2). Phylogenetic analysis revealed that the isolates under current study formed five different clades (highlighted in red) and were supported with good bootstrap values (Figure 7). Isolate AR10 formed a distinct clade A with *S. deccanensis*, AL5 formed clade B with *S. azureus*, AR26 formed clade C with *S. tuirus*, AR1 formed clade D with *S. rochei*, and AL7 formed clade E with *S. geysiriensis*, with *Pseudomonas fluorescens* as the out group.

**Table 1.** Actinobacterial isolates for their ability to function as antagonist with various antifungal mechanisms.

| S.No | Isolate Code | Antagonistic Activity | | | | | | Antifungal Mechanisms | | | | | Total Assessment Points Out of 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mycelial Growth Inhibition (%) | | | | | | Halo Zone Diameter (cm) | | | | | |
| | | Dual Culture Assay | | | Paired Antibiosis Assay | | | | | | | | |
| | | *C.s* | *C.t* | *F.o* | *C.s* | *C.t* | *F.o* | Siderophore | Amylase | Cellulase | Chitinase | Protease | |
| 1. | AR1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 9 |
| 2. | AR10 | 2 | 2 | 1 | 1 | 2 | 2 | 2 | 1 | 1 | 1 | 0 | 15 |
| 3. | AR26 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 0 | 17 |
| 4. | AL5 | 1 | 1 | 1 | 2 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 10 |
| 5. | AL7 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 0 | 13 |
| 6. | AFE2 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 9 |

Mycelial Growth inhibition percentage (1 = 30–54.9%; 2 = 55–74.9%; 3 = 75–95%); Lytic enzyme production was evaluated with 1 point and siderophore with 2 points each; *C.s*: *C. scoville*; *C.t*: *C. truncatum*; *F.o*: *F. oxysporum.*

**Table 2.** 16S ribosomal RNA partial sequence analysis of actinobacterial isolates and their closest BLASTN matches with NCBI database supplementary.

| S. No. | Isolate Name | Isolation Source | NCBI Accession Number | Base Pair Length | Closest 16S rRNA Sequence Match (BLASTN) | | Per Cent Identity (%) |
|---|---|---|---|---|---|---|---|
| | | | | | Organism and Strain | Base Pair Length | |
| 1. | AR1 | Rhizosphere, Pudukottai | OM883984 | 1358 bp | *Streptomyces rochei* AL14 | 1448 | 99.70 |
| 2. | AR10 | Rhizosphere, Karaikal | ON692910 | 1458 bp | *Streptomyces deccanensis* WJA64 | 1462 | 99.73 |
| 3. | AR26 | Rhizosphere, Salem | ON140212 | 1432 bp | *Streptomyces tuirus* PAS9 | 1461 | 99.72 |
| 4. | AL5 | Phyllosphere: Leaf, Coimbatore | ON692752 | 1486 bp | *Streptomyces azureus* NRRL B-2655 | 1516 | 100.00 |
| 5. | AL7 | Phyllosphere: Leaf, Trichy | ON692754 | 1466 bp | *Streptomyces geysiriensis* DSD176 | 1466 | 100.00 |

### *3.7. Biocontrol Potential of Liquid Formulation and Methanol Extract of S. tuirus AR26*

Healthy chilli fruits inoculated with *C. scovillei*, *C. truncatum*, *F. oxysporum* and the co-inoculation of three pathogens produced typical fruit rot symptoms in the form of lesions of up to 2.5, 2.2, 2.6 and 2.9 cm, respectively, seven days after inoculation with the pathogens. Fruits that were not inoculated with the pathogens (healthy control) did not develop fruit rot symptoms, indicating that *C. scovillei*, *C. truncatum* and *F. oxysporum* were the causative agent of the anthracnose disease. Chilli fruits inoculated with the liquid formulation of *S. tuirus* AR26 caused no symptoms or damage to the fruits, indicating its non-pathogenic nature and biocontrol ability (Table 3). The liquid formulation of *S. tuirus* AR26 at both the concentrations 5 mL/L and 10 mL/L caused significant reductions in disease symptom when compared to the pathogen-inoculated control. Application of the liquid formulation of *S. tuirus* AR26 at 10 mL/L completely (100%) suppressed the fruit rot lesions caused by *C. truncatum*, *F. oxysporum* and *Cscovillei* + *C. truncatum* + *F. oxysporum.* *C. scovillei* inoculated fruits recorded 87.9% disease reduction with a corresponding lesion size of 0.30 cm when compared to the *C. scovillei* inoculated control (2.48 cm) (Figure 8). The liquid formulation at 5 mL/L concentration reduced the lesion size by 70.85%, 82.68%, 67.32% and 77.08%, respectively for *C. scovillei*, *C. truncatum*, *F. oxysporum* and the co-inoculation of all the three pathogens with corresponding lesion size of 0.73 cm, 0.38 cm, 0.85 cm and 0.63 cm. Irrespective of pathogens, the metabolites in the methanol extract of antagonist also had significant inhibitory effect on the suppression of fruit rot lesions on chilli fruits compared to the pathogen-inoculated control (Table 4). However, the percentage inhibition of the fruit rot lesion by the antagonist metabolites was significantly lower than the active culture formulation of *S. tuirus* AR26. Antagonist metabolites reduced the lesions up to 70.10%, 62.45%, 53.08% and 44.85% caused by *C. truncatum*, *C. scoviellei*, *F. oxysporum* and co-infection of three pathogens, respectively.

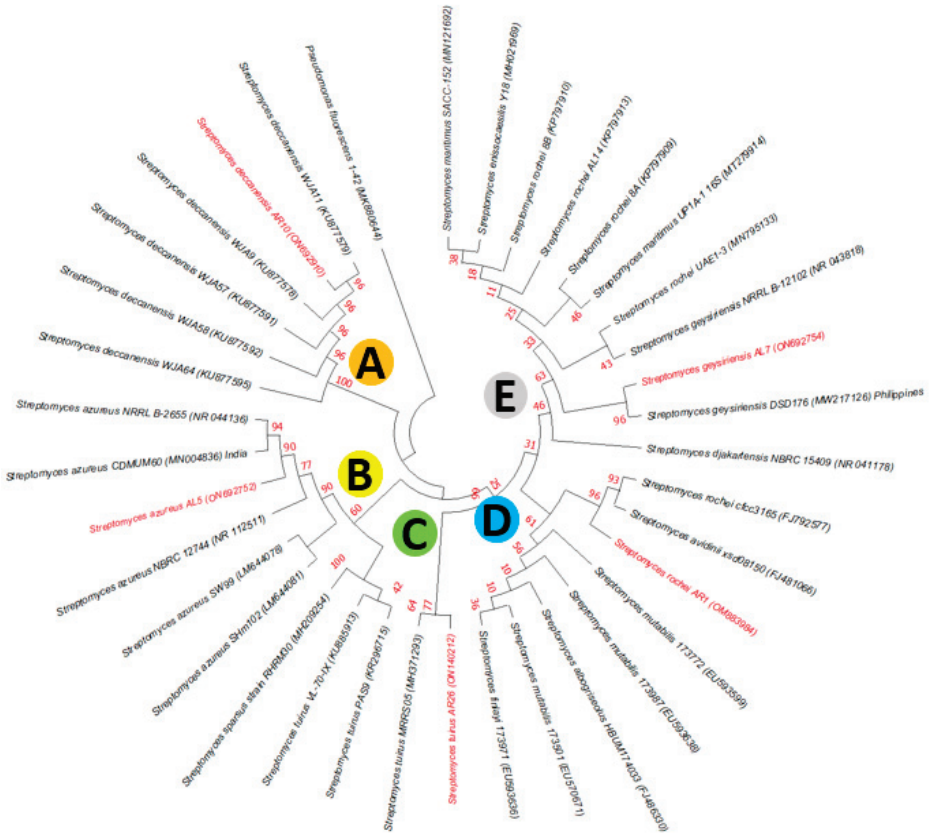



**Figure 7.** Phylogenetic tree representing the evolutionary relationships of five potent antagonistic actinobacterial isolates isolated from chilli plants. Neighbour joining (NJ) phylogenetic tree constructed from 16S rRNA sequences shows the position of five potent actinobacterial isolates (highlighted in red) and all isolates belong to the genera *Streptomyces*. Bootstrap values (expressed as percentages of 1000 replications) are shown at the nodes. *Pseudomonas fluorescens* 1-42 (MK88064) was used as an outgroup. GenBank accession numbers are given in parenthesis.

**Table 3.** Antifungal efficacy of liquid formulation of *Streptomyces tuirus* AR26 against chilli fruit rot pathogens.

| S. No | Treatments/Pathogens | Lesion Diameter (cm) | Per cent Disease Reduction (%) |
|---|---|---|---|
| 1. | T1: Healthy (uninoculated) control | 0.00 | 100.00 $^{a}$ (89.71) |
| 2. | T2: Antagonist inoculated control (10 mL/L) | 0.00 | 100.00 $^{a}$ (89.71) |
| 3. | T3: *Colletotrichum scovillei* inoculated control | 2.48 | 0.00 $^{c}$ (0.29) |
| 4. | T4: *Colletotrichum truncatum* inoculated control | 2.18 | 0.00 $^{c}$ (0.29) |
| 5. | T5: *Fusarium oxysporum* inoculated control | 2.60 | 0.00 $^{c}$ (0.29) |
| 6. | T6: Co-inoculation of *C. scovillei*, *C. capsici* and *F. oxysporum* | 2.88 | 0.00 $^{c}$ (0.29) |
| 7. | T7: *C. scovillei* + *S. tuirus* (5 mL/L) | 0.73 | 70.85 $^{d}$ (57.34) |
| 8. | T8: *C. scovillei* + *S. tuirus* (10 mL/L) | 0.30 | 90.32 $^{b}$ (80.17) |
| 9. | T9: *C. truncatum* + *S. tuirus* (5 mL/L) | 0.38 | 82.68 $^{c}$ (65.75) |
| 10. | T10: *C. truncatum* + *S. tuirus* (10 mL/L) | 0.00 | 100.00 $^{a}$ (89.71) |
| 11. | T11: *F. oxysporum* + *S. tuirus* (5 mL/L) | 0.85 | 67.32 $^{d}$ (55.41) |
| 12. | T12: *F. oxysporum* + *S. tuirus* (10 mL/L) | 0.00 | 100.00 $^{a}$ (89.71) |

**Table 3.** *Cont.*

| S. No | Treatments/Pathogens | Lesion Diameter (cm) | Per cent Disease Reduction (%) |
|---|---|---|---|
| 13. | T13: Co-inoculation of *C. scovillei*, *C. capsici* and *F. oxysporum* + *S. tuirus* (5 mL/L) | 0.63 | 77.08 $^{c,d}$ (61.53) |
| 14. | T14: Co-inoculation of *C. scovillei*, *C. capsici* and *F. oxysporum* + *S. tuirus* (10 mL/L) | 0.00 | 100.00 $^{a}$ (89.71) |
| | CD (0.05) | 0.167 | 8.040 |
| | SE (d) | 0.082 | 3.960 |

The values are the mean of three replications. The means in a column followed by the same superscript letters are not significantly different at $p = 0.05$. Values in parenthesis are arc sine transformed.

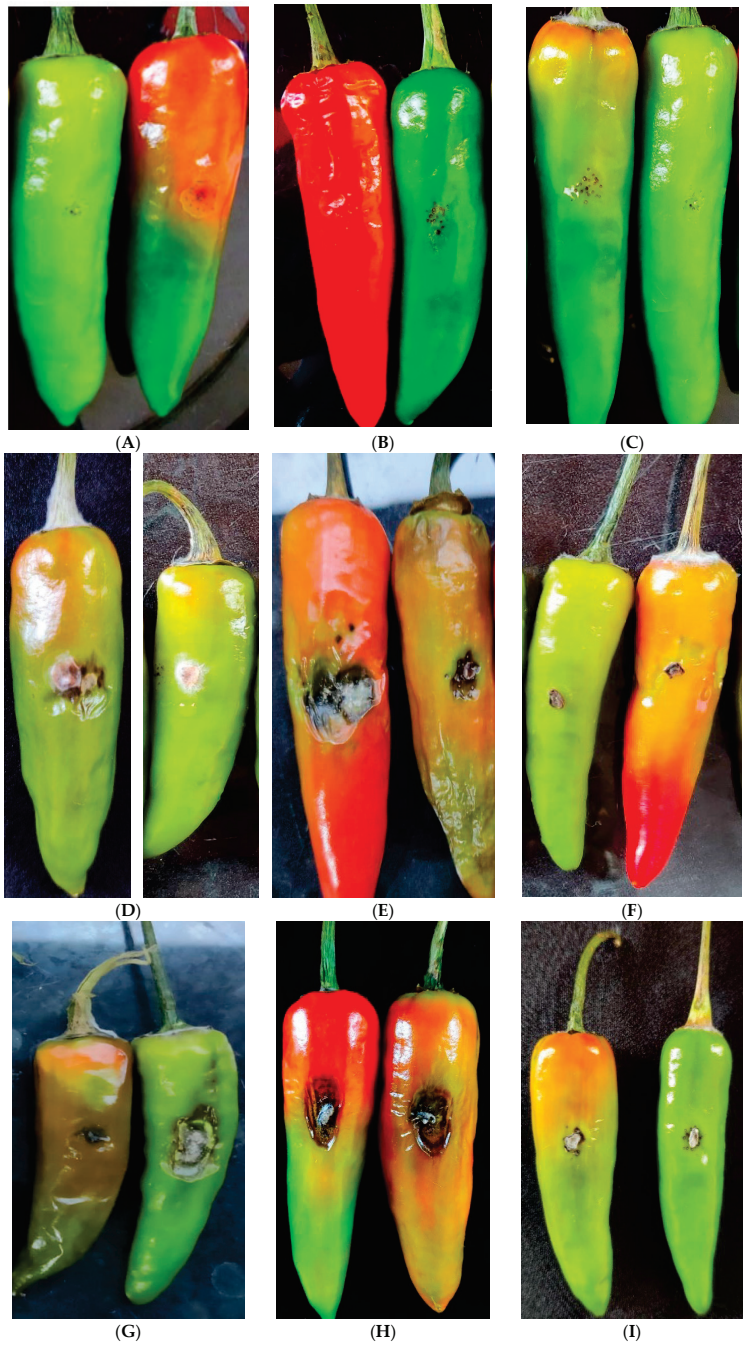



**Figure 8.** *Cont.*

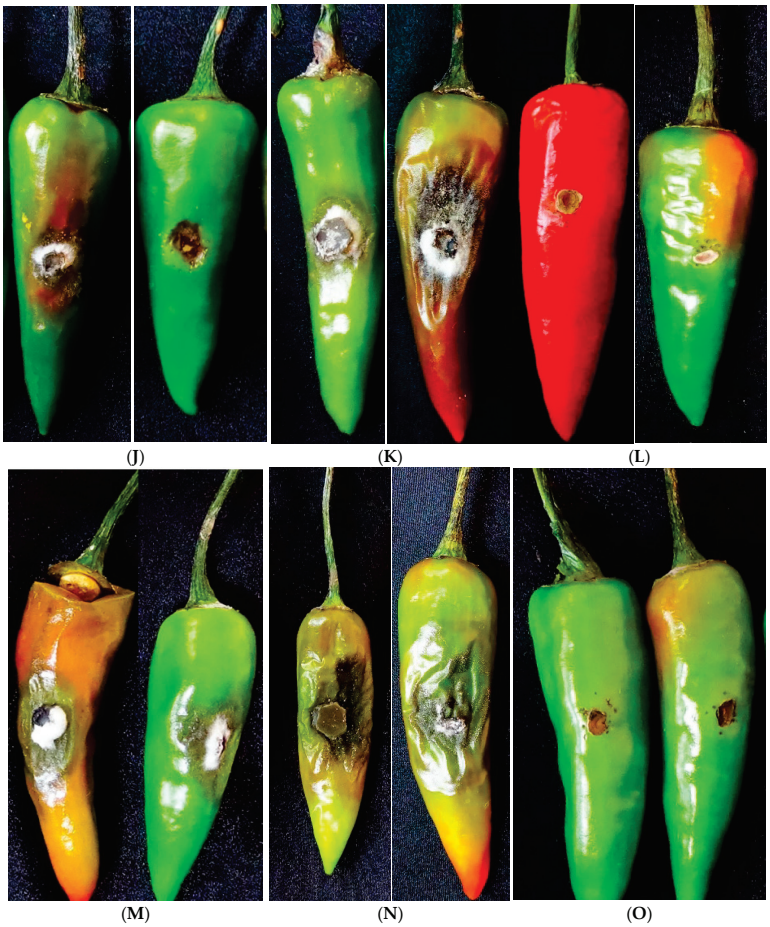



**Figure 8.** Antifungal efficacy of liquid formulation and methanol extract of *S. tuirus* AR26 against chilli fruit rot pathogens. (**A**) Fruits inoculated with sterile distilled water; (**B**) fruits inoculated with methanol alone; (**C**) fruits inoculated with liquid formulation of *S. tuirus* AR26 at 10 mL/L; (**D**) fruits inoculated with *C. scovillei* and methanol extract of *S. tuirus* AR26; (**E**) fruits inoculated with *C. scovillei* alone; (**F**) fruits inoculated with *C. scovillei* and liquid formulation of *S. tuirus* AR26 at 10 mL/L; (**G**) fruits inoculated with *C. truncatum* and methanol extract of *S. tuirus* AR26; (**H**) fruits inoculated with *C. truncatum* alone; (**I**) fruits inoculated with *C. truncatum* and liquid formulation of *S. tuirus* AR26 at 10 mL/L; (**J**) fruits inoculated with *F. oxysporum* and methanol extract of *S. tuirus* AR26; (**K**) fruits inoculated with *F.oxysporum* alone; (**L**) fruits inoculated with *F. oxysporum* and liquid formulation of *S. tuirus* AR26 at 10 mL/L AR26; (**M**) fruits inoculated with *C. scovillei*, *C. truncatum*, *F. oxysporum* and methanol extract of *S. tuirus* AR26; (**N**) fruits inoculated with *C. scovillei*, *C. truncatum*, *F. oxysporum* alone; (**O**) fruits inoculated with *C. scovillei*, *C. truncatum*, *F. oxysporum* and liquid formulation of *S. tuirus* AR26 at 10 mL/L.

**Table 4.** In vivo antifungal efficacy of methanol extract of *S. tuirus* AR26 against chilli fruit rot pathogens.

| S. No | Treatments/Pathogens | Lesion Diameter (cm) | Per cent Disease Reduction (%) |
|---|---|---|---|
| 1. | T1: Healthy (uninoculated) control | 0.0 | 100.00 $^{a}$ (89.71) |
| 2. | T2: Methanol extract inoculated control | 0.0 | 100.00 $^{a}$ (89.71) |
| 3. | T3: *C. scovillei* inoculated control | 2.93 | 0.00 $^{f}$ (0.29) |
| 4. | T4: *C. truncatum* inoculated control | 2.50 | 0.00 $^{f}$ (0.29) |
| 5. | T5: *F. oxysporum* inoculated control | 2.35 | 0.00 $^{f}$ (0.29) |

**Table 4.** *Cont.*

| S. No | Treatments/Pathogens | Lesion Diameter (cm) | Per cent Disease Reduction (%) |
|---|---|---|---|
| 6. | T6: Co-inoculation of *C. scovillei*, *C. capsici* and *F. oxysporum* | 3.40 | 0.00 $^{f}$ (0.29) |
| 7. | T7: *C. scovillei* + Methanol extract of *S. tuirus* | 1.10 | 62.45 $^{C}$ (52.22) |
| 8. | T8: *C. truncatum* + Methanol extract of *S. tuirus* | 0.75 | 70.10 $^{b}$ (56.99) |
| 9. | T9: *F. oxysporum* + Methanol extract of *S. tuirus* | 1.10 | 53.08 $^{d}$ (46.79) |
| 10. | T10: Co-inoculation of *C. scovillei*, *C. capsici* and *F. oxysporum* + methanol extract of *S. tuirus* | 1.88 | 44.85 $^{e}$ (46.04) |
| | CD (0.05) | 0.326 | 3.129 |
| | SE (d) | 0.159 | 1.525 |

The values are the mean of three replications. The means in a column followed by same superscript letters are not significantly different at $p = 0.05$. Values in parenthesis are arc sine transformed.

## 4. Discussion

The use of synthetic fungicides is a common practice among farmers for many decades for the management of chilli fruit rot disease; however, this can cause several ill effects to the environment and living creatures. The most urgent and necessary activity of human society is to eliminate the use of fungicides in food crops [66] like chilli which are directly consumed by people. Hence, protecting crops with safe biocontrol agents will not only address concerns about fungicide residues in fresh and processed products, but also increase the export value of fungicide-free food products in domestic as well as world markets. Furthermore, antagonistic microbe–plant interactions reduce the dependence on chemical pesticides by upto 20% [67]. Native actinobacteria adopting a dual role as a biocontrol agent and biofertilizer is a more sustainable, promising and versatile candidate towards the eco-friendly management of plant disease with multiple benefits to society and the ecosystem as a whole.

Hence, in the present study, around 52 actinobacterial isolates were screened for their antagonistic activity against the chilli fruit rot pathogens. Among which, six isolates AR1, AR10, AR26, AL5, AL7 and AFE2 exerted strong antifungal activity against all three pathogens with an inhibition zone of >2 cm and belonged to the genus *Streptomyces*. It was evidenced from previous literature that several species of *Streptomyces* have emerged as biocontrol agents that are safe alternatives to synthetic fungicides for the management of phytopathogens [68,69]. There is ample scientific evidence indicating the successful interaction of various *Streptomyces* spp. with chilli plants to curtail the infection of fruit rot pathogens both at pre- and post-harvest levels. Shahbazi et al. [70] reported that *Streptomyces rochei* strain P42 displayed the highest inhibitory activity against *C. acutatum*, *C. capsici* and *C. gloeosporioides*. *S. griseocarneus* R132 inhibited the development of anthracnose symptom in chilli fruits [47], and likewise the application of *S. violaceoruber* fermentation broth reduced the incidence of the chilli anthracnose under greenhouse conditions [30].

The results of the present study also revealed that a higher proportion of native rhizospheric actinobacteria exert strong antagonistic activity against *C. scovillei* and *C. truncatum* compared to phyllospheric and endophytic isolates. Similar results were also highlighted by Shahbazi et al. [70], who reported that out of 66 native rhizosphere strains of streptomycetes, 16 strains showed very strong to moderate inhibition against *C. acutatum*, *C. capsici* and *C. gloeosporioides*. Many researchers have reported that diverse species of actinobacteria are recognized to play a crucial function in the rhizosphere by suppressing pathogenic species, as well as promoting the growth and multiplication of beneficial microbes. *Streptomyces* is one of the most dominant and promising biocontrol bacterial genera of plant diseases which efficiently colonise the plant rhizosphere and are known to produce over two-third of antibiotics with the ability to inhibit a wide range of phytopathogens [71,72]. Hyder et al. [73] stated that eight native rhizospheric bacterial isolates obtained from chilli plants were

found to exert antifungal activity against damping pathogen *Phytophthora capsici* in vitro and in vivo.

Based on the dual culture and paired antibiosis assay, the actinobacterial isolate AR26 obtained from chilli rhizosphere, which was subsequently identified as *Streptomyces tuirus*, was found to be the most effective isolate in inhibiting the mycelial growth of all the three tested pathogens. This finding is in accordance with the results of Chaudhry [74] who reported that *S. tuirus* strongly inhibited carrot cavity spot pathogen *Pythium violae* including various other pathogens such as *Phytophthora spinosum*, *Phytopythium helicoides*, *Fusarium oxysporum*, *Fusarium falciforme*, *Fusarium solani*, *Sclerotium rolfsii*, and *Sclerotinia sclerotiorum*. Scanning electron micrographs of the interaction of *S. tuirus* with fruit rot pathogens in the dual culture plate revealed mycelial deformities like shrinkage, distortion and aggregation of *C. scovillei*, *C. truncatum* and *F. oxysporum* hyphae, in contrast to dense, smooth and regular mycelium in the control plate. Xu et al. [75] also observed severe morphological and internal abnormalities such as the shrinkage and aggregation of *Magnaporthe oryzae* hyphae when treated with the culture filtrate of rice endophyte *Streptomyces hygroscopicus* OsiSh-2.

*S. tuirus* also exhibited positive results for most of the antifungal bioassays under study. It is the most potent antagonist to produce almost all the tested extracellular hydrolytic enzymes, most prominently cellulase and chitinase which are reported to be the important hydrolytic enzymes responsible for the biocontrol ability of an antagonist. High chitinase-producing strains are more antagonistic to fruit-rotting pathogens compared to low-chitinase-producing strains [76]. Jha and Modi [77] and Bhattacharyya et al. [78] pointed out that the genus *Streptomyces* is an efficient producer of various lytic enzymes, which plays an important role in the biological control of plant diseases by degrading the cell wall of phytopathogenic fungi made up of chitins and glucans. It is also evident from the earlier reports that *Streptomyces* spp. are significantly responsible for the suppression of plant diseases through the production of chitinase, glucanase [40] and protease [79]. Shahbazi et al. [70] stated that the production of hydrolytic enzymes, especially chitinases, can be considered as a potential antagonistic mechanism against chilli anthracnose pathogens. Therefore, the production of these enzymes will help to select potential actinobacterial isolates for the biological control of the tested pathogens.

*S. tuirus* AR26 is also a highly efficient synthesizer of siderophore which is considered to be one of the most important mechanisms for the biocontrol of plant pathogens [80], in which the antagonist inhibits pathogen growth by depriving it of the available iron in the environment [81]. Hence, it is possible that the siderophore-producing ability of *S. tuirus* AR26 might also have contributed to the suppression of mycelial growth of all the tested pathogens. It is similar to the finding of Liotti et al. [47] who reported the possible role of siderophore of *S. griseocarneus* R132 in the biocontrol of *F. oxysporum* in chilli.

Volatile, non-volatile and thermostable compounds of the *S. tuirus* isolate AR26 also reported significant antifungal activity, particularly volatile organic compounds, which recorded the maximum antifungal activity against the fruit rot pathogens. Many *Streptomyces* spp. were reported to produce various volatile compounds that were effective against the anthracnose disease in various crops [82]. The volatile compounds from *Streptomyces philanthi* RM-1-138 and *Streptomyces* spp. are highly potent for the biocontrol of chili anthracnose caused by *C. gloeosporioides* PSU-NY8 [14] and cucumber anthracnose caused by *C. orbiculare* [83] respectively in the post- harvest pathosystem. Metabolites produced by *Streptomyces* include bioactive compounds such as macrolide, benzoquinones, aminoglycosides, polyenes, and nucleoside antibiotics that are involved in the suppression of various phytopathogens [84,85].

The results of detached fruit assay revealed that application of active antagonists in the form of a liquid bio-formulation was found to be most effective against all the three pathogens compared to methanol extracts. The active culture of the antagonist *S. tuirus* AR26 in the liquid bio formulation caused a significant reduction in the expression of fruit rot symptom, ranging from 87.9% to as high as 100%. It completely suppressed the

expression of symptoms caused by *C. truncatum*, *F. oxysporum* and *C. scovillei* + *C. truncatum*, *F. oxysporum* in chilli fruits, which is approximately 30%, 50% and 55% higher than the suppression by the methanol extract. Our finding is in line with the research findings of Sadeghian et al. [50] who also reported that active antagonists as practical formulations seem more effective compared to crude extracts against the bitter rot of apple fruits caused by *C. gloeosporioides*.

Therefore, the inhibition of fruit rot pathogens observed in this study might be due to the antagonistic potential of *S. tuirus* AR26 through the production of antifungal compounds, siderophores, chitinase or through the synergistic action of all these mechanisms. It has been documented in earlier findings that the antifungal ability of actinobacteria might be due to the synergistic activity of two or more antagonistic mechanisms. Furthermore, Evangelista-Martínez [86] also reported that the *Streptomyces* sp. CACIA-1.46HGO strain inhibited the hyphal growth of many fungal plant pathogens by the production of secondary metabolites, extracellular enzymes and probably by the combined effect of these mechanisms. It is well understood from the findings of Yasmin et al. [87] who reported that the antagonistic activity of *Pseudomonas* spp. E227, E233, Rh323, *Serratia* sp. Rh269 and *Bacillus* sp. might be due to the production of siderophores, lytic enzymes and HCN or the synergistic interaction of these two or with other metabolites.

## 5. Conclusions

The management of chilli fruit rot disease still continues to be the focus of intensive research. Though there are several ways of managing this disease, none of the methods were found to be completely successful when applied alone. Hence a preliminary attempt was made to screen the antifungal activity of native actinobacteria against fruit rot pathogens under in vitro conditions. Current results confirmed the potentiality of native actinobacterial isolate *S.tuirus* AR26 to be exploited as a biointensive component under an integrated disease management strategy. The actinobacteria *S. tuirus* AR26 exhibited multifarious biocontrol mechanisms such as the production of volatile, non-volatile and thermostable compounds, competition for iron through the synthesis of siderophores, and production of extracellular lytic enzymes such as chitinase and cellulases. Hence, *S. tuirus* AR26 has a great scope for evaluating its biocontrol potential against chilli fruit rot disease under field conditions as well against a broad spectrum of post-harvest plant pathogens. Larger investigations in the future will demonstrate such possibilities. As farmers become increasingly aware of the concept of sustainable agriculture and organic farming, use of this actinobacteria based bio-formulation will definitely address concerns about ecologically sustainable and socially acceptable long-term solutions to tackle notorious fruit rot pathogens.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/life13020426/s1, Figure S1: Paired antibiosis assay for antifungal activity of actinobacterial isolates against chilli fruit rot pathogens; Figure S2: Relative ability of actinobacterial isolates to produce siderophore and extracellular hydrolytic enzymes; Figure S3: Screening of actinobacterial isolates for amylase activity; Figure S4: Screening of actinobacterial isolates for cellulase activity; Figure S5: Screening of actinobacterial isolates for chitinase activity; Figure S6: Screening of actinobacterial isolates for protease activity; Figure S7: Screening of actinobacterial isolates for siderophore production; Table S1: In vitro antifungal activity of actinobacterial isolates against chilli fruit rot pathogens.

**Author Contributions:** Conceptualization, R.R., K.P., L.R., R.A., L.P., T.R. and G.K.; methodology, R.R., L.R., K.P., R.A. and L.P.; validation, R.R., K.P., L.R. and R.A.; formal analysis, R.R.; investigation, R.R.; K.P. and L.R.; resources, K.P., L.R., R.A., L.P., T.R. and G.K.; data curation, R.R.; writing—original draft preparation, R.R.; writing—review and editing, R.R., K.P. and R.A.; visualization, R.R., K.P. and L.R.; supervision, K.P.; project administration, K.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The dataset supporting the conclusions of this article are included within the article and its additional files.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Physiological and Anatomical Responses of Faba Bean Plants Infected with Chocolate Spot Disease to Chemical Inducers

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**Abstract:** Plant diseases are biotic stresses that restrict crop plants' ability to develop and produce. Numerous foliar diseases, such as chocolate spots, can cause significant production losses in *Vicia faba* plants. Certain chemical inducers, including salicylic acid (SA), oxalic acid (OA), nicotinic acid (NA), and benzoic acid (BA), were used in this study to assess efficacy in controlling these diseases. A foliar spray of these phenolic acids was used to manage the impacts of the biotic stress resulting from disease incidence. All tested chemical inducers resulted in a significant decrease in disease severity. They also enhanced the defense system of treated plants through increasing antioxidant enzyme activity (Peroxidase, polyphenol oxidase, β-1, 3-glucanase, and chitinase) compared to the corresponding control. Healthy leaves of faba plants recorded the lowest ($p < 0.05$) values of all antioxidant activities compared to those plants infected by *Botrytis fabae*. Moreover, the separation of proteins using SDS-PAGE showed slight differences among treatments. Furthermore, foliar spray with natural organic acids reduced the adverse effects of fungal infection by expediting recovery. The SA (5 mM) treatment produced a pronounced increase in the upper, lower epidermis, palisade thickness, spongy tissues, midrib zone, length, and width of vascular bundle. The foliar application with other treatments resulted in a slight increase in the thickness of the examined layers, especially by benzoic acid. In general, all tested chemical inducers could alleviate the adverse effects of the biotic stress on faba bean plants infected by *Botrytis fabae*.

**Keywords:** chocolate spot disease; *Botrytis fabae*; faba bean; antioxidant enzymes; protein banding and anatomy

**Citation:** Alnefaie, R.M.; EL-Sayed, S.A.; Ramadan, A.A.; Elmezien, A.I.; El-Taher, A.M.; Randhir, T.O.; Bondok, A. Physiological and Anatomical Responses of Faba Bean Plants Infected with Chocolate Spot Disease to Chemical Inducers. *Life* **2023**, *13*, 392. https://doi.org/10.3390/life13020392

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 6 December 2022
Revised: 20 January 2023
Accepted: 23 January 2023
Published: 31 January 2023



## 1. Introduction

Faba bean (*Vicia faba*) is Egypt's most important legume crop and is widely produced throughout the Mediterranean as a protein source for human and animal consumption [1]. The high protein content of faba bean ranges from 25 to 35 percent and has been credited with its nutritional value. The seeds are also high in thiamin, tocopherols, niacin, and folic acid and are notably high in calcium and iron [2]. At the same time, its cultivation increases the amount of nitrogen in the soil [3].

Chocolate spot disease of faba bean is caused by *Botrytis fabae* and *B. cinerea* and is considered the most important disease in the Northern region of the Egyptian Nile Delta [4], which has relatively low temperature and, at the same time, high relative humidity that favor the spread of this disease [5]. The disease causes a severe yield loss reaching 60–80%

among the susceptible cultivars [6]. It decreases the total carbohydrates, nitrogen, nucleic acid, and protein contents of the yielded seeds [7]. Under stress conditions, the production of reactive oxygen species (ROS) (known for signaling intermediates during abiotic and biotic stress conditions) increases and causes plant oxidative stress [8]. In fact, ROS damages cellular membranes in the processes of lipid peroxidation and are also able to cause harmful effects on DNA, proteins, and chlorophyll [9]. Plants produce several major antioxidant enzymes, for example, superoxide dismutase (SOD), which has an essential role in singlet oxygen and scavenging ROS from the cytosol, mitochondria, and chloroplasts in the cell [10].

Utilization of chemical inducers is a new approach in fungal and bacterial infections control within an environmentally friendly defense system in crop plants. These substances induce resistance throughout the signal transduction system, which promotes the production of specific enzymes that catalyze biosynthetic responses to form resistance compounds such as polyphenols, and pathogenesis-related proteins that enhance the plant resistance to pathogens [11]. Salicylic acid (SA) is an endogenous growth regulator that works as a phenolic non-enzymatic antioxidant (a defense mechanism in plants against stress conditions) that helps plants to regulate some physiological activities [12]. The SA also plays a vital role in the plant growth and development, seed germination, pigmentation, photosynthesis, transpiration rate, ion uptake and transport, fruit yield, glycolysis, and induces changes in leaf anatomy and chloroplast ultrastructure [13]. The use of SA significantly reduced chocolate spot disease severity in faba beans caused by *B. fabae* [14,15].

From the anatomical point of view, Cárcamo et al. [16] on *Zea mays*, L., Nour et al. [17] on bean and Gomaa et al. [18] on *Lupinus termis* L., reported that SA minimized the harmful effects of stress conditions. Applying SA improved anatomical measurements of cell wall, epidermis, fiber strands, cortex, xylem and phloem tissues, the parenchymatous area of the pith and vessel diameter, midvein, and leaflet lamina. Benzoic acid (BA) is a natural antioxidant organic acid also considered a biosynthetic precursor of SA [19]. It works as a key intermediate in shikimate and phenyl propanoid pathways. Shikimic acid is a precursor of many alkaloids, aromatic amino acids, and indole derivatives that improve plant growth and productivity [20] and provide plants with abiotic stress tolerance [21].

Moreover, oxalic acid (OA) is crucial in controlling fungal infection [22] since fungal mutants deficient in OA production were non-pathogenic to common bean plants [23]. Decreasing OA accumulation by using fungal mutants or the overexpression of oxalate oxidase leads to ROS generation, allowing the plant to activate some defense responses [24]. At later stages of pathogen infection, OA reduces ROS production [25]. As a result, at an advanced stage of *Sclerotinia sclerotiorum*, the plant antioxidant system most likely plays a role in inhibiting ROS formation [26]. Nicotinic acid (NA), known also as niacin, nicotinamide, and vitamin $B_3$, is a known component of the pyridine dinucleotide coenzymes NADH and NADPH, which are involved in a variety of enzymatic oxidation-reduction events in plant cells [27]. Nicotinamide is a growth-regulating substance that can modify various physiological features of plants in small amounts [28]. Moreover, nicotinamide is a stress-related chemical that causes and controls the activity of the secondary metabolic accumulation process and/or defensive metabolism expressed in plants [29]. Niacin may be utilized to improve stress tolerance in kiwi fruit when exposed to short-term stressful conditions [30]. Furthermore, foliar spray with the niacin solution increased NADPH and $NADP^+$ levels and decreased both $O_2^-$ generation and $H_2O_2$ content for a short time.

The present study examines the protective effects of selected organic acids and resistance inducers in controlling the chocolate spot disease of faba bean plants and studying their effects on the antioxidant defense system.

## 2. Materials and Methods

Chocolate spot disease of faba bean was surveyed at six Egyptian Governorates, namely El-Beheira (Nubaria and Kafr-Eldawar), Kafer El-Sheik (Sakha), Gharbiya (Tanta), Minufiya (Serce-Alian), Sharkia (Zagazig) and Qalubia (Qalub). The severity of the choco-

late spot disease of faba bean in Egypt varied by local weather (temperature and humidity) in each Governorate.

The survey was conducted during 2019/2020 and 2020/2021 growing seasons, where the survey was chosen to coincide with the flowering, fruiting, and late fruiting stages of faba bean when the disease reached its peak [31,32]. The survey of the examined sites started at one corner of each field and transected in an M-shaped pattern for approximately 800 paces, stopping at ten equally spaced spots along the way for sampling.

### *2.1. Studies on the Causal Pathogens*

#### 2.1.1. Isolation of Chocolate Spot Pathogens

Samples of faba bean leaves naturally infected with chocolate spot disease symptoms were collected from the studied locations at the flowering stage. The infected leaves were cut into small pieces (5 mm), each containing a single lesion. The infected tissues were sterilized by soaking in 5% sodium hypochlorite for two minutes, then washed thoroughly several times with sterilized distilled water and dried between two layers of sterilized filter paper. The surface sterilized pieces were transferred onto potato dextrose agar (PDA) plates at the rate of five pieces/plate. All plates were incubated at 20 ± 1 °C for 5–7 days. The isolated fungi were purified using the hyphal tip technique [33].

#### 2.1.2. Identification of Isolated Fungi

Isolated fungi were identified as described by Moussa et al. [34] according to their morphological and microscopical characteristics. The identification was carried out at the Department of Mycology, Survey and Identification Unit, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt. Pure cultures of each isolate were kept on PDA slants at 4 °C for further studies.

#### 2.1.3. Pathogenicity Test

Pathogenicity test was carried out using seven local isolates of *B. fabae*, i.e., (Nubaria, Kafer-Eldawer, Sakha, Tanta, Serce-Alian, Zagazig, and Qalub) under greenhouse conditions.

##### Inoculum Preparation

Isolates of *B. fabae* were grown on leaves of faba bean extract agar media. For spore suspension preparation, the medium was added into sterilized Petri dishes prior to solidification. Then the solidified media in plates were inoculated on equal discs (5 mm) of each test isolate and incubated at a temperature of 20 °C ± 1 for a period of 12 days [35] under alternating light (12 h) and darkness (12 h) procedure in automatically incubated to boost the production of spores. For replication, a total of ten plates were used for each isolate. When the incubation period passed, 10 mL of distilled sterilized water was added to the plates and then brushed carefully using a rubber brush. Three layers of cheesecloth were used to filter the suspension in order to eliminate the residues of mycelia. A Spencer Haemacytometer slide was used to count the number of spores/mL in the spore suspension, and then the spores/mL rate was adjusted to approximately $2.5 \times 10^5$ of *B. fabae.*

##### Plant Preparation

Faba bean susceptible cultivar Giza 429 (*Vicia faba*, L.) used in these experiments were obtained from the Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. Four abiotic inducers (SA, NA, OA, and BA) were obtained from Sigma Company.

Faba bean plants were grown in plastic pots (20 cm), each planted with eight seeds and thinned to five plants/pot with five pots specified for each treatments under greenhouse conditions. After forty-five days from sowing, each group of faba bean plants was sprayed until runoff of abiotic inducers 24 h before inoculation with *B. fabae* at the rates of 1, 3, and 5 mM for salicylic acid (SA), 1, 2, and 3 mM for Nicotinic acid (NA) and Oxalic acid (OA). Furthermore, Benzoic acid (BA) was applied at 0.8, 1.6, and 3.2 mM. All examined

materials were firstly dissolved in 2 mL of 100% dimethyl sulfoxide (DMSO) and then adjusted to the final concentration using sterilized water for each inducer to examine the possibility of alleviating the adverse effects of chocolate spot. The sprayed plants were covered with polyethylene bags for two days before spraying with *B. fabae* spore suspension ($2.5 \times 10^5$ spores/mL), about 10 mL/each pot. Whereas the control plants were sprayed with 10 mL sterilized water only.

Pathogenicity Assessment and Development of Choloate Spot Disease

The inoculated plants were examined for chocolate spot disease infection. The diease severity were recorded after 2, 3, and 5 days of spray *B. fabae* inoculation. This test was done under greenhouse conditions following Bernier et al. [36]. Moreover, four abiotic inducers, SA, NA, OA, and BA, were used as comparison treatments. Each treatment was repeated three times, and the experimental design was a randomized complete block design under open field conditions to investigate their effectiveness against chocolate spot disease severity in faba bean. The inducers were sprayed twice, first at 15 days (at the 1st leaf stage) while the other was at 30 days (at the 6th leaf) from sowing. After that results were recorded with natural infection at 15, 30, and 50 days after the second spray treatment in the two successive seasons (2019/2020 and 2020/2021).

Determination of Chocolate Spot Disease Severity

The disease severity was recorded after 2, 3 and 5 days of inoculation using the scale (0–9) using the following equation:

$$\text{Disease severity \%} = \Sigma(n \times v)/9N \times 100 \quad (1)$$

whereas: n = number of plants in every grade, v = numerical grade, N = total number of examined plants, and 9 = maximum disease grade.

### *2.2. Biochemical Analysis*

Antioxidant activity of some enzymes performed on the tested inducers best concentration (5 mM for SA, 3 mM for both OA and NA and 3.2 mM BA) which were noticiable in disease severity results. Treated and untreated samples were taken before spraying and 6, 12, 24, and 48 h after spraying. A known weight of *vicia faba* leaves which was extracted in 10 mL of 100 mM phosphate buffer (pH 6.8) and kept at 4 °C overnight. The extract was centrifuged at 5000 rpm for ten minutes and reserved to assay the activities of enzymes [37].

#### 2.2.1. Peroxidase (POX) Assay

The POX activity was assayed according to [38]. Aliquot of 0.2 mL plant enzyme extract was reacted with 5.8 mL of phosphate buffer (50 mM; pH 7.0), 2.0 mL pyrogallol (20 mM) and 2.0 mL hydrogen peroxide (20 mM). The increase in absorbance was determined within 60 s against a reagent without enzyme at 470 nm using a spectrophotometer. The amount of crude enzyme that converts one mM of hydrogen peroxide in one minute at room temperature equals one unit of enzyme activity.

#### 2.2.2. Polyphenol Oxidase (PPO) Assay

The PPO activity was assayed according to Atrooz [39]. A volume of 2.0 mL extract of plant enzyme was reacted with 1.2 mL of phosphate buffer (pH 6.8) and 0.6 mL catechol (2%). The blank tube has only the substrate and the buffer. Thenafter, all samples incubated for 5 min. and the reaction stopped by adding 1 mL of $H_2SO_4$ and the optical density was read at wavelength 430 nm by a spectrophotometer at intervals of 20 min for 100 min. The activity of PPO was expressed as the change in the absorbance of the mixture every 0.5 min. period.

#### 2.2.3. B-1, 3 Glucanase Assay

The method of Abeles and Forrence [40] was used to determine B-1, 3 glucanase activity. Laminarin was used as substrate and dinitro salicylic acid as reagent to measure the reducing sugars. Plant enzyme extract (0.5 mL) was added to 0.5 mL of 0.05 M potassium acetate buffer (pH 5) containing 2% Laminarin. The mixture was incubated for 60 min at 50 °C. The reaction was stopped by adding one ml of dinitrosalicylic acid reagent and heating the tubes for 5 min at 100 °C. The tubes were cooled and 3 mL of distilled water were added before assay. The optical density was adjusted at 500 nm. B-1, 3 glucanase activity was expressed as mM glucose equivalent released gram fresh weight tissues/60 min.

#### 2.2.4. Chitinase Assay

Twenty five grams of chitin was milled, suspended in 250 mL of 85% phosphoric acid ($H_3PO_4$) and stored at 4 °C for 24 h, then blended in 2 L of distilled water using a warning blender and the suspension was centrifuged. This washing procedure was repeated twice. The colloidal chitin suspension in the final wash was adjusted to pH 7.0 with 1 N NaOH, separated by centrifugation and the pelted colloidal chitin was store at 4 °C. The determination was carried out according to the method of [41]. One mL of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) in a test tube, then one ml of enzyme extract was added and mixed by shaking. Tubes were kept in a water bath at 37 °C for 60 min, then cooled and centrifuged before assaying. Reducing sugar was determined by adding 1 mL of supernatant with 1 mL of dintrosalicylic acid and 3 mL distilled water in the test tubes and boiled in water bath for 5 min and then cooled, then determined at 540 nm. Chitinase activity was expressed as mM N-acetyl glucose amine equivalent released gram fresh weight tissue/60 min.

#### 2.2.5. Protein Profile

The electrophoretic protein banding pattern of faba bean leaves. 0.2 g were extracted with 1 mL of protein bufferand kept in the freezer overnight and then vortexed for 15 s and centrifuged at 5000 rpm at 4 °C for 15 min. Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed [42], The molecular weight of the isolated proteins was estimatedusing standard molecular weight markers (standard protein markers, 35–320 kDa; Sigma, St. Louis, MO, USA). The protein bands were stained with silver nitrate following the method described by Sammons et al. [43].

### *2.3. Anatomical Studies*

Samples were prepared for anatomical investigation according to the method proposed by Nassar and El-Sahhar [44]. One a square centimeter of the terminal leaflet was removed, and it was dehydrated in a succession of solutions with ethyl alcohol concentrations ranging from 50% to 100%. The samples were then embedded in paraffin wax (the melting point of paraffin wax range is 58–62 °C using xylol as a solvent). Sections were cut at a thickness of 15 microns using a rotary microtome and then mounted on slides using egg albumin as an adhesive agent. The slides were subjected to a declining sequence of ethyl alcohol solutions ranging from 100% to 50% ethyl alcohol concentrations. The anatomical characters (Each value represents five sections with five readings each) of faba been leaflet including the upper epidermal layer (µm), lower epidermal layer (µm), palisade tissue thickness (µm), spongy tissue thickness (µm), length of the vascular bundle (µm), and width of the vascular bundle (µm) While in the anatomical study, the percentage (%) was calculated to show the increase or decrease attributed to the control.

### *2.4. Statistical Analysis*

A significant differences test among means of five replicates was performed at a signifi cance level of $p < 0.05$ using the LSD (Least Significant Difference) test [45] using the SPSS software. However, under open field conditions, three replicates were used to compare mean differences.

## 3. Results

### *3.1. Severity of Chocolate Spot Disease*

Four abiotic inducers, including SA, NA, OA, and BA were investigated for their effects on the severity of chocolate spot disease on faba bean plants grown in greenhouse conditions (Figure 1).

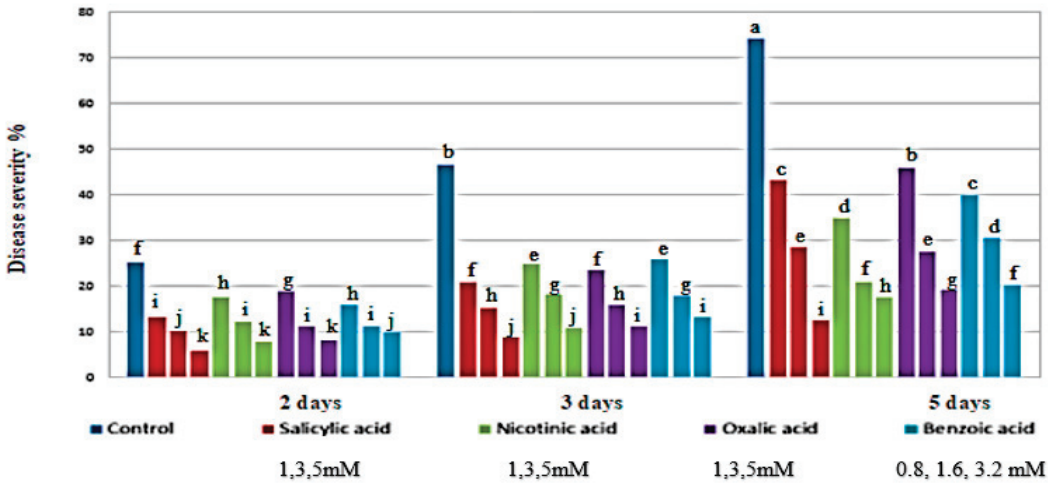


**Figure 1.** Effect of spraying faba bean plants with varying concentrations of organic acids on the severity of chocolate spot disease under greenhouse conditions. Letters a to k represent significant levels ($p < 0.05$), i.e., a treatment with the letter "a" is significantly different from "b", and "b" is significantly different from "c", and so forth. If two treatments have the same letter, they are not significantly different from each other.

The presented results indicated that all tested abiotic inducers clearly decreased disease severity. There was a positive correlation between the reduction of disease severity and increments in abiotic inducer concentrations. Plants treated with salicylic, nicotinic, oxalic, and benzoic acids reduced chocolate spot disease severity by 83.2, 76.6, 74.2, and 72.8%, respectively, at the highest used concentration.

### *3.2. Development of Chocolate Spot Disease*

Effects of four abiotic inducers (SA, OA, NA, and BA) on chocolate spot disease severity in faba bean, compared to the control, after 15, 30, and 50 days of treatment with inducers under open field conditions are illustrated in Figure 2.

Results showed that spraying faba bean plants with the tested abiotic inducers were able to manage chocolate spot disease using the tested treatments during the two studied seasons. The result indicated that disease severity increased by increasing the plant age. Whereas among abiotic inducers, salicylic acid at a concentration of 5 mM gave the highest reduction through the two seasons by 81.73 and 79.59%, respectively, followed by nicotinic acid at 3 mM concentrations that led to 73.99 and 71.32% reduction, respectively. Benzoic acid at the concentration of 3.2 mM resulted in the lowest reduction in the two seasons by 60.70 and 56.98%, respectively.

### *3.3. Antioxidant Enzymes Activity*

The effect of selected chemical inducers (SA: 5 mM, OA: 3 mM, BA: 3.2 mM, and NA: 3 mM) in addition to the untreated control on the activity of enzymes (Peroxidase, polyphenol oxidase, β-1, 3-glucanase, and chitinase enzymes) were presented in Figure 3. It is worth mentioning that the antioxidant activity was increased with increasing the action time of the content of all tested enzymes till 24 h and then decreased compared to the corresponding control. The healthy faba bean leaves recorded significantly ($p < 0.05$) the lowest value of all antioxidant action compared to the infected plants with *B. fabae*.

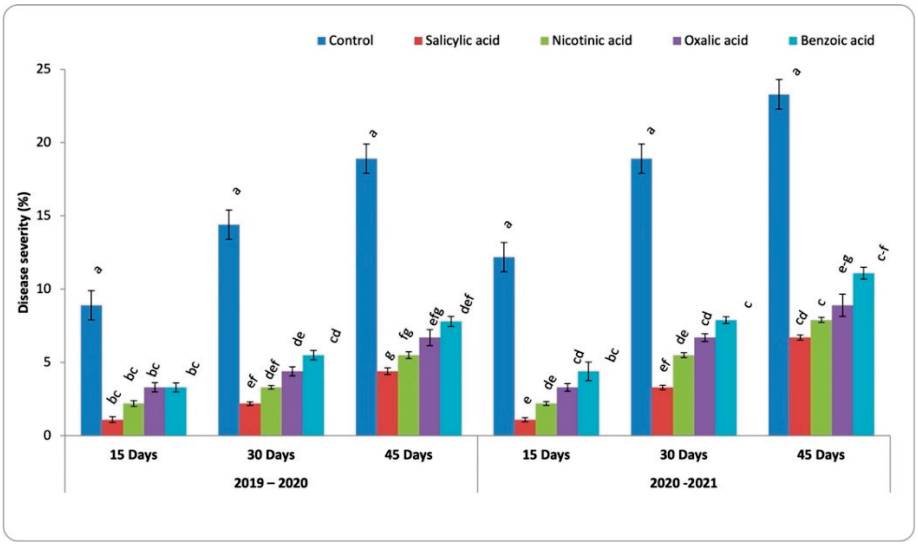


**Figure 2.** Effect of spraying faba bean with various concentrations of organic acids on the development of chocolate spot disease in field conditions in the two successive seasons. Letters a to k represent significant levels ($p < 0.05$), i.e., a treatment with the letter "a" is significantly different from "b", and "b" is significantly different from "c", and so forth. If two treatments have the same letter, they are not significantly different from each other.

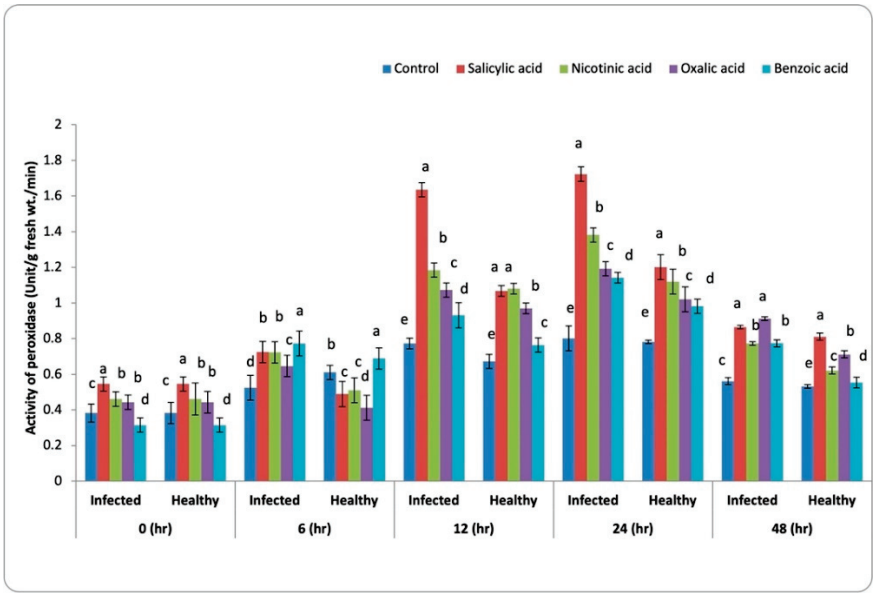


**Figure 3.** Activity of peroxidase (Unit/g fresh wt./min) in faba bean leaves infested with *B. fabae* plants treated with chemical inducers. LSD 5% for healthy and infected plants at 0, 6, 12, 24, and 48 h are 0.012, 0.012, 0.021, 0.004, 0.018, 0.005, 0.00.016, 0.010, and 0.021, respectively. Letters a to k represent significant levels ($p < 0.05$), i.e., a treatment with the letter "a" is significantly different from "b", and "b" is significantly different from "c", and so forth. If two treatments have the same letter, they are not significantly different from each other.

### 3.3.1. Peroxidase Activity (POX)

The obtained values of peroxidase presented in Figure 3 showed a gradual increase with time intervals 0, 6, 12, 24, and 48 h in chemical inducers treated faba bean plants. Foliar spray of faba bean infested with *Botrytis fabae* with various organic acids resulted in a significant increase ($p < 0.05$) of peroxidase activity compared with the untreated control. The maximum increase in peroxidase activity was recorded after 24 h for all treatments. The most pronounced increase was obtained with SA, followed by NA, OA, and BA. Also,

peroxidase activity in healthy plants recorded a reduced value compared with infected plants with time intervals in all treatments.

### 3.3.2. Polyphenol Oxidase (PPO) Activity

Data presented in Figure 4 indicated that treating faba bean plants infected with *B. fabae* as a foliar treatment with different abiotic inducers resulted in an increase of polyphenol oxidase compared with the untreated infected control.

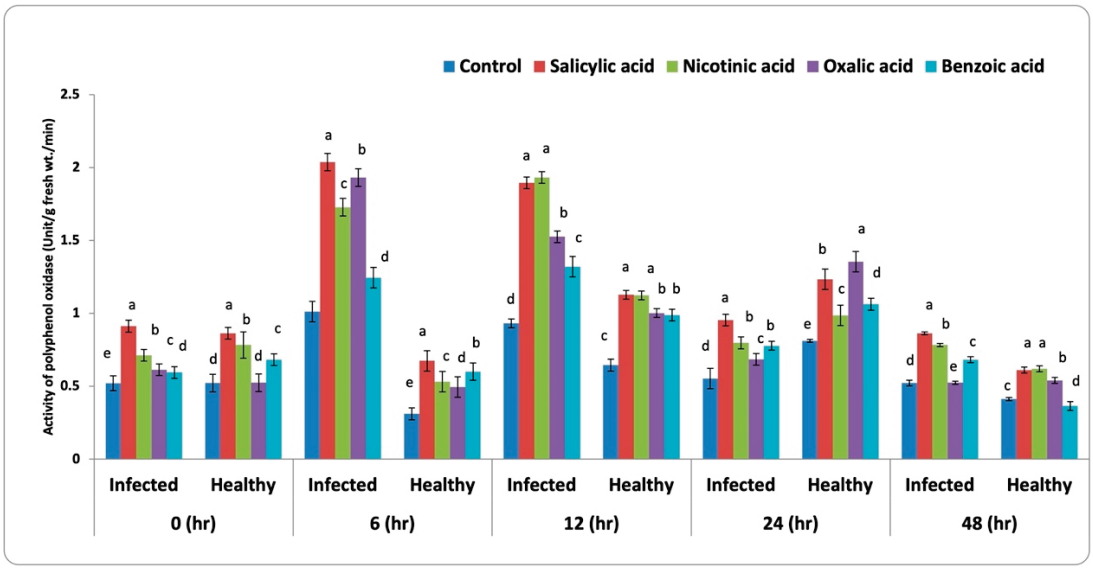


**Figure 4.** Activity of polyphenol oxidase in faba bean leaves infested with *B. fabae* plants treated with chemical inducers. LSD (5%) for infected and healthy plants at 0, 6, 12, 24, and 48 h are 0.009, 0.008, 0.062, 0.044, 0.099, 0.010, 0.039, 0.011, 0.014, and 0.037, respectively. Letters a to k represent significant levels ($p < 0.05$), i.e., a treatment with the letter "a" is significantly different from "b", and "b" is significantly different from "c", and so forth. If two treatments have the same letter, they are not significantly different from each other.

### 3.3.3. β-1,3-Glucanase Activity

Data presented in Figure 5 showed a significant increase in the β-1, 3-glucanase activity in all treatments during the examination periods compared to the control in both infected and healthy plants. Some treatments recorded the highest increase in β-1, 3-glucanase activity after 12 h, while other treatments recorded the highest values after 24 h. For infected faba bean plants, OA and SA caused the maximum increase in β-1, 3-glucanase after 12 h, followed by BA. Meanwhile, SA recorded the maximum increase after 24 h, followed by OA then BA came in the third order. The increase of β-1, 3-glucanase activity was more than two-fold of the untreated control. In the case of healthy faba bean plants, all chemical inducers produced the highest increase in β-1, 3-glucanase activity after 24 h, where OA came in the first order, followed by NA, then SA, and BA treatments. After that, the activity reduced slightly at 48 h but was still higher than the untreated control.

### 3.3.4. Chitinase Activity

A perusal of data in Figure 6 showed that the infected and healthy faba bean foliar treated with different abiotic inducers was associated with increased chitinase activity compared with the untreated corresponding control. All treatments increased significantly ($p < 0.05$) chitinase activity. Salicylic acid recorded the maximum increase in this concern after 24 h of both infected and healthy plants, followed by benzoic and oxalic acids, respectively. Meanwhile, nicotinic acid recorded the lowest value compared to untreated plants (control).

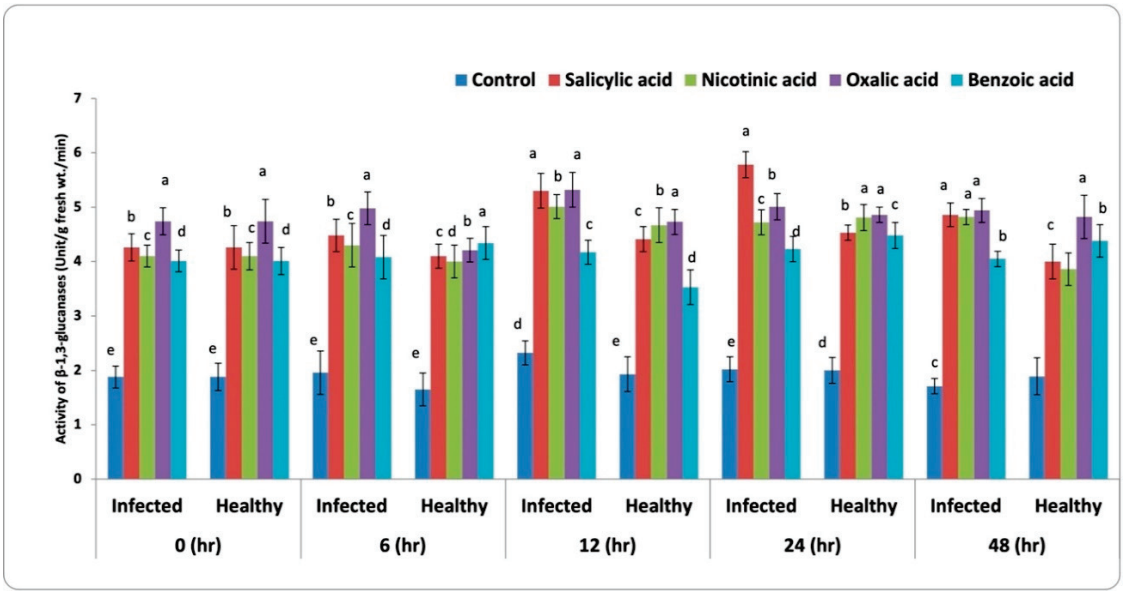


**Figure 5.** Activity of β-1,3-glucanases in faba bean leaves infested with *B. fabae* treated with chemical inducers. LSD (5%) for infected and healthy plants at 0, 6, 12, 24, and 48 h are 0.096, 0.096, 0.104, 0.061, 0.122, 0.099, 0.083, 0.012, 0.011, and 0.060, respectively. Letters a to k represent significant levels ($p < 0.05$), i.e., a treatment with the letter "a" is significantly different from "b", and "b" is significantly different from "c", and so forth. If two treatments have the same letter, they are not significantly different from each other.

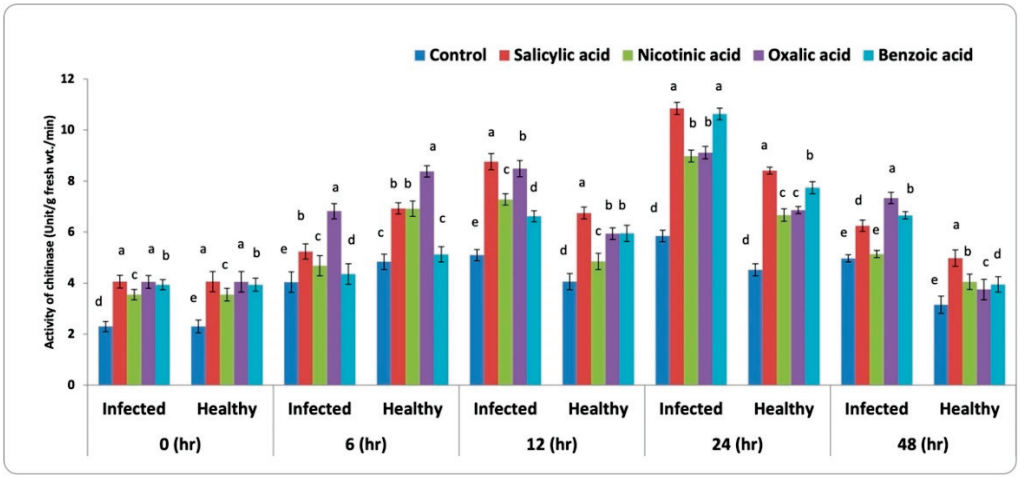


**Figure 6.** Activity of chitinase in leaves of faba bean infested with *B. fabae* plants treated with chemical inducers. LSD 5% for infected and healthy plants at 0, 6, 12, 24, and 48 h are 0.011, 0.011, 0.120, 0.101, 0.103, 0.066, 0.079, 0.086, 0.105, and 0.049, respectively. Letters a to k represent significant levels ($p < 0.05$), i.e., a treatment with the letter "a" is significantly different from "b", and "b" is significantly different from "c", and so forth. If two treatments have the same letter, they are not significantly different from each other.

### *3.4. Protein Electrophoretic Banding Patterns*

A banding pattern of the soluble proteins in infected and healthy leaves of faba bean that belongs to the cultivar Giza 429 infected with *B. fabae* and treated with selected chemical inducers. The inducers included salicylic acid (5 mM), oxalic acid (3 mM), benzoic acid (3.2 mM) and nicotinic acid (3 mM), via SDS-PAGE as presented in Table 1. A total of 27 polypeptides of faba bean leaves displayed heterogeneity compared to control. The infected and treated plants with some chemical inducers with molecular weights (MWs) ranged from 35 to 320 kDa after 24 and 48 h of application and 43 kDa in response to oxalic

and benzoic acids only after 24 h from the application. Meanwhile, after 48 h from the application, data showed a new protein polypeptide bands appeared at Mwt. of 172, 104, 59, 44, and 35 kDa due to all organic acid treatments (tested chemical inducers) compared to the control plants. The number if protein bands increased due to the salicylic, nicotinic, oxalic, and benzoic acid treatments, which recorded the numbers of 8, 9, 16, and 14 bands after 24 h from the application. Meanwhile, the protein bands 11, 11, 13, and 11 were recorded after 48 h from the application. The most pronounced increases were observed in response to oxalic acid treatment, which recorded 16 bands after 24 h and 13 after 48 h of application, followed by benzoic acid, which recorded 14 and 11 bands, respectively, after 24 and 48 h. It is evident from the obtained data that all the treatments of faba bean plants with organic acids cause the appearance of polypeptide protein bands with Mwt. of 59 and 51 kDa.

**Table 1.** Separation of soluble proteins (SDS-PAGE) in faba bean leaves treated with various chemical inducers against *B. fabae*.

| Band No. | Marker | After 24 h from Application | | | | | | After 48 h from Application | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Molecular Weight (kDa) | Control | | SA | NA | OA | BA | Control | | SA | NA | OA | BA |
| | | Healthy | Infected | | | | | Healthy | Infected | | | | |
| 1 | 320 | + | + | + | + | + | + | + | + | + | + | + | + |
| 2 | 301 | − | − | + | − | − | − | − | − | − | − | − | − |
| 3 | 399 | − | − | − | − | + | − | − | − | − | − | − | − |
| 4 | 297 | − | + | − | − | − | − | − | − | − | − | − | − |
| 5 | 176 | − | − | − | + | + | + | − | − | − | − | − | − |
| 6 | 172 | − | − | + | − | + | + | − | − | + | + | + | + |
| 7 | 137 | − | − | − | − | − | − | + | − | + | + | − | + |
| 8 | 130 | − | − | − | − | + | + | + | + | − | − | + | − |
| 9 | 117 | + | + | − | − | + | + | − | − | − | − | − | − |
| 10 | 116 | − | − | − | − | − | − | − | − | − | − | − | + |
| 11 | 104 | − | + | + | + | − | − | − | + | + | + | + | + |
| 12 | 98 | + | − | − | − | + | − | − | − | − | − | − | + |
| 13 | 85 | − | − | − | + | + | + | − | − | − | − | + | − |
| 14 | 84 | − | − | − | − | − | + | + | + | + | − | − | − |
| 15 | 78 | + | − | − | − | − | − | − | − | − | + | + | − |
| 16 | 76 | − | − | − | − | + | + | − | − | − | − | − | − |
| 17 | 66 | + | − | − | − | − | − | + | + | − | + | + | + |
| 18 | 65 | − | + | + | + | + | + | − | − | + | − | − | − |
| 19 | 62 | − | − | − | − | + | − | − | − | − | + | − | − |
| 20 | 59 | − | − | − | + | + | + | − | + | + | + | + | + |
| 21 | 51 | − | − | + | + | + | + | + | + | + | + | + | + |
| 22 | 44 | − | − | − | − | − | − | − | − | + | + | + | − |
| 23 | 43 | − | + | + | − | + | + | + | + | − | − | − | − |
| 24 | 42 | − | − | − | − | − | − | − | − | + | − | + | − |
| 25 | 41 | − | − | − | + | + | + | + | + | − | − | − | + |
| 26 | 40 | − | + | + | + | + | + | − | − | − | − | + | − |
| 27 | 35 | − | − | − | − | − | − | − | + | + | + | + | + |
| | Total bands | 5 | 7 | 8 | 9 | 16 | 14 | 8 | 10 | 11 | 11 | 13 | 11 |

+ = Presence of band and − = Absence of band.

Data in Figure 7 showed the appearance of a new band at Mwt. of 176, 85, 65, 59, 51, and 41 in response to NA, OA, and BA, and also 172, 130, and 77 kDa.

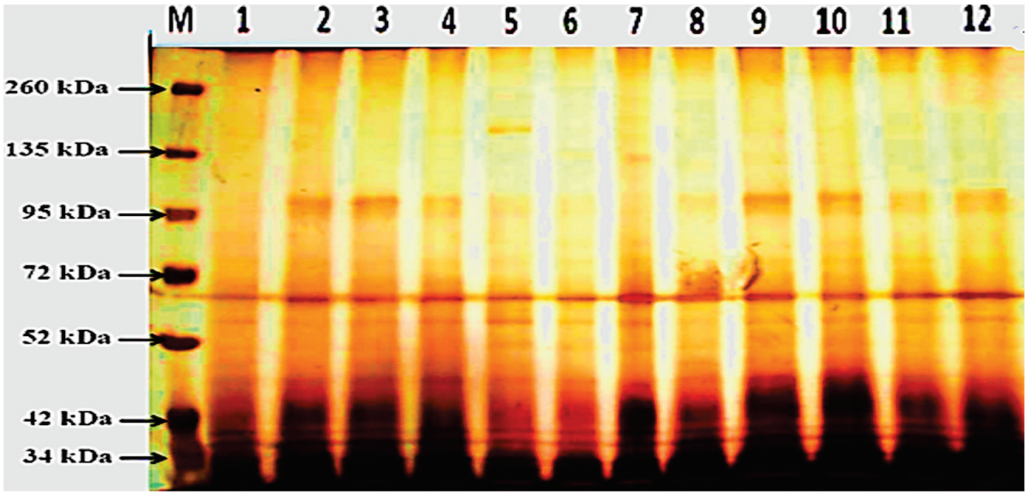


**Figure 7.** Separation of soluble proteins (SDS-PAGE) in faba bean plant leaves treated with chemical inducers against *B. fabae*.

The infected faba bean plants with *Botrytis fabae* induced a *de novo* synthesis of new polypeptide bands that appeared at molecular weights 297, 104, 66, 59, and 43 kDa after 24 h of application and also at 104, 59, and 35 kDa after 48 h of application. At the same time, there is a disappearance of 3 protein bands with molecular weight 98, 78, and 66 after 24 h of application, and after 48 h, only one band (OA treatment) was absent at Mwt 137 kDa.

Lane M: Marker, (1) Healthy control after 24 h from the application, (2) Infected control after 24 h from application, (3) Salicylic acid after 24 h from the application, (4) Nicotinic acid after 24 h from the application, (5) Oxalic acid after 24 h from the application, (6) Benzoic acid after 24 h from application, (7) Healthy control after 48 h from the application, (8) Infected control after 48 h from the application, (9) Salicylic acid after 48 h from the application, (10) Nicotinic acid after 48 h from the application, (11) Oxalic acid after 48 h from application and (12) Benzoic acid after 48 h from the application.

### *3.5. Anatomical Characteristics*

Microscopically counts and measurements of specific histological characteristics in transverse-sections through the blade of mature leaflet of faba been plant, benzoic, and salicylic acids presented in Figures 8 and 9a–c. The SA (Figure 9c) recorded the highest value with an increase in leaf thickness by (+30.1%) more than the control (Figure 9a). Such increase in leaf thickness corresponds with the enhancement recorded on the upper and lower epidermal layer thickness as well as palisade and spongy tissues by +42.8, +33.3, +28.0 and +28.5%, respectively more than the control. Such response also resulted in a clear appearance and arrangement of spongy and palisade tissue parenchymatous cells as compared to the control. However, foliar applications with BA (Figure 9b) recorded the lowest value with increased leaf thickness by (+15.8%) more than the control. Such increase in leaf thickness is related to the increment recorded in the upper and lower epidermal layer thickness as well as palisade and spongy tissues by (+28.5%, +22.2%, +12.0%, and +17.8%) respectively, more than the control.

This effect is associated with vast intercellular spaces between both palisade and spongy tissues parenchymatous cells. Furthermore, parenchymatous cells of upper and lower epidermal layers, as well as palisade and spongy tissues, are bigger in size and rounded in shape under treatment with SA. Clearly, Figures 8 and 9a–c revealed that foliar application with SA recorded the highest value with increased thickness of the midrib zone by (+38.9%) more than the control. Such an increase in midrib zone thickness corresponds with the enhancement recorded on the length and width of the vascular bundle as well

as the diameter of xylem vessels by +29.1, +21.4, and +45.4%, respectively, more than the control.

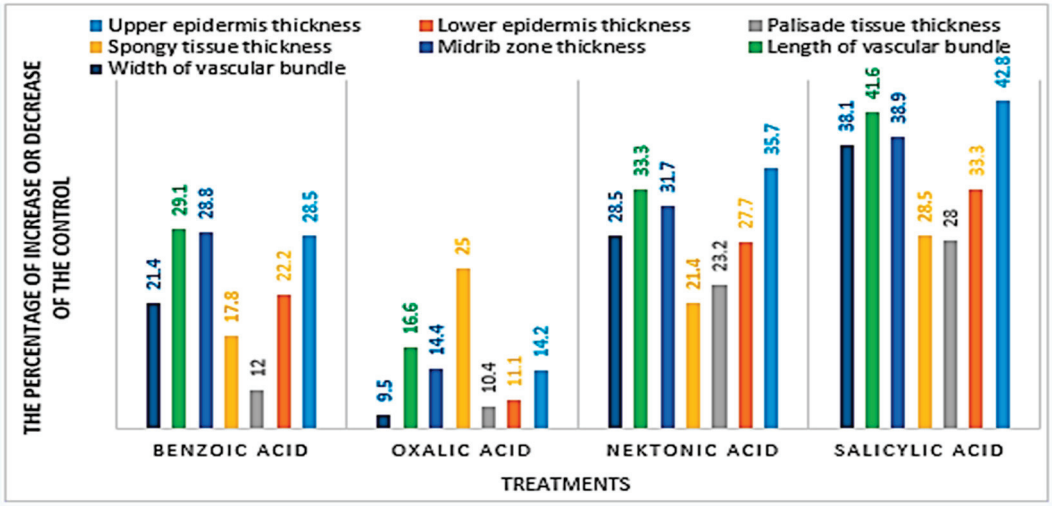


**Figure 8.** Effect of foliar application with chemical inducers on the anatomical characteristics of faba bean (*Vicia faba* L.) terminal leaflet under chocolate spot disease stress.

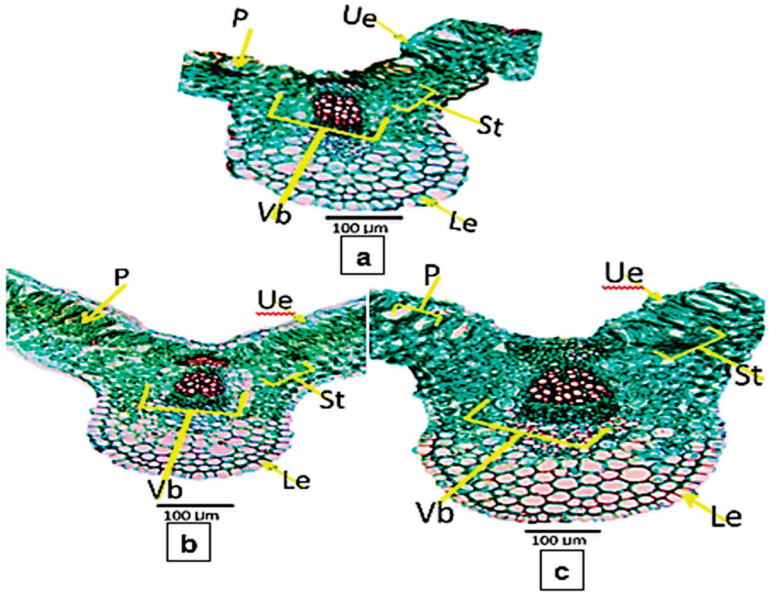


**Figure 9.** Transverse-sections through blade of the terminal leaflets of faba bean (*Vicia faba* L.) under chocolate spot disease: (**a**) Untreated plant (Control), (**b**) Plant treated with benzoic acid and (**c**) Plant treated with salicylic acid. Abbreviations: Le = Lower epidermis; P = Palisade tissue; St = Spongy tissue Ue = Upper epidermis and Vb = Vascular bundle.

In addition, the area occupied by collenchymatous cells behind the main vascular bundle is occupied by larger sizes and more layers of collenchymatous cells. Such effect corresponds with clear development and differentiation of the main vascular bundle's elements, especially xylem vessel elements. Also, metaxylem vessels also changed from a rounded shape, as recorded in the control, to an elongated shape in the treated plants. This response is mainly due to the increment that occurred in the area occupied by vascular bundle elements, which appeared in the area coupled by xylem vessels, cambium, and phloem as compared to the untreated plants.

## 4. Discussion

### *4.1. Severity of Chocolate Spot Disease*

The greenhouse experimental results indicated that pre-treated faba bean plants with abiotic inducers foliar treatment resulted in a significant reduction in disease severity of *B. fabae* compared with untreated control. Using bioagent isolates revealed antagonistic activity against *B. fabae* due to the production of protease, lipase, IAA, and ammonia. Moreover, bioagents release tricalcium phosphate (TCP) which promote multiple plant growth characteristics [46]. Abiotic inducers; SA, NA, OA, and BA gave a reduction of 83.2, 76.6, 74.2, and 72.8% at the highest concentration of each treatment. Among all foliar treatments, SA spray had the highest reduction of 83.2% at 5 mM of 5-day treatment. A second highest reduction of 80.9% was observed at a 3-day treatment with SA with 5 mM concentration. This was followed by NA application that reduced disease severity by 76.7% and 76.6% in 3-day and 5-day, respectively. The third highest reduction in severity was observed with OA application that reduced the severity by 75.8% and 74.2% in 3-day and 5-day treatments, respectively. While the fourth highest reduction was obtained with BA application at 5 mM, which reduced the disease severity by 72.8% and 71.5% in 5-day and 3-day treatments, respectively.

This agrees with Metwaly [47], who studied the evaluation of some chemical inducers, i.e., ascorbic, citric, salicylic acids, and calcium chloride (as a nutrient salt) to control faba bean chocolate spot disease. The study concluded that all tested organic acids significantly reduced the in vitro mycelial growth of the pathogenic fungus (*B. fabae*), and complete growth inhibition was recorded when 2500 ppm concentration was applied [47]. SA was the most effective one, followed by ascorbic acid. Plant treatment with organic acids and calcium chloride under greenhouse and/or field conditions led to a significant effect on controlling the disease and increasing phenols content as well as the activity of oxidative enzymes [6]. The results also agreed with the results of salicylic, citric, ascorbic, and oxalic acids as effective chemical inducers.

Under field conditions, plants treated with SA significantly reduced the disease severity in comparison with the control treatment. SA was highly efficient in controlling the fungal disease compared to other treatments that provided limited partial protection. In this regard, Thakur et al. [48] explained that chemical inducers are elicitors' compounds that activate plant chemical defense. Activation of a variety of biosynthetic pathways was observed in treated plants, which depended upon the used compound. Commonly, the studied elicitors include SA, methyl salicylate, benzothiadiazole, BA, and chitosan, with a key role in phenolic acid production as well as activation of several plant enzymes involved in defense mechanisms. Also, Zian et al. [49] observed that using SA led to a significant decrease in the root rot and wilt diseases of lupine. They observed that the SA leads to an increase in the activity of chitinase, β-1, 3-glucanase, peroxidase, and polyphenoloxidase. Morever, the induced resistance caused by some abiotic (BTH) and/or biotic inducers might provide a practical, eco-friendly management approach to chocolate spot disease when they are combined with suitable agronomic practices [50]. In their study, applying 0.3 and 0.5 mM benzothiadiazole as the foliar treatment significantly reduced the severity of chocolate spot disease.

### *4.2. Antioxidant Enzymes Activity*

The antioxidant activities by enzymes, such as peroxidase, polyphenol oxidase, β-1, 3-glucanase, and chitinase in the leaves of faba bean plants increased significantly under biotic stress (Figure 3a–d). In this regard, Gholami et al. [51] reported that these antioxidant enzymes were thought to be part of a preservation system that reduced oxidative damage caused by the increased formation of reactive oxygen species (ROS) due to biotic and abiotic stress. Higher content of $H_2O_2$ is detoxified at stress conditions through catalase and glutathione peroxidase [52].

Interestingly, the antioxidant system in plants includes enzymes that scavenge ROS, such as ascorbate peroxidase, catalase, peroxidase, and superoxide dismutase [26]. Sairam

and Srivastava [53] determined that plants having higher levels of either inducible or constitutive enzymes showed to be more resistant to oxidative harmful effect. OA appears to have a biphasic effect on ROS metabolism since it suppresses ROS buildup at first and activates their generation at later phases of pathogen infection [25]. Application of bioagents was characterized by the expression of cyclolipopeptides (cLP) genes, encoding for induced resistance factors, as well as the production of lipopeptides, indoleacetic acid, siderophores, hydrocyanic acid, and extracellular enzymes such as amylase, protease, pectinase, and cellulase [54].

Pretreatment of faba bean plant (infected and healthy plants) with the studied natural organic acids improved stress tolerance by increasing the tested antioxidant enzyme activities compared to control. Furthermore, Sharma and Dubey [55] observed that a viable protection against oxidative damage was produced by this antioxidant system. This enhances active oxygen species lifetime in the cellular environment. The defense mechanism of plant β-1, 3-glucanases is directly through suppressing ROS buildup and/or hydrolyzing the fungal pathogen cell walls along with chitinase isozymes. Laboratory studies showed that fungal pathogens were directly affected by β-1, 3-glucanases through deteriorating b-1, 3/1, 6-glucans. Also the chitinases produced their effects via the C1 and C4 bond that are consecutive N-acetylglucosamines of chitins in the cell walls of fungi [56]. The plants treated with salicylic acid increased the activity of enzymes such as catalase, peroxidase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase. [57]. Peroxidase belongs to oxido-reductase enzymes and contributes to oxidation-reduction reactions.

Concerning the effect of benzoic acid on the antioxidant enzyme system, a similar result was obtained by Zhang et al. [58] on tomato seedlings and Amist and Singh [59] on wheat seedlings, who found that the increasing antioxidant enzymes seem to protect plants from oxidative stress. Hassanein et al. [60] found that 100 ppm of nicotinamide (either soaking or spraying) increased the content of antioxidant enzymes (Superoxide dismutase, peroxidase, and catalase) and decreased lipid peroxidation of *Zea mays* plants. Also, in this regard exogenous application of methyl jasmonate increased the activity of antioxidant enzymes, improved photosynthetic pigments and PSII efficiency. This resulted in enhanced growth of pea plants under Cd stress. The improved traits included increments in the fresh and dry weights of shoots and roots. Thus, the mitigating effect of methyl jasmonate was due to its role in cellular redox balance and photosynthetic mechanism of plants under Cd stress conditions [54].

### *4.3. Changes in Protein Electrophoretic Patterns*

The data clearly demonstrated that, in general, after 24 h from the application, there was an absence of some polypeptide bands at molecular weights 117 in response to salicylic acid and 98, 78, and 66 kDa in response to nicotinic acid treatments. In this respect, the disappearance of protein bands and also the synthesis of a new group of soluble proteins were observed by many authors due to biotic and abiotic stress. The absence of some bands may result from inherited effects of infection with *B. fabea*, which explains the basis of the mutational event on the regulatory genes that prevent or attenuate transcription [61].

It is obvious from the obtained results (Figure 4 and Table 1) that all treatments of faba bean plant with organic acids cause the appearance of the polypeptide protein band with Mwt. Of 59 and 51 kDa. In this regard, Spreitzer and Savucci [62] discovered that the 51 kDa band, that might be associated with Ribulose-1, 5-bisphosphate carboxylase activase (Rubisco activase), was elevated. By promoting the separation of firmly bound sugar-phosphates from Rubisco in an ATP-dependent mechanism, this enzyme could modify the Ribulose-1 activity, 5-bisphosphate carboxylase/oxygenase (Rubisco), a crucial enzyme involved in the initiation of photosynthetic and photorespiratory carbon metabolic processes. Enhancing Rubisco's capability has important implications for plant productivity and resource use efficiency [63]. Moreover, these total proteins have osmoprotectant functions and protective effects on cellular structures [64].

Amino acids are primary metabolites that play essential roles in plant immunity against many pathogens. The variation in plant tissues amino acid quantity may determine the chance of environment for the pathogenic attackers like fungi, bacteria, and viruses. This finally strengthens plant defense to resist pathogenic attack effectively or surrender before vigorous infection [65]. Morphological and structural barriers, chemical substances, proteins, and enzymes are all examples of biotic stress protection mechanisms. By preserving products and giving them strength and stiffness, they confer tolerance or resistance to biotic stressors. In addition, amino acids serve an important function in stress response and secondary metabolism in plants [66]. For scavenging ROS resulting from biotic stress (infection with *B. fabae*), the plant has a protective system against the devastating oxidative reaction. The protective system includes osmoprotectants (total soluble protein) and antioxidative enzymes, which are known to play a crucial role in defense mechanisms, and all of them are proteins and can appear as protein bands. In this regard, Ramadan et al. [67] on flax plant found that benzoic acid increased total soluble protein and free amino acids. Also, Mahgoob and Talaat [68] found that foliar application of nicotinic acid at 50 mg/L significantly increased total protein contents of rose geranium.

The appearance of *de novo* synthesis of new polypeptides band and/or increase the band density 77, 59, 51 and 41 may represent an antioxidant enzyme (chitinase and β-1, 3-glucanase) which is considered as defense proteins to protect the plant from pathogens. In ripe cherimoya fruits (*Annona cherimola* Mill.), Goni et al. [69] found a highly expressed constitutive chitinase (at 27 kDa), as well as the creation of a unique acidic chitinase (at 26 kDa) and a 1, 3-glucanase (51 kDa). Likewise, a new basic chitinase at 33 kDa was observed in the post-ripening stage of fruits that were stimulated, as well as another basic constitutive β-1, 3-glucanase at the molecular weight of 76 kDa. In vitro, extracts of these acidic and basic proteins suppressed the growth of *B. cinerea*, a necrotrophic fungal pathogen in grape leaves. At the berries' harvest stage, defense proteins were dominant, particularly various chitinase and β-1, 3-glucanase isoforms that support fruit ripening. Also, Shukry [70] explained that the hydrin is defined at the protein band with Mwt. of 40 kDa. These proteins play a preventative role during water stress through their function as ion traps in dehydrating cells and insulating ions as their concentration increases.

### *4.4. Anatomical Characteristics*

The present experimental results (Figures 8 and 9a–c) clear that foliar application with salicylic acid at 3 mM on faba been plant led to the highest increase in the thickness of the upper, lower epidermis, leaf blade thickness, palisade, or spongy tissue midrib zone, length, and width of the vascular bundle and. Also, foliar application with salicylic acid produced great increase in the most mentioned layers more than the control. Although the foliar application with all treatments led to a slight increase in the thickness of the examined layers was recorded especially by benzoic acid, compared to the untreated plants. In this regard, Ali et al. [71] on maize (*Zea mays*, L.) reported that treatment with citric acid led to increasing the upper and lower epidermal layers, length of the vascular bundle, and mesophyllic tissue, while the width of vascular bundle was similar to the control. Concerning foliar application with salicylic acid, the results showed an increase of thickness in both palisade and spongy tissues; these results are in agreement with Gomaa et al. [18], who observed that foliar spraying of the cultivar 'Giza 2, Egyptian lupine, with salicylic acid increased the thickness of leaflets lamina and midvein. This is due to an increase in the thickness of spongy and palisade tissues as well as to increase in the midvein bundle size. Some investigators confirmed the present findings using salicylic acid on other field crop plants, for instance, Cárcamo et al. [16] on *Zea mays*, L., Nour, et al. [17] on bean, Gomaa et al. [18] on *Lupinus termis* L. and Khalil, et al. [72] on pea plants. They found that salicylic acid application increased the thickness of the midvein and lamina of the studied plant leaves. Also, Maddah et al. [73] found that spraying salicylic acid with 0.1 mM increased the stomata number, but watering plants with 1.5 mM of salicylic acid damages the parenchyma tissues in leaves, the tissue of sclerenchyma in stems, and

xylem in the root, the exogenous spraying of SA might alleviate the inhibitory impact of salinity stress on the growth, physiological and anatomical features, and the productivity of cowpea plants El-Taher et al. [74].

## 5. Conclusions

This study investigates the positive role of organic acids in alleviating abiotic stress conditions, which that can be beneficial for improving the performance of faba bean plants to increase the productivity of land area units. It was found that the uses of these phenolic materials not only eliminate the pathogens but also improve the characteristics of the plant to resist diseases (Chocolate spot disease). Whereas among abiotic inducers, salicylic acid at the concentration (5 mM) gave the highest reduction (control chocolate spot disease) through the two seasons respectively. This research will assist the researcher in uncovering crucial regions of secondary metabolites defense causative agents of plants against abiotic stress that many researchers have yet to investigate.

**Author Contributions:** Conceptualization, R.M.A. and T.O.R.; data curation, S.A.E.-S., A.A.R., A.I.E. and A.B.; formal analysis, A.B. and T.O.R.; funding acquisition, T.O.R. and A.M.E.-T.; investigation, R.M.A. and A.A.R.; methodology, A.M.E.-T., S.A.E.-S. and A.I.E.; project administration, T.O.R. and A.B.; resources, R.M.A. and A.A.R.; software, A.I.E., S.A.E.-S. and A.M.E.-T.; supervision, T.O.R. and A.B.; validation, R.M.A. and A.A.R.; visualization, A.M.E.-T. and S.A.E.-S.; writing—original draft, R.M.A. and A.M.E.-T.; writing—review and editing, T.O.R. and A.B. All authors have read and agreed to the published version of the manuscript.

**Funding:** Partial support is provided to T.O.R. by the National Institute of Food and Agriculture, CSREES, U.S. Department of Agriculture, Massachusetts Agricultural Experiment Station (MAES), under Project MAS00045.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data will be made available with a reasonable request to the corresponding authors.

**Acknowledgments:** We thank Emad A. Mady at the University of Massachusetts for reviewing and providing valuable comments on the manuscript. We also thank Mohamed F.M. Ibrahim at Ain Shams University for reading and commenting on the manuscript's original version.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# The Role of Heat Acclimation in Thermotolerance of Chickpea Cultivars: Changes in Photochemical and Biochemical Responses

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**Abstract:** This study was conducted to determine the effects of heat stress on the physiological and biochemical responses of chickpea (*Cicer arietinum* L.; Diyar and Küsmen-99) cultivars that are both heat acclimated and non-acclimated. The seedlings were grown in soil for 15 days and then exposed to heat stress (35 °C, 5 days) after heat acclimation (30 °C, 2 days) or non-acclimation (25 °C, 2 days). Chlorophyll *a* fluorescence (ChlF) measurements were analyzed using the JIP test. Heat acclimation had no significant effect on ChlF parameters. Seedlings exposed to higher temperatures by acclimation were more tolerant in terms of ChlF parameters and Diyar had a better photochemical activity of photosystem II (PSII). Heat stress resulted in a decrease in electron transport efficiency, quantum yield, photosynthetic performance, and driving force in both chickpea cultivars, while K-band, L-band, and quantum yield of dissipation increased, especially in the non-acclimated cultivars. Additionally, ion leakage (RLR), malondialdehyde (MDA) content, and $H_2O_2$ synthesis increased in the cultivars, while water content (RWC), chlorophyll ($a + b$) content, and carotenoid content of the cultivars decreased. On the other hand, the cultivars attempted to eliminate reactive oxygen species (ROS) by increasing the content of anthocyanins and flavonoids and the activity of antioxidant enzymes (SOD and POD) under heat stress. Heat acclimation alleviated the negative effects of heat stress on each cultivar's water content, chlorophyll and carotenoid content, membrane damage, photosynthetic activity, and antioxidant defense systems. The results of this study showed that, by providing heat acclimation more effectively, Diyar was better able to cope with the biochemical and physiological alterations that could be resulted from heat stress.

**Keywords:** *Cicer arietinum* L.; chlorophyll *a* fluorescence transient; physiological and biochemical traits; high temperature

**Citation:** Arslan, Ö. The Role of Heat Acclimation in Thermotolerance of Chickpea Cultivars: Changes in Photochemical and Biochemical Responses. *Life* **2023**, *13*, 233. https://doi.org/10.3390/life13010233

Academic Editors: Hakim Manghwar, Wajid Zaman and Balazs Barna

Received: 11 December 2022
Revised: 11 January 2023
Accepted: 12 January 2023
Published: 13 January 2023



## 1. Introduction

Climate change increases the generation and dispersion of abiotic stresses that pose a serious risk to crop production [1]. Heat is an abiotic stress factor that limits plant development and crop yield. Heat stress is described as a temperature increase that exceeds a particular level over a period of time and irreversibly damages plant growth [2]. A temporary temperature rise of 10–15 °C above ambient temperatures is evaluated as heat stress [3]. When plants are exposed to heat stress, it inhibits plant growth and production by causing physiological and biochemical disorders in plants [4]. Heat stress leads to the denaturation and aggregation of proteins [2], disruption of membrane structures [5], inhibition of photosynthesis [6], deterioration of photosynthetic pigments [7], and alterations in antioxidant enzymes [8]. The main reason for these adverse effects is the negative effect of heat stress on photosynthetic activity. Photosystem II (PSII) is the most heat sensitive in the photosynthetic apparatus, and PSII activity is significantly reduced under heat stress [9]. Chlorophyll *a* fluorescence (ChlF) transients (OJIP), which can be used to determine the extent of photosynthetic responses of plants to heat stress, are a reliable, non-invasive and powerful tool for assessing photosynthetic electron transport. The signals recorded by ChlF allow the determination of the physiological state of plants, calculation of specific

biophysical parameters, quantum yields, and probabilities that determine changes in PSII units, electron transport chain, and photochemical reactions by light [10–14]. Analysis of ChlF has been widely used in numerous studies to investigate various plant responses under heat stress, including rice [15], alfalfa [9], exotic weeds [16], tall fescue [7], barley [6], and maize [3]. The imbalance between the absorption and consumption of light energy due to heat stress leads to overexcitation of thylakoid membranes, resulting in photoinhibition. Heat stress leads to excessive energy loading of thylakoid membranes and eventually photoinhibition due to the imbalance between light energy absorption and utilization [17]. Photoinhibition is mainly due to the overproduction and accumulation of reactive oxygen species (ROS) such as hydroxyl radical ($OH^-$), superoxide radical ($O_2^-$), and hydrogen peroxide ($H_2O_2$) [18]. Subsequently, the presence of excessive amounts of ROS leads to oxidative stress and oxidative stress damages all cellular structures, especially membranes [9]. To alleviate the ROS-induced oxidative injury, plants generate antioxidant defense systems (enzymatic and non-enzymatic) to scavenge the overproduced ROS [19]. The enzymatic antioxidant defense system includes several antioxidant enzymes: superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase, etc. Non-enzymatic antioxidants include the metabolites: ascorbate, carotenoids, anthocyanins, flavonoids, etc. The antioxidant defense mechanism in plants is part of the adaptation to heat and its strength correlates with the acquisition of thermotolerance. Thermotolerance can be achieved by heat acclimation with exposure to a non-lethal heat treatment [5]. Heat acclimation is increased tolerance to the physical and physiochemical exceedances of heat stress. This complex process, which involves physiological and biochemical alterations in plants, including rearrangements in the lipid composition of membranes, changes in the content of compatible metabolites, synthesis and accumulation of antioxidants and protective proteins, changes in hormone levels, and modifications of gene expression [20,21]. Even when heat acclimation is successful, plant susceptibility to heat stress varies with plant genotype and developmental stage; however, susceptibility is largely affected by genotype and species variability, as well as mostly intra- and inter-species variations [22].

Chickpea is a heat-sensitive cool season legume, as its potential yield decreases at temperatures above 35 °C [8]. The main growing areas of chickpea are in the arid and semi-arid zones of the world and due to climate change, it will be inevitable that the potential yield of chickpea will decrease due to the increase in the intensity and duration of exposure to high temperatures. Since the chickpea is an economically and agriculturally valuable crop, it was very important to investigate the responses of this crop to heat stress and heat acclimation, which our research group had previously studied under chilling [23,24], freezing [25,26] and drought conditions [26,27]. Karacan et al. [26] studied 18 chickpea cultivars using a multi-criteria decision making method to rank them according to their cumulative tolerance to cold and drought stress conditions, using physiological and biochemical analysis data from previous studies. According to the research results, when chickpea cultivars were ranked according to these two stress responses, Diyar scored quite differently from the other cultivars and was classified as tolerant, while Küsmen-99 was classified as moderately tolerant with an average score. Therefore, the heat stress responses of these two cultivars, classified as drought and cold tolerant (Diyar) and moderately tolerant (Küsmen-99), were investigated. To this end, two chickpea cultivars (Diyar and Küsmen-99) were subjected to heat stress (35 °C for 5 days) with or without heat acclimation (30 °C for 2 days) to understand the interaction between heat tolerance and heat acclimation on PSII photochemical activity, pigments, membrane stability, and defense mechanisms. The objective of this study was to (1) elucidate the physiological mechanisms, especially the photochemical activity of PSII and antioxidant defense systems in chickpea under heat stress; (2) explain the mitigating effects of heat acclimation on the mechanisms damaged by heat stress; (3) compare the thermotolerance of the cultivars studied; (4) determine the role of the correlation between oxidative stress and endogenous defense systems in the thermotolerance of the cultivars.

## 2. Materials and Methods

Seeds of chickpea (*Cicer arietinum* L.) cultivars (Diyar and Küsmen-99) were obtained from the Central Research Institute of Field Crops in Ankara, Turkey. To prevent fungal infections, to which chickpea is frequently exposed, seeds were treated with pesticides [Benomyl and Thriam (0.3 g per 100 g of seed)] and were sown in pots (3 seeds each) containing 325 g of air-dried soil. The soil had the following characteristics: Texture, clay [28]; water holding capacity, 20.1% [29]; pH, 7.54 [30]; EC, 258 μS $cm^{-1}$ [31]; N, 1.48 g $kg^{-1}$ [32]; P, 16.25 mg $kg^{-1}$ [33]; and K, 464 mg $kg^{-1}$ [33]. 100 μg $g^{-1}$ $NH_4NO_3$ and 100 μg $g^{-1}$ $KH_2PO_4$ were added to the soil, because the N, P, and K levels were found to be insufficient for chickpea. Plants were grown for 15 days in a growth chamber under good irrigation, at 25 ± 1 °C/20 ± 1 °C (day/night), a 16/8 h (day/night) photoperiod, a relative humidity of 60 ± 5%, and a light intensity of 250 μmol $m^{-2}$ $s^{-1}$ and then randomly divided into the following groups to conduct the experiments:

$C_0$ and C, 17- and 22-day-old control seedlings grown under control conditions (25 ± 1 °C/20 ± 1 °C);

A, 17-day old heat-acclimated seedlings (grown under control conditions for 15 days, then exposed to 30 ± 1 °C/25 ± 1 °C for 2 days);

A + S, 22-day-old heat-treated acclimated seedlings (heat-acclimated and then exposed to 35 ± 1 °C/30 ± 1 °C for 5 days);

S, 22-day-old heat-treated non-acclimated seedlings (grown for 17 days under control conditions, then exposed to 35 ± 1 °C/30 ± 1 °C for 5 days).

The central leaves of the seedlings were used for the experimental analyses.

Since no statistically significant difference was found between the 17- and 22-day-old control groups ($C_0$ and C) in all physiological and biochemical analyses examined, the results of the study were evaluated using the 22-day-old control group (C).

### *2.1. Polyphasic Chlorophyll a Fluorescence (ChlF) Measurement*

ChlF transients were determined in dark-adapted leaves (6 replicates) using a Handy PEA fluorimeter (Plant Efficiency Analyser, Hansatech Instruments Ltd., Norfolk, UK). After a 30-min dark adaptation, leaves were irradiated with light (3000 μmol $m^{-2}$ $s^{-1}$) for one second and the intensity of fluorescence at 20 μs ($F_0$), 300 μs ($F_K$), 2 ms ($F_J$), 30 ms ($F_I$), and maximum fluorescence ($F_P$) were determined [10]. The JIP test parameters were calculated from obtained fluorescence intensities. The effects of heat stress on cultivars were assessed based on relative fluorescence between the steps O and K [20 and 300 μs, respectively = $V_{OK} = (F_t - F_0)/(F_K - F_0)$], O and J [20 μs and 2 ms, respectively = $V_{OJ} = (F_t - F_0)/(F_J - F_0)$] and I and P [30 ms and at the peak P of OJIP, respectively = $V_{IP} = (F_t - F_I)/(F_P - F_I)$] were normalized and given as the kinetic difference $V_{OK} = V_{OK(treatment)} - V_{OK(control)}$, $V_{OJ} = V_{OJ(treatment)} - V_{OJ(control)}$ and $V_{IP} = V_{IP(treatment)} - V_{IP(control)}$, respectively [10,11]. The efficiencies and quantum yields of fluorescence were also calculated: $\varphi_{P0}$, $(1 - F_0/F_M$ or $F_V/F_M)$, maximum quantum yield of primary photochemistry; $\psi_{E0}$, $(1 - V_J)$, probability that a trapped exciton moves an electron into the electron transport chain beyond $Q_A^-$; $\varphi_{E0}$, $[(1 - F_0/F_M) \times \psi_{E0}]$, quantum yield for electron transport; $\varphi_{D0}$, $(DI_0/ABS)$, quantum yield of energy dissipation; $\varphi_{R0}$, $(\varphi_{P0} \times \psi_0 \times \delta_{R0})$, the quantum yield of electron transport from $Q_A^-$ to the PSI end electron acceptors; $\delta_{R0}$, $(1 - V_I)/(1 - V_J)$, the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end final electron acceptors. The performance indexes ($PI_{ABS}$ and $PI_{TOTAL}$) were calculated from the components to determine the difference between the cultivars $PI_{ABS}$, $[(RC/ABS) - [\varphi_{P0}/(1\ \varphi_{P0})]\ [\psi_0/(1 - \psi_0)]$, performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors; $PI_{TOTAL}$, $PI_{ABS}$ $[(\delta_{R0}/(1 - \delta_{R0})]$, performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors; DF, $\log(PI_{ABS})$, driving force on absorption basis [10,11].

### 2.2. Water Content and Pigment Analysis

To determine the percent relative water content (RWC) of leaf segments (R = 0.5 cm and 6 replicates), fresh leaves were weighed (FW) and then incubated in 10 mL distilled water for 24 h to determine the saturated weight (SW), and the leaves were dried at 80 for 48 h, their dry weight (DW) was determined, and the RWC was calculated as (%) = [(FW − DW)/(SW − DW)] × 100 [34]. After extraction of the leaves (0.1 g with 6 replicates) in 100% acetone, they were measured spectrophotometrically (at wavelengths 470, 644.8, and 661.6 nm), and the content of chlorophyll (Chl) (*a* + *b*) and carotenoids (*x* + *c*) (mg $g^{-1}$ FW) was calculated [35]. To determine anthocyanin content (mg $g^{-1}$ FW) and flavonoid content (%), fresh leaf samples (0.1 g with 3 replicates) were ground in acidified methanol [methanol:water:HCl (79:20:1)] and measured at wavelengths of 530 and 657 nm for anthocyanin and 300 nm for flavonoid, respectively. Anthocyanin was calculated according to the method of Mancinelli et al. [36]. Flavonoid was calculated as a percentage of the content of 22-day-old control plants (C) [37].

### 2.3. Relative Leakage Ratio, MDA, and $H_2O_2$ Contents

Relative leakage ratio (RLR) was measured indirectly as leakage of UV-absorbing substances according to the method of Redmann et al. [38]. Five leaf segments (R = 0.5 cm) with three replicates were kept in 10 mL distilled water for 24 h and measured at 280 nm ($A_1$). Samples were treated in liquid nitrogen and shaken for another 24 h in incubation water. The samples were measured at 280 nm ($A_2$) and the RLR was calculated as $A_1/A_2$ [38]. Malondialdehyde (MDA) content (nmol $g^{-1}$ FW) was determined (0.1 g leaf samples with 3 replicates) as described by Hodges et al. [39] and MDA was calculated using the extinction coefficient (157 $mM^{-1}$ $cm^{-1}$). To determine the amount of $H_2O_2$ (nmol $g^{-1}$ FW), leaf samples (0.1 g and 3 replicates) were extracted in 0.1% TCA with 0.1 M Tris-HCl (pH 7.6). The extracts were treated with potassium iodide reagent and kept in the dark for 90 min. Samples were measured at 390 nm and calculated using the standard curve [40].

### 2.4. Antioxidant Enzyme Activities

Soluble protein was extracted from leaves (0.5 g with 3 replicates) to determine the enzyme activities. The Bradford method [41] was used to determine the protein concentration and the leaf samples were extracted in the corresponding extraction buffer. 1 mL of buffer solution (9 mM Tris-HCl and 13.6% glycerol) was added to the powdered samples with liquid nitrogen and the total SOD activity (EC 1.15.1.1) (U mg $protein^{-1}$) was determined [42]. The buffer solution of the leaves homogenized for POD (EC 1.11.1.7) and CAT (EC 1.11.1.6) included 100 mM potassium phosphate buffer (pH 7.0), 2% PVP, and 1 mM $Na_2EDTA$. The POD activity was determined by measuring the oxidation of guaiacol (ε = 26.6 mM $cm^{-1}$) by $H_2O_2$ (nmol $H_2O_2$ $min^{-1}$ mg $protein^{-1}$) at 470 nm [43]. The CAT activity was calculated as nmol $H_2O_2$ $min^{-1}$ mg $protein^{-1}$, with the absorbance values at 240 nm decreasing according to the dissociation of $H_2O_2$ [44].

### 2.5. Statistical Analysis

The research experiments were conducted in a completely randomized design. The experiment was laid out in three replicates with 90 plants in a total of 30 pots, and mean values (*n* = 3 or 6) were obtained for each treatment. Analysis of variance (*ANOVA*) was performed for all data obtained from the experiments. The variability of data among cultivars and treatments was calculated using the least significant difference (LSD) test at the 95% probability level ($p < 0.05$). *SPSS* v 20.0 (Chicago, IL, USA) was used for all research data analyzes.

## 3. Results

### 3.1. Effect of Heat Stress on Chickpea ChlF Rise and ChlF Parameters

The OJIP transients measured as ChlF rises in dark-adapted control and stressed chickpea leaves were determined by plotting them on a logarithmic time scale (Figure 1).

The OJIP rise reflects three reduction processes in the electron transport chain (O-J, J-I, and I-P phases) [6,10]. The O-J rise contains information about the antenna size and indicates the reduction on the acceptor side of PSII [45]. The J-I phase refers to the kinetic properties required for the reduction and/or oxidation of the plastoquinone pool (PQ) [46]. The I-P phase represents the re-reduction of plastocyanin and the acceptor side of PSI [6,46]. Exposure to 35 °C significantly altered the shape of the typical OJIP curves seen in controls. The reduction in fluorescence intensity was more pronounced in both heat-acclimated (A + S) and non-acclimated (S) treatments of Küsmen-99 (Figure 1B). The S treatment caused the disappearance of the J-I and I-P phases, while the P level approached the O-J phase, indicating photochemical inhibition of PSII. A similar effect was determined in the heat-acclimated stress treatment (A + S) of Küsmen-99.

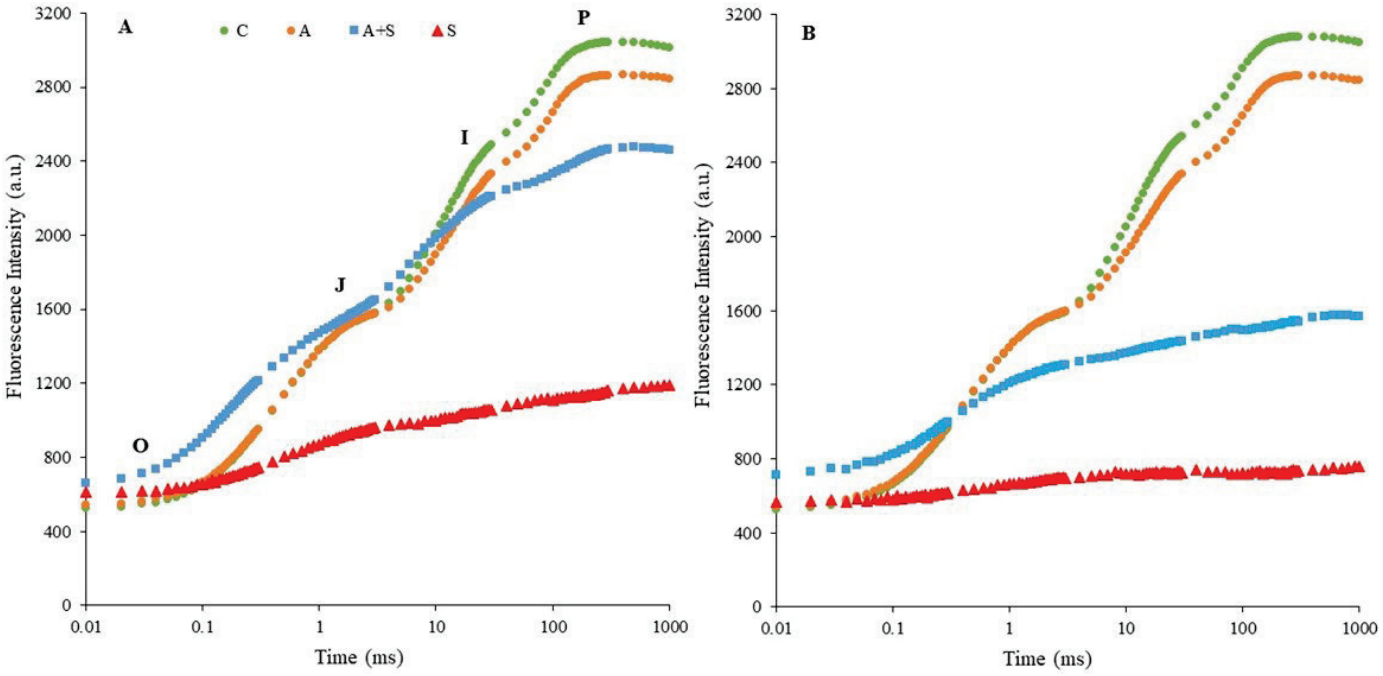


**Figure 1.** Induction curves of polyphasic ChlF in chickpea cultivars ((**A**,**B**), Diyar and Küsmen-99, respectively) exposed to heat stress with or without heat acclimation. The transients are plotted on a logarithmic time scale (10 µs to 1 s). The mean values of the OJIP transients are plotted, $n = 6$.

ChlF parameters, which provide information about photosynthetic fluxes and quantify the PSII and PSI behaviors are derived from ChlF transients. The parameters representing the relative values of controls were shown by spider plot graphics (Figure 2). Exposure to heat acclimation (30 °C for 2 days, A) resulted in slight changes in both cultivars compared to corresponding controls. However, significant changes in almost all selected ChlF parameters were determined in both cultivars exposed to heat stress (35 °C for 5 days), whether acclimated (A + S) or non-acclimated (S), compared to the controls. Heat stress resulted in a similar extent increase in both $V_{OK}$ and $V_{OJ}$ parameters in Diyar and Küsmen-99 (Figures 2A and 2B, respectively). The $V_{OK}$ and $V_{OJ}$ parameters are expressed as L- and K-bands, respectively, and reflect the inactivation of the oxygen-evolving-complex (OEC). The $V_{IP}$ values decreased when the cultivars were subjected to 35 °C, except S treatment of Diyar (Figure 2A). The decrease in $V_{IP}$ values (G-band) indicates limitations in electron transport on the PSI acceptor side. The maximum quantum yield of the photochemistry of PSII ($\varphi_{P0} = TR_0/ABS = F_V/F_M$) of chickpea cultivars reduced in both acclimated and non-acclimated heat stress treatments (Figure 2). The highest $\varphi_{P0}$ decreases were determined in A + S (38%) and S (72%) treatments of Küsmen-99 (Figure 2B). The parameter $\psi_{E0}$ ($ET_0/TR_0$) explains the probability that captured exciton moves the electron further in the electron transport chain than $Q_A^-$. The highest decreases in $\psi_{E0}$ values were determined

in Küsmen-99 during heat stress, especially heat acclimated (41% of control). The $\varphi_{E0}$ value that defines the quantum yield efficiency that captured exciton moves electron to the electron transport chain ($\varphi_{E0} = ET_0/ABS$), declined markedly in all cultivars due to heat stress treatments, and the highest decline of $\varphi_{E0}$ results was determined in A + S (63%) and S (81%) treatments of Küsmen-99 (Figure 2B). Heat treatments led to marked increases in the quantum yield of dissipation ($\varphi_{D0} = DI_0/ABS$) values of both cultivars (Figure 2). Küsmen-99 exhibited the highest increment of A + S and S treatments 2.6- and 3.1-fold of control, respectively. Heat reduced quantum yield of electron transport from $Q_A^-$ to the PSI end electron acceptors ($\varphi_{R0} = RE_0/ABS$) values in cultivars, mainly in S treatment of Küsmen-99 (76%). The parameter $\delta_{R0}$ ($RE_0/ET_0$), which reflected the probability that electron was transferred from intersystem electron carried to electron acceptors at PSI acceptor side was significantly increased by heat stress treatments in all cultivars, except 19% decrease in the A + S treatment of Diyar (Figure 2). The cultivars exhibited a gradual decrease in the values of performance indexes ($PI_{ABS}$ and $PI_{TOTAL}$) in both heat acclimation and heat stress treatments (Figure 2). In determining PSII behavior, $PI_{ABS}$ refers to energy absorption, capture, and conversion in electron transport steps. Heat acclimation led to a significant decrease in $PI_{ABS}$ of both Diyar and Küsmen-99 (21% and 25%, respectively). Additionally, the highest reduction was determined in non-acclimated heat stress treatment of the cultivars, Diyar (94%) and Küsmen-99 (98%). The $PI_{TOTAL}$ parameter includes additional electron steps to $PI_{ABS}$, and PSI refers to the measure for performance up to the reduction of final electron acceptors. The extent of the reductions of the $PI_{TOTAL}$ was remarkable in both A + S and S treatments for Diyar (84% and 87%, respectively) and Küsmen-99 (91% and 96%, respectively). Likewise, the $PI_{ABS}$ and $PI_{TOTAL}$, the total driving force for photosynthesis ($DF = \log PI_{ABS}$) values of cultivars declined gradually with heat stress treatments (Figure 2). Among the cultivars, Küsmen-99 had the highest reductions, especially for the S treatment (5-fold of the corresponding control).

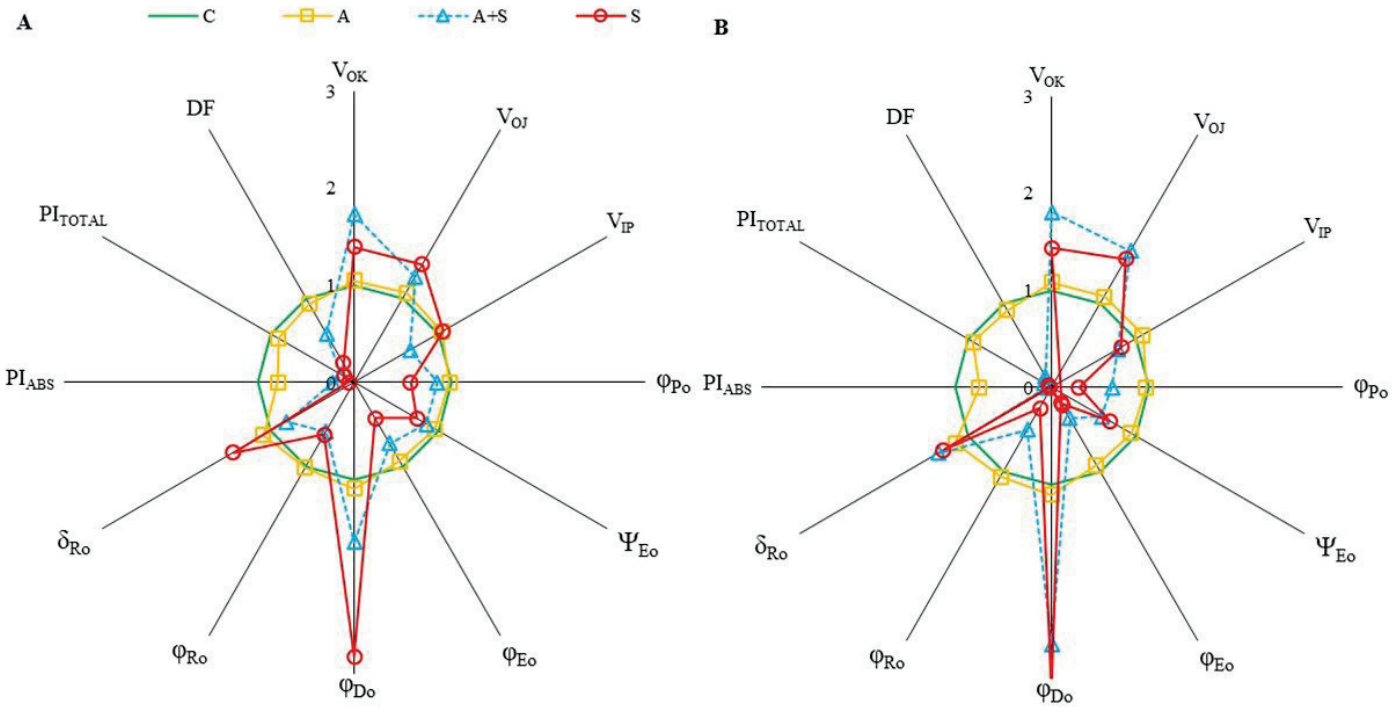


**Figure 2.** The radar-plot presentation of selected OJIP parameters in chickpea cultivars ((**A**,**B**), Diyar and Küsmen-99, respectively) exposed to heat stress with or without heat acclimation. The mean values of the parameters were plotted in relation to the corresponding controls, $n = 6$.

### 3.2. Effect of Heat Stress on Chickpea Water and Pigment Contents

The relative water content (RWC) of the leaves of the cultivars declined sharply in all heat treatments, including heat acclimation (Diyar and Küsmen-99, 9% and 20%, respectively) (Table 1). Exposure to 35 °C with heat acclimation (A + S) resulted in significant reductions (Diyar and Küsmen-99, 35% and 46%, respectively), while the non-acclimated heat stress treatment (S) resulted in the highest reductions (Diyar and Küsmen-99, 41% and 57%, respectively). All heat treatments significantly reduced the Chl (*a* + *b*) content of the cultivars (Table 1). The extent of Chl (*a* + *b*) reduction caused by heat acclimation was not as great as that by heat stress treatments. Heat acclimation led 17% and 12% reduction in control levels for Diyar and Küsmen-99, respectively. In addition, the magnitude of the reduction in Chl (*a* + *b*) content for the A + S and S treatments was 23% and 33%, respectively, for Diyar and 48% and 56%, respectively, for Küsmen-99. Similarly, all treatments resulted in a gradual decrease in the carotenoid content of the cultivars. The A + S treatment resulted in a 47% and 57% reduction in carotenoid content of Diyar and Küsmen-99, respectively, with the highest reduction determined in the S treatment of Diyar (60%) and Küsmen-99 (66%). In contrast to the results for Chl (*a* + *b*) and carotenoids, anthocyanin and flavonoid contents of cultivars subjected to heat treatments significantly increased (Table 1). The increase in anthocyanin content was more pronounced in all treatments (A, A + S, and S) of Diyar (3.3-, 5.7- and 4.9-fold of the corresponding control, respectively), while the highest flavonoid content was determined in the heat treatments (A + S and S) of Küsmen-99 (87% and 85%, respectively).

**Table 1.** Relative water content (RWC) (%), chlorophyll (Chl) (*a* + *b*) (mg $g^{-1}$ FW), carotenoid (mg $g^{-1}$ FW), anthocyanin (mg $g^{-1}$ FW), and flavonoid (%) content of chickpea cultivars subjected to heat treatments.

| Cultivars | Treatment | RWC | Chl (*a* + *b*) Content | Carotenoid Content | Anthocyanin Content | Flavonoid Content |
|---|---|---|---|---|---|---|
| Diyar | C | $65^{1} \pm 1^{a}$ | $74 \times 10^{-3} \pm 0.0^{a}$ | $159 \times 10^{-4} \pm 0.0^{a}$ | $117 \times 10^{-6} \pm 0.0^{a}$ | $100 \pm 0^{a}$ |
| | A | $59 \pm 2^{b}$ | $62 \times 10^{-3} \pm 0.0^{b}$ | $119 \times 10^{-4} \pm 0.0^{b}$ | $387 \times 10^{-6} \pm 0.0^{b}$ | $111 \pm 3^{b}$ |
| | A + S | $42 \pm 1^{c}$ | $57 \times 10^{-3} \pm 0.0^{c,f}$ | $84 \times 10^{-4} \pm 0.0^{c}$ | $665 \times 10^{-6} \pm 0.0^{c}$ | $144 \pm 8^{c}$ |
| | S | $38 \pm 2^{c,e}$ | $50 \times 10^{-3} \pm 0.0^{d}$ | $64 \times 10^{-4} \pm 0.0^{d}$ | $574 \times 10^{-6} \pm 0.0^{d}$ | $152 \pm 5^{d}$ |
| Küsmen-99 | C | $67 \pm 1^{a}$ | $67 \times 10^{-3} \pm 0.0^{e}$ | $141 \times 10^{-4} \pm 0.0^{a}$ | $259 \times 10^{-6} \pm 0.0^{e}$ | $100 \pm 0^{a}$ |
| | A | $54 \pm 1^{d}$ | $59 \times 10^{-3} \pm 0.0^{f}$ | $116 \times 10^{-4} \pm 0.0^{b}$ | $330 \times 10^{-6} \pm 0.0^{f}$ | $94 \pm 1^{e}$ |
| | A + S | $36 \pm 1^{e}$ | $35 \times 10^{-3} \pm 0.0^{g}$ | $61 \times 10^{-4} \pm 0.0^{d,e}$ | $442 \times 10^{-6} \pm 0.0^{g}$ | $187 \pm 6^{f}$ |
| | S | $29 \pm 1^{f}$ | $29 \times 10^{-3} \pm 0.0^{h}$ | $48 \times 10^{-4} \pm 0.0^{e}$ | $434 \times 10^{-6} \pm 0.0^{g}$ | $185 \pm 3^{g}$ |
| LSD 5% | | 4 | $3 \times 10^{-3}$ | $13 \times 10^{-4}$ | $55 \times 10^{-6}$ | |

[1] Each value is presented as the mean ± SEs, $n = 6$ (for RWC, Chl and carotenoid) or 3 (for anthocyanin and flavonoid). Different letters indicate significant differences between treatments and cultivars at $p < 0.05$ according to LSD 5%.

### 3.3. Effect of Heat Stress on Chickpea Membrane Integrity and Lipid Peroxidation

The heat acclimation period did not cause any membrane damage in the leaves of cultivars according to the relative leakage ratio (RLR) and malondialdehyde (MDA) results (Figure 3). Heat treatments, both heat acclimated and non-acclimated, led to a dramatic increase in the RLR, indicating loss of membrane integrity in the leaf cells of chickpea cultivars (Figure 3A). RLR increased 4.9- to 5.6-fold in Diyar and 10- to 10.9-fold in Küsmen-99 under A + S and S treatments, respectively. Similar results were obtained for MDA contents that reflect the lipid peroxidation of cellular membranes. The MDA levels increased 2.1- and 2.8-fold in heat treatments (A + S and S, respectively) in Diyar and 5.1- and 6.2-fold in Küsmen-99 compared with control (Figure 3B).

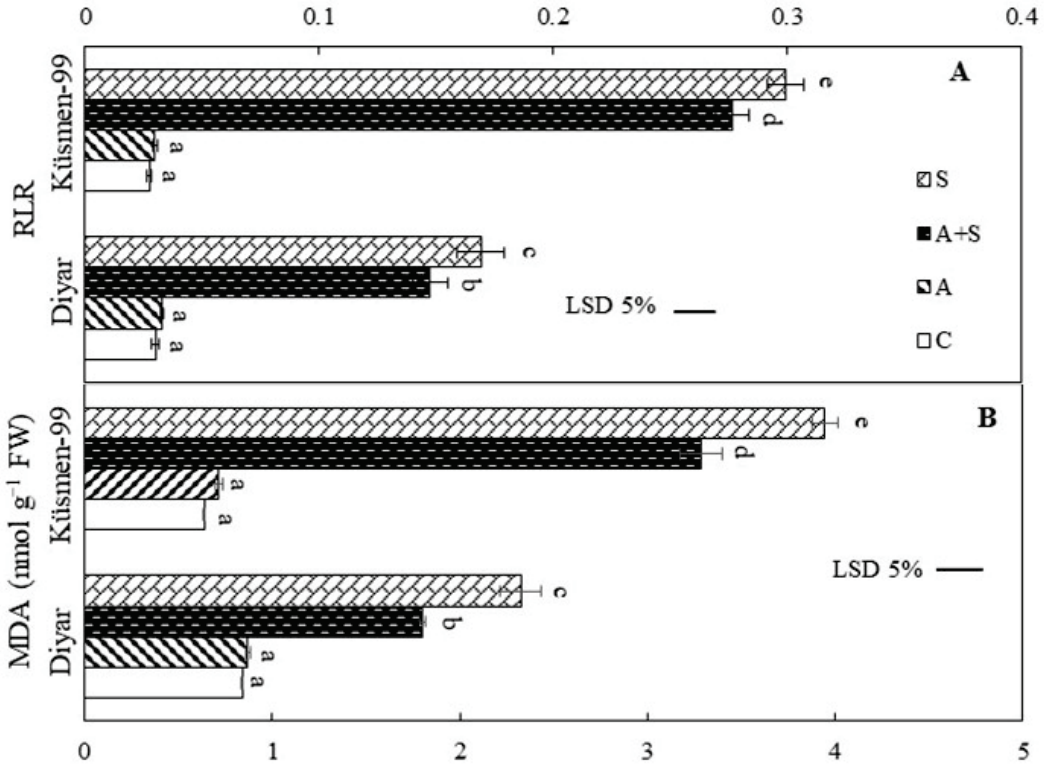


**Figure 3.** Heat stress with or without heat acclimation resulted in changes in RLR (**A**) and MDA contents (**B**) in chickpea cultivars. The values are presented as the mean ± standard error (SE), $n = 3$. The bars and different letters indicate significant differences between treatments and cultivars at $p < 0.05$ according to the LSD test.

### *3.4. Effect of Heat Stress on Chickpea $H_2O_2$ Content and Antioxidant Enzyme Activities*

The $H_2O_2$ content of the cultivars increased during heat stress treatments, indicating oxidative stress (Figure 4A). While the increase during heat acclimation (A) treatment was not significant in the Diyar cultivar, A treatment caused a 32% increase in Küsmen-99. Moreover, heat stress treatments caused a gradual increase in the $H_2O_2$ content of Diyar and Küsmen-99, especially in S treatment, by 31% and 84%, respectively. Superoxide dismutase (SOD) activity increased markedly in all heat-treated (A, A + S, and S) chickpea cultivars, although it was more pronounced in Diyar (3.9-, 6.7-, and 7.1-fold, respectively, of the corresponding control) (Figure 4B). Similar to SOD, all heat treatments resulted in a significant increase in peroxidase activity (POD) of both cultivars compared to the corresponding controls (Figure 4C). However, the increase in activity between A + S and S treatments in Diyar and A and A + S treatments in Küsmen-99 proved to be insignificant compared to each other. In contrast to SOD and POD, the catalase (CAT) activity of cultivars declined sharply in heat stress treatments, regardless of whether they were acclimated or not, while heat acclimation (A) treatment did not cause any significant change in CAT activity (Figure 4D). However, when acclimated and non-acclimated heat treatments (A + S and S) were compared, no significant differences in the CAT activity were determined for either cultivar.

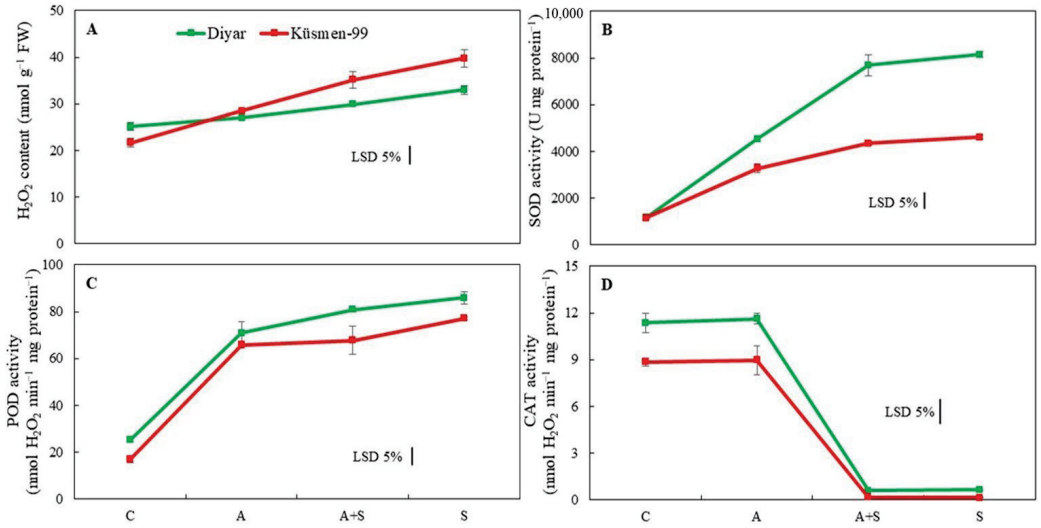


**Figure 4.** Heat stress with or without heat acclimation induced changes in $H_2O_2$ content (**A**) and antioxidant enzyme activities (SOD, (**B**); POD, (**C**) and CAT, (**D**)) of chickpea cultivars. The values are presented as the mean ± standard error (SE), $n = 3$. The bars indicate significant differences between treatments and cultivars at $p < 0.05$ according to the LSD test.

## 4. Discussion

The objective of the study was to elucidate the physiological and biochemical mechanisms involved in the tolerance of chickpea cultivars to heat stress, either acclimated or non-acclimated. Plants develop different tolerance mechanisms to overcome the deleterious effects of high-temperature stress, especially when acclimated to heat [5]. The effects of heat acclimation on the biochemical and physiological mechanisms of two *Cicer arietinum* L. cultivars subsequently exposed to higher temperatures were studied. Photosynthetic responses to rising temperatures play a critical role in regulating plant heat tolerance. One of the most important responses in regulating plant heat tolerance is the photosynthetic response that the plant develops as temperatures rise. The most heat-sensitive components of the electron transfer chain are the units responsible for photosynthesis and, in particular, the oxygen-evolving complex (OEC) of PSII [6]. Heat stress inhibits photosynthesis by altering the redox balance of electron transport reactions [6,47]. In this study, chickpea cultivars exhibited higher $F_O$ values, especially when heat acclimated at 35 °C. Elevated $F_O$ values represent increased damage to chloroplasts due to heat stress, resulting in the inhibition of energy transfer to PSII and reduced quantum efficiency of PSII [22]. Fluorescence densities $F_J$, $F_I$, and $F_P$ also decreased in phases J, I, and P of cultivars exposed to heat stress. These changes reflect the inhibition of electron transport from the OEC to the PQ pool. The curves show that the I and P phases decreased similarly in both cultivars during heat acclimation (A). In addition, significant differences were determined between the cultivars in both heat stress treatments when the ChlF curves were examined. Non-acclimated heat treatments (S) in both cultivars and acclimated stress treatment of Küsmen-99 made the OJIP curves to disappear on the logarithmic scale. Remarkably, the effects of heat stress on the OJIP curve were much more pronounced in the S treatments. The results show that OEC and reduction/reoxidation of $Q_A$ and $Q_B$ are more susceptible to high temperatures in Küsmen-99 than in Diyar. Similar effects of high temperature on photoinhibition of PSII have been reported previously [15,48]. The differential effects of heat on PSII could be due to different cyclic electron flow capacities around PSI [15], which could result from the genetic variation of cultivars. Heat stress treatment resulted in an increase in $V_{OK}$ and $V_{OJ}$, indicating the alteration of L- and K- bands, respectively. The presence of the L-band provides information about the utilization of excitation energy, while the K-band refers to the stable electron transfer from OEC to $P680^+$ and subsequently to $Q_A^-$ [10,12]. Higher K-band is a heat indicator to predict plant response to heat [16]. Küsmen-99 showed

higher L- and K-bands than Diyar in acclimated and non-acclimated heat treatments. It has been reported that an increased L-band indicates a loss of connectivity between the reaction centers and their antenna complexes, while elevated K-band value represents an inhibition of OEC due to Mn-complex injury [6,10]. In contrast to the results of $V_{OK}$ and $V_{OJ}$, the levels of $V_{IP}$ decreased in all treatments, except non-acclimated Diyar. The $V_{IP}$ indicates the changes in the G-band related to electron transfer from PSII to PSI [13]. Thus, these bands (K-, L-, and G-) formed as a result of the heat stress showed that the light reactions of photosynthesis, particularly the acceptor side of PSII were negatively affected. Moreover, heat stress markedly changed the efficiency and quantum yield of PSII ($\varphi_{P0}$, $\psi_{E0}$, $\varphi_{E0}$, $\varphi_{D0}$, $\varphi_{R0}$, and $\delta_{R0}$) of chickpea cultivars. Among the efficiency and quantum yield parameters, $\varphi_{P0}$, $\psi_{E0}$, $\varphi_{E0}$, and $\varphi_{R0}$ were lower, whereas $\varphi_{D0}$ and $\delta_{R0}$ were higher in both acclimated and non-acclimated heat treatments. The only exception was the $\delta_{R0}$ parameter in the acclimated heat treatment of Diyar. The increased values of $\varphi_{D0}$ indicated that the trapped energy was probably radiated as heat energy and the connection between the photosynthetic systems was broken [11]. The results showed that electron transfer from PSII was inhibited by heat on both the electron donor and acceptor sides. All heat treatments caused photoinhibition in both cultivars and photoinhibition of PSII was alleviated by heat acclimation. Chickpea cultivars exposed to heat treatments exhibited reductions in both $PI_{ABS}$ and $PI_{TOTAL}$. Changes in the efficiencies and quantum yields of the photosystem could be the reason for alterations in the performance indexes, which are multiparametric expressions for successive steps in primary photochemical reactions. While $PI_{ABS}$ describes the part up to the reduction of intersystem electron transport of photons absorbed by the PSII reaction centers, $PI_{TOTAL}$ describes the part up to the reduction of the PSI final electron acceptor [10,12]. Reductions in performance indexes and DF indicate impairment of the photochemical activities of the reaction centers. The main reason for these disruptions in photochemical activities as a result of heat stress is oxidative stress, which results from the increased formation of ROS in the thylakoid membranes [49].

Heat stress causes wilting, curling and yellowing of leaves as well as a reduction in plant biomass, suggesting that heat stress causes plants to reduce growth and trigger stomatal closure to prevent water loss [4,22]. RWC gradually declined under heat-stress temperatures. Previous reports showed that heat-tolerant wheat [50] and alfalfa [22] cultivars had the highest water content. Since Diyar had higher RWC values, the cultivar could be classified as heat tolerant. Treatments with heat acclimation had higher RWC values than treatments without acclimation, suggesting that the heat acclimation period may play an important role in maintaining the homeostasis of heated cells. When plants are exposed to any stress, this stress is accompanied by oxidative stress. It is well known that chloroplasts are the main source of ROS generation under stress conditions due to the limitation of electron transport [51]. Since chlorophyll is a necessary pigment for photosynthesis, the varying amount of total chlorophyll is a decisive indicator of the level of photosynthesis in plants [4]. The Chl ($a$ + $b$) content of cultivars was drastically reduced by heat temperature, regardless of whether acclimated or non, especially in Küsmen-99. Similar results occurred in chickpea plants under heat stress, and this damage to pigment was found to be due to the photooxidation of chlorophyll [8]. Carotenoids, non-enzymatic antioxidants, protect chlorophyll from photooxidation [3]. In this study, the carotenoid content of the cultivars declined in all heat treatments. It was found that the content of photosynthetic pigments was higher in heat-tolerant chickpea genotypes than in the other genotypes [52]. Since Diyar always contains more chlorophyll and carotenoids in heat treatments, the results of this study are consistent with the literature. Contrary to chlorophyll and carotenoids, the increased levels of anthocyanins and flavonoids were determined in heat treatments. Anthocyanins and flavonoids act as antioxidants in plants and maintain chloroplast functionality by protecting chlorophyll from photoinhibition under heat-stress conditions. However, the reduced photosynthetic efficiency of chickpea under heat treatment indicated that the light screening role of anthocyanins and the antioxidant roles of flavonoids was not sufficient to prevent the overexcitation of chloroplasts, especially at Küsmen-99.

Heat stress leads to lipid peroxidation of cell membranes and membrane injury in numerous plants [5,19,22,53]. The heat stress treatments led to a marked increase in RLR and MDA results of the cultivars, but this increase remained lower increase due to heat acclimation. The increase in the RLR ratio indicates electrolyte leakage and loss of membrane stability. The excessive accumulation of MDA is due to the induction of lipid peroxidation in cell membranes by ROS, which is formed and accumulated by heat stress. Oxidative stress, which occurs as a result of increased ROS production and accumulation due to metabolic disorders, is known to be an important indicator of stress in plants. $H_2O_2$ is one of the ROS and is highly toxic to plant tissues [5]. Chickpea cultivars were found to have significantly increased $H_2O_2$ levels under heat stress. However, the increase in $H_2O_2$ levels was lower in acclimated cultivars than in non-acclimated cultivars. Although $H_2O_2$ is a stimulant to increase antioxidant capacity under stress conditions, its presence in cellular components above a threshold level is an indicator of oxidative stress [19]. Plants have antioxidant defense mechanisms to prevent excessive ROS production and improve tolerance to oxidative stress. It was found that plants with high levels of antioxidant enzymes also have a high tolerance to ROS-induced oxidative damage [4]. The results showed that both cultivars exhibited higher activity of antioxidants in all heat stress treatments, except CAT. Disturbances in the photochemical activity of PSII due to heat stress may lead to increased production of superoxide radicals ($O_2^-$), the secondary product of the electron transfer chain, and thus to an increase in the activity of SOD [54]. SOD plays a role as the first defense against ROS, by converting $O_2^-$ to $O_2$ and $H_2O_2$ [55]. Subsequently, POD, one of the antioxidants that detoxify $H_2O_2$, removes $H_2O_2$ by oxidizing components such as phenolic compounds and/or antioxidants. In the present study, although both SOD and POD activities increased with heat stress, the elevated RLR, MDA, and $H_2O_2$ levels indicated insufficient to remove oxidative stress in cultivars. In addition, ascorbate peroxidase (APX) and glutathione reductase (GR) are enzymes of the Halliwell–Asada pathway, one of the metabolic systems responsible for the detoxification of $H_2O_2$. It was reported that the activities of APX and GR increased in maize exposed to high temperatures [56]. Moreover, heat tolerance at lethal temperatures was found to be associated with increased activities of SOD and APX [57]. In contrast to SOD and POD, the activity of CAT exhibited a pronounced decline under heat treatments. CAT degrades $H_2O_2$ to $H_2O$ and $O_2$ and is primarily located in peroxisomes. The decrease in the activity of CAT under heat stress is due to the photoinactivation of catalase and decreased catalase synthesis. In addition, high $H_2O_2$ may lead to a decrease in the activity of CAT due to substrate inactivation. The reduced activity of CAT may contribute to $H_2O_2$ accumulation, which leads to lipid peroxidation under heat-stress conditions [53].

According to the research results, it was found that heat acclimation in chickpea cultivars increased heat tolerance at higher temperatures to which the cultivars were later exposed. The increased tolerance was found to be associated with the enhancement of protective mechanisms such as anthocyanins, flavonoids, and antioxidant enzymes. Acquisition of thermotolerance by prior heat acclimation reduced cellular leakage and membrane injury. Therefore, oxidative damage and heat injury was reduced in treatments subjected to heat acclimation. Tolerance differences among cultivars became more evident in seedlings exposed to heat stress, especially acclimation. Diyar, which is known to be cold and drought tolerant, responded similarly to heat stress as the acclimated Küsmen-99, although it was not acclimated in all parameters. However, heat tolerance of both cultivars increased significantly with acclimation. According to the polyphasic chlorophyll *a* fluorescence data, Diyar showed photosynthetic activity under heat stress that approached the control by acclimation, whereas Küsmen-99 did not improve photosynthetic activity. This could be due to the fact that Diyar is more successful in maintaining water, chlorophyll, and carotenoid content and increases its anthocyanin content under heat-stress conditions. In addition, the increase in antioxidant enzyme efficiencies while maintaining membrane damage and the lower $H_2O_2$ content are physiological changes that make Diyar more tolerant to heat stress. The cultivar Diyar was more successful than Küsmen-99 in coping with the negative effects of heat stress.

**Funding:** This research was supported by the Scientific Research Unit (Project FEN-BAP-A-220413-39) of Giresun University.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data are contained within the article.

**Acknowledgments:** The author would like to thank Yasemin Ekmekçi for her guiding contributions to the research.

**Conflicts of Interest:** The author declares no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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# Regulatory Mechanisms of Plant Growth-Promoting Rhizobacteria and Plant Nutrition against Abiotic Stresses in Brassicaceae Family

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**Abstract:** Extreme environmental conditions, such as abiotic stresses (drought, salinity, heat, chilling and intense light), offer great opportunities to study how different microorganisms and plant nutrition can influence plant growth and development. The intervention of biological agents such as plant growth-promoting rhizobacteria (PGPRs) coupled with proper plant nutrition can improve the agricultural importance of different plant species. Brassicaceae (Cruciferae) belongs to the monophyletic taxon and consists of around 338 genera and 3709 species worldwide. Brassicaceae is composed of several important species of economical, ornamental and food crops (vegetables, cooking oils, forage, condiments and industrial species). Sustainable production of Brassicas plants has been compromised over the years due to several abiotic stresses and the unbalanced utilization of chemical fertilizers and uncertified chemicals that ultimately affect the environment and human health. This chapter summarized the influence of PGPRs and nutrient management in the Brassicaceae family against abiotic stresses. The use of PGPRs contributed to combating climate-induced change/abiotic factors such as drought, soil and water salinization and heavy metal contamination that limits the general performance of plants. Brassica is widely utilized as an oil and vegetable crop and is harshly affected by abiotic stresses. Therefore, the use of PGPRs along with proper mineral nutrients management is a possible strategy to cope with abiotic stresses by improving biochemical, physiological and growth attributes and the production of brassica in an eco-friendly environment.

**Keywords:** microorganisms; stressful conditions; sustainability; abiotic stresses; nutrition; Brassicaceae

**Citation:** Jalal, A.; da Silva Oliveira, C.E.; Galindo, F.S.; Rosa, P.A.L.; Gato, I.M.B.; de Lima, B.H.; Teixeira Filho, M.C.M. Regulatory Mechanisms of Plant Growth-Promoting Rhizobacteria and Plant Nutrition against Abiotic Stresses in Brassicaceae Family. *Life* **2023**, *13*, 211. https://doi.org/10.3390/life13010211

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 23 November 2022
Revised: 29 December 2022
Accepted: 5 January 2023
Published: 11 January 2023



## 1. Introduction

Brassica is one of the most important and economical vegetables of the Brassicaceae family [1] and includes several species (*Brassica oleracea*, *Brassica rapa*, *Nasturtium officinale*, *Raphanus sativus*, *Diplotaxis tenuifolia* and *Eruca vesicaria*), containing secondary metabolites and beneficial contents of putative health-promoting compounds [2]. Brassicaceae are a rich source of primary and secondary metabolites (amino acids, sugars, indoles, phenolics and glucosinolates) that help in the production of antioxidants [3,4] to promote tolerance to biotic and abiotic stresses [5]. Brassicaceae are emergently adapting as a research model crop in plant science due to their interaction with biotic and abiotic stresses as their high defensive mechanisms and a series of alterations in metabolites allow them to survive under climatic extremes [6]. Therefore, proper management practices are needed when encountering extreme environmental conditions (drought, salinity, temperature, heavy metals and nutrients deficiency) and to ensure optimal plant growth and productivity [7].

Abiotic stresses disturb plant physiology and metabolism, which leads to the reduction of plant growth and productivity [8]. The growth, yield and quality of Brassica grown in arid and semi-arid areas were extremely affected by drought conditions [9]. In addition, nutrient limitation is another vulnerable condition that alters plant growth, production and quality. Plants adapt different physiological and biochemical functions to adjust to extreme challenges and avoid injuries under abiotic stresses [10]. Macronutrients mobilize and assimilate along with organic compounds that could improve plant growth and development and mitigate plant abiotic stresses [11]. The absorption of chromium (Cr), zinc (Zn), iron (Fe) and manganese (Mn) was increased with chelating agents of low molecular weight, which led to the improvement of oil content in *Brassica juncea* up to 35% [12]. The imbalanced utilization of macro and micronutrients may cause metal toxicity in several crop plants [13]. However, Brassica species deal with the hyper-accumulation of these nutrients by improving biochemical processes and the mobilization of nutrients through the roots–shoot system [14]. In addition, the root rhizosphere is influenced by different biotic and abiotic factors including soil and root type and plant species and age. Hence, plant growth-promoting rhizobacteria (PGPR) are classified into several groups on the basis of their capacities and taxonomical status. These bacteria activate several mechanisms that alter soil organic matter to an instantly available form [15], as well as the regularization and transformation of soluble sugars, proline, amino acids and mineral nutrients in the soil above plant parts, thus improving nutrient accumulation in nutrient-deficient soils [16].

The plant and bacteria association promotes nutrient uptake and assimilation, which favors the plants' tolerance to biotic and abiotic stresses [17]. Plants and microbial communities are the components of similar limited resources with a different relationship. However, plants assist microbial communities with available nutrients from the soil rhizosphere [18] and improve nitrogen mineralization, which can enhance the uptake of other nutrients for a higher performance and yield of plants [19]. The positive association (symbiosis) and negative association (pathogenesis) of the plant rhizosphere microbial community can affect nutrient availability and resource partition, thus increasing or reducing crop production, respectively [18,20]. The positive association of the microbial community increases their activities in the rhizosphere of host plants, which can improve the soil organic matter (SOM) content and nutritional status of the plant [21]. Beneficial bacteria are the first soil-borne communities that alter and re-adjust in stressful environments for their survival; however, their activities and configurations are the first affected factors under stress [22]. The plant growth-promoting rhizobacteria community is vulnerable to stressful conditions of low water potential and nutrient availability that may be reflected in the form of physiological stress in the plants [23].

The eco-physiological and functional activities of nutrients and PGPRs need proper attention and extensive research to improve plant tolerance to abiotic stresses. Therefore, this review highlighted the interaction between plant growth-promoting rhizobacteria and mineral nutrition and their influence on the tolerance to abiotic stresses in the Brassicas plant species.

## 2. Adverse Effects of Abiotic Stress in Plants

Abiotic stresses are the foremost confining factors for agricultural productivity. Crop plants overcome the drastic external pressure of intrinsic mechanisms caused by environmental and edaphic conditions that affect the growth, development and productivity of plants [24,25]. The sustainable production of vegetables such as Brassicas around the world has been compromised due to several harsh environmental conditions and the unbalanced use of synthetic fertilizers and uncertified chemicals over the years that affect the environment and human health and led to inadequate climatic conditions. Abiotic stresses consist of drought, low/high temperature, salinity, light intensity, flooding, heavy metals toxicity and nutrient starvation. The extensive use of chemicals, macro and micronutrients, non-essential elements and radionuclides are the main sources of metal toxicity in soil [13,25]. Brassicaceae are capable plant species that deal with the hyper-accumulation of heavy met-

als through their biochemical expression, acquisition and re-mobilization in roots [13,14]. Waterlogging/flooding is an excess of soil water that can reduce oxygen availability in plant root systems and thus negatively affect crop growth and yield [26]. Flooding has negatively affected lipid biosynthesis and the yield of several rapeseed varieties [27].

Cold stress is associated with chilly weather (0–15 °C) and frosty weather (<0 °C) that leads to the disturbance of the photosynthetic process and reduces the primary production of *B. oleracea* [28]. Cold stress impairs metabolic and enzymatic activities that can disrupt the cell membrane and cause seed rotting in Brassica plants [29,30]. Light radiation (low or high) affects plant morphology and the root–shoot ratio [31]. Exposure of broccoli (*B. oleracea*) to ultraviolet (UV) light can increase ascorbic acid [32,33]. High light causes photoinhibition of the photosystem and protein degradation in *B. rapa* plants [34]. In short, abiotic stresses alter several internal functions of plants by disturbing homeostasis, physio-biochemical and molecular attributes, such as water and nutrient use efficiency and assimilation, osmotic adjustment, disruption of membrane integrity and enzymatic activities, as well as reduction in photosynthetic efficiency [29,31,34]. The abiotic stresses and their consequences are summarized in Figure 1.

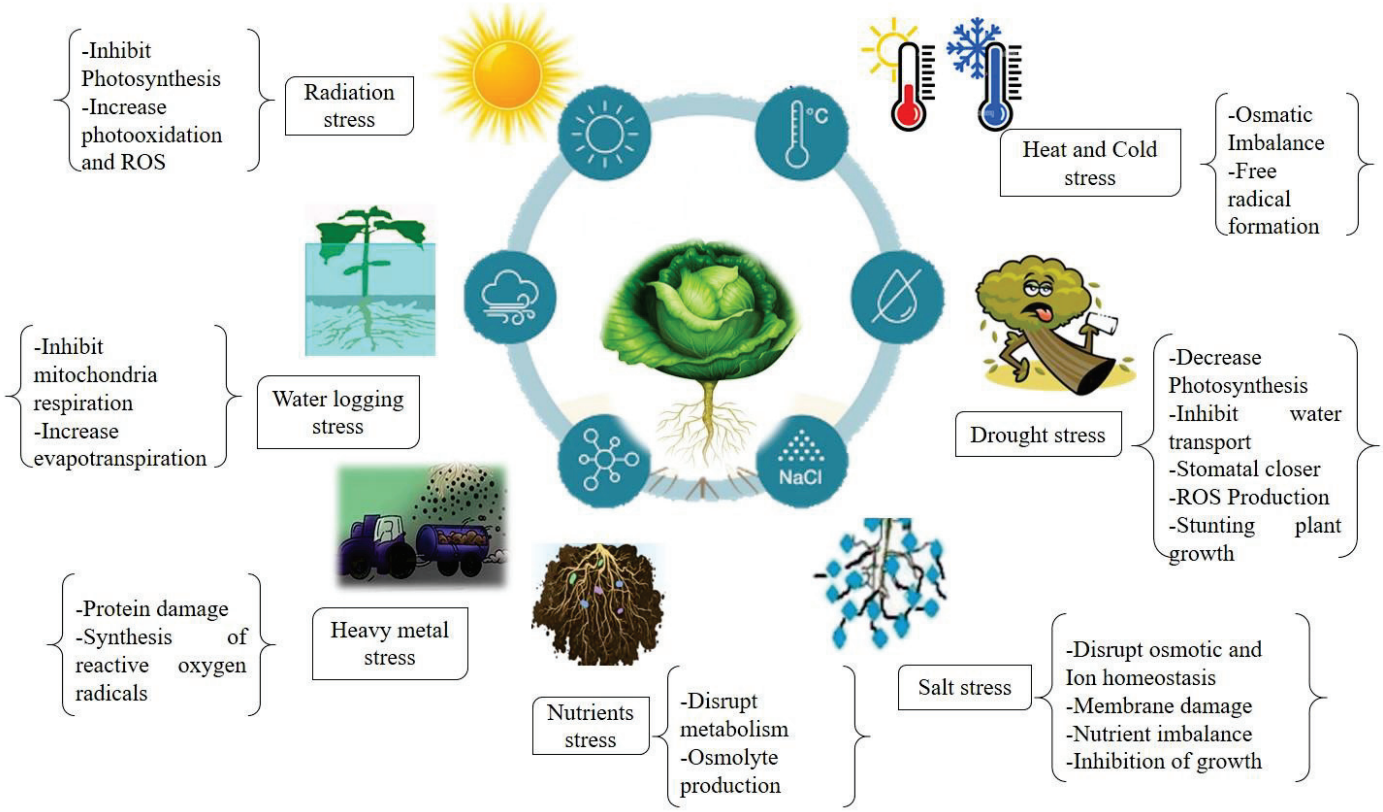


**Figure 1.** Effects of abiotic stresses and their consequences on Brassicaceae.

## 3. Use of Plant Growth-Promoting Rhizobacteria to Mitigate Adverse Effects of Abiotic Stress

In recent years, the contribution of rhizosphere microorganisms to increasing plant growth and crop productivity as well as tolerance to biotic and abiotic stresses without causing pathogenicity have been discussed in the literature [35]. Several genera of plant growth-promoting rhizobacteria (PGPR) including *Azospirillum*, *Bacillus*, *Rhizobium*, *Pseudomonas* and *Bradyrhizobium* showed positive interactions with different vegetables species [36,37]. Several previous studies highlighted the capacity of different PGPRs in biological nitrogen fixation ($N_2$) [38,39], increasing the availability of iron (Fe) [40], phosphorus (P) and zinc (Zn) solubilization and transportation [41,42]. The PGPRs also improved the

performance and growth of plants through the production of phytohormones such as gibberellins, ethylene, cytokinin, auxins and salicylic acid [43,44].

The use of PGPRs has contributed to combating climate-induced changes (abiotic factors) such as uneven rainfall (drought), soil and water salinization and heavy metal contamination that limit the general performance of plants [44,45]. These microorganisms improve soil fertility and structure, which contribute to a successful adaptation of the plant under stressful conditions [45]. Researchers have been focused on the use of these microorganisms with emphasis on bacteria of the genera *Azospirillum*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Bradyrhizobium*, *Herbaspirillum* and *Burkholderia* [36,38].

PGPRs exist in the rhizosphere and tissues of plants, which may adapt multiple mechanisms including the synthesis and exudation of phytohormones (indole-3-acetic acid (AIA)), cytokinin, ethylene and gibberellins [46]; synthesis of plant growth-regulators including nitric oxide [47]; abscisic acid [48]; polyamines such as spermidine and spermine [49]; increase solubilization and availability of nutrients [50,51]; increase nitrate reductase activity and nutrient use efficiency [38,52]; biocontrol of phytopathogens and diseases [53]; and protection of plants against water and saline stress and toxic chemical elements of the soil [54]. In addition to assisting in biological nitrogen fixation, PGPRs have the ability to enhance cell membrane stability of the leaf and reduce the rate of leaf abscission during drought stress conditions [55]. Several PGPRs improve the tolerance capability of plants by producing certain phytohormones [56] that can be used for heavy metal remediation, mobilization or immobilization from soil into plant tissues [57,58]. These microbes also utilized 1-aminocyclopropane-1-carboxylic acid (ACC) to prevent ethylene production [59] and mitigate stresses by endophytic biota, which were caused due to high radiation and light stress [60]. Plant growth-promoting rhizobacteria adapted several mechanisms to improve the growth and development of the plants of the Brassicaceae family under abiotic stresses (Figure 2).

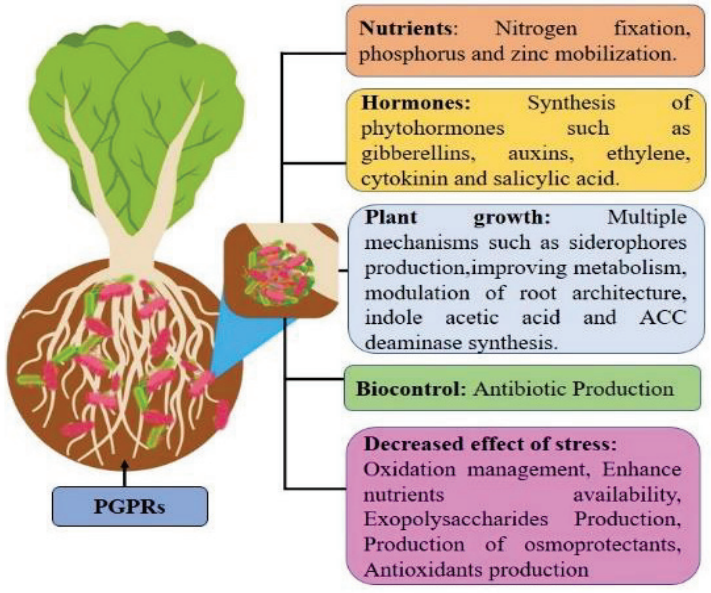


**Figure 2.** Role of plant growth-promoting rhizobacteria in Brassica species against abiotic stresses.

Plant growth-promoting rhizobacteria (PGPR) promote plant tolerance to abiotic stresses through the adaptation of several mechanisms as well as down- or up-regulating stress genes [61]. The inoculation of rapeseed plants with *Pseudomonas* sp. and *Azospirillum* sp. mitigate salt stress [62] by increasing the solubilization and availability of macro- and micronutrients for better uptake in the above-ground part of the host plant [63,64]. PGPRs prominently improved root–shoot fresh and dry weights, leaf area, chlorophyll and several growth-promoting hormones, which ultimately improved the seedling growth of *B. oleracea*

and *B. napus* [65,66]. Flooding is another abiotic stress that harshly reduces antioxidant activities; however, inoculation with bio-fertilizers (*Azotobacter chroococcum*, *Azospirillum* spp. and *Pseudomonas* spp. and *Azospirillum* spp., *Pseudomonas fluorescens* and *Basillus subtilis*) via seeds and foliar efficiently alleviate flooding affects in canola by increasing growth and yield [67]. In this context, the supply of these rhizobacteria or PGPRs to plants of the Brassicaceae family brought benefits to their cultivation in abiotic conditions (Table 1).

**Table 1.** Summary of the positive effects of PGPR in mitigating unfavorable abiotic stress conditions in Brassicas (2008–2020).

| Crops | Abiotic Stresses | Positive Effect of PGPR | Reference |
|---|---|---|---|
| Radish | Salinity | *Bacillus subtilis*, *B. atrophaeus* and *B. spharicus* reduced osmotic effects of salinity to improve production. | [68] |
| Radish | Salinity | *Bacillus subtilis* and *Pseudomonas fluorescens* improved morphological and biochemical attributes as well as hormonal levels of plants. | [69] |
| Rapeseed | Drought | Inoculation of rapeseeds with *Pseudomonas fluorescens* or *P. putida* improved yield, 1000-grain weight, grains/pod, pods and branches/plant. | [70] |
| Rapeseed | Heavy metals | Use of *Bacillus megaterium* reduced soil Ni concentrations through the activity of IAA and solubilization of P. | [71] |
| Rapeseed | Salinity | *Pseudomonas* sp. and *Azospirillum brasilense* mitigated harmful effects of salinity by increasing leaf water content, activity of antioxidant enzymes, leaf area, osmolyte production, productivity and leaf nutrient concentrations. | [72] |
| Rapeseed | Heavy metals | Use of *Pseudomonas* sp. A3R3 and *Psychrobacter* sp. SRS8 reduced Zn toxicity in the soil due to the production of hormones and siderophore activity. | [73] |
| Rapeseed | Heavy metals | Use of *Arthrobacter* sp., *Bacillus altitudinis* SrN9, *B. megatherium* reduced soils cadmium contamination by producing IAA and siderophores. | [64] |
| Cabbage | Salinity | *Azotobacter chroococcum* minimized salt stress by increasing root development and IAA. | [74] |
| Cabbage | Drought | Inoculation with *Bacillus megaterium*, *Pantoea agglomerans* and *Brevibacillus choshiensis* improved physiology of membrane integrity and increased accumulation of osmolytes, antioxidant enzymes, hormonal production, decreased electrolyte leakage and production of ROS-eliminating enzymes | [75] |
| Canola | Salinity | *E. cloacae* improved tolerance to saline soils by promoting root–shoot growth and increasing production of phytohormones. | [76] |
| Turnip | Heavy metals | *B. megaterium* reduced soil contamination with cadmium and lead by the synthesis of IAA and siderophore activity. | [77] |
| Turnip | Drought and phytotoxicity of Zn and Cu | Inoculation with *Pseudomonas libanensis* TR1 and *Pseudomonas reactans* Ph3R3 reduced phytoremediation of metals polluted soils and increased relative water content by the synthesis of IAA, 1-aminocyclopropane-1-carboxylate deaminase, and siderophore. | [78] |

## 4. Plant Nutrition to Mitigate Adverse Effects of Abiotic Stress on Brassicas

Plants develop extensive adaptive and/or resistance mechanisms to sustain productivity and survival under stressful conditions. However, adequate nutrient application is an imperative tool to meet the Sustainable Development Goals to attain food and nutritious security and promote sustainable productivity under climate extremes [79]. Optimization of nutrient content (macro- and micronutrients, secondary nutrients and heavy metals) in

soil and plant systems have been reported to enhance crop adaptation to resilience conditions, as these are structural elements of several co-factors and enzymes. Nutrients assist structures' stability of protein and alleviate reactive oxygen species (ROS) production. The versatility of nutrient application under severe environmental conditions has significantly improved the yield and quality traits of various crops [80].

Fertilizers are considered the most important and crucial inputs to achieve greater crop growth and production in modern agriculture [81]. Plants require NPK and other essential micronutrients such as iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), molybdenum (Mo), nickel (Ni), chlorine (Cl) and boron (B) in very small quantities for better performance and yield. These elements are collectively considered as essential for humans and animals and their deficiency can affect their metabolic, physical and mental development. Macro- and micronutrients play a critical role in the effectiveness of several biological compounds and enzymes for the proper functioning of different metabolic processes. The relevance of macronutrients' essentiality for higher yield and nutritional status has been increasing over several decades [82]. Ensuring that plants are well-fed with essential nutrients is a cost-effective strategy with the capacity to mitigate abiotic stresses and enhance productivity [79,81]. The effect of macro- and micronutrients on different functions of Brassicaceae crops promotes plant growth and increases tolerance to abiotic stresses (Figure 3).

### *4.1. Macronutrients*

Macronutrients are considered to be significant drivers for enhancing the yield and quality parameters of crop plants. Traditional fertilizer application in a field may not fulfill the demands of individual plants while over and/or under application causes soil quality degradation, groundwater pollution and reduction in productivity. Leaf nutrition of rapeseeds is an important factor to optimize fertilization and productivity, alongside contributing to commercial and environmental profits [83]. Better management of macronutrient fertilizers can improve plant growth and yield under stressful conditions. The nutrients and their functions in the crop plants are discussed below in detail.

#### 4.1.1. Nitrogen

Nitrogen (N) is the most needed nutrient for most cultivated plants, and it directly affects plant development and yield [84–89]. Nitrogen is the main constituent of the atmosphere, but its availability is still one of the main limiting factors for the productivity of terrestrial ecosystems including agro-ecosystems [90]. Nitrogen plays an important role in plant nutrition and development [87], such as the synthesis and production of phytohormones, co-enzymes, nucleic acids, secondary metabolites, chlorophyll and proteins content [91].

Several studies have reported that N fertilization promoted different species of Brassicaceae including oilseed producer crops such as rapeseed (*B. napus*) [92,93], brown mustard (*B. juncea*) [94,95] and turnip rape (*B. rapa*) [96] and horticultural crops such as radish (*Raphanus sativus*) [97], cauliflower (*B. oleracea* L. var. botrytis) [98,99], cabbage (*B. oleracea* L. var. Capitata) [100,101], broccoli (*B. oleracea* L. (var. italica) [102,103], kale (*B. oleracea* L. var. sabellica) [104,105] and arugula (*Eruca vesicaria* subsp. Sativa) [106].

Abiotic stress conditions alter the N metabolism of Brassicaceae plants [94], negatively affecting N uptake and assimilation, N use efficiency (NUE), photosynthetic capacity and plant growth [107], particularly under prolonged (24 h) stress exposure [108]. The interaction of N fertilization and abiotic conditions plays an important role in determining the potential of plant development and abiotic stress tolerance. Stress relief depends on the type of N fertilization; applying ammonium ($NH_4^+$) to plants resulted in a stronger tolerance to heat stress as compared to the fertilization with nitrate ($NO_3^-$) [109]. In addition, N fertilization can compensate for the negative effects of abiotic conditions by facilitating carbon partitioning, cell membrane stability, osmoregulation and antioxidative mechanisms that could improve plant growth and development as well reduce leaf senescence under extreme environmental conditions [110].

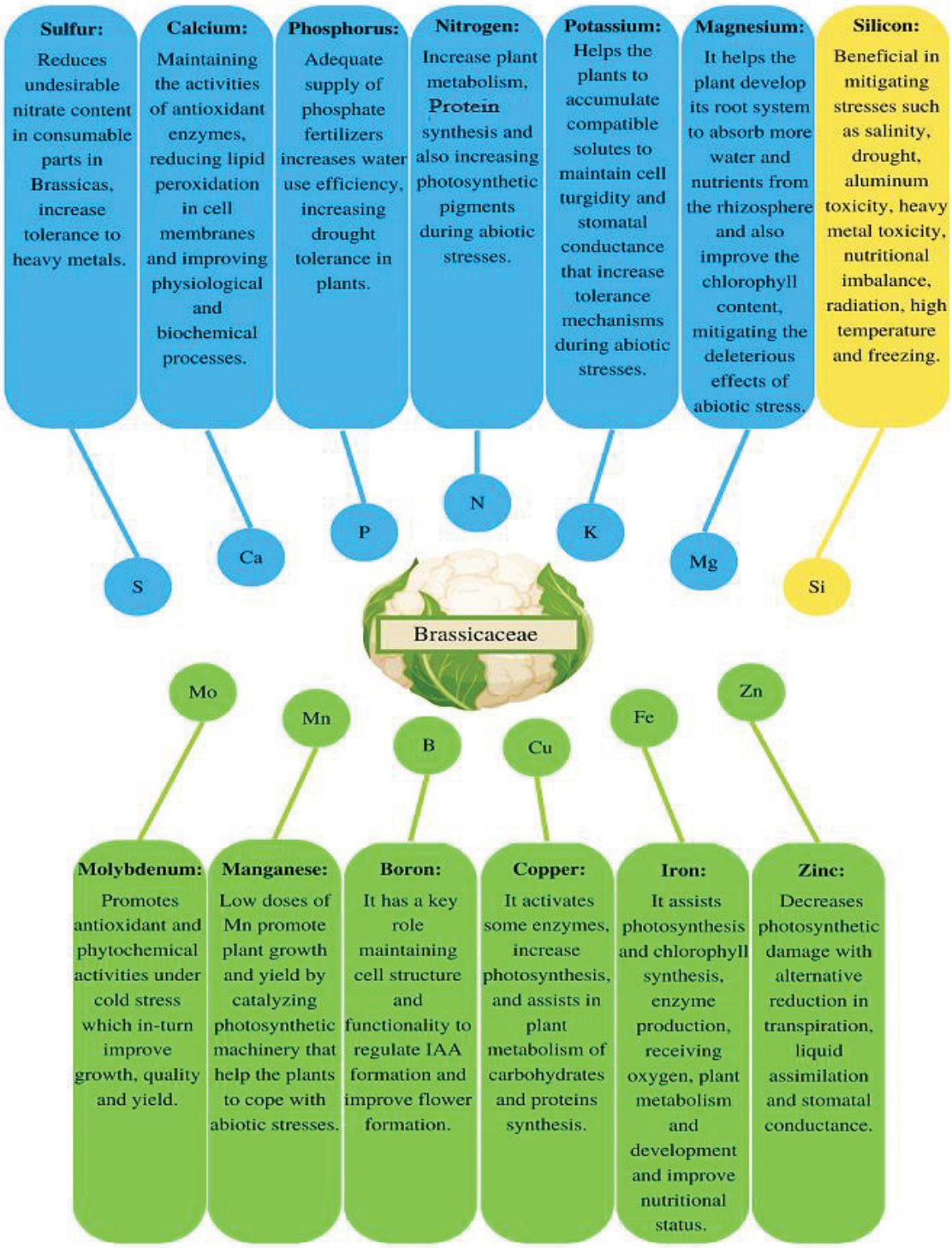


**Figure 3.** Effect of macronutrients (indicated in blue color), silicon (orange color) and micronutrients (green color) on different functions of Brassicaceae crops.

#### 4.1.2. Phosphorus

Phosphorus (P) is a primary macronutrient with a structural function in plants. It is involved in drivers of metabolic functions including respiration, energy storage and transportation, production of nucleic acid, membrane stability, catalyze enzymes activities, redox reactions and contribution to carbohydrate metabolism [111]. As with other plant families, P is one of the important nutrients for the Brassicaceae family that directly affects its development and productivity [112]. Holzschuh et al. [113] studied different doses of P

fertilizer in Brassicas and reported that the species of this family are highly demanding of P availability in the soil, especially broccoli (*B. oleracea* var. itálica) and cauliflower (*B. oleracea* var. botrytis). The optimal management of P fertilization in vegetables is essential for their proper growth, development and yield [112]. Phosphorus deficiency in soil and plants directly affects vegetable vigor, establishment and root development, thus disrupting water use efficiency [114]. Several plants of the Brassicas species have the capability to tolerate and respond to various types of stresses through hormonal stimulation, ion exchange, antioxidant enzymes and the activation of signaling flow in their metabolic and genetic boundaries that mitigate stressed conditions [115].

Application and management of appropriate P fertilization has increased water use efficiency against drought stress [116,117]. Jones et al. [118] indicated that adequate soil P contents compensate for the impact of drought stress on the growth and yield of plants. Application of P source fertilizers may reduce the drastic effects of water scarcity during pollen formation or the reproductive stage that could increase flower and pod production, resulting in a greater yield and high protein content in grains [119]. Phosphate fertilizers improved the performance of *B. juncea* under salt stress by increasing plant dry mass and P uptake while lowering the $Na^+/K^+$ ratio [114]. Phosphorus fertilization adapts different mechanisms that immobilize the metal content in soil [120] by reducing their dissolution under the low pH range of soil, hence leading to the reduction of the bioavailability and uptake of metals by plants [121]. Phosphate fertilization increases the pH of soil solution to constrain absorption of heavy metals, as their availability decreases with increasing P fertilization [122].

#### 4.1.3. Potassium

Plants develop a wide range of adaptive and resistive strategies that sustain productivity and survival under stressful conditions. Plant tissues may adjust osmotic potential through the absorption of various compatible osmolytes such as inorganic ions, carbohydrates, organic acids and free amino acids [123,124]. Plants adjust osmotic potential by regulating stomatal conductance, photosynthesis, leaf turgidity and plant growth rate under drought, salt and high-temperature stresses through potassium (K) osmolytes [125]. Potassium is one of the major inorganic osmolytes that enable osmotic regulation and adjustment during stress conditions. Potassium ion absorption protects plants from harmful impacts of different stresses including drought, salinity, metal toxicity and high or cold temperatures by osmotic adjustment and maintenance of stomatal conductance, protecting cell integrity and increasing photosynthesis as well as via the detoxification of reactive oxygen species [123].

In addition, K is a crucial element for the distribution of photo-assimilates in root systems [126] that protects plants against most abiotic stresses including metal toxicity such as Cd-induced oxidative damage [127], Zn toxicity [128], NaCl toxicity [129], drought stress [130] and high radiance incidence [131]. Potassium supplementation increases the adjustment of stomata, which regulates carbohydrate formation and the growth of *Nicotiana rustica* during stress conditions [125]. Samar-Raza et al. [132] reported that application of K fertilizer under drought stress enhanced the tolerance of wheat (*Triticum aestivum* L.) by reducing toxic elements' absorption and enhancing physiological efficiency and yield [133].

#### 4.1.4. Calcium

Calcium (Ca) plays a vital role in the physiological functions of plants and acts as a second messenger element of external signals for the higher performance of plants. It has an essential role in the structure and stabilization of the cell wall and membrane, regulating metabolic, enzymatic and hormonal processes [134]. The alteration in free cytosolic $Ca^{2+}$ ion contents is validated during naturally occurring abiotic stimulants (low and high temperature/light, tensions, high osmotic and oxidative tensions, also during biotic stimulants (nodulation aspects and fungal drivers)) [135]. It also has an explicit function in the performance and maintenance of plant development and detoxification

of heavy metals [136]. The main function of $Ca^{+}$ ions under heavy metal stress is to maintain the activities of antioxidant enzymes, reducing the peroxidation of lipids in the cell membrane and improving the physio-biochemical processes of plants [127,137].

#### 4.1.5. Magnesium

Magnesium ($Mg^{2+}$) is an essential nutrient for plant growth [138], regulating cell membrane stability, carbon fixation, chlorophyll synthesis, carbohydrate transport, enzymatic activities and reproductive process [139–141]; thus, it helps plants to adapt defensive mechanisms against abiotic stresses [142]. Plants under Mg nutrition improve root growth and root surface area that increase water and nutrient uptake from the rhizosphere and enhance transportation of photo-assimilates and carbohydrate synthesis, which can mitigate drought-stress-induced deleterious changes [143]. Magnesium transports carbohydrates from roots to shoots and helps in the fixation of photosynthetic $CO_2$ during the reproductive growth stage under salt stress. The efficiency of Mg foliar fertilization is right-away associated with the distribution of nutrients within plants [144]. Nutrient solution with Mg fertilization improved the shoot growth of *B. rapa* L. var. pervirdis under cadmium (Cd) toxicity [145].

Deficiency of Mg is one of the common nutritional syndromes in plants, which may have drastic impacts on agricultural productivity and quality [146] and lead to morphological and physiological abnormalities of plants [147]. Plants produce antioxidants and antioxidative defensive enzyme activities, especially ascorbic acid during the stress of Mg deficiency [148]. The glutathione-producing ascorbate-determined $H_2O_2$ scavengers are responsible for ascorbic acid that can enable the plants to detoxify ROS production to protect plants from climate extremes [149]. Glutathione homeostasis can be regulated through the over-production of glyoxalase genes that can help the plants to sustain Mg content during stressful conditions and increase tolerance to metalliferous soil [150,151].

Magnesium transporters are also involved in metal transport. Under the low Mg content, nickel ($Ni^{+}$) is well-cited for the suppression of electron flow and impairing photosynthesis functions by replacing $Mg^{2+}$ in chlorophyll fragments. Adequate fertilizer of Mg alleviates the $Ni^{+}$ effect in the root rhizosphere that may reduce the negative probability of Ni at the outer surface of the plasma membrane by replacing the targeted ionic binding site [152]. The Mg transporter (AtMHX) from Arabidopsis acts as an $H^{+}$ exchanger with Zn and Mg and is confined to the vacuole membrane [153]. The AtMGT1 protein derived from AtMGT (transporter gene of Arabidopsis) family in the plasma membrane exhibited greater attraction to $Mg^{2+}$ ion, which helped in the re-distribution of $Ni^{+}$, $Ca^{2+}$, $Fe^{3+}$, $Mn^{2+}$ and $Cu^{2+}$, when they are present in high concentrations [154].

#### 4.1.6. Sulfur

Sulfur (S) is among the very active macronutrients in plant metabolism, which is why it is recognized alongside nitrogen (N) as a key nutrient for plant development [155]. Sulfur is used by plants to assimilate with a variety of organic compounds that are essential for the growth, development and mitigation of plant stress [11,156]. It is also responsible for making vegetables softer and adding greater commercial value [157]. Sulfur is predominantly found in the soil and is one of the main nutrients that is absorbed by plants in the form of sulfate anion ($SO_4^{2-}$) from organic matter and a small proportion from the atmosphere in the form of sulfuric gas [158].

Kohlrabi (*B. oleracea* L.) is one of the crucially demanding S vegetables of the Brassicas family, which absorbs 1.5 kg S $ton^{-1}$ of yield. Sulfur deficiency can inhibit leaf formation and change young leaves' color from dark green to light green or yellowish. Proper S fertilization in kohlrabi (*B. oleracea* L.) improves tuber yield and reduces the undesirable nitrate content in consumable parts [159]. Canola (*B. napus* L. var. Oleifera) is also one of the most demanding S vegetables in reproductive phases as compared to other winter crops, as it exports a large amount of S to the grains [160]. Sulfur is one of the known nutrients that performs an imperative role in the tolerance to heavy metal toxicity [161].

Chromium is actively transported across the plasma membrane and appears to be mediated by transporters, which are primarily responsible for sulphate uptake [162,163]. This suggests the action of this molecule inhibit the absorption of heavy metals that are toxic to plants (Table 2).

**Table 2.** Summary of the positive effects of N, P, K, S, Mg, Ca and Si fertilization in mitigating abiotic stress conditions in Brassicas (2004–2020).

| Crops | Abiotic Stresses | Macronutrients and Si Positive Effects | Reference |
|---|---|---|---|
| Radish | Zn toxicity | $Mg^{2+}$ enhanced uptake and translocation of Zn, as well as alleviated Zn toxicity. | [164] |
| Mustard | Salinity | Nitrogen maintains synthesis of proline and ethylene to combat drastic impacts of salinity on photosystem. | [94] |
| Rapeseed | Cd toxicity | Nutrient solution of Mg fertilization improved shoot growth of *B. rapa* under cadmium (Cd) toxicity. | [145] |
| | Salinity | Nitrogen ameliorates salinity effects by improving growth attributes, physio-biochemical attributes (total chlorophyll, water content, stomatal conductance, K / Na ratio, carbonic anhydrase activity and malondialdehyde) and yield attributes (seeds $pod^{-1}$, pod and yield $plant^{-1}$). | [165] |
| | Cd toxicity | Silicon increased photosynthetic pigments and reduced inhibitory effects of Cd on root elongation. | [166] |
| | Salinity | Higher proline accumulation and photosynthetic efficiency increased plant growth with S fertilization. | [167] |
| | Heavy metals | Cadmium and lead have negative effects on P, Ca, Mn and Fe content root and leaves dry mass. | [168] |
| | Heavy metals | Application of Ca increases tolerance to Cd in mustard plants by restoring morphological and biochemical attributes. | [137] |
| | As toxicity | Silicon modulated root elongation with development of both primary and lateral roots. | [169] |
| | Cr toxicity | Silicon reduced transportation of Cr from root to shoot and photosynthetic activity by increasing net photosynthetic rate, chlorophyll, and carotenoid content. | [170] |
| | Salinity | Silicon increased plant growth, antioxidant activity (catalase, peroxidase and superoxide dismutase) and proline content. | [171] |
| | Heavy metals | Application of S mediated antioxidant enzymes in the plant, contributing to phytoextraction of potentially toxic elements (cadmium and zinc) from contaminated soils, helping in phytoremediation process of the soil. | [172] |
| | Drought | Potassium fertilization improved relative water content, stomatal conductance, relative chlorophyll index, and productivity. | [173] |
| | Salinity | Silicon nutrition ameliorated the lethal impacts of salinization in canola by lowering Na absorption, maintaining root cell integrity, reduced lipid peroxidation, enhancing the scavenging capability of ROS and decreased lignification. | [174] |
| | Drought | Fertilization with $K_2SO_4$ alleviated deleterious effects of water stress by stimulating productive characteristics (pods $plant^{-1}$, seeds $pod^{-1}$ and grain yield). | [130] |
| | Drought | Nitrogen improved proline production to maintain water balance and integrity of proteins, enzymes and cell membranes. | [107] |
| | Oxidative stress | Nitrogen and S reducing reactive oxygen species production. | [175] |

**Table 2.** *Cont.*

| Crops | Abiotic Stresses | Macronutrients and Si Positive Effects | Reference |
|---|---|---|---|
| | Salinity | Silicon application prevented toxic ions (Na and Cl) accumulation while maintaining K, P and Fe content in plants. | [176] |
| | Drought | Silicon increased shoot–root biomass, total chlorophyll content, activity of superoxide dismutase and catalase while reducing lipid peroxidation. | [177] |
| | Salinity | Phosphate fertilizers improved plant performance under salt stress by lowering $Na^+/K^+$ ratio and increasing P uptake. | [114] |
| | Drought | Nitrogen increased plant height, number of branches, number of fruits per plant, thousand seed weights and crude protein. | [178] |
| | Cd toxicity | Silicon reduced oxidative damage in plants by increasing antioxidant components and methylglyoxal detoxification system that enhance tolerance to Cd stress. | [179] |
| | Drought | Silicon improved antioxidants enzymes, ascorbate and glutathione pool, glyoxalase systems and proline by increasing protective role and maintaining redox status of plants. | [126] |
| | Oxidative stress | Silicon improved biomass, N uptake and chlorophyll content. Also, decreased oxidative stress by reducing hydrogen peroxide and malondialdehyde production. | [180] |
| | Haze condition | Nitrogen increased shoot biomass and photosynthetic productivity. | [181] |
| Canola | Salinity | Calcium-fortified composted animal manure alleviate oxidative stress, improvement in growth, physiology and mineral nutrition | [182] |
| | Salinity | Increased activity of phosphatase enzymes and reduced phosphate levels in plants. | [183] |
| | Salinity | Potassium fertilization mitigates the effects of salinity by confining Na absorption, activating cellular compartmentalization of excess $Na^+$ in cell vacuole. | [129] |
| | Heavy metals | Sulfur application increases lipid peroxidation and activities of antioxidant enzymes. | [184] |
| | Drought | Fertilization of Ca allows plants to resist drought by improving antioxidant capacity, oil quality and essential fatty acids (linolenic acid and linolenic acid) in seeds. | [185] |
| | Drought | Potassium mitigated the effect of water deficiency by increasing water and nitrogen use efficiency, improving chlorophyll index, leaf area index, cell membrane integrity and productivity. | [186] |
| Cabbage | Drought | Nitrogen increased harvest index and dry matter production. | [187] |
| | Cd toxicity | Silicon alleviated Cd toxicity by increasing activities of antioxidant enzymes and shoot and root biomass. | [188] |
| | Salinity | Potassium fertilization improved absorption of total soluble free amino acids and proteins, proline content, regulated activities of antioxidant and improved gas exchange traits. | [189] |
| Broccoli | Heavy metals | Calcium fertilization mitigates $ZnSO_4$ toxicity by increasing total phenolic content and antioxidant capacity of sprouts. | [190] |
| | Salinity | Nitrogen increased photosynthetic capacity and vitamin C content. | [191] |
| | $NH_4^+$ toxicity | Silicon alleviated $NH_4^+$ toxicity in cauliflower by increasing physical integrity of membranes while increasing water use efficiency in broccoli. | [192] |
| | Drought | Co-application of macro- and micronutrient and biostimulants increased nutritional status of broccoli plants in water deficient conditions. | [193] |
| | High luminosity | A positive correlation between Fe, Mg and Ca, and high light was observed. | [194] |

**Table 2.** *Cont.*

| Crops | Abiotic Stresses | Macronutrients and Si Positive Effects | Reference |
|---|---|---|---|
| Arugula | Drought | Potassium mitigated the effect of water deficiency by increasing water and nitrogen use efficiency, improving chlorophyll index, leaf area index, cell membrane integrity and productivity. | [195] |
| | Drought | Silicon improved gas exchanges capacity. | [196] |
| Kale | Drought | Silicon reduced water loss, increased shoot biomass and plant height. | [197] |
| Turnip | Heavy metals | Polypeptide Ca has a dual function in competitive inhibition in cadmium-contaminated agricultural land. | [198] |

### *4.2. Silicon*

Silicon (Si) is the second most abundant chemical element after oxygen in the earth's crust [199,200]; however, it is still not available directly to plants and is commonly adsorbed with oxides and silicates, affecting plant nutritional status [180,201–203]. In addition, the low dissolution of Si in the soil decreases its availability; thus, it occurs in a very low amount [204].

Plants uptake Si mainly from dissoluble mono-silicic acid ($H_2SiO_4$), a noncharged molecule which plays a significant role to increase plant resistance to abiotic and biotic conditions [205–207]. Silicon is distributed via xylem in the form of hydrated amorphous silica/silica bodies ($SiO_2.nH_2O$) and pledged to the epidermis of cell membrane. After deposition to the cell membrane, Si is no longer available for further distribution into the above-soil parts of the plants [208]. The transport of $H_4SiO_4$ occurs in a similar direction to transpiration (mass flow). Therefore, drought conditions increase the deposition of Si in the regions of leaf epidermis to protect water from high transpiration [209].

All soil-grown plants had Si constituents ranging from 0.1 to 10% of dry weight of plants [180,210]. However, Si is classified as a beneficial element, with it being an imperative element for several crops, specifically rice (*Oryza sativa* L.) and sugarcane (*Saccharum officinarum* L.). Moreover, its role has been well documented for the performance, growth and development of different Gramineae family crops [180,206,207,211–213]. This chemical element has been reported to be beneficial in mitigating abiotic stresses including heavy metal toxicity, salinity, high temperature, drought, radiation, aluminum toxicity, lodging, nutrient imbalance, wounding and freezing [214,215]. Rapeseed is one of the most studied plants of the Brassicaceae family regarding Si application to alleviate abiotic stress conditions [211], with the most common improvements reported in plant resistance to cold stress conditions, as well as the formation of larger seeds [216]. Table 2 summarizes the studies with Si fertilization in Brassicaceae plants under abiotic stress in the last decade (2004–2020).

### *4.3. Micronutrients*

Micronutrients (zinc (Zn), iron (Fe), manganese (Mn), molybdenum (Mo), boron (B), copper (Cu) and chlorine (Cl)) improve plant health, water use efficiency, biomass production and provide systemic response against abiotic stresses [217–219]. Whereas plant growth-promoting rhizobacteria (PGPR) promote plant growth and tolerance to abiotic stresses by adapting and altering certain mechanisms, the production of ACC (1-aminocyclopropane-1-carboxylate) deaminase reduces ethylene synthesis, as well as alters phytohormones and antioxidative enzymes synthesis, and improves nutrient uptake [115,220].

Micronutrients may influence directly or indirectly the stress affecting plants due to their role in several enzymatic and metabolic activities [221]. Abiotic stress such as drought harshly impairs mineral nutrient translocation from soil to plant parts [222,223]. The Brassicaceae family is one of the most nutrient-demanding plant species, which is highly affected by inadequate nutrients application [224]. Therefore, deficiency of micronutrients

disrupts the net-assimilation rate and stomatal conductance, electron transportation in photosynthesis, chlorophyll content, root–shoot ratio and antioxidant activities of cabbages, turnip and canola under abiotic stresses [225–231]. Salinity is a critical challenge to high production, physiological and biochemical attributes and nutrient uptake in Brassicaceae species [232]. Brassicas adapted certain mechanisms and variations, especially physiological variations to cope with salinity [233]. Salinization in plant systems can be ameliorated with foliar nutrient spray and rhizosphere micronutrient availability and uptake [234]. The accumulation of sodium ($Na^+$) and chlorine ($Cl^-$) ions increases osmotic potential and decreases water availability and nutrient uptake through plant roots [235].

Several studies regarding the Brassicaceae family indicated that most of the species grown on contaminated soils with high accumulation of nutrients (Zn and Cu) and non-essential metals (Pd, Cd, Ni and Cr) [236–239]. Plants of *B. juncea* have the ability to accumulate high amounts of Cd, Cu, Ni, Cr, Zn, Fe, Co, Pb and Se from metal-contaminated sites [240–242]. Rapeseed subjected to early waterlogging stress resulted in higher accumulation of Mn, Fe, Zn and Cu in the leaves and caused toxicity [243]. Zinc is one of the efficient nutrients in the reduction of heat stroke by improving biochemical activities and superoxide dismutase (SOD) content in *B. rapa* [244,245]. Boron and Mn application in winter rapeseed (*B. napus*) positively influenced pod production, photosynthetic rate, N-metabolism, antioxidant activities and improved N and Ca contents in seeds [227,246]. High UV-B radiation may alter nutritional status, disturb plant cell metabolism, increase pathogens and disease tolerance [247], whereas light-emitting diodes (short duration blue light) enhanced phytochemical activities and micronutrient (Zn, Mn, Mo, B, Na, Fe and Cu) concentration in Broccoli (*B. oleacea* var. italica) [248,249] (Table 3).

**Table 3.** Summary of the positive effects of micronutrients fertilization in mitigating unfavorable abiotic stress conditions in Brassicas (2005–2020).

| Crops | Abiotic Stresses | Micronutrients Effects | Reference |
|---|---|---|---|
| Rapeseed | Cold temperature and high light radiation | Boron removal, mobilization and partitioning into root–shoot and younger leaves of plant were imperatively reduced in chilling temperature and intensive light. | [250] |
| | Drought | Drought drastically reduced Zn contents and led to photosynthetic damages with alternative reduction in transpiration, net assimilation and stomatal conductance and act as Cu-Zn SOD enzyme. | [251] |
| | Salinity | Protein and micronutrients were improved in order of Mn > Zn > Cu > Fe in aerial parts with application of N and Zn. | [252] |
| | Waterlogging | Waterlogging severely impaired growth and nutrients accumulation and ATP synthesis in plants. | [253] |
| | Waterlogging | Early waterlogging stress resulted in higher accumulation of Mn, Fe, Zn and Cu in the leaves and causes toxicity. | [243] |
| Turnip | Drought | Boron deficiency is increased under drought stress and led to the disturbance of electron transportation in photosynthesis, lowering chlorophyll content and root–shoot ratio. | [228] |
| | Drought | Drought drastically reduced Zn contents and led to photosynthetic damages with alternative reduction in transpiration, net assimilation and stomatal conductance. | [226] |
| Canola | Salinity | Micronutrients (Fe, Mn and Cu) contents in plant aerial parts were improved under salt stress | [254] |
| | Drought | Boron deficiency is increased under drought stress and led to the disturbance of electron transportation in photosynthesis, lowering chlorophyll content and root–shoot ratio. | [229] |
| | Drought | Yield and yield components were improved with lower dose of foliar Fe and Mn. | [231] |

**Table 3.** *Cont.*

| Crops | Abiotic Stresses | Micronutrients Effects | Reference |
|---|---|---|---|
| Mustard | Heavy metals | Accumulate high amount of Cd, Cu, Ni, Cr, Zn, Fe, Co, Pb and Se from contaminated sites. | [241] |
| | Heavy metals | High levels of Cd decrease micronutrients (Mn, Fe, Cu and Zn) content. | [255] |
| Broccoli | High light radiation | Short duration of blue light-emitting diodes (LED) prominently improves phytochemical components, essential micronutrient (B, Fe, Zn, Mn, Mo, Na and Cu) and macronutrients (Ca, P, K, Mg and S). | [248] |
| | Salinity | Salinity reduced yield and boron accumulation in aerial parts of plant. | [256] |
| | Hight light radiation | Short duration blue light enhanced different phytochemical activities, micronutrients (Zn, Mn, Mo, B, Na, Fe and Cu) concentration and also macronutrients (Ca, P, K, S and Mg) concentration in plants. | [248,249] |
| | Salinity | Biofertilizers improve Fe availability and also Ca and Mg content in plants. | [257] |
| Chinese cabbage | Cold temperature | Molybdenum promotes antioxidant and phytochemical activities, improve growth, quality and yield. | [258] |
| *A. thaliana* | High light radiation | Zinc prevents photo-inhibitory damages to photosynthetic apparatus by producing ROS and enhancing carotenoid contents plant leaves. | [259] |

## 5. Conclusions

Based on the updated literature, this review highlighted the importance of adequate and balanced nutrition against abiotic stresses in Brassicas species to ensure food and nutritious security. Proper management of macronutrients, micronutrients and silicon under certain conditions of abiotic stress could improve nutritional and physiological status, thus resulting in higher productivity and quality of Brassicas plants. Balanced application of macro- and micronutrients mitigates abiotic-stress-induced changes in Brassicas plant species by stimulating absorption and accumulation mechanisms for better survival.

The use of plant growth-promoting rhizobacteria (PGPR) has a critical role in combating climate-induced changes such as uneven rainfall (drought), soil and water salinization and heavy metal contamination, which limit the general performance of Brassicas plant species. Among the PGPRs, the genera *Azospirillum*, *Bacillus*, *Pseudomonas*, *Herbaspirillum* and *Burkholderia* are well studied for increasing plant nutrition, tolerance to pathogens and climate extreme conditions, and hence could improve plant performance and productivity in adverse growing conditions. Therefore, inoculation with PGPRs can increase productivity of Brassicas grown under abiotic stress conditions.

In the future, attention needs to be paid to the response of Mg and micronutrient application on crop resilience under different abiotic stresses. Dose-response management and multiple interactions of nutrients and heavy metals still need further investigation. Bio-fortification via foliar spray of micronutrients is a cost-effective strategy in alleviating global food and nutritious security which requires future advances and intensified research. The intervention of nano-fertilizers on the basis of integrated evidence is required to reduce the gap. The expansion of enhanced detection, tracking and monitoring strategies may be the best early detection technique for abiotic stresses which can also control yield losses and lethal impacts on the nutritional security of crops.

**Author Contributions:** Conceptualization, A.J. and M.C.M.T.F.; methodology, A.J. and C.E.d.S.O.; software, C.E.d.S.O. and F.S.G.; validation, A.J., F.S.G. and C.E.d.S.O.; formal analysis: A.J., I.M.B.G.; resources, M.C.M.T.F.; data curation, A.J., C.E.d.S.O., P.A.L.R. and B.H.d.L.; writing—original draft preparation, A.J. and F.S.G. writing—review and editing, M.C.M.T.F. and F.S.G.; visualization, B.H.d.L. and I.M.B.G.; supervision, M.C.M.T.F.; project administration, A.J. and M.C.M.T.F.; funding acquisition, A.J. and M.C.M.T.F. All authors have read and agreed to the published version of the manuscript.

**Funding:** This review received funding from The World Academy of Science (TWAS) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), for the first author's doctoral fellowship (CNPq/TWAS grant number: 166331/2018-0), and the productivity research grant (award number 311308/2020-1) of the corresponding author.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** No new data were created or analyzed in this study. Data availability is not applicable to this article.

**Acknowledgments:** The authors thank São Paulo State University (UNESP) for providing the technology and support as well as CNPq for the financial support.

**Conflicts of Interest:** The authors declare no conflict of interest.

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*Review*

# Free Radicals Mediated Redox Signaling in Plant Stress Tolerance

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**Abstract:** Abiotic and biotic stresses negatively affect plant cellular and biological processes, limiting their growth and productivity. Plants respond to these environmental cues and biotrophic attackers by activating intricate metabolic-molecular signaling networks precisely and coordinately. One of the initial signaling networks activated is involved in the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS). Recent research has exemplified that ROS below the threshold level can stimulate plant survival by modulating redox homeostasis and regulating various genes of the stress defense pathway. In contrast, RNS regulates the stress tolerance potential of crop plants by modulating post-translation modification processes, such as *S*-nitrosation and tyrosine nitration, improving the stability of protein and DNA and activating the expression of downstream stress-responsive genes. RSS has recently emerged as a new warrior in combating plant stress-induced oxidative damage by modulating various physiological and stress-related processes. Several recent findings have corroborated the existence of intertwined signaling of ROS/RNS/RSS, playing a substantial role in crop stress management. However, the molecular mechanisms underlying their remarkable effect are still unknown. This review comprehensively describes recent ROS/RNS/RSS biology advancements and how they can modulate cell signaling and gene regulation for abiotic stress management in crop plants. Further, the review summarizes the latest information on how these ROS/RNS/RSS signaling interacts with other plant growth regulators and modulates essential plant functions, particularly photosynthesis, cell growth, and apoptosis.

**Keywords:** ROS; RNS; RSS; signaling; post-translational modification; stress tolerance

**Citation:** Rai, K.K.; Kaushik, P. Free Radicals Mediated Redox Signaling in Plant Stress Tolerance. *Life* **2023**, *13*, 204. https://doi.org/10.3390/life13010204

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 24 November 2022
Revised: 5 January 2023
Accepted: 9 January 2023
Published: 10 January 2023



## 1. Introduction

In the 21st century agriculture and various climatic stresses, such as high temperature, drought, and salinity, have redundantly affected crop growth and productivity, prompting severe threats to global food security for ever-growing global populations [1]. In the Asian continent, a rainfed agriculture system is usually standard and is followed by most farmers. These climatic stresses have become a daunting challenge that has imposed severe repercussions on crop health, thereby ultimately affecting its productivity to a certain extent and leading to livestock death [2]. In addition to sessile, plants are constantly exposed to these climate extremes that instigate various cellular, physiological, biochemical, and molecular responses. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) act as integral components of signal transduction processes regulating vital functions in plants exposed to climate extremes [3]. Initially, ROS and RNS are considered toxic molecules where elevated levels provoke oxidative stress in plants leading to cellular damage and death [4]. However, several recent reports have highlighted that ROS and RNS also function as signaling molecules (when their generation is critically maintained below a threshold level by antioxidative systems), catalyzing several oxidation reactions, thereby modulating vital signaling cascades [5].

Redox chemistry is inextricably engaged in the generation, regulation, and sustainment of life on earth by exhilarating reduction–oxidation (redox) reactions essential for driving crucial cellular and metabolic processes such as photosynthesis, respiration, and other biochemical reactions in diverse life forms [6]. Interestingly, recent reports have restricted the involvement of ROS and RNS in stimulating thermodynamically favorable reactions that are essential for sustaining life, including (i) their ability to enhance metabolic reactions, (ii) regulate enzymatic and non-enzymatic antioxidants, (iii) cross barriers (membranes) to activate signaling cascades, and (iv) provide a source of energy (electrons) to defend against oxidative stress [7]. In the recent decade, a plethora of research has been conducted to assess the positive side of ROS/RNS signaling in plants' growth, development, and defense response. A concomitantly large body of literature has pinpointed their exemplary role [8].

Reactive sulfur species (RSS) is a term still to be entered into the general scientific vocabulary due to its low expression and lack of consideration of its role in signal transduction [9,10]. Early prebiotic life forms, i.e., before photosynthesis, likely thrived under a sulfur-rich environment, and several reports claimed that when life originated, approx. 3.8 billion years ago, RSS was the first reactive molecule that influenced expansion [9]. Before photosynthesis, RSS, mainly hydrogen sulfide ($H_2S$) released from volcanic eruptions and other geothermal activity, served as building blocks for nucleic acid biosynthesis and protein synthesis for early life forms, such as Beggiatoa, pupfish, giant tubeworms, and mollusks [10]. Researchers have also confirmed the involvement of RSS in providing reducing powers for fixing $CO_2$ via the Calvin cycle in various green and purple sulfur bacteria [6,11]. Furthermore, a large body of literature has implicated the significant role of RSS in initiating oxidation reactions, thereby controlling redox homeostasis, cell signaling, and defense response in plants [12].

Recent studies have corroborated that ROS, RNS, and RSS have similar chemical structures, yet RSS is more versatile and reactive. In addition, ROS/RNS-mediated biosynthesis of RSS under an oxidative environment is its unprecedented source [9]. Due to its similar chemical nature, RSS triggers oxidation reactions by modifying cysteine sulfur and produces an identical effector response as ROS and RNS. Still, the reaction of RSS is more prominent and stable for a much longer duration [13]. Recent discoveries have spectated the growth stimulatory effect of RSS on plant growth and development under stress conditions [6,11,12]. The functional mechanism by which RSS exerts a change stimulatory effect in plants has been unearthed by a few researchers who identified that RSS signaling stimulates post-translational modification of cysteine residues (Cys-SSH) that regulate the expression of stress-responsive genes/proteins [13].

Further, RSS-induced sulfidation, or sulfhydration of proteins, accentuates critical metabolic pathways that stimulate the biosynthesis of secondary metabolites and enzymatic antioxidants, thereby improving the physiology and morphology of plants exposed to climate extremes [12]. Despite their versatility and stability, RSS chemistry, biosynthesis, and plant functions remain anonymous to date. Several excellent review articles have been published on ROS and RNS signaling in plants [9,10]. Discussing them in detail will result in the lengthening of the context. Therefore, readers are requested to consider the articles mentioned above. This review provides an in-depth understanding of RSS biosynthesis in plants and its chemical and biological properties in regulating the functions of proteins/genes and signaling pathways under stress conditions. In addition, this review also examines how RSS interact with other reactive oxidants, such as ROS and RNS, and their combinatorial effect in regulating the growth and metabolism of plants.

## 2. Chemical Biology of RSS in Plants

In recent years, RSS has been proclaimed to be inexorably interlinked with all life forms from its inception to the present day [14]. Several studies have confirmed that ROS and RSS are chemically similar and are often grouped under the category of chalcogens, i.e., both belong to group 16 of the periodic table [15]. Concomitantly, ROS and RNS mediate the production of RSS under oxidative environments, which is a potential route

for RSS biosynthesis [9]. RSS, like ROS, is also categorized as free radicals and non-radicals. Non-radicals are comprised of thiol (RSH), disulfide (RSSR), sulfenic acid (RSOH), and thiosulfate (RSOSR), whereas free radicals are composed of thiyl-radical ($RS^{\cdot}$) [14]. Various organisms such as plants, bacteria, fungi yeasts, nematodes, and humans employ different chemical reactions at intracellular and molecular levels, thereby affecting physiological and molecular processes in the concerned organisms [15].

During the prebiotic era, hydrogen sulfide ($H_2S$) was the primary energy source catalyzing significant steps for incepting life that drove the evolution [16]. In a study, Wachtershauser's group tried to mimic the conditions before photosynthesis, where organic carbon and nitrogen molecules in the presence of $H_2S$ catalyze the formation of thiosulfoxide, which is then converted to persulfide via one-electron oxidation reactions [17]. Correspondingly, upon its subsequent exposure to additional thiosulfoxide, persulfide produces polysulfides ($H_2Sn$) capable of catalyzing oxidative metabolism in purple and green sulfur bacteria [17]. Thiosulfoxide, persulfide, and polysulfide can be stored and recycled/reduced back to $H_2S$ to accentuate future oxidation reactions, thus confirming their capability to induce oxidation/reduction reactions for stimulating various signaling pathways [18]. In contrast, ROS and RNS do not possess such ability; they cannot be stored or reused in signaling or related pathways because they function as "one and done".

However, despite dissimilarity, RSS exhibits higher similarity with ROS than RNS because both are chalcogens with six electrons in their valence shell [19]. Nonetheless, RSS is considered the most versatile, promiscuous, and stable reactive oxidant of its counterparts, i.e., ROS/RNS. Due to higher electronegativity, outer shell electrons in oxygen are near the nucleus, as in sulfur, where electrons are farther from the nucleus [9]. Furthermore, the most stable oxidation state for oxygen is $-2$ and 0; however, it may also exist in a less stable form of $-1$, +1, and +2; on the other hand, the most stable oxidation state for sulfur ranges from $-2$ to +6 [14]. Additionally, sulfur contains more than 30 allotropes compared to oxygen, which has fewer than 10, further attesting to the flexibility of RSS under extreme environments [9]. Strikingly, ROS and RSS exist in various forms with similar chemical and functional properties, i.e., ROS is produced from the one-electron reduction of oxygen (Table 1). In contrast, RSS is produced from one-electron oxidation of $H_2S$ [10]. ROS, such as hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), perhydroxy radical ($HO_2$), and singlet oxygen ($^1O_2$), are exclusively involved in signaling under stress conditions [10]. Below, Equation (1) depicts the generation of ROS via a single oxygen reduction process:

$$O_2 \overset{+/-e}{\longleftrightarrow} O_2^{\cdot -} \overset{+/-e}{\longleftrightarrow} H_2O_2 \overset{+/-e}{\longleftrightarrow} OH^{\cdot} \overset{+/-e}{\longleftrightarrow} H_2O \quad (1)$$

While perhydroxy radical ($HO_2$) and singlet oxygen ($^1O_2$) are less intense, signaling molecules often scavenged into peroxide and oxygen are relatively impermeable across membranes. They have a short half-life, are unstable, and are less reactive.

In contrast, 'RSS, which are produced by single-electron oxidation of $H_2S$, are often composed of thiyl radical ($HS^{\cdot}$), hydrogen persulfide ($H_2S_2$), and persulfide radical ($S_2^{\cdot -}$) (Table 1). This biosynthetic reaction ends with the formation of elemental sulfur; the steps of which are depicted in Equation (2):

$$S_2 \overset{+/-e}{\longleftrightarrow} S_2^{\cdot -} \overset{+/-e}{\longleftrightarrow} H_2S_2 \overset{+/-e}{\longleftrightarrow} HS^{\cdot} \overset{+/-e}{\longleftrightarrow} H_2S \quad (2)$$

**Table 1.** The enzymes involved in the generation of ROS, RNS, and RSS in plants.

| | Enzymes | Reaction Catalysed | Cellular Location | Uniprot/Gene | References |
|---|---|---|---|---|---|
| **ROS** | Cytochrome C oxidases and alternative oxidases | $O_2 \overset{+/+e^-}{\leftrightarrow} \mathbf{O_2}^{\cdot -}$ (Mehler's reaction) | Thylakoid membrane | A0A1P8AZ61/ AT2G43780, Q39219/AT3G22370 | [20] |
| | Cytochrome C oxidases and alternative oxidases | $O_2^{\cdot -} + Fe^{3+} \rightarrow {}^{1}\mathbf{O_2} + Fe^{2+}$ | Thylakoid membrane/mitochondria | A0A1P8AZ61/ AT2G43780, Q39219/AT3G22370 | [20] |
| | Cytochrome C oxidases and superoxide dismutase | $2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + \mathbf{H_2O_2}Fe^{3+}$ (Haber- Weiss reaction) | Thylakoid membrane/ peroxisomes | A0A1P8AZ61/ AT2G43780, P24704/AT1G08830 | [20] |
| | Cytochrome C oxidases and alternative oxidases | $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \mathbf{OH}^{\cdot}$ (Fenton reaction) | Thylakoid membrane | A0A1P8AZ61/ AT2G43780, Q39219/AT3G22370 | [20] |
| **RNS** | Nitrate reductase | $NO_2^{\cdot} + NADPH \rightarrow \mathbf{NO}^{\cdot}$ | Peroxisomes | P11035/ AT1G37130, | [8,21] |
| | NOS-like activity, aldehyde oxidase, sulfite oxidase, and xanthine dehydrogenase | $L-Arg + NOS$ cofactors (FAD, MOCO) $\rightarrow \mathbf{NO}^{\cdot}$ | Chloroplast, mitochondria | Q7G191/AT1G04580, Q8GUQ8/ AT4G34890 | [8,21] |
| | Peroxidase | $Hydroxyurea + H_2O_2 \rightarrow \mathbf{NO}^{\cdot}$ | Chloroplast, mitochondria | Q9SMU8/AT3G49120 | [8,21] |
| | Amidoxime reducing components | $NO_2^{\cdot} + Cyt\ C\ (red) \rightarrow \mathbf{NO}^{\cdot}$ | Chloroplast, mitochondria | LOC9299625 | [8,21] |
| **RSS** | L-cysteine desulfhydrase | $L-Cys + H_2O \rightarrow \mathbf{H_2S} + NH_3 +$ pyruvate | Cytosol | Q9MIR1/At3g62130 | [11] |
| | D-cysteine desulfhydrase | $D-Cys + H_2O \rightarrow \mathbf{H_2S} + NH_3 +$ pyruvate | Mitochondria | A1L4V7/At3g26115 | [11] |
| | Sulfite reductase | $SO_3^{-2} + e- \rightarrow \mathbf{H_2S} + 3H_2O$ | Chloroplast | Q9LZ66/At5g04590 | [11] |
| | Cyanoalanine synthase | $L-Cys +$ hydrogen cyanide $\rightarrow \mathbf{H_2S}$ | Mitochondria | Q9S757/At3g61440 | [11] |
| | Cysteine synthase 1 | $L-Cys +$ acetate $\rightarrow \mathbf{H_2S}$ | cytosol | P47998/At4g14880 | [11] |

Abbreviations: $O_2$: molecular oxygen, $O_2^{\cdot -}$: superoxide anion, $Fe^{3+}$: ferric ion, $^1O_2$: singlet oxygen, $Fe^{2+}$: ferrous ion, $H_2O_2$: hydrogen peroxide, $OH^-$: hydroxyl anion, $OH^{\cdot}$: hydroxyl radical, $NO_2^{\cdot}$: nitric dioxide, NADPH: nicotinamide adenine dinucleotide phosphate dehydrogenase, $NO^{\cdot}$: nitric oxide radical, L-Arg: L-arginine, FAD: flavin adenine dinucleotide, MOCO: molybdenum cofactor, Cyt C: cytochrome C, L-Cys: L-cysteine, $H_2S$: hydrogen sulfide, $NH_3$: ammonia, $SO_3^{-2}$: sulfite ion.

## 3. RSS Biosynthesis in Plants

In plants, the genus Allium provides an intriguing insight into the biosynthetic pathway of RSS from natural sulfur agents [22]. The first step in RSS production is the biosynthetic transformation of sulfoxides to thiosulfinates, i.e., allin to allicin catalyzed by the alliinase enzyme C-S-lyase type enzyme of 103 kDa with two similar subunits of 448 amino acids [22,23]. The alliinase enzyme contains pyridoxal 5′-phosphate (PLP) at the active site, enhancing the enzyme's efficiency when the substrate is present at a low level, i.e., allin. In the presence of allin, alliinase catalyzes the formation of highly reactive aldimine, resulting in the generation of sulfenic acid (RSOH) and a PLP amino acylates complex [22]. The complex becomes detached from the RSOH and combines with ammonia and pyruvate to facilitate the formation of thiosulfinate (RSOSR), such as allicin/water [9].

Thiyl radicals, a free radical RSS, are synthesized via single-electron oxidation of the thiol group occurring under oxidative stress conditions, where a proton from the S-atom is removed before or after electron dissemination leading to the formation of disulfide [22]. A notable example of thiyl free radical formation is seen during the glutathionylation of several proteins in plants, where thiyl radical intermediates are provoked by ROS/RNS and other enzymatic antioxidants to generate RSS radicals [23]. Furthermore, researchers have indicated that thiol/disulfide exchange between RSSR and RSH can potentially shift the redox potentials of RSH in a powerful way that can oxidize RS (O) SR from four thiol radicals (R'SH) which are later converted to thiol and R'SSR [24].

Thiosulfinates are highly reactive non-radical RSS that can react with any molecule containing the thiol (R'SH) group to form sulfenic acid and disulfide (RSSR) [24]. Additionally, disulfides can be oxidized by themselves under stress conditions or can be corrupted by ROS/RNS to thiosulfinates and later into thiosulfonates (RD (O)2SR), thus validating the involvement of ROS/RNS in RSS biosynthesis [23]. Concomitantly, ROS/RNS-mediated oxidation of the S-atom of sulfenic acids results in sulfinic acid formation. The S-atom of the latter has a higher oxidation state than the former, which can cause further oxidation of sulfinic acid to sulfonyl radicals, which are considered the progenitors of RSS biosynthesis in plants [23].

Interestingly, sulfenic acids also have the potential to oxidize thiols to disulfides and, at the same time, can initiate the reduction of cumene and linoleate and, therefore, are also called ultimate reductants [24]. Like superoxide anions, sulfenic acids also possess electrophilic and neutrophilic properties, thus acting as powerful oxidizing and reducing agents leading to the formation of RSS even by reducing sulfur-containing molecules or by oxidizing sulfur atoms from cell proteins, such as cysteine and methionine [13]. A large body of literature has indicated that sulfenic acid-mediated oxidation of protein-thiols could result in the generation of RSOH, RS (O)2H), RS (O)3H, and RSSR [6]. Sulfenic acids are pivotal RSS molecules that can readily react with thiols to form disulfides, exponentially increasing thiol-containing proteins' catalytic efficiency. In addition, sulfenic acids are included upon hydrolysis of nitrosothiols (nitric oxide donors), modulating critical biological processes and signal transduction.

## 4. RSS Signaling in Plants

The functional mechanism by which RSS works as a signaling molecule is relatively analogous to that of ROS/RNS signaling; nonetheless, the former is more intricate and perplexing at the biochemical and molecular levels [22]. Several researchers have established that ROS and RSS exploit cysteine residues as the "junction" to mediate redox signaling, sensing, and catalysis of critical metabolic functions [23]. Recent studies have corroborated that RSS function as a signaling molecule after interacting with thiol (-SH) containing proteins (cysteine and methionine), thus regulating persulfidation through post-translational modification (PTMs). Furthermore, RSS can modulate various PTMs such as *S*-glutathionylation, *S*-nitrosation, *S*-cyanylation, and *S*-acylation upon interaction with RNS, glutathione, cyanide, and fatty acids [13]. The profound biochemical implication of RSS in plant cells has delineated its intricate interaction with various antioxidant enzymes, such as glutathione reductase, peroxidase, and sulfite oxidase, thus mediating sulfur metabolism in plant peroxisomes [25]. Correspondingly, this sulfite oxidase, in conjunction with the catalase enzyme, catalyzes the conversion of sulfite to sulfate, thus resulting in the generation of ROS [26]. The existence and inception of RSS have been recently mapped in peroxisomes of root tips and guard cells of Arabidopsis using confocal laser scanning microscopy (CLSM) and proteomic techniques [13,27].

Furthermore, researchers have also mapped the position of corresponding protein genes involved in RSS biosynthesis and observed that the majority of them were present on chromosomes 1, 2, 3, and 5 (Figure 1A,B) along with the protein/genes of ROS and RNS biosynthesis in Arabidopsis [28]. In addition, conserved motif analysis and gene sequence alignments demonstrated that all the reactive species producing enzymes/proteins have

a highly conserved arrangement of motifs with varying sequences (Figures 1C and 2A) [29]. The phylogenetic and gene co-expression analysis (Figure 2B,C) of the major reactive species producing enzymes/proteins diverge from one another as they evolve from a common ancestor, and co-expression of the corresponding genes is essential for activating the transcription of stress-responsive genes and transcription factors [28,29].

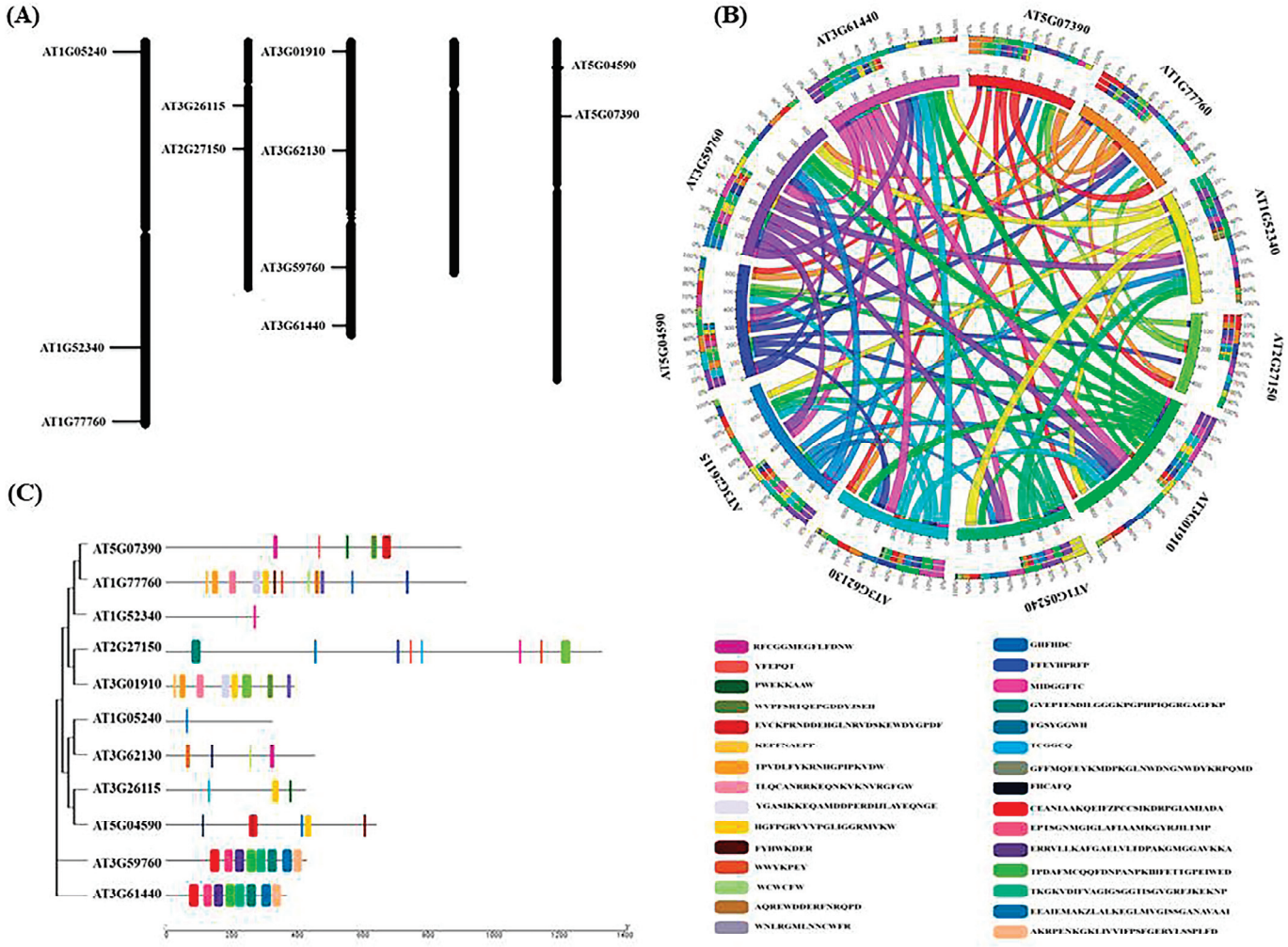


**Figure 1.** Chromosomal localization, synteny analysis, and motif elucidation of important ROS-, RNS-, and RSS-producing genes in model plant *Arabidopsis thaliana*. (**A**) Chromosomal localization, (**B**) synteny analysis, and (**C**) conserved motifs are represented in different color boxes. AT5G07390/RBOHA: respiratory burst oxidase homolog protein A, AT1G77760/NIA1: nitrate reductase 1, AT1G52340/ABA2: ABA xanthoxin dehydrogenase, AT2G27150/AAO: abscisic-aldehyde oxidase, AT3G01910/SOX: sulfite oxidase, AT1G05240/POX: peroxidase 1, AT3G62130/LDES: L-cysteine desulfhydrase, AT3G26115/DDES: D-cysteine desulfhydrase 2, AT5G04590/SIR: assimilatory sulfite reductase, AT3G59760/CYSC1: cysteine synthase, AT3G61440/QASC: nifunctional L-3-cyanoalanine synthase/cysteine synthase C1.

Scientists have only recently uncovered that the peroxisomal enzymes are the primary targets of the persulfidation of thiol-containing proteins leading to the generation of RSS under stress conditions [30]. Later, in vitro analysis in Arabidopsis and *Capsicum annum* unraveled the mechanistic insight into the enzymatic pathway of RSS biosynthesis, where catalase functions both as oxidase and reductase to catalyze the synthesis of $H_2S$ [30]. Apart from catalase and peroxidase, superoxide dismutase can generate persulfide by stimulating the reaction between O2 and $H_2S$ in mitochondria and peroxisome, thus validating that apart from ROS/RNS, these organelles also serve as the site of RSS biosynthesis [31]. Still, scientists are trying to delve deeper and further to identify the precise biochemical and

metabolic pathway involved in the biosynthesis of RSS in plants, as well as to unravel the mechanism by which they interact with other signaling molecules [13]. Few studies have identified possible enzymatic routes of RSS biosynthesis in the different subcellular compartments of plants, such as cytosol, chloroplast, mitochondria, and peroxisomes. They postulated that the enzymes such as D-cysteine desulfhyfrase, cyano alanine synthase, sulfite reductase, cysteine synthase, and cysteine synthase-like modulate RSS signaling in plants exposed to climate extremes [13,25].

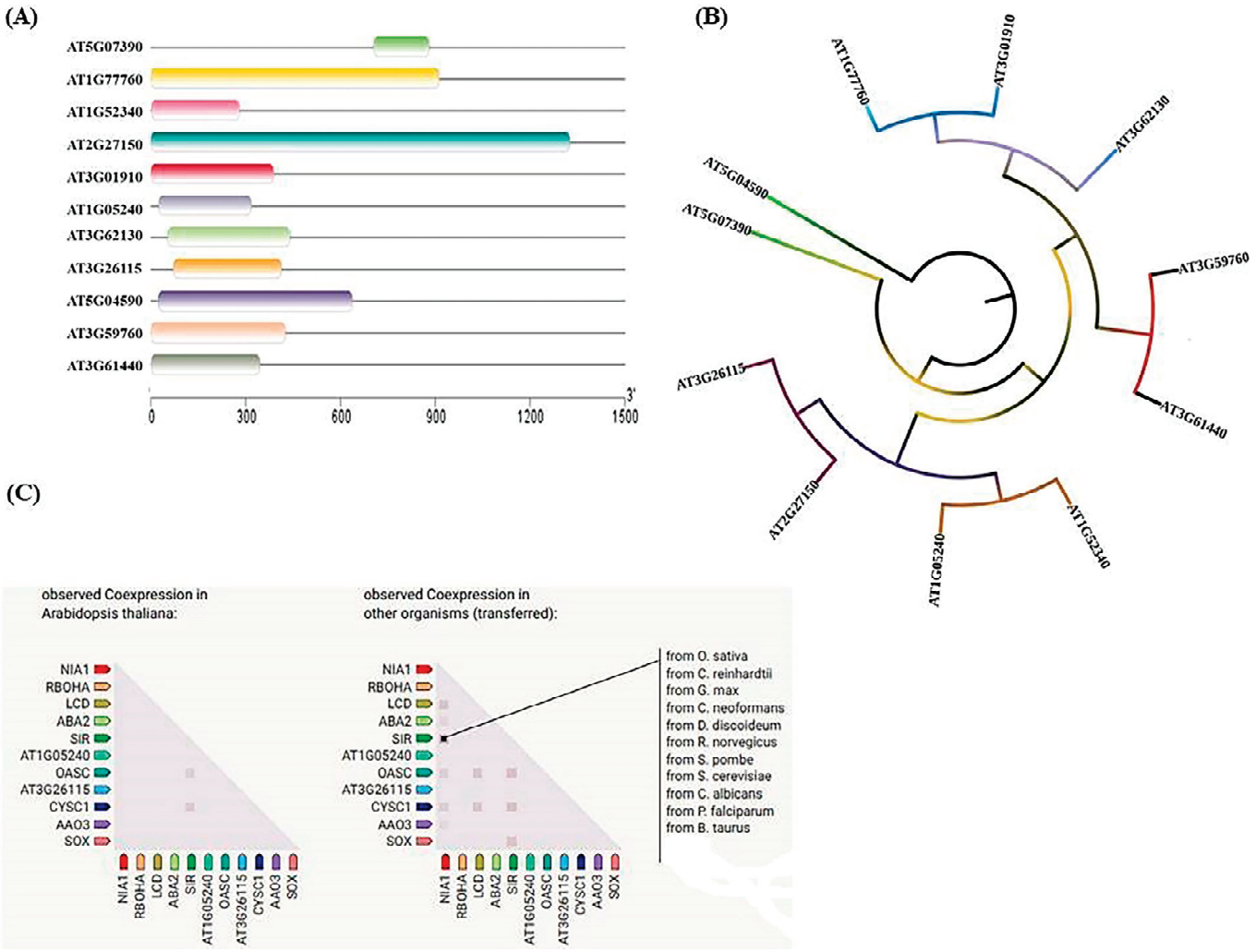


**Figure 2.** Basic sequence analysis, phylogenetic relationship, and co-expression prediction of important ROS-, RNS-, and RSS-producing genes in the model plant *Arabidopsis thaliana*. (**A**) Gene sequence, (**B**) phylogenetic analysis, and (**C**) co-expression of putative genes using TBtools and iTOL software. AT5G07390/RBOHA: respiratory burst oxidase homolog protein A, AT1G77760/NIA1: nitrate reductase 1, AT1G52340/ABA2: ABA xanthoxin dehydrogenase, AT2G27150/AAO: abscisic-aldehyde oxidase, AT3G01910/SOX: sulfite oxidase, AT1G05240/POX: peroxidase 1, AT3G62130/LDES: L-cysteine desulfhydrase, AT3G26115/DDES: D-cysteine desulfhydrase 2, AT5G04590/SIR: assimilatory sulfite reductase, AT3G59760/CYSC1: cysteine synthase, AT3G61440/QASC: bifunctional L-3-cyanoalanine synthase /cysteine synthase C1.

Plant peroxisome is the house of various signaling molecules such as glutathione, *S*-nitrosoglutathione, hydrogen peroxide, and sulfite oxidase that are explicitly involved in the RSS biosynthesis and metabolism [32]. Recent discoveries have indicated that RSS signaling profoundly mediated the peroxidation of cysteine sulfur resulting in the formation of sulfenyl via a process known as sulfenylation. Sulfenylation is analogous

to persulfidation, a method capable of accentuating RSS-mediated redox sensing and signaling, thus modulating protein folding, histone modification, DNA–protein interaction, and other regulatory functions [33]. One of the mechanisms by which RSS signaling works is that both sulfenylation and persulfidation can reduce hydrogen peroxide to water, which in turn induces the restoration of protein thiols, thus provoking their interaction with other low molecular weight thiols (thioredoxins and reduced glutathione) [34]. The interaction between persulfidated proteins and low molecular weight thiols results in the generation of polysulfide, which is actively involved in the storage and transportation of sulfur molecules capable of initiating RSS signaling [33]. RSS signaling is also incepted upon the NADPH-mediated reduction of persulfidated proteins, which upon interaction with peroxisomal ROS, results in the generation of RSS [35]. Some researchers have also advocated accentuating RSS signaling by incorporating cysteine persulfides into proteins via ROS-mediated PTMs [30]. Furthermore, peroxide signaling is often associated with unresolved conundrums regarding RSS signaling, yet it is hypothesized peroxide signaling acts as a relay between peroxidase and thiol proteins. Correspondingly, the resulting reaction product (oxidants) establishes a complex with the peroxidase proteins, thus categorizing the oxidant signals involved in the modulation of RSS signaling [36].

## 5. Crosstalk between ROS/RNS/RSS in Plant Defense

Plant stress signaling comprehends an intricate network of various signaling molecules that function synergistically or antagonistically to regulate multiple physiological and metabolic processes under specific environmental conditions [37]. Several plant growth regulators, such as salicylic acid, auxin, jasmonic acid, ethylene, abscisic acid, polyamines, and melatonin, are involved in modulating a broad range of plant functions [6]. Apart from these, the gasotransmitters, such as $H_2S$ and NO, have also been recognized as an indispensable combination for regulating plants' physiological and stress-related processes (Table 2) with high efficacy and stability compared to their natural counterparts [6]. A molecule can act as a signal only when its level reaches beyond the optimum threshold, driven by complex processes occurring inside the cell. Despite the complex network, all the reactive species, whether ROS, RNS, or RSS, diverge at some metabolic checkpoints where they encounter identical targets responsible for their production, accumulation, and scavenging [18]. When these signaling molecules (they may contain reactive species or plant growth regulators) are exogenously applied, it results in the transient accumulation of compounds that exaggerate the endogenous level of ROS, RNS, or RSS [37]. This transient accumulation of reactive species could have synergistic or antagonistic effects before or after stressful events.

Recent studies have corroborated that the exogenous application of these signaling molecules is integrally involved in regulating complex networks (Table 2) such as redox status, stomatal movement, root development, and apoptosis in plants exposed to climate extremes [38,39]. However, the effects of these signaling molecules are primarily dependent upon their dosage, subcellular compartment, duration of the application, and their relative flux in the treated plant cell/tissue [40]. A large body of literature exists on the stimulatory role of $H_2S$ and NO in plants exposed to biotic/abiotic stresses, which has been summarized in recent review articles [6,41–43]. Therefore, this crosstalk will provide current updates on the involvement of $H_2S$ and NO in modulating plant functioning at the molecular level, which is imperative for understanding the role of these gasotransmitters in stress alleviation.

**Table 2.** Major regulatory effects of $H_2O_2$, NO, and $H_2S$ on the physiological and metabolic process in plants exposed to climate extremes.

| Processes | Functional Mechanisms | Stress Conditions | Pathway Involved | References |
|---|---|---|---|---|
| Seed germination | | | | |
| $H_2O_2$ | Break seed dormancy and promote seed germination by initiating protein carbonylation | Drought, heat, and salinity | GA signaling pathway | [44,45] |
| NO | Regulate seed germination and pollen tube growth by activating catabolic enzymes of ABA and GA biosynthesis | Osmotic and heavy metals | ABA and GA signaling pathways | [46] |
| $H_2S$ | Promote seed germination of wheat plants by enhancing amylase and esterase activities | Heavy metal and salt | Antioxidant defense pathway | [47,48] |
| Stomatal movement | | | | |
| $H_2O_2$ | Induce stomatal closure by regulating the activity of NADPH oxidase and control the level of ROS modulates' stomatal opening by invoking plant peroxisome—specific autophagy | Drought and pathogen attack | ABA and antioxidant signaling pathways | [49,50] |
| NO | Promote stomatal closure by inducing tyrosine nitration and *S*-nitrosation of ABA receptors | Drought and salinity | ABA and antioxidant signaling pathways | [51,52] |
| $H_2S$ | Induce stomatal closure by affecting the activities of ion channels via inducing sulfhydration | Drought and cold | ABA and MAPK signaling | [51,53] |
| Root organogenesis | | | | |
| $H_2O_2$ | Instigate de novo root organogenesis by acting downstream of auxin and $Ca^{2+}$ signaling | Oxidative stress | Auxin signaling pathway | [54,55] |
| NO | Modulate root organogenesis by activating the expression of MYB and BHLH transcription factors | Oxidative stress | Auxin and jasmonic acid signaling pathways | [54] |
| $H_2S$ | Induce adventitious root formation by acting as upstream of IAA and NO signaling | Heat and heavy metal | Auxin and abscisic acid signaling pathways | [56,57] |
| Leaf senescence/ fruit ripening | | | | |
| $H_2O_2$ | Delay leaf senescence/fruit ripening by regulating the ascorbate-glutathione cycle | Oxidative stress | Antioxidant defense pathway | [58] |
| NO | Delay induced leaf senescence/fruit ripening by enhancing the expression of stress-responsive genes, ACC synthase, and oxidase enzymes | Salt stress | Antioxidant and ABA signaling pathways | [59,60] |
| $H_2S$ | Delay leaf senescence/fruit ripening by regulating the expression of the *Des1* gene | Oxidative stress | Abscisic acid signaling pathways | [56,61] |

**Table 2.** *Cont.*

| Processes | Functional Mechanisms | Stress Conditions | Pathway Involved | References |
|---|---|---|---|---|
| Post-translational/ epigenetic regulation | | | | |
| $H_2O_2$ | Induce oxidative posttranslational modifications, DNA methylation, and histone modification, thus stimulating plant stress response | Abiotic/biotic stress | Stress defense pathway | [62,63] |
| NO | Induce tyrosine nitration and *S*-nitrosation and epigenetic regulation of various small and long non-coding RNAs, thereby regulating plant immune system | Abiotic/biotic stress | Stress defense pathway | [63,64] |
| $H_2S$ | Induce *S*-Sulfhydration of cysteine residues, thereby activating plant tolerance | Abiotic/biotic stress | Stress defense pathway | [65] |

Abbreviation: $H_2O_2$: hydrogen peroxide, NO: nitric oxide, $H_2S$: hydrogen sulfide, GA: gibberellic acids, ABA: abscisic acid, ROS: reactive oxygen species, NADPH: nicotinamide adenine dinucleotide phosphate dehydrogenase, MAPK: mitogen-activated protein kinase, BHLH: basic helix loop helix, MYB: myeloblastosis transcription factor, IAA: indole acetic acid, Des1: l-cysteine desulfhydrase, Ash: ascorbate, Gsh: glutathione, ACC: 1-Aminocyclopropane 1-carboxylic acid.

### *5.1. Regulation of Gene Expression*

The functional analogy of ROS, RNS, and RSS has been extensively studied by applying them exogenously to analyze their potential in modulating growth and defense-related processes in plants exposed to environmental cues [18]. Recent studies have shown that both NO and $H_2S$ are potentially involved in regulating seed dormancy and germination via modulating ABA signaling [51]. Molecular investigation revealed that NO exerts this effect by activating PYR/PYL/RCAR (pyrabactin resistance 1/PYR, such as the regulatory component of ABA receptor), SnRK2 (SNF1-related protein kinase), and ABI5 (abscisic acid insensitive 5) protein, whereas $H_2S$ signaling stimulated seed germination in Arabidopsis by modulating the expression of L-DES (L-cysteine desulfhydrases) protein [66,67]] Recent investigations in Arabidopsis indicated that $H_2S$ signaling results in the accumulation of constitutive photomorphogenesis 1 (COP1) in the nucleus that stimulates the degradation of elong hypocotyl 5 (HY5). The increased degradation of HY5 resulted in decreased expression of the ABI5 gene, thereby enhancing seed germination under heat stress [68]. In another study, the combinatorial effect of NO and $H_2S$ signaling regulated the adventitious rooting in tomato plants by activating the expression of auxin-related genes (ARF4 and ARF16) and cell cycle-related genes (CYCD3, CYCA3, and CDKA1) [69]. Exogenous application of NaHS leads to the activation of transcription and activity of L-DES1 that stimulates the $H_2S$ biosynthesis, which in turn activates the germination of Arabidopsis seeds [70]. In mutant (lcd/des1 defective) plants, exogenous application of NaHS does not induce the germination of the Arabidopsis seeds. In wild plants (lcd/des1 induced), NaHS-mediated increase expression of L-DES1 protein activated the $H_2S$ biosynthesis have successfully maintained the GSH/GSSG ratio within the cells that may have triggered alternative oxidase (AOX) mediated cyanide resistant respiration to regulate seed germination [70]. Similar observations have also been made where the exogenous application of NO and $H_2S$ positively modulated the seed germination and lateral root formation in cucumber and maize plants by triggering the expression of the heme oxygenase-1 (HO-1) gene and by reorganizing the arrangement of F-actin bundles [71–73].

Another complex process that is sophistically regulated via signaling molecules (ABA, ethylene, and $K^+/Ca^{2+}$) is the opening/closing of stomata, and recent investigations have confirmed the role of NO and $H_2S$ signaling in controlling guard cell function [67,74].

Previous investigations have concluded that NO signaling modulates stomatal movement by regulating the expression of SNF-1 (sucrose nonfermenting 1) and SnRK2.6 genes [75]. In contrast, $H_2S$ signaling negatively regulated the opening of stomata via inducing NO-mediated activation of 8-mercapto-cGMP, which restricts the inward flow of K+ in guard cells [76,77]. Recent investigations have reported $H_2S$ signaling downstream of mitogen-activated protein kinase 4 (MAPK4), thereby inducing ABA-mediated stomatal closure in response to drought stress [78]. A study concluded that the exogenous application of NaHS significantly altered the drought-tolerant capacity of mapk4 and slac1 mutant plants compared to wild-type Arabidopsis plants. $H_2S$ signaling could not effectively regulate the lcd/des1 gene expression, which has affected the ABA-mediated stomatal movement in mutant plants [78]. Similarly, the exogenous application of $H_2O_2$ induced $H_2S$ signaling by activating the expression of the L-DES1 gene significantly regulated the salt stress tolerance in *Vicia faba* plants by altering the movement of guard cells [79]. Recently, researchers have confirmed that the NO can modulate ABA-induced stomatal closure by activating the GSNO reductase gene, which in turn causes differential regulation of nitrate reductase genes (NIA1 and NIA2) and CLE 9 peptide (Clavato3/Embryo Surrounding region) [52]. The differential accumulation of NIA genes alters the ABA-induced activity of $K^+$ inward and activates the outward flow of K+ in wild-type Arabidopsis plants. In contrast, the down-regulation of NIA genes abolishes the inward movement of $K^+$ [52].

Several studies have demarcated NO and $H_2S$ signaling in leaf senescence and fruit ripening. Previous works have reported a substantial role of exogenous NO in reducing ethylene production and ABA-induced senescence in rice and strawberry plants [80,81]. NO regulated ethylene production and senescence by modulating the expression of NOS-like enzymes, leading to differential accumulation of endogenous NO [82]. Later, researchers indicated that exogenous application NO evidently interacts and activates SA and JA signaling, which induces the expression of NOS-like enzymes, thereby delaying leaf senescence upon upregulation of the antioxidant defense system [83]. Recently, $H_2S$ signaling in regulating leaf senescence was analyzed by inducing mutation in an $H_2S$-producing enzyme encoding L-cysteine desulfhydrase 1 (L-DES1). They concluded that mutant (Des1) Arabidopsis plants showed enhanced susceptibility to drought stress and accelerated leaf senescence [84].

In contrast, over-expressed Arabidopsis plants (OE-DES1) exhibited more tolerance to drought and delayed leaf senescence [84]. The effect of LCD1 on the regulation of leaf senescence was explored in wild-type, mutant, and overexpressed tomato plants. The mutant LCD1 plants showed early development of leaf senescence, whereas overexpressed LCD1 plants exhibited delayed leaf senescence compared to wild-type tomato plants. Further investigations revealed that the delayed leaf senescence in overexpressed tomato plants was due to the downregulation of various chlorophyll degradation genes, such as NYC1, PAO, PPH, and SGR1, and senescence-associated genes (SAGs), along with active scavenging of ROS [85].

Fruit ripening is governed by a complex physiological process fine-tuned via various signaling molecules, plant growth hormones, and environmental stimuli [86]. In recent years, extensive studies have been conducted to unravel the mechanism of regulation of fruit ripening mediated via reactive species (ROS/RNS/RSS). Exogenous application of NO and $H_2S$ differentially regulated the endogenous level of NO and $H_2S$, accentuating fruit ripening in *Capsicum annum* fruits. The differential regulation of endogenous NO and $H_2S$ levels altered the activity of the NADP isocitrate dehydrogenase enzyme, thus promoting fruit ripening in pepper plants [87]. A year later, they conducted a different study to reveal that an increase in the endogenous NO and $H_2S$ levels differentially regulated the fruit ripening process in *Capsicum annum* fruits by downregulating the expression of the NADP malic enzyme and the 6-phsphogluconate dehydrogenase enzyme [88].

Furthermore, Zhu et al. [89] reported the synergistic effect of NO and $H_2S$ on the inhibition of peach fruit ripening during storage. They concluded that the combined application of NO and $H_2S$ caused a significant reduction of ethylene production, i.e.,

it decreased the expression of ACC synthase and ACC oxidase enzyme and cell wall metabolism to delay fruit ripening in peach plants. Interestingly, the exogenous application of strigolactones regulated the endogenous level of NO and $H_2S$ by upregulating NOS-like enzymes and L-cysteine desulfurase, which concomitantly delayed the ripening of strawberries [90]. They further concluded that the delayed development of strawberries was due to differential regulation of the antioxidant defense system mediated via increased NO and $H_2S$ during storage.

### *5.2. Regulation of Post-Translational Modification*

Recent research has confirmed that ROS/RNS/RSS regulates the expression of various stress-responsive genes and modulates specific proteins' activity via post-translational modifications (PTMs) [6]. PTMs such as Persulfidation, tyrosine nitration, S-nitrosylation, and carbonylation are activated upon exogenous application of RSS/RNS/ROS, thereby influencing plant growth and developmental processes under stress conditions [37]. Cysteine thiols are a highly nucleophilic group that regulate a plethora of protein regulatory functions, such as their catalytic efficiency, stability, and interaction with ligands upon reacting with ROS/RNS/RSS [91]. Persulfidation, previously known as *S*-sulfhydration, is a reversible oxidative PTM process where the thiol group (-SH) is converted into persulfide form (-SSH) in a complex reaction, in which protein thiol reacts with an anionic and protonated form of $H_2S$ or reactive species-mediated interaction of protein thiols with inorganic polysulfides [91]. The advent of ultra-throughput proteomic approaches and advancement in mass spectrophotometry has led to the identification of several proteins undergoing persulfidation [91]. Exogenous application of $H_2S$ (NaHS) coupled with proteomic analysis identified 1000 differentially regulated proteins in Spinacia oleracea plants, of which few were persulfidated [92]. Similarly, researchers employed the biotin switch method with liquid chromatography–tandem mass spectrometry (LC-MS). They identified 106 to 2015 (using the modified switch method) persulfidated proteins in Arabidopsis leaves [12]. In addition, exogenous $H_2S$ persulfidated the mitogen-activated protein kinase 4 (MAPK4), which causes up to a 10-fold expression of MAPK4, leading to the enhanced tolerance of Arabidopsis plant's chilling stress [93]. Concomitantly, the exogenous application of NaHS reverses the adverse effect of heavy metals inducing oxidative damage in tomato plants by inducing the persulfidation of enzymatic antioxidants such as CAT, APX, GR, and SOD [94]. $H_2S$-mediated persulfidation significantly regulated the expression of the L-DES1 gene, which modulated the ABA signaling in guard cells, leading to the opening and closing of stomata [68]. They further concluded that $H_2S$ mediated persulfidation of Open Stomata 1/SNF1-related protein kinase 2.6 (SnRK2.6) increases its ability to interact with another transcription factor, such as DREB and ABA-responsive factor 2 (ABF2), to promote stomatal closure [68]. Interestingly, $H_2S$-mediated persulfidation of ABI4, RBOHD, L-DES1, ATG4, and ATG18a have improved plant cellular and physiological processes under stressful conditions by preventing reversible protein oxidation and DNA damage [95–97].

*S*-nitrosation is a PTM process essentially regulating discrete signaling functions in plants ranging from seed germination to fruit maturation and is also mechanistically involved in regulating abiotic/biotic stress tolerance [51,98,99]. Much of the NO-derived PTMs in plants are accentuated by tyrosine nitration followed by *S*-nitrosation, which involves adding nitric oxide radical to protein thiols to generate *S*-nitrosoglutathione (GSNO) [13,64]. Extensive research on NO-mediated PTMs has been conducted in Arabidopsis plants, where the researchers have identified several *S*-nitrosated proteins after treating them with a NO donor (GSNO) [100]. Similar reports have also been published [101–103] where they used a combination of BSM and affinity tags to identify targets of *S*-nitrosation proteins in Arabidopsis plants exposed to biotic and abiotic stresses. *S*-nitrosation and tyrosine nitration was involved in heavy metal-stressed mutant and wild-type Arabidopsis plants [104]. In their study, the researcher identified that *S*-nitosothiol signaling was responsible for enhancing NADPH-dependent thioredoxin reductase activity, which is involved in the regulation of hydrogen peroxide, and further application of

LC-MS/MS analysis indicated that heavy metal-induced *S*-nitrosation of APX resulted in enhanced tolerance to stressed plants [104]. Ripening of *Capsicum annum* fruits is also differentially regulated by NO-mediated PTMs [105]. They exploited BSM, nano-liquid chromatography, and mass spectrometry to identify that Cys377 of peroxisomal catalase potentially undergoes *S*-nitrosation, thereby modulating ROS/RNS levels in fruits.

Further, Silva et al. [106] identified potential targets and substrates for *S*-nitrosation in plants. Their study determined that cytosolic and plastid glutamine synthetase is differentially regulated by *S*-nitrosation, where Cys369 is a possible site. Later, [107] postulated that the exogenous application of NO-mediated nitration of tyrosine residues and *S*-nitrosation of cysteine residues differentially affected vital amino acid residues involved in the binding of FAD and molybdenum cofactors in Arabidopsis plants. Conversely, *S*-nitrosation of protein thiols has modulated tomato plants' stress defense response against *Phytophthora infestans* [108]. Their study identified decreased activity of the *S*-nitrosoglutathione reductase enzyme in both tolerant and susceptible genotypes.

In contrast, *S*-nitrosation protein was increased in the tolerant genotypes as the infection progressed. Correspondingly, proteomic approaches identified tolerant plants exhibiting increased protein-*S*-nitrosation and showed enhanced accumulation of osmolytes, metabolites, and enzymatic and non-enzymatic antioxidants [108]. Recently, time-course gene expression analysis and non-denaturing PAGE have confirmed that PTMs such as tyrosine nitration, *S*-nitrosation, and glutathionylation heterologously regulated the expression of APX enzyme in the fruits of *Capsicum annum* plants [109].

Carbonylation is an irreversible PTM mediated by various ROS such as hydroxyl radical ($HO^{\cdot}$), superoxide anion ($O_2^{\cdot -}$), and hydrogen peroxide ($H_2O_2$) that potentially add aldehyde/ketone group into side chains of specific amino acids [110]. Carbonylation is a complex chemical process driven in conjunction with lipid/sugar derivatives and is basically of two types, viz. primary/secondary carbonylations [111]. Primary protein carbonylation is often modulated via metal-associated oxidation of Lys, Pro, and Thr residues or through $\alpha$-amidation of glutamyl residue to form aldehyde and ketone derivatives [110]. Conversely, Secondary protein carbonylation is driven by adding reactive carbonyl species (RCS) to Cys, His, and Lys residues of specific proteins [112]. A large body of literature has confirmed that the protein carbonylation is exaggerated upon an increase in cellular ROS levels that differentially regulate plant cell growth and physiology under stress conditions [111,112]. The effect of protein carbonylation has been profoundly studied in Arabidopsis plants, where researchers have reported that the protein carbonylation first increases and then decreases with the age of the plants, thereby drastically affecting the reproductive and senescence phase [113]. Researchers used liquid chromatography–tandem mass spectrometry to identify carbonylation sits in *Phaseolus vulgaris* plants [114]. They further concluded that approx. 238 proteins were carbonylated, of which maximum effect was observed in two essential nodule proteins, i.e., glutamine synthetase and glutamate synthase, that conversely regulated cell metabolism and nodule senescence in legume plants [114].

Furthermore, a proteomics approach was employed to identify carbonylated proteins in premature, mature, and desiccated seeds of *Medicago truncatula* [115]. The PM34 protein, a protein responsible for seed growth and also known to possess cellulase activity, was carbonylated more readily than other proteins. Effect of As accumulation on protein carbonylation was analyzed in *Oryza sativa* root and shoots [116]. Researchers concluded that increased As accumulation drastically enhanced lipid peroxidation and protein carbonylation, affecting rice plants' yield. Likewise, ROS-mediated protein carbonylation of enzymatic and non-enzymatic antioxidants invariably decreases seed vigor and other physiological activity of the seed of wheat plants [117]. Similarly, [118] identified 180 carbonylated proteins in ABA-treated *Arabidopsis thaliana* leaves using affinity chromatography that differentially regulated stomatal closure upon ABA supplementation.

*S*-acylation, previously known as *S*-palmitoylation, is a reversible PTM that instigates the palmitate group's addition to the specific Cys residues of soluble/membrane proteins [119]. *S*-acyl transferases are the main catalyzing this reaction, having conserved

the Asp-His-His-Cys motif and formed a thioester bond in the target proteins [120]. Numerous studies have identified and quantified *S*-acylated proteins in Arabidopsis and poplar plants using a proteomics approach coupled with BSM labeling [119,121]. Researchers have corroborated that the proteins which are most commonly *S*-acylated in plants growing under climate extremes are calcium-dependent protein kinases (CDPK), mitogen-activated protein kinases (MAPK), ATPases, calcineurin-B-like proteins, and several integral proteins [122,123]. *S*-acylation of several receptors, such as kinases proteins adjacent to the transmembrane domain, differentially regulated the flagellin perceiving receptor kinases 2 (FLS2) and associated signaling function [124]. Interestingly, another study concluded that *S*-acylation of plant immune receptor proteins such as P2K1, FLS2, and CERK1 activated the defense response of Arabidopsis plants more prominently compared to their corresponding non-mutant plants [125]. Recently, *S*-acylation of Alpha/Beta Hydrolase domain-containing protein 17-like acyl protein thioesterases invariably affected cell death/apoptosis in Arabidopsis and *Nicotiana tabaccum* plants [126]. They further concluded that protein-*S*-acyltransferases and de-*S*-acylation enzymes are progressively involved in regulating subcellular localization, stability, and activity of enzyme proteins. Likewise, the *S*-acylation of FLS2 controlled and stabilized the ligand-induced receptor kinase complex, thereby triggering plant innate immune response against pathogen attack [127]. *S*-acylation-mediated regulation of plant immune receptors (DORN1, LecRK-1.9, and ATP receptors) inhibited bacterial invasion in Arabidopsis plants via P2K1-mediated autophosphorylation and protein degradation [128].

### *5.3. Regulation of Epigenetic Modification*

Epigenetic modifications, such as DNA methylation and histone modifications, are potentially involved in the modulation of growth, development, and defense process of plants exposed to climate extremes [129]. The epigenetic modifications exert these stimulatory/inhibitory functions by regulating gene expression or performing chromatin remodeling dynamics strongly influenced by ROS/RNS/RSS signaling [129,130]. Accumulating shreds of evidence have indicated that reactive species can potentially affect the post-replication modification of DNA molecules by methylating cytosine at the five positions or adenine at the N4 or N6 positions [131]. Several excellent review papers have comprehensively described plant methylation and histone modifications [129–131]. Therefore, this section is mainly confined to ROS/RNS/RSS mediated regulation of epigenetic modifications influencing plant growth and development under stress conditions. These reactive species accentuate epigenetic mechanisms of gene regulation by modulating the functioning of various proteins/enzymes such as histone deacetylases, DNA methylases, Demeter1, a repressor of silencing 1 and Demeter-like 2 and 3 [132,133].

Researchers have corroborated that these reactive species directly interact with the Fe-S cluster of Demeter proteins, resulting in the generation of oxidative stress in plants, thus revealing the linkage between redox signaling and epigenetic modification [134]. Some of the pioneering studies in redox regulation of epigenetic modification in transgenic rice plants indicated that plants over-expressing OsSRT1 (silent information regulator 2 (SIR2)-related HDAC gene) exhibited less DNA methylation and oxidative damage compared to wild-type plants [135]. A decade later, Zhang et al. [136] confirmed the above notion and showed that OsSRT1 enhanced the tolerance to oxidative of transgenic plants by differentially regulating the expression of glyceraldehyde-3-phosphate dehydrogenase that had prevented the excessive generation of reactive species, thereby inhibiting DNA damage. Since then, a large body of literature has confirmed that differential regulation of ROS/RNS/RSS signaling can modulate epigenetic modification, such as acylation and nitrosation in Arabidopsis and rice plants via reactive species-mediated development of oxidative stress and decreased expression of HDA19 and HDA9 [33].

Likewise, the exogenous application of salicylic acid and nitric oxide accentuated ROS scavenging, thereby inducing DNA de-methylation and enhancing high-temperature tolerance in *Lablab purpureus* L. plants [41,42]. Reactive species-mediated PTMs, such as acylation

and nitrosation, have also been implicated in regulating secondary metabolite synthesis and the formation of crown roots in rice plants [33]. In another study, the exogenous application of nitric oxide efficiently modulated the ROS/RNS signaling, thereby improving the tolerance of lettuce plants exposed to metal stress by decreasing DNA methylation and activating the expression of stress-responsive genes [137]. Furthermore, the knockdown of argonaute 2 (OsAGO2) protein in rice anthers exaggerated ROS biosynthesis leading to increased methylation levels, abnormal growth, and apoptosis [138]; whereas an overexpression of hexokinase 1 (OsHXK1) differentially regulated the ROS level and tapetal apoptosis, leading to a significant decrease in the methylation level of the concerned promoter.

An increasing body of literature has demonstrated DNA methylation's involvement in fruit ripening, which was analyzed recently by [139]. They concluded that the treatment of grape plants with azacitidine significantly lowered the methylation rate in grapefruit compared to non-treated plants and enhanced the expression of the superoxide dismutase enzyme, thus establishing a link between ROS metabolism and epigenetic regulation. Recently, non-thermal plasma (NTP) treatment of seedlings of *Glycine max* displayed alteration in methylation patterns [140]. Researchers employed two doses of NTP that differentially regulated the ROS/RNS mediated methylation pattern that accentuated seed vigor and germination, resulting in improved seedling growth. In addition, genome-wide methylome status under the influence of $H_2O_2$ production was analyzed in transgenic tobacco plants [141]. They identified that overexpressing the CchGLP gene of transgenic plants stimulated endogenous ROS/RNS signaling, thereby enhancing its biotic and abiotic stress tolerance. Several recent findings have argued and confirmed the multifaceted role of ROS/RNS/RSS in epigenetically regulating gene expression and stress tolerance in plants. Still, this regulation's molecular mechanism is far-fetched. Therefore, future efforts must be diverted toward unraveling the functional mechanism by which ROS/RNS/RSS strengthens the plant's innate immunity against biotic/abiotic stresses.

## 6. Potential Biotechnological Application in Agriculture

In the recent decade, the plant science community has diverted its attention toward un-earthing the functional mechanisms by which ROS/RNS/RSS play a substantial role in regulating plant growth, development, and stress amelioration (Figure 3) [37]. Researchers have confirmed that these ROS/RNS/RSS regulate important cellular, physiological and biological processes in plants after interacting with other phytohormones [4,7]. A large body of literature has contemplated the role of NO during seed germination, fruit development, maintaining ion/osmotic homeostasis, and coping with biotic/abiotic stresses [6,11,13]. Furthermore, NO has also been known to differentially regulate the virulence of bacterial and fungal pathogens by acting as an antimicrobial or antifungal agent [142]. In addition, studies have also delineated that the perception of NO gas by microorganisms, such as bacteria and fungi, can induce transcriptional reprogramming of genes involved in metabolic and virulence systems, thereby regulating their adaptation [143]. Correspondingly, the genes involved in NO perception and signaling have been identified in both gram-positive and gram-negative bacteria [142]. The potential biotechnological application of NO in agricultural or horticultural crops depends on the donor molecules, application, tissue type, environment, and possible interaction with other secondary metabolites [142]. The most frequently used NO donor is sodium nitroprusside, and the application of foliar, seed soaking, or irrigation has tremendously improved plant growth and development under stress conditions [41]. Increasing evidence has contemplated that exogenous application of NO (Figure 3) can explicitly break seed dormancy and improve seed vigor, an essential prerequisite for attaining better growth and increasing crop yield [144,145]. Several studies have found that applying NO donors can effectively modulate seed dormancy even at low concentrations compared to the mechanical and chemical methods of breaking seed dormancy, such as $H_2SO_4$ and NaOCl [146,147].

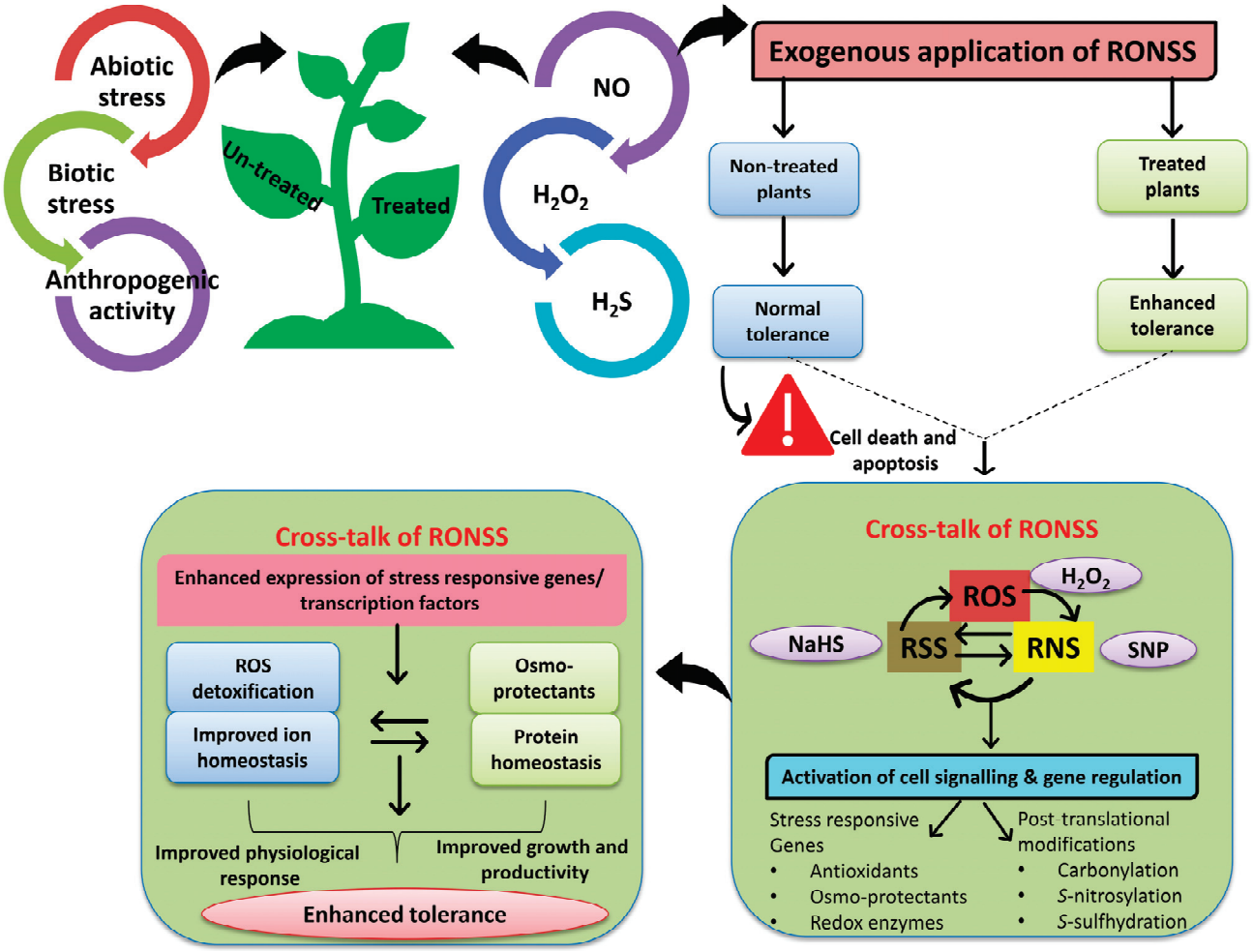


**Figure 3.** Crosstalk of ROS, RNS, and RSS in cell signaling, gene regulation and their role in ameliorating abiotic/biotic stress tolerance in plants. Exogenous application of SNP (NO donor), NaHS ($H_2S$ donor), and $H_2O_2$ results in the activation of a plethora of signaling pathways, thus regulating cell signaling and activation of several downstream stress-responsive genes/transcription factors leading to improved stress tolerance.

Recent investigations have shed light on the functional mechanisms by which NO mediates regulation of seed dormancy/germination by activating PTMs, such as tyrosine nitration of abscisic acid receptors, such as *S*-nitrosylation SnRK2s and ABI5, followed by degradation of ethylene-responsive factors [66]. In another study, Nagel et al. [146] identified novel loci in barley plants capable of regulating NO signaling, thereby regulating seed dormancy and preharvest sprouting. They concluded that the HvGA20ox1 gene is upregulated by NO signaling, alleviating dormancy and initiating flowering in barley plants [146]. Similarly, the significance of $H_2S$ in helping seed dormancy has been reported by various researchers where exogenous $H_2S$ upregulated the expression of COP1/HY5 stimulated seed germination in heat-stressed Arabidopsis plants [68]. The implication of exogenous application explicitly improves seed germination and seedling growth in heat-stressed *Zea mays* by regulating the expression of antioxidants and osmolyte biosynthesis [148].

Interestingly, the exogenous application of $H_2S$ resulted (Figure 3) in the persulfidation of ethylene biosynthetic enzymes, thereby decreasing the sensitivity of ethylene receptors and leading to enhanced seed germination due to the expression of antioxidative enzymes under stress conditions [149]. Recently, the antagonistic effect of the exogenous application of $H_2S$ has also been observed by [18]. The authors confirmed that $H_2S$-mediated persulfidation has also improved seed dormancy/germination by downregulating the expression of DES1 and ABI4 genes.

NO is also known to regulate fruit ripening and post-harvest shelf life by antagonizing ethylene signaling and contributing to shelf life extension [150]. Exogenous application of NO in post-harvest stages of *Magnifera indica* showed decreased membrane damage, ethylene production, and polygalacturonase activities, prolonging the fruits' shelf life without losing quality [151]. Likewise, the exogenous application of NO positively regulated the physiological weight loss, antioxidants, lipooxygenase, and pectin methylesterase activities, improving the quality/shelf life of *Prunus persica* fruits [152]. In addition, exogenous application of $H_2O_2$ and NO to guava and tomato fruits exhibited delayed ripening and minimum loss in TSS and ascorbic content, thus improving the storage life of these fruits under storage conditions [153]. Similarly, the exogenous application of $H_2S$ has been demarcated to improve the shelf life and quality of tomato post-harvest fruits by inhibiting the protease activity and decreasing the ERF and beta-amylase gene expression with higher nutritional contents [154]. In another study, exogenous application of $H_2S$ differentially regulated the expression of cell wall modifying related genes and critical genes involved in pectinase enzyme, thereby preventing rapid fruit softening in the fruits of *Fragaria chiloensis* [155]. Recently, exogenous application of $H_2S$ on the activity of various biochemical markers linked to fruit ripening in the fruits of banana and pointed gourd in the post-harvest stage was analyzed to detect their quality and shelf life. They unraveled that exogenous application of $H_2S$ significantly lowered membrane damage, lignification, and chlorophyll loss and correspondingly maintained higher PAL and PPO activity, thereby increasing the shelf life of the fruits without compromising their quality and extending their marketing duration [156,157].

NO and $H_2S$ have been known to modulate legume rhizobia symbiosis, a critical process in agriculture, as it accounts for the availability of biological nitrogen in the soil [158]. NO is believed to regulate two crucial aspects of nitrogen fixation in legume plants (i) by controlling the rhizobial infection and (ii) by activating nodule organogenesis, both of which are significantly regulated upon the expression of nodulation factors (nod genes) [158]. Increasing evidence has confirmed the regulatory role of NO as a signaling molecule for promoting legume–bacteria interaction and nodule development [159–162]. Exogenous application of NO to *Phaseolus vulgaris* and *Glycine max* stimulated the nodule formation, as indicated by the over-accumulation of nod factors [163]. In addition, studies have suggested that NO and ROS positively regulate legume–rhizobia interaction and nodule formation via ROS/RNS-mediated differential expression of ROS/RNS-producing enzymes [163].

Furthermore, the exogenous application of NO and $H_2S$ compellingly enhanced the heavy metal stress tolerance of legume–rhizobia symbiosis by increasing the soil enzyme activity and bacterial diversity, thereby improving their growth and productivity [158]. Likewise, the exogenous application of NO regulated the expression of phytoglobin 1.1 in *Medicago truncatula*, accentuating the symbiosis by controlling nodule development and nitrogen fixation [158]. ROS/RNS/RSS amplifier effect in stimulating legume–rhizobia symbiosis is mainly due to the various PTMs catalyzed by these reactive species that activate essential enzymes, such as thiol peroxidase and phytoglobin genes, that efficiently form nodules in legume crops [6]. Future application of these reactive species in a precise and controlled way can potentially regulate crop growth and productivity by regulating many distinct physiological and biological processes.

## 7. Conclusions

Earlier, ROS/RNS/RSS has been described as toxic molecules affecting various metabolic and regulatory functions in plants exposed to several biotic and abiotic stress conditions. With the advent of technological breakthroughs, different redox biologists have implicated their role in regulating critical cellular and metabolic processes in plants exposed to climate extremes. When these redox regulators are maintained below a threshold level, they control many signal transduction pathways by mediating PTMs and epigenetic modification in plants. PTMs, such as carbonylation, *S*-nitrosation, tyrosine nitration, and persulfidation, instigate dynamic signaling of different components of enzymatic and non-enzymatic defense

systems at gene and protein levels. The precise regulation induced by these redox regulators at the biochemical and molecular level strengthens plant innate immune response, thereby maintaining the growth and productivity of plants under adverse growing conditions.

Moreover, researchers have also reported that these redox molecules essentially regulate DNA methylation and histone modification in plants, thus significantly impacting the transcription and activities of various genes and enzymes. Nevertheless, the precise mechanisms by which ROS/RNS/RSS regulate these epigenetic marks and other biochemical and molecular processes under stress conditions need further investigation. Furthermore, in-depth explorations of how these redox regulators interact with other signaling molecules and plant growth regulators must be unraveled. The unraveling of molecular mechanisms underlying the regulatory functions of ROS/RNS/RSS will open a new realm for formulating new biotechnological strategies for their possible application in agriculture for fostering new/improved cultivars in the era of climate change.

**Author Contributions:** K.K.R. conceived the idea, prepared all the figures and tables, and wrote the final draft of the manuscript; P.K. critically reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors are also thankful to the anonymous reviewers for their careful reading.

**Conflicts of Interest:** The authors declare that no conflicts of interest exist.

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# Comparative Proteomics Analysis between Maize and Sorghum Uncovers Important Proteins and Metabolic Pathways Mediating Drought Tolerance

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**Abstract:** Drought severely affects crop yield and yield stability. Maize and sorghum are major crops in Africa and globally, and both are negatively impacted by drought. However, sorghum has a better ability to withstand drought than maize. Consequently, this study identifies differences between maize and sorghum grown in water deficit conditions, and identifies proteins associated with drought tolerance in these plant species. Leaf relative water content and proline content were measured, and label-free proteomics analysis was carried out to identify differences in protein expression in the two species in response to water deficit. Water deficit enhanced the proline accumulation in sorghum roots to a higher degree than in maize, and this higher accumulation was associated with enhanced water retention in sorghum. Proteomic analyses identified proteins with differing expression patterns between the two species, revealing key metabolic pathways that explain the better drought tolerance of sorghum than maize. These proteins include phenylalanine/tyrosine ammonia-lyases, indole-3-acetaldehyde oxidase, sucrose synthase and phenol/catechol oxidase. This study highlights the importance of phenylpropanoids, sucrose, melanin-related metabolites and indole acetic acid (auxin) as determinants of the differences in drought stress tolerance between maize and sorghum. The selection of maize and sorghum genotypes with enhanced expression of the genes encoding these differentially expressed proteins, or genetically engineering maize and sorghum to increase the expression of such genes, can be used as strategies for the production of maize and sorghum varieties with improved drought tolerance.

**Keywords:** proteomic analysis; drought stress; maize; sorghum; drought tolerance

**Citation:** Ali, A.E.E.; Husselmann, L.H.; Tabb, D.L.; Ludidi, N. Comparative Proteomics Analysis between Maize and Sorghum Uncovers Important Proteins and Metabolic Pathways Mediating Drought Tolerance. *Life* **2023**, *13*, 170. https://doi.org/10.3390/life13010170

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 14 November 2022
Revised: 31 December 2022
Accepted: 3 January 2023
Published: 6 January 2023



## 1. Introduction

*Sorghum bicolor*, commonly known as sorghum, and *Zea mays*, commonly called maize, are two of the major staple cereals in the world belonging to the Panicoideae subfamily in the family Gramineae [1]. Under drought conditions, sustained crop production is necessary to ensure global food security and requires accelerated crop breeding to develop drought-tolerant crops [2]. Sorghum is a candidate for this breeding effort due to its adaptation to drought [3]. The completed sequencing of the sorghum genome makes it a key model system for understanding the drought-responsive molecular mechanisms in plants [4]. Sorghum ranks as the fifth most significant crop across the globe after maize, rice, wheat and barley [5,6]. It is cultivated for food, feed and biofuel production. In Africa, sorghum is the second major grain after maize, with an annual production of approximately 20 million tons in the continent, which contributes one-third of the global crop production [6]. Globally, maize is the third main cereal in terms of harvested area [7]

and serves as a staple in sub-Saharan Africa [8]. Maize is the main grain crop grown in South Africa, with approximately 12.2 million tons produced annually in the country [9]. It also acts as a source of biofuel and starch.

Drought is considered as one of the most significant natural hazards, and its intensity and frequency are projected to increase due to global warming [10]. According to recent reports, drought has affected 2.3 billion people across the world, with African communities affected the most as the continent accounts for 40% of the world total [11,12]. Based on annual rain fall data, South Africa is one of the 30 driest countries in the world [13]. In the last four decades, drought has become more prevalent in the country, negatively affecting agriculture and magnifying food insecurity in the region [14]. Among the major threats to crop production, drought is the most significant [15]. It has been reported that over the period from 1983 to 2009, three-quarters of the cultivated areas of key crops in the world, including maize, rice, soybean and wheat, experienced yield losses due to droughts [16]. The crop yield losses per drought event during that period were 7% for maize and soybean, 8% for wheat and 3% for rice. Improving sustainable crop production under conditions of limited water supply is important to meet the increasing food demand of the world's growing population [17]. In arid or semi-arid regions, such as South Africa, screening of the adaptive responses to drought in crops is essential to the improvement of crop production under water deficit. Drought tolerance indices can be obtained by assessing the molecular responses to water deficit in crop plants, providing more insight into the mechanisms that may improve drought tolerance in the plants.

Recent advances in proteomic approaches have significantly improved the identification of a wide range of proteins in living cells [18]. This aspect is particularly important and useful for crop science. This is because it may augment the understanding of the molecular mechanisms that regulate the processes involved in the determination of the yield and nutrient content in crops. Advances in proteomics will help in elucidating how the yield and nutrient content are affected by adverse conditions such as stress resulting from drought [19]. Proteomics, one of the key tools of the post-genomic era [20], offers sensitive identification of the proteins associated with drought responses in plants [21–24].

Although several studies have already reported proteomic analyses of drought stress responses in sorghum and maize separately, no comparative studies have been performed to identify differences in the molecular events underpinning the greater drought tolerance of sorghum than maize; and therefore, this is the first such cross-species comparison at the proteome level. Bridging this knowledge gap on the adaptive responses to drought across these two species has the potential to enhance drought tolerance in both plant species, since there are differences in the level of drought sensitivity/tolerance within the genotypes of each of the two species. Thus, despite the greater drought tolerance in sorghum than in maize, drought-sensitive sorghum genotypes with various other desirable agronomic traits will benefit if their drought tolerance is improved. A previous study in our laboratory demonstrated the ability of sorghum to sustain growth better than maize under water deficit stress [25]. Furthermore, the study showed that the contrasting responses between the two species were associated with differences in reactive oxygen species (ROS) accumulation and antioxidant enzyme activities [25]. To further understand the molecular events that determine the contrasting responses to water deficit between the two species, a proteomics approach was used to compare the changes in protein expression in the two species under drought. This study measured the relative water content and proline levels in maize and sorghum in response to water deficit and assessed the changes in protein expression of the two plant species under water deficit using label-free quantitative proteomic analysis. This study proposes that the higher level of drought tolerance in sorghum than maize is driven by molecular mechanisms associated with differences in the expression of specific proteins involved in drought responses.

## 2. Materials and Methods

### *2.1. Seed Germination and Plant Growth*

The experiment was carried out in a greenhouse at the University of the Western Cape, South Africa (33°55′51.3″ S 18°37′29.2″ E). Sorghum [*Sorghum bicolor* (L.) Moench cv. Superdan] (purchased from Agricol, Brackenfell, South Africa) and maize [*Zea mays* (L.) cv. Borderking] (purchased from McDonalds Seeds, Pietermaritzburg, South Africa) were disinfected with 0.35% (*v*/*v*) sodium hypochlorite for 10 min, followed by 5 rinses using sterile distilled water. Surface sterilized seeds were sown in vermiculite (Windell Hydroponics, Western Cape, South Africa) that had been wetted with 1X nutrient solution [Nitrosol®, Fleuron (Pty) Ltd., Johannesburg, South Africa] at room temperature for 3 days to allow their germination. Seedlings were transplanted into cylindrical acrylic tubes (10 cm diameter and a height of 100 cm, with the length covered in foil) containing 7.8 L of Promix Organic (Windell Hydroponics, South Africa), which was saturated with 1% fertilizer (*v*/*v*) [Nitrosol®, Fleuron (Pty) Ltd., Johannesburg, South Africa]. The plants were grown at 29 °C during the day (16 h light) and at 19 °C during the night (8 h dark), with a photon flux density reaching 400 μmol $m^{-2}$ $s^{-1}$ during the day phase. Plants received 500 mL of tap water every second day until the V1 stage of growth.

### *2.2. Water Deficit Treatment*

Two sets of plants, namely well-watered plants (WW, for which there were ten maize plants and ten sorghum plants) and others subjected to water deficit (WD, for which there were ten maize plants and ten sorghum plants) were used in this study. The WW (control) plants were irrigated with water (500 mL) at intervals of two days until they were harvested (V8 stage of vegetative growth). Five WW maize plants and five WW sorghum plants that showed uniform growth (height, leaf number and morphological appearance) within each of the two species were selected for further analyses. To induce water deficit that simulates drought, WD plants were provided with 100 mL of water (20% of the water supplied to WW plants) once a week, which was stopped when plants reached V3 stage of growth. The WD plants were grown henceforth without further water supply until they showed signs of drought stress (three to four of the oldest leaves turned brown). This corresponded to 40 days after complete water withholding for maize and 55 days after water withholding for sorghum. At these points of water deficit treatment, only five WD maize plants and only five WD sorghum plants exhibiting uniformity in growth within the species were selected for further analyses. The four youngest leaves from maize and sorghum, which were still green, were harvested from each of the selected WW and WD plants. At the time of harvest, maize and sorghum plants were all green and looked healthy, except for the two oldest leaves (at the bottom) in plants grown under water deficit, for which these two leaves were turning dry and started browning, and additionally the four youngest leaves of maize (but not sorghum) showed visible leaf rolling despite still being green and looking healthy. A sample of the Promix Organic growth medium was taken at a depth of 30 cm from the surface of the medium and used to measure the water potential of the growth medium on a WP4C Water PotentioMeter (Meter Group, Pullman, WA, USA) to assess the water status of the soil at the time of harvest, since this is essential in interpretation of the responses of the plants to water deficit. The harvested plant material was rapidly frozen in liquid $N_2$, ground into a fine powder and stored at −80 °C until further processing.

### *2.3. Relative Water Content*

The youngest fully expanded leaf from each of the selected five plants were used to measure leaf relative water content (RWC). Segments (10 cm long) from the tip of each leaf were obtained and their fresh weights were determined by weighing the segments on a fine weighing balance. The leaf segments were incubated for 4 h in Petri dishes filled with distilled water under ambient light. The turgid weight was measured after blotting off the excess water on the leaf surface. Thereafter, the leaves were dried in an oven at 60 °C for 72 h, after which their dry weights were immediately recorded. The formula

RWC = [(FW − DW) ÷ (TW − DW)] × 100 was used for calculation of the RWC, where FW is the fresh weight, DW is the dry weight and TW is the turgid weight.

### *2.4. Proline Content*

Proline content was measured from frozen tissue of the four youngest fully expanded leaves from each of the selected five plants, based on a microplate method for small tissue amounts [26]. For these measurements, plant tissue (100 mg) was mixed with 500 µL of sulfosalicylic acid (3% $C_7H_6O_6S$) and centrifuged for 5 min at 13,000× *g* at room temperature. In a 2 mL microcentrifuge tube, the supernatant (100 µL) was mixed with a reaction mixture (500 µL) consisting of 20% (*v*/*v*) of the 3% sulphosalicylic acid extract (i.e., 100 µL), 40% (*v*/*v*) glacial acetic acid ($CH_3COOH$) and 40% (*v*/*v*) acidic ninhydrin ($C_9H_6O_4$). After mixing, the reaction solution was incubated for 60 min at 100 °C. After cooling in ice for 5 min, the reaction solution was mixed with 99.9% toluene (1 mL of $C_6H_5CH_3$) and incubated at room temperature for 5 min. Absorbance of the solution at 520 nm was measured using a POLARstar Omega multimode microplate reader (BMG Labtech, Offenburg, Germany). A standard curve was prepared with L-proline and used to determine proline content [26].

### *2.5. Protein Extraction*

A modified SDS/phenol extraction method previously described by Wang et al. [27] was used for total soluble protein extraction. The experiment consisted of five independent biological replicates of each species under well-watered and water deficit conditions. Leaf tissue (1 g) was added to 0.5 g polyvinylpolypyrrolidone (PVPP) in a pre-cooled mortar and ground into fine powder with a pestle in liquid nitrogen. The powder was homogenized with 2 mL of 10% TCA/acetone (*w*/*v*) and split equally between two microcentrifuge tubes (one for SDS-PAGE gel analysis and one for label-free liquid chromatography/mass spectrometry analysis). The homogenate was centrifuged for 20 min at 13,000× *g* at 4 °C. This was followed by washing of the pellet twice with pre-cooled ammonium acetate (1 M) in methanol (80% *v*/*v*) and three times with pre-cooled 80% (*v*/*v*) acetone. The supernatant was discarded after each wash. After air drying, the pellet was dissolved in 0.5 mL of buffer containing 2% (*w*/*v*) sodium dodecyl sulphate (SDS), Tris-HCl (0.1 M, pH 8.0), phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1 mM, 5% (*v*/*v*) β-mercaptoethanol (BME) and 30% (*w*/*v*) sucrose (Sigma, St. Louis, MI, USA). The suspension was mixed with 0.5 mL of phenol (Tris-buffered, pH 8.0) and centrifuged at 4 °C for 20 min at 13,000× *g*. The phenolic layer was taken and mixed with cold 80% (*v*/*v*) methanol, which contained ammonium acetate at a final concentration of 0.1 M. The samples were incubated at 4 °C overnight to precipitate the extracted proteins. The mixture was centrifuged for 20 min at 4 °C at 13,000× *g*. The pellet was washed with cold ammonium acetate (0.1 M, prepared in methanol), followed by a second wash with cold acetone (80% *v*/*v*). After removal of the acetone, the pellet was vacuum-dried in a desiccator at room temperature. The protein pellet for 1-D SDS-PAGE was solubilized in 100 µL of solubilization buffer made up of 2 M thiourea, 4% (*w*/*v*) 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS), 7 M urea and dithiothreitol (DTT) at a final concentration of 20 mM. The second set of pellets was used for the proteomic analysis. The concentration of solubilized proteins was determined using the Bradford method [28]. The quality of the extracted proteins was assessed using electrophoresis on 1-D SDS-PAGE.

### *2.6. Preparation of Protein Samples for LC–MS/MS Analysis*

#### 2.6.1. Solubilization and Quantification of Proteins

Protein pellets from above were resuspended in solubilization buffer [50 mM triethylammonium bicarbonate (TEAB), 2% SDS] and incubated for 5 min at 95 °C. Solubilized proteins were clarified by centrifugation for 5 min at 10,000× *g*. Solubilized proteins were quantified using the QuantiPro BCA assay kit as described by the manufacturer (Sigma).

#### 2.6.2. On-Bead Protein Digestion and HILIC Enrichment

Magnetic beads for hydrophilic interaction liquid chromatography (HILIC) were rinsed twice, for 1 min each time, with 250 µL of washing solution consisting of 15% acetonitrile (ACN) and ammonium acetate (100 mM) at pH 4.5. The beads were dissolved in a loading buffer containing 30% ACN and ammonium acetate (200 mM) at pH 4.5. All of the subsequent steps described hereafter were carried out using a Hamilton MassSTAR robotic liquid handling system (Hamilton, Switzerland). Protein samples (50 µg each) were added to a protein LoBind plate (Merck, Rahway, NJ, USA). Prior to trypsin digestion, proteins were reduced with 10 mM Tris (2-carboxyethyl) phosphine (TCEP) at 60 °C for 1 h and alkylated with 10 mM methyl methanethiosulphonate (MMTS) for 15 min at room temperature. After reduction and alkylation, HILIC magnetic beads were added to the samples in an equivalent volume and incubated for 30 min on a plate shaker at room temperature at 900 rpm. The beads were washed twice with 500 µL of wash buffer (95% ACN) for 1 min each before trypsin digestion. Trypsin (Promega, Madison, WI, USA) was added at a 1:10 ratio (trypsin:protein), followed by incubation on a shaker at 37 °C at 900 rpm for 4 h. The resulting peptides were collected and dried under vacuum, followed by resuspension in 0.1% trifluoroacetic acid (TFA) and desalted. The desalted digests were vacuum-dried once again and subsequently resuspended in loading buffer (2.5% ACN, 0.1% formic acid (FA) prior to analyses.

#### 2.6.3. Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS)

Peptides were subjected to LC–MS/MS analyses on a Q-Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), which was directly coupled to a nano-HPLC system (Dionex Ultimate 3000). The peptides were dissolved in 2% ACN and 0.1% formic acid (Sigma) and injected into a column (C18 trap) in 3.5% solvent B (0.1% FA, 0.1% ACN) at a flow rate of 5 µL/min for 4 min. Peptides were chromatographically separated on a C18 column (PepAcclaim). Peptides were eluted using a multi-step LC gradient generated at 300 nL/min flow rate as follows: 3.5–9% Solvent B over 6 min, 9–24.6% Solvent B over 45.5 min, 24.6–38.7% Solvent B over 2 min, 38.7–52.8% Solvent B over 2.1 min and 52.8–85.4% solvent B over 0.4 min. The gradient was held at 85.4% solvent B for 10 min, returned to the starting condition (3.5% solvent B), which was held for 15 min. The mass spectrometry system was performed with the capillary temperature set at 320 °C on positive ion mode (at +1.95 kV electrospray). Details of data acquisition on the Q Exactive quadrupole Orbitrap mass spectrometer, which was fitted with a higher-energy collisional dissociation (HCD) cell, are shown in the Supplementary Table S1.

### *2.7. Bioinformatics*

#### 2.7.1. Data Source

In this experiment, a total of 20 LC–MS/MS runs (81 min runs) were conducted on the Thermo Q-Exactive mass spectrometer. Three extra LC–MS/MS runs represented pools of all samples. A total of 30,000 MS/MS spectra were obtained for each plant species. Protein sequence databases downloaded from the Phytozome protein database on 11 October 2017, held 88,760 proteins for maize and 47,121 proteins for sorghum.

#### 2.7.2. Peptide and Protein Identification Pipeline

Raw data files (spectra) were converted into mzML using the ProteoWizard 3.0 msConvert tool [29]. Peak-Picking and Zlib compression were employed. Database searching employed the MS-GF+ search engine [30] to identify the potential peptides shaped by semi-tryptic specificity, and a 20 ppm precursor tolerance was applied. The data retrieval results were refined by IDPicker (version 3.1) [31] to produce a 2% peptide–spectrum match (PSM) false discovery rate (FDR), with two unique peptides required for each protein.

NCBI BLAST 2.5.0+ makeblastdb [32] was used to index the FASTA sequence databases for ortholog identification between the two species. The blastp program was used to query

each sorghum sequence in the maize database and to query each maize sequence in the sorghum database. The generated ortholog files were read in R statistics script and a minimum bit score of 50 was applied. Matches that exceed this threshold were considered true. In cases where multiple matches were found, only the hit with the highest bit score value was retained. Ortholog data and spectral counting tables from IDPicker were read in a script in the R statistical environment to align the spectral count row for each sorghum protein with the spectral count row for the orthologous maize protein. When maize and sorghum orthologs were split into different rows (for example, in the case of paralogs for one species but not the other), the two rows were merged to form one joint row. Proteins lacking orthologs or those with unidentified orthologs were not subjected to further analyses.

#### 2.7.3. Statistical Analysis

Five biologically replicated comparisons of contrasting cohorts (control and water deficit) were used, with target-decoy searching employed to limit aggregate PSM error to 1%. The spectral count data were compared in an R statistics script, with a minimum information criterion of 10 spectra per protein being set, after which a Quasi-Poisson regression was conducted with treatment (well-watered/water deficit) and species variables. Values for multiple testing were corrected via the Benjamini–Hochberg FDR method [33]. If a protein had a q-value < 0.05, it was considered significantly different, with 5% of the claimed changes expected to be false positives.

One-way analysis of variance (ANOVA) was used to analyze the physiological and biochemical results and their significance was determined using the Tukey–Kramer method at a 5% significance level.

#### 2.7.4. Gene Ontology and KEGG Analysis

Differentially expressed proteins were functionally annotated using the Blast2GO program implemented in the OmicsBox v2.2.4 software [34]. The sequence information of the differentially expressed orthologs were obtained from the UniProtKB website in FASTA format on 19 September 2022. Protein sequences were searched, using the basic local alignment search tool (BLAST), against sequences in NCBI (National Center for Biotechnology Information) via the BlastP search algorithm to determine similarity matches. The BLAST search was carried out using the default parameters with a maximum of 20 hits, at an expectation value of $1.0 \times 10^{-3}$, with 33 as the high-scoring segment pair (HSP) length cutoff and 0 as the HSP-hit coverage, with application of a low complexity filter. Sequences that received the best BLAST hits were mapped and annotated using default settings (annotation cutoff 55, Go weight 5, e-value $1.0 \times 10^{-6}$, HSP-hit coverage cutoff 0 and hit filter 500). Proteins were annotated according to gene ontology (GO) by 'level 2' on the basis of molecular function (MF), biological process (BP) and cellular component (CC). Protein sequences were scanned for conserved domains against signatures in InterPro using the InterProScan tool, which was an inbuilt program of Blast2GO. Annotated sequences were linked to metabolic pathways via Enzyme Commission (EC) numbers using the KEGG (Kyoto Encyclopedia of Genes and Genomes) extension of Blast2GO.

## 3. Results

### *3.1. Drought Induced the Accumulation of Free Proline in Maize and Sorghum*

At the time of harvesting, the water potential of the soil in which the plants were grown under well-watered conditions was −0.12 MPa for the soil used to grow maize and −0.14 MPa for the soil used to grow sorghum, which was not statistically different between the two sets of soil (Figure 1a). This was different for the soil water potential at the time of harvesting of the plants grown under water deficit, where the soil water potential in the maize experiments was −0.75 MPa and it was a statistically different value from the −0.98 MPa obtained from the soil used in the sorghum experiment (Figure 1a). The exposure to drought stress significantly influenced the physiological and biochemical traits

of both plant species, as reflected by the decreased relative water content in maize under the water deficit treatment (Figure 1b). In response to water deficit, maize showed a sharp decrease in relative water content (30%), whereas the relative water content in sorghum leaves did not significantly decrease, as shown in Figure 1b. Free proline content was significantly higher in maize leaves (an increase by 60%) than in sorghum leaves (increase was limited to 50%), as depicted in Figure 1c. Interestingly, under drought conditions, the accumulation of free proline in sorghum roots increased by 60% whereas it increased only by 40% in maize roots (Figure 1d).

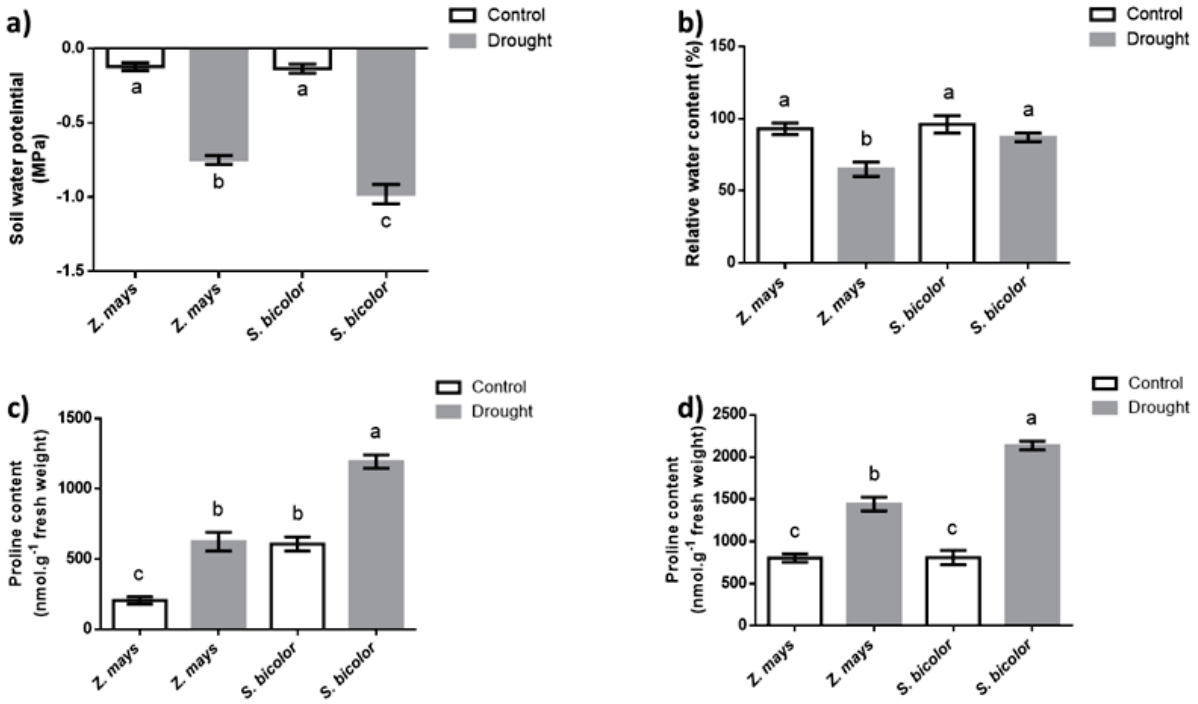


**Figure 1.** Changes in soil water potential (**a**), relative water content (**b**), and free proline accumulation in leaves (**c**) and roots (**d**) of maize and sorghum under water deficit. Data presented are means (±SE) of five independent experiments (n = 5). Bars with different letters are significantly different at $p \leq 0.05$.

### *3.2. Differentially Regulated Proteins between Sorghum and Maize*

An initial quality control via SDS-PAGE established that protein degradation was minimal (Supplementary Figure S1). The LC–MS/MS analysis yielded 3154 distinct peptides, including 2752 entries for maize and 2794 for sorghum. There were no matches for 718 (23%) peptides in both plant species for which an ortholog had been named. Proteins named in the ortholog map were constituted from 945 peptides (30%). Furthermore, 416 proteins (13%) consisted of orthologs of both plant species. Of the identified peptides, there were 1070 entries (34%) that consisted of a protein for which another orthologous protein was identified. These 1070 peptides formed 535 rows, which joined the maize spectra from one of the composite rows and the sorghum spectra from a different row. This improved the number of protein sequences for which the sorghum profile and maize profile could be matched, from 416 to 951 distinct proteins (+129% improvement). The Quasi-Poisson model (based on a cut-off of at least 10 spectral counts with a q-value less than 0.05) revealed that 207 orthologs differed in abundance between the two species, irrespective of the treatment (Supplementary Table S2). Among the 207 orthologs, 4 proteins (Table 1) were differentially expressed between maize and sorghum in response to drought. These four differentially expressed orthologs thus define the different responses of the two species under water deficit stress. Therefore, the relative differential expression of these proteins between maize and sorghum as well as their functional fates in response to drought stress are further addressed herein.

**Table 1.** Protein orthologs with differential expression between sorghum and maize in response to water deficit stress.

| Accession | Description | Log2 Fold Change | | Q-Value Species | Q-Value Treatment |
|---|---|---|---|---|---|
| | | Maize | Sorghum | | |
| GRMZM2G141473_P01/ Sobic.001G062300.1.p | Indole-3-acetaldhyde oxidase | 2.6 | 1.1 | 0.000187049 | 0.044375583 |
| GRMZM2G319062_P01/ Sobic.007G068500.1.p | polyphenol oxidase I, chloroplastic-like | 3.0 | 1.9 | 0.000564461 | 0.009718389 |
| GRMZM2G074604_P01/ Sobic.004G220300.1.p | phenylalanine/tyrosine ammonia-lyase | −0.9 | −1.0 | 0.000403801 | 0.00363421 |
| GRMZM2G152908_P01/ Sobic.001G344500.2.p | sucrose synthase 2 | −1.5 | −2.3 | 0.000451047 | 0.000119933 |

A negative sign indicates a decrease in protein expression. Maize proteins are depicted in the top accession number (starting with GRMZM) and sorghum proteins are depicted in the bottom accession number (starting with Sobic).

### *3.3. Gene Ontology and KEGG Pathway Annotation*

Differentially expressed orthologs between maize and sorghum (Table 1) were characterized according to Gene Ontology (GO). As shown in Figure 2, GO enrichment analysis revealed that the metabolic process and cellular process were the most represented biological processes, followed by the response to stimulus and biological regulation. Within the molecular function category, catalytic activity and binding were the most enriched. According to the cellular component GO terms, the differentially expressed proteins were mainly localized in the cellular anatomical entity.

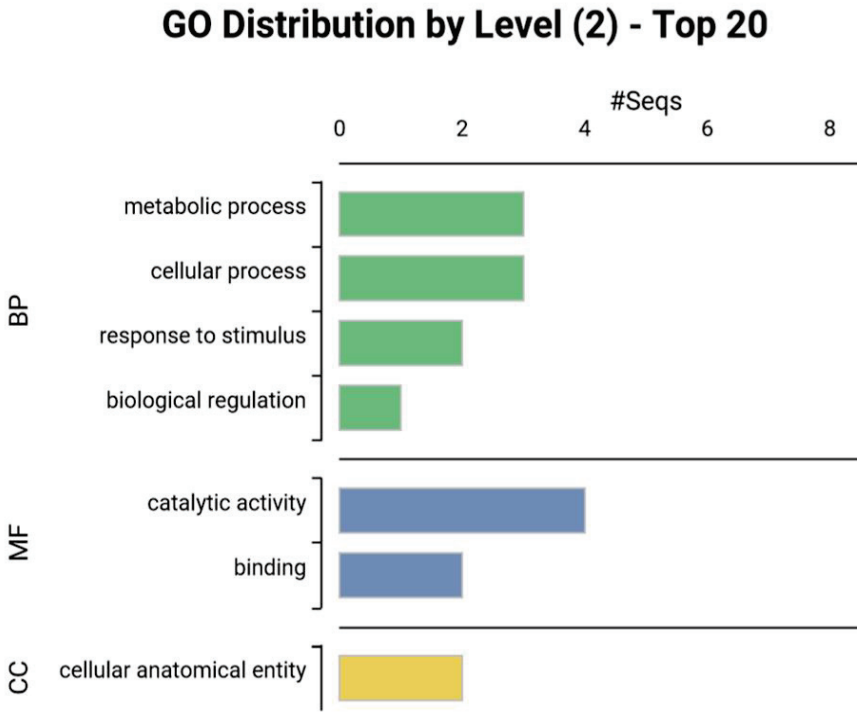


**Figure 2.** Gene ontology (GO) analysis of proteins differentially regulated between maize and sorghum under water deficit stress as determined by Blast2GO according to GO distribution by level 2. Molecular function (MF); cellular component (CC); biological process (BP).

To better understand the functions of the differentially regulated proteins between maize and sorghum, orthologs were assigned to different metabolic pathways via the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The KEGG pathway anal-

yses showed that the differentially expressed proteins were associated with the biosynthesis of phenolic acids, biosynthesis of indole acetic acid, sucrose and D-fructose metabolism, ROS scavenging and biosynthesis of melanin-related compounds (Figure 3).

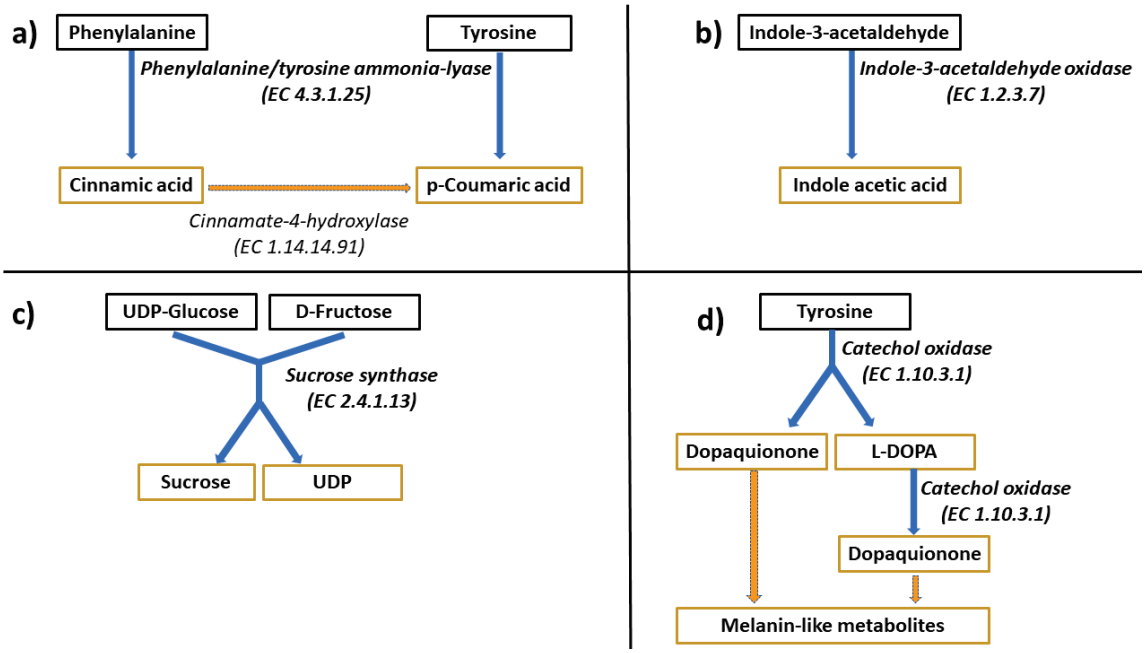


**Figure 3.** KEGG pathway analysis revealing proteins differentially regulated between maize and sorghum under water deficit stress. Phenylalanine and tyrosine metabolism affecting phenolic biosynthesis via phenylalanine/tyrosine ammonia lyase (PTAL) is illustrated (**a**), along with conversion of indole-3-acetyldehyde to indole acetic acid by indole-3-acetaldehyde oxidase (**b**), production of sucrose/D-fructose from UDP glucose via a reaction catalyzed by sucrose synthase (**c**) and production of DOPA quinone via a reaction catalyzed by catechol oxidase to metabolize tyrosine towards synthesis of melanin-like compounds (**d**). Metabolites encased in black rectangles are substrates for the enzymes (described by italicized bold font, with indicated Enzyme Commission numbers). Metabolites enclosed in gold rectangles are products of the indicated enzymes. The gold dashed arrow or its associated enzyme (in italicized regular font, not bold) indicates a section of a metabolic pathway for which the catalyzing enzyme was not identified as differentially regulated between maize and sorghum but for which the products of the identified differentially regulated proteins are substrates for downstream proteins in the pathway.

## 4. Discussion

### *4.1. Proline Accumulation in Sorghum Roots Was Associated with Improved Water Retention*

The reduction of RWC in maize leaves, which did not occur in sorghum, indicated better water retention ability in sorghum under drought than in maize. A similar observation was reported by Hasan et al. (2017) [35], where drought stress significantly decreased the leaf RWC in maize, while no significant effect was observed in sorghum. Compatible solutes act as osmoprotectants and mediate osmotic adjustment in plants under water deficit [36]. Among them, free proline is the most common osmolyte occurring in plants grown under water deficit [37]. Therefore, high accumulation of proline can enhance water retention capacity [38]. In this study, water deficit increased the proline content in the roots and leaves of both species. However, compared to maize, sorghum demonstrated a greater increase in proline content in the root. Such enhanced proline accumulation in the sorghum roots would result in a higher degree of decrease in water potential in sorghum roots than in maize roots, which would allow for better water uptake from the soil by sorghum roots than maize roots, and hence sustain shoot water status longer in sorghum than maize.

### *4.2. Gene Ontology and KEGG Pathways*

A combination of gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses can link physiological changes to molecular pathways, which can facilitate the identification of the pathways mediating the effects of environmental stresses in the plant [39]. In this study, some differentially expressed proteins between maize and sorghum were involved in various cellular process, metabolic process, catalytic activity and stimulus response. Proteins with catalytic function act as pivotal regulators involved in multiple processes of plant development and responses to environmental changes, through modulation of downstream protein activities [40]. Exploring the function of the enzymes and their associated pathways could provide deeper insight into the mechanisms underlying sorghum adaptation to drought stress. To gain this understanding, the pathways with potential roles in plant stress responses are further discussed. Phenylalanine/tyrosine ammonia-lyase (EC 4.3.1.25) plays a key role in the phenylpropanoid biosynthesis pathway (ko00940, Table 2 and Figure 3) and was downregulated in both maize and sorghum (0.9- and 1.0-fold, respectively). Three enzymatic activities are central to the phenylpropanoid biosynthesis pathway. This includes the non-oxidative elimination of ammonia from L-phenylalanine and L-tyrosine by phenylalanine/tyrosine ammonia-lyases (PAL/PTALs) to produce trans-cinnamic acid and p-coumaric acid, respectively. In the second step, cinnamic acid 4-hydroxylase (C4H) catalyzes the hydroxylation of trans-cinnamic acid to 4-coumarate.

**Table 2.** Functions of the enzymes differentially expressed between *Zea mays* and *Sorghum bicolor* in response to drought.

| Enzyme Code (EC) | Name of Enzyme | Sequences | Substrates | Products |
|---|---|---|---|---|
| 1.2.3.7 | Indole-3-acetaldhyde oxidase | GRMZM2G141473_P01/ Sobic.001G062300.1.p | Indole-3-acetaldehyde | Indole acetic acid |
| 1.10.3.1 | Polyphenol oxidase I, Catechol oxidase | GRMZM2G319062_P01/ Sobic.007G068500.1.p | Tyrosine | L-DOPA Dopaquionone |
| 4.3.1.25 | Phenylalanine/Tyrosine ammonia-lyase | GRMZM2G074604_P01/ Sobic.004G220300.1.p | Phenylalanine Tyrosine | Cinnamic acid p-Coumaric acid |
| 2.4.1.13 | Sucrose synthase 2 | GRMZM2G152908_P01/ Sobic.001G344500.2.p | UDP-Glucose D-Fructose | Sucrose UDP |

EC represents the Enzyme Commission number. Maize proteins are depicted in the top accession number (starting with GRMZM) and sorghum proteins are depicted in the bottom accession number (starting with Sobic). UDP is uridine diphosphate, L-DOPA is L-3,4-dihydroxyphenylalanine.

Lastly, 4-coumarate is activated by 4-coumarate-CoA ligase (4CL) to form 4-coumaroyl-CoA [41]. The p-Coumaroyl-CoA enters different downstream pathways, which leads to the biosynthesis of numerous compounds with antioxidant properties, including monolignol, coumarin, stilbene and flavonoids. In a previous study, salt stress increased the expression of PTAL in *Zea mays* [42]. These reports contradicted the results observed both in our study and another preceding study [43] on stressed *Medicago sativa* L., where a decreased abundance of PAL was correlated with elevated levels of cinnamic acid. Our recent assessment of the maize response to water deficit suggested that drought leads to altered levels of phenolic acids, driven by changes in the expression of genes encoding cinnamate 4-hydroxylase and p-coumaric acid 3-hydroxylase [44]. Thus, assessing the levels of phenolic acids and flavonoids in sorghum and maize will contribute to the understanding of how enzymes in the phenylpropanoid biosynthesis pathway influence the responses to drought in these two C4 plant species.

Indole-3-acetaldehyde oxidase (IAA oxidase, EC 1.2.3.7), which is part of the tryptophan biosynthetic pathway (ko00380, Table 2 and Figure 3), was over-expressed in both species. Although *Zea mays* showed a greater fold change (2.6) than *Sorghum bicolor* (1.1) in response to water deficit, indole-3-acetaldehyde oxidase abundance was higher in sorghum than in maize irrespective of whether the plants were grown in well-watered or water deficit conditions (Supplementary Table S2). Therefore, it can be proposed that the higher expression of indole-3-acetaldehyde oxidase in sorghum could lead to greater production of metabolites associated with the tryptophan biosynthetic pathway. Hence, this would mean that the greater abundance of this enzyme in sorghum than in maize translates to higher products of this pathway in sorghum compared to maize. In the tryptophan

biosynthesis pathway, indole-3-acetaldehyde (IAAl) is oxidized by IAAl oxidase to produce indole-3-acetic acid (IAA) [45]. Indole-3-acetic acid is the most commonly occurring auxin in plants. Auxin is a key hormone that plays vital roles in plant growth and development, which include cell division, cell differentiation and cell elongation [46]. As a regulator, auxin mediates the signaling pathways in plant responses to stress [47]. Given that indole-3-acetaldehyde oxidase produces indole-3-acetatic acid, we suggest that sorghum tolerance to drought is mediated by the higher accumulation of indole-3-acetate in sorghum upon water deficit. This is based on compelling evidence showing that high levels of indole-3-acetate lead to drought tolerance [48]. Such indole-3-acetate-mediated drought tolerance occurs via the activation of genes related to auxin, abscisic acid and jasmonic acid biosynthesis [48].

Sucrose synthase (EC 2.4.1.13) was linked to the starch and sucrose metabolism pathway (ko00500, Table 2 and Figure 3). Sucrose synthase showed a decrease in abundance (1.5- and 2.3-fold) in maize and sorghum, respectively. In plants, sucrose synthase is involved in the hydrolysis of sucrose, leading to the production of UDP-glucose and D-fructose (or ADP-glucose) [49]. A recent study demonstrated that the activity of sucrose synthase was decreased in sorghum when grown under osmotic stress [50]. As organic osmolytes, sucrose or D-fructose have an important role in regulating the osmotic gradient in cells to maintain water status in plants [51]. Interestingly, the decrease in sucrose synthase expression under drought was higher in sorghum than in maize. The higher reduction in sucrose synthase expression in sorghum may be linked to the greater water retention capacity in sorghum than maize under water deficit, thus implying that sucrose synthase activity is only required in cases where water deficit stress is experienced in the plant to necessitate osmotic adjustment through sucrose or D-fructose.

Polyphenol oxidase I (EC 1.10.3.1), which catalyzes the initial reactions in the tyrosine metabolism pathway (ko00350, Table 2 and Figure 3), was upregulated in both plant species in response to water deficit stress. Polyphenol oxidases possess catechol oxidase activity. Even though the expression of polyphenol oxidase I/catechol oxidase increased in both maize and sorghum by 3.0- and 1.9-fold, respectively, it was considerably higher in sorghum under both water treatments (WW and WD). Therefore, its metabolic products are likely more in sorghum than in maize under both water status conditions. Catechol oxidase can regulate the biosynthesis of melanins and other polyphenolic compounds by catalyzing the oxidation of DOPA to DOPA–quinone [52]. The adaptive role of catechol oxidase during plant exposure to drought is not yet well known. However, the evidence reporting that hydrogen peroxide is utilized as a cofactor in the oxidation of DOPA and dopamine during the process of melanogenesis has been presented [53]. These observations were supported by research demonstrating that catechol oxidase has the catalytic activity of catalase [54]. According to these authors, two catechol oxidase isoforms (39 kDa and 40 kDa) from sweet potato (*Ipomoea batatas*) were tested for catalase activity by applying $H_2O_2$ as a substrate. Their results showed that the 39-kDa protein exhibits catalase enzymatic activity, but not the 40-kDa protein. Furthermore, it was proposed that the catalytic mechanism is based on the binding of two molecules of hydrogen peroxide to the active site of the enzyme [54]. Therefore, catechol oxidase can act as a ROS scavenger by detoxifying hydrogen peroxide into $O_2$ and $H_2O$, as catalase does, and/or impart plant stress tolerance through the production of phenolic compounds, which regulate important defense mechanisms in plants against water deficit stress. Furthermore, given that catechol oxidase is a phenol oxidase and the increased activity of phenol oxidase is associated with improved drought tolerance [55], the enhanced drought tolerance in sorghum can be attributed partly to the more pronounced abundance of catechol oxidase observed in sorghum than in maize. Therefore, the greater abundance of catechol oxidase in sorghum under both water conditions possibly contributes to the better ability of sorghum to withstand water deficit than maize.

## 5. Conclusions

In this study, drought stress reduced the RWC of maize leaves but not sorghum leaves. In addition, *Sorghum bicolor* displayed a considerable increase in free proline content in roots and showed better capability to maintain water status than *Zea mays*. This supports

the notion that *Sorghum bicolor* withstands water stress better than *Zea mays*. Importantly, the leaf proteome profiling revealed different response patterns in these two cereal crops. Furthermore, our findings indicate that the better drought tolerance of sorghum than maize involved the regulation of some enzymes, with PTAL, sucrose synthase, indole-3-acetaldehyde oxidase and catechol oxidase being among these enzymes. Proteins with PTAL activity are required for the synthesis of cinnamic acid and p-coumaric acid, and the observed changes in PTAL abundance implied a role of phenolic acids in drought tolerance. As an osmolyte, sucrose plays an important role in plant osmotic regulation, enabling sorghum to retain water better than maize. In short, the higher decrease in sucrose synthase expression in sorghum is possibly related to its ability to maintain water status better than maize under drought. The differential water deficit-induced expression of indole-3-acetaldehyde oxidase may positively contribute towards the growth of sorghum despite the water limitation. Alterations in catechol oxidase, which has catalase activity, could also contribute to efficient scavenging of stress-induced ROS in sorghum compared to maize, and this may involve downstream products of the phenol oxidase-like activity in the catechol oxidase. This study thus identified proteins whose encoding genes could be targeted for the improvement of maize and sorghum tolerance to drought, as represented in the schematic proposed for conferring drought tolerance (Figure 4).

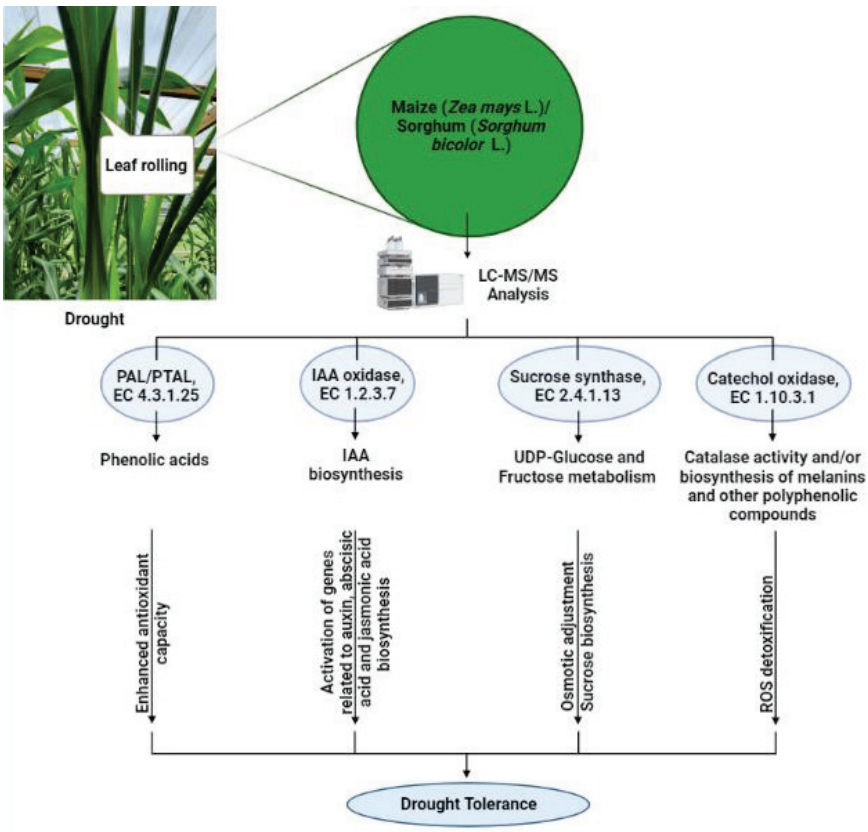


**Figure 4.** Proposed mechanism through which proteins differentially regulated between maize and sorghum under water deficit stress lead to drought tolerance. Drought-induced phenylalanine and tyrosine metabolism leading to phenolic acid biosynthesis via phenylalanine/tyrosine ammonia lyase (PAL/PTAL, i.e., PTAL) enhances antioxidant activity. When activation of indole acetic acid oxidase occurs, auxin biosynthesis is enhanced and can act coordinately with the biosynthesis of abscisic acid and jasmonic acid to regulate plant responses to water deficit, leading to drought tolerance. Furthermore, improved drought tolerance can be achieved by regulation of sucrose synthase to enhance osmotic adjustment through sucrose and D-fructose metabolism, and through catechol oxidase-mediated detoxification of ROS that can be coupled to biosynthesis of melanin-related and other phenolic compounds. BioRender (biorender.com) was used to create the figure.

Such drought tolerance can be achieved through marker assisted selection to select varieties of maize and sorghum with expression profiles of these genes that follow patterns of expression in drought-tolerant genotypes of sorghum, or through altering the expression of these genes in maize and sorghum through genetic engineering to achieve similar patterns of their expression as in drought-tolerant sorghum genotypes. Such biotechnological approaches are important for sustaining maize and sorghum production during drought, which will contribute positively to food security. This is because these crops are critical for food security in Africa and globally, based on their extensive use as food for humans and feed for animals, in addition to their industrial uses (mainly as starch and biofuel). The use of only one sorghum and only one maize genotype in this study limits the number of proteins that can be associated with drought responses in the two species. This limitation is also prohibitive in concluding whether the changes observed in the water deficit-induced differences in protein expression between sorghum and maize are associated with drought tolerance or drought sensitivity. To resolve this limitation, future work will involve the screening of several genetically diverse genotypes of sorghum and maize to include a number of drought-sensitive and drought-tolerant genotypes of both species and subject these diverse genotypes to similar proteomic analysis. This will allow for the identification of regulated proteins based on whether such proteins are upregulated or downregulated in the drought-sensitive or the drought-tolerant genotypes, and thus enable us to distinguish between proteins associated with tolerance from those associated with sensitivity to drought. Despite these limitations, this study clearly shows which subset of proteins and pathways are important in distinguishing the responses of maize from those of sorghum in water deficit conditions.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/life13010170/s1, Table S1: Details of LC–MS/MS data acquisition. Table S2: List of orthologs with different abundances between maize and sorghum. Well-watered (wet), water deficit (dry). Figure S1: SDS-PAGE profiles of *Zea mays* (a) and *Sorghum bicolor* (b) leaf proteins. The proteins (10 μg) were loaded in 12% SDS-PAGE gels, where lane M is the molecular weight marker; protein samples (from five independently obtained replicates) were loaded on lanes 1–10. Well-watered treatments are denoted as WW and water deficit treatments are denoted as WD.

**Author Contributions:** Conceptualization, N.L.; methodology, A.E.E.A., N.L., L.H.H. and D.L.T.; software, D.L.T. and A.E.E.A.; validation, D.L.T., N.L. and A.E.E.A.; formal analysis, D.L.T. and A.E.E.A.; investigation, A.E.E.A., L.H.H., N.L. and D.L.T.; resources, N.L. and D.L.T.; data curation, A.E.E.A., D.L.T. and N.L.; writing—original draft preparation, A.E.E.A.; writing—review and editing, A.E.E.A., L.H.H., N.L. and D.L.T.; visualization, A.E.E.A. and D.L.T.; supervision, N.L., L.H.H. and D.L.T.; project administration, N.L.; funding acquisition, N.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Research Foundation (NRF) of South Africa (Grant Number 87743) and the Department of Science and Innovation/National Research Foundation Centre of Excellence in Food Security (Grant Number 170201).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data are available upon request in addition to data provided in the supplementary material.

**Acknowledgments:** We would like to acknowledge the Centre for Proteomics & Genomics (CPGR) for the LC–MS/MS runs.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Role of Sodium Nitroprusside on Potential Mitigation of Salt Stress in Centaury (*Centaurium erythraea* Rafn) Shoots Grown In Vitro

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**Abstract:** Soil salinity is one of the most common abiotic stressors that affects plant growth and development. The aim of this work was to investigate the influence of sodium nitroprusside (SNP), a donor of nitric oxide (NO), on the physiological response of common centaury (*Centaurium erythraea*) shoots grown under stress conditions caused by sodium chloride (NaCl) in vitro. Centaury shoots were first grown on nutrient medium containing different SNP concentrations (50, 100 and 250 μM) during the pretreatment phase. After three weeks, the shoots were transferred to nutrient media supplemented with NaCl (150 mM) and/or SNP (50, 100 or 250 μM) for one week. The results showed that salinity decreased photosynthetic pigments, total phenolic content and DPPH (1,1-diphenyl-2-picrylhydrazyl radical) concentration. The activities of antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX), were also reduced under salt stress. However, MDA concentration was decreased, while $H_2O_2$ and proline content did not drastically change under the stress conditions caused by NaCl. Exogenous application of SNP altered the biochemical parameters of centaury shoots grown under salt stress. In this case, increased photosynthetic pigment content, total phenolics and proline content were noted, with reduced MDA, but not $H_2O_2$, concentration was observed. In addition, the exogenous application of SNP increased the degree of DPPH reduction as well as SOD, CAT and POX activities.

**Keywords:** common centaury; salinity stress; oxidative stress; antioxidative protection; sodium nitroprusside

**Citation:** Trifunović-Momčilov, M.; Stamenković, N.; Đurić, M.; Milošević, S.; Marković, M.; Giba, Z.; Subotić, A. Role of Sodium Nitroprusside on Potential Mitigation of Salt Stress in Centaury (*Centaurium erythraea* Rafn) Shoots Grown In Vitro. *Life* **2023**, *13*, 154. https://doi.org/10.3390/life13010154

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 6 December 2022
Revised: 28 December 2022
Accepted: 3 January 2023
Published: 5 January 2023



## 1. Introduction

Environmental conditions are rarely ideal and plants are constantly exposed to various types of stress during their life cycle. Stress can be defined as a factor that decreases the rate of physiological processes that negatively affects growth, development and plant productivity [1]. In the context of energy consumption, stress can be observed as a state in which reduced energy production is directed towards stress-defense processes rather than growth and development [2]. In natural conditions, plants are mainly exposed to a combination of different stress-inducing factors that interact with each other and modify their individual effects accordingly. Salt stress is one of the major abiotic factors limiting crop productivity. According to Shrivastava and Kumar [3], more than 50% of lands are affected by salinity, while salinized areas have a tendency to increase by 10% every year. Since almost all food originates from soil, it is more than clear what problem salinization presents to the food supply [4]. In addition to natural salinization, which is the accumulation of dissolved salts in the soil to the levels that interfere with agricultural production and environment, there is also secondary salinization that occurs as a result of anthropogenic influences [5].

Salt stress disrupts plant homeostasis in two ways. First, high concentrations of salt in the soil prevent water uptake by the roots, while the accumulation of salts in plants, primarily $Na^+$ and $Cl^-$ ions, further leads to toxic effects [6]. During the initial phase of defense against salt stress, water deficit and osmotic stress causes a decrease in cell division rate in leaves, root and shoot meristems [7]. Osmotic stress also leads to stomatal closure and reduction in photosynthesis efficiency [8]. The next phase of the plant's defense against salt stress occurs due to accumulation of toxic ions, leading to damage of cell membranes' structure and function, inhibition of enzyme activity and finally, plant productivity [9]. Secondary oxidative stress follows immediately after primary stressors, osmotic stress and ion toxicity. Oxidative stress is a complex chemical and physiological phenomenon that occurs as a result of intensive production and accumulation of reactive oxygen species (ROS) which, due to high reactivity, damage proteins, lipids and nucleic acids [10]. By damaging the lipids, the integrity and functions of membranes deteriorate. These fragmentation products can further damage proteins and nucleic acids, thereby interfering with the normal functioning of receptors, enzymes and membrane channels, resulting in cell death [11]. Accordingly, lipid oxidation, also known as lipid peroxidation, is one of the markers of oxidative stress.

Since prolonged exposure to stress leads to cell death, plants have developed numerous mechanisms that enable growth in different stress conditions. Tolerance to salt stress is a complex phenomenon involving numerous regulatory processes such as stomatal opening, changes in hormonal balance, activation of antioxidant defense systems, osmotic adjustment, maintenance of water balance, export of toxic ions or their compartmentalization in vacuoles [12]. Antioxidant defense mechanisms are divided into two groups, non-enzymatic and enzymatic. Both groups of antioxidants are involved in protecting cellular components from oxidation as well as conversion of ROS into less reactive forms. In addition to ascorbic acid, glutathione, tocopherols, polyamines and phenols, proline and glycine betaine are the most important non-enzymatic components [9]. Proline is one of the essential amino acids, with great importance in protein synthesis. The accumulation of proline in plant cell results after different disturbances to the external environment [13]. In addition, proline is known to regulate the expression of genes important for mitochondrial stability, cell division and cell death [9,14,15]. Several different enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferases (GST), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR), act as part of the plant antioxidant defense system [16]. In addition, SOD is considered one of the major enzymatic systems that scavenges stress-generated free radicals in plants while other enzymes such as CAT and POX, work in close synchrony with SOD to prevent the formation of more harmful ROS through the Haber–Weiss reaction.

Nitric oxide (NO) is a paramagnetic molecule with an unpaired $\pi*$ electron, which can easily diffuse through membranes [17]. Initially, NO was considered an air pollutant that inhibits plant growth and denatures DNA, damages lipids, and decreases intensity of photosynthesis and respiration [18]. Today, however, it is known that NO is an important molecule in redox signaling, participates in the control of numerous physiological processes and plays an important role in establishing resistance to pathogens and regulating the plant's response to abiotic stress [19–21]. Sodium nitroprusside (SNP), a common NO donor, plays diverse roles in plant growth and development. Numerous studies have confirmed the protective role of SNP during salt stress conditions in tomato [22], cucumber [23], orange [24], cotton [25], alfalfa [26], apple [27], wheat [28] and lentil [29] plants.

Centaury (*Centaurium erythraea* Rafn) is medicinal plant that is widely used in traditional medicine as an antidiabetic, antipyretic, antiflatulent and detoxifying agent [30]. Various bioactive compounds isolated from the aerial part of centaury have shown different therapeutic properties [30–36]. Among the species belonging to the *Centaurium* genus, centaury is the plant species to which the greatest attention has been paid during recent years. The first and most important reason is the relatively easy manipulation of this plant species,

which makes it an excellent model system for studying genetic transformation, secondary metabolites and salt stress physiology [37–40]. Moreover, centaury has recently shown to possess great developmental plasticity and the ability to induce somatic embryogenesis in root and leaf cultures [41]. A previous report described the salinity-stress response of centaury shoots and roots grown in vitro [38]. In this work, we investigated whether exogenous application of SNP can alleviate the effects of stress caused by NaCl in centaury shoots grown in vitro.

## 2. Materials and Methods

### *2.1. Plant Material, Culture Conditions and Experimental Design*

Mother stock cultures of centaury plants were used as the primary plant material. The centaury shoots were cultured in vitro, on half-strength MS medium ($\frac{1}{2}$MS, [42]) solidified with 0.7% agar and supplemented with 3% sucrose as well as 100 mg $L^{-1}$ *myo*-inositol. The medium was adjusted to pH 5.8 with NaOH/HCl and autoclaved at 121 °C for 25 min. All in vitro cultures were grown at 25 ± 2 °C and a 16/8 h light/dark photoperiod ("Tesla" white fluorescent lamps, 65 W, 4500 K; light flux of 47 µmol $s^{-1}$ $m^{-2}$). During the three-week long pretreatment, the centaury shoots were first placed on four types of $\frac{1}{2}$MS nutrient media containing different SNP concentrations (0, 50, 100 or 250 µM). After pretreatment, the centaury shoots were transferred to fresh $\frac{1}{2}$MS nutrient media supplemented with NaCl (0 or 150 mM) and/or SNP (0, 50, 100 or 250 µM) and cultured for one week (Figure 1). All experiments were repeated three times.

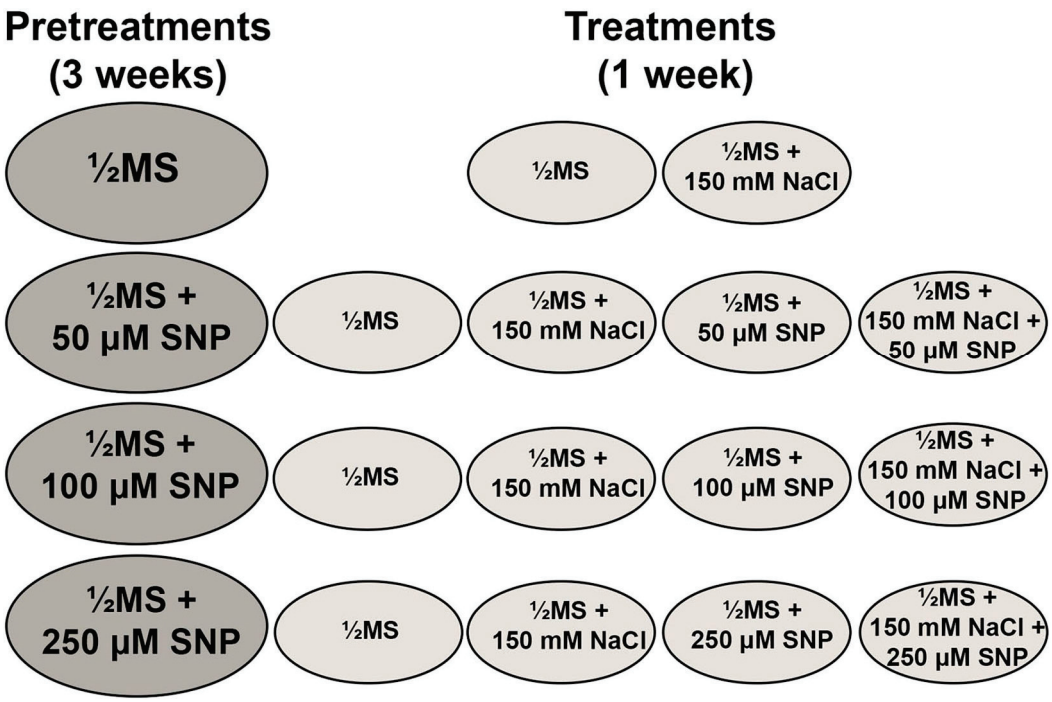


**Figure 1.** Schematic of experimental design including different SNP pretreatments and NaCl and/or SNP treatments.

### *2.2. Quantification of Photosynthetic Pigments*

Isolation of total chlorophyll (*Chl*) and carotenoids was accomplished from the leaves collected from the bottom part of the centaury rosette after four weeks of cultivation. Total *Chl* and carotenoid content were extracted using 96% ethanol as proposed by Lichtenthaler [43] and previously described in detail by Trifunović-Momčilov et al. [39]. The absorbance of the photosynthetic pigments was measured using a UV–visible spectrophotometer (Agilent 8453, Life Sciences, Santa Clara, CA, USA).

### *2.3. Estimation of Oxidative Stress Biomarkers*

The level of lipid peroxidation was measured as malondialdehyde (MDA) concentration by the procedure described by Heath and Packer [44], while $H_2O_2$ concentration was determined as described by Velikova et al. [45]. In both assays, 0.1% trichloroacetic acid was used and detailed protocols were previously described by Trifunović-Momčilov et al. [38]. The spectrophotometric determination of MDA and $H_2O_2$ were measured using an ELISA Micro Plate Reader (LKB 5060–006, Winooski, VT, USA).

### *2.4. Estimation of Nonenzymatic Antioxidants*

Free proline content was determined by the ninhydrin reaction which consists of the reaction of proline and ninhydrin reagent (2,2-dihydroxyindane-1,3-dione) resulting in a yellow reaction product [46]. Proline extraction and measurement was performed according to a modified method by Carillo and Gibon [47] and described in detail by Trifunović-Momčilov et al. [38].

Total polyphenol content was determined using the Folin–Ciocalteu test (FC test) based on reaction of polyphenols from plant tissues and Folin–Ciocalteu reagents forming a blue-colored complex that can be spectrophotometrically quantified. This method was previously described by Singleton et al. [48]. The plant material (200 mg) was homogenized in liquid nitrogen and extracted with 96% ethanol. The homogenate was incubated for 60 min at room temperature and then centrifuged for 15 min. The supernatant was further mixed with the FC reagent solution, which was previously prepared by adding distilled water to the FC reagents in a volume ratio of 2:1. The reaction mixture was quickly vortexed and 20% $Na_2CO_3$ was added. After 90 min at room temperature in darkness, the absorbance was measured at 765 nm. In this assay, gallic acid was used as a phenol standard.

The antioxidant activity in the centaury shoots was determined after evaluation of stable DPPH radical concentrations. The samples were prepared using the same method as the FC test. In the reaction with antioxidants, the DPPH radical is converted to a non-radical form through reduction by hydrogen ions. After homogenization and centrifugation of the supernatant, methanol and DPPH reagent solution were added. The reaction mixture was incubated at room temperature in the dark. After 60 min, the degree of reduction of the DPPH radical was estimated through an absorbance measurement at 520 nm. The scavenging capacity of the DPPH radical was calculated using the following equation: $(\%) = [1 - (A1 - A0)] \times 100$ where A1 is the absorbance of the sample and A0 is the absorbance of the blank reaction.

For the spectrophotometric determination of all nonenzymatic antioxidants, an ELISA Micro Plate Reader (LKB 5060–006, Winooski, VT, USA) was used.

### *2.5. Estimation of Enzymatic Antioxidants*

Centaury shoots were homogenized in potassium phosphate extraction buffer containing insoluble polyvinylpolypyrrolidone, dithiothreitol and phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 4 °C for 5 min and the protein content was determined from the supernatant according to Bradford [49] using bovine serum albumin as the standard. The quantification of SOD, CAT and POX was also performed.

SOD activity was determined spectrophotometrically using a modified method from Beyer and Fridowich [50]. The reaction mixture contained potassium phosphate buffer, ethylenediaminetetraacetic acid, methionine, nitroblue tetrazolium chloride (NBT) and riboflavin. The reaction mixtures were added to the samples, which were then illuminated for 1–2 min and the absorbance was measured at 540 nm. One unit of SOD activity is the amount of sample required for 50% inhibition of NBT photoreduction and is presented as the specific activity (U/mg). SOD activity was spectrophotometrically detected using an ELISA Micro Plate Reader (LKB 5060–006, Winooski, VT, USA).

CAT activity was determined spectrophotometrically using the method from Aebi [51]. This method is based on monitoring the kinetics of the consumption of $H_2O_2$, which can be detected by measuring the absorbance (at 240 nm) of the reaction mixture consisting

potassium phosphate buffer, $H_2O_2$ and enzyme extract. One unit of CAT activity is defined as the amount of enzyme required to degrade 1 μM of $H_2O_2$ in 1 min and is indicated as μM $min^{-1}$ mg $protein^{-1}$ (U/mg protein).

POX activity was determined spectrophotometrically using the method from Kukavica and Veljović-Jovanović [52]. The reaction mixture contained potassium phosphate buffer and pyrogallol as the enzyme substrate. The POX-catalyzed oxidation of pyrogallol to purpurogallin in the presence of $H_2O_2$ was monitored by absorbance determination at 430 nm. Enzyme activity is indicated as μM $min^{-1}$ mg $protein^{-1}$ (U/mg protein). The absorbances of the CAT and POX reactions were measured with a UV–visible spectrophotometer (Agilent 8453, Life Sciences, USA).

### *2.6. Statistical Analysis*

The effect of different SNP pretreatments/treatments on the biochemical parameters of centaury shoots, after four weeks of culture, were evaluated using standard two-factor analysis of variance (ANOVA). All analysed parameters were measured using three biological samples per treatment. In addition, the absorbances of all supernatants were measured in triplicate for each sample. The results are presented as mean ± SE. The comparisons between the mean values were made using a Fisher LSD (the least significant difference) post-hoc test, calculated at a confidence level of $p \leq 0.05$.

## 3. Results

### *3.1. The Effect of SNP on Photosynthetic Pigments Content during Salt Stress in C. erythreae Shoots*

The centaury shoots successfully survived four weeks on $\frac{1}{2}$MS media supplemented with different combinations of SNP (0, 50, 100 or 250 μM) and/or NaCl (150 mM). Control centaury shoots grown on NaCl-free medium developed the usual rosette morphology and dark green oval leaves (Figure 2). Pretreatments with 50 and 100 μM SNP altered the color of the leaves to light green. Pretreatment with 250 μM SNP caused leaf tip curling and desiccation as well as yellowing of the leaves and chlorosis of the entire shoot. After this pretreatment, and especially in combination with SNP, and NaCl, the highest number of yellow leaves was observed. Unlike other pretreatments/treatments, only after the pretreatment with 250 μM SNP, most centaury shoots did not spontaneously develop roots.

Leaf chlorosis is one of the most common symptoms of stress caused by NaCl due to decreased photosynthetic pigments and is also an important indicator of the physiological state of the plants. Therefore, the content of photosynthetic pigments was determined in two control groups of shoots that were grown on $\frac{1}{2}$MS NaCl-free medium throughout the whole experimental period, then in medium supplemented with 150 mM NaCl, as well as in shoots grown on different SNP pretreatments and NaCl and/or SNP treatments. In the second control group of centaury shoots not exposed to SNP during the pretreatment, NaCl decreased total *Chl* content ~21% in comparison to the first control group grown on $\frac{1}{2}$MS NaCl-free medium (Figure 3a). In addition, pretreatment with 50 μM SNP significantly decreased the total *Chl* in shoots grown on NaCl-free medium in comparison to the control group of shoots grown on the same medium. Conversely, the combination of 50 μM SNP pretreatment and then treatments with NaCl and 50 μM SNP, increased total *Chl* content ~20% in comparison to the control group of shoots grown on NaCl-supplemented medium, as well as in comparison to the control shoots from the appropriate treatment. Pretreatment with 50 μM SNP in combination with treatment including NaCl and 50 μM SNP together, also reduced total *Chl* content to the lowest level in this experimental group. The application of 100 μM SNP in the pretreatment, did not lead to significant changes in total *Chl* content in comparison to the control group centaury shoots grown on medium with NaCl. The 250 μM SNP pretreatment did not show any positive effects, and decreased total *Chl* content in comparison to the control group of shoots grown on medium with NaCl. It was interesting to note that the lowest total *Chl* content was detected in centaury shoots exposed to treatments including both NaCl and SNP after the appropriate SNP

pretreatments. It was also found that increased SNP concentrations in the pretreatment were negatively correlated with decreased total *Chl* content after the corresponding SNP and NaCl treatments.

The effect of the different SNP pretreatments and NaCl and/or SNP treatments on total carotenoid content is shown in Figure 3b. In the control groups of century shoots, NaCl decreased the total carotenoid content 27% in comparison to the control group of shoots grown on NaCl-free medium. Pretreatment with 50 µM SNP halved the total carotenoid content in shoots grown on NaCl-free medium in comparison to the control group of shoots grown on the same medium. Conversely, pretreatments with 150 and 250 µM SNP did not significantly change the total carotenoid content in comparison to the control group of shoots grown on NaCl-free medium. In shoots grown on medium supplemented with NaCl, pretreatments with 100 and 250 µM SNP increased the total carotenoid content 31 and 52%, respectively, in comparison to control group of shoots grown on the same medium. In addition, in comparison to the control group, the application of 100 and 250 µM SNP as pretreatments in combination with the same SNP concentrations in the treatments, influenced a significant increase in total carotenoid content. Furthermore, pretreatments with 100 and 250 µM SNP, followed by treatments with the same SNP concentrations and NaCl together, resulted in a significant increase in total carotenoid content in comparison to the control group of shoots grown on medium supplemented with NaCl.

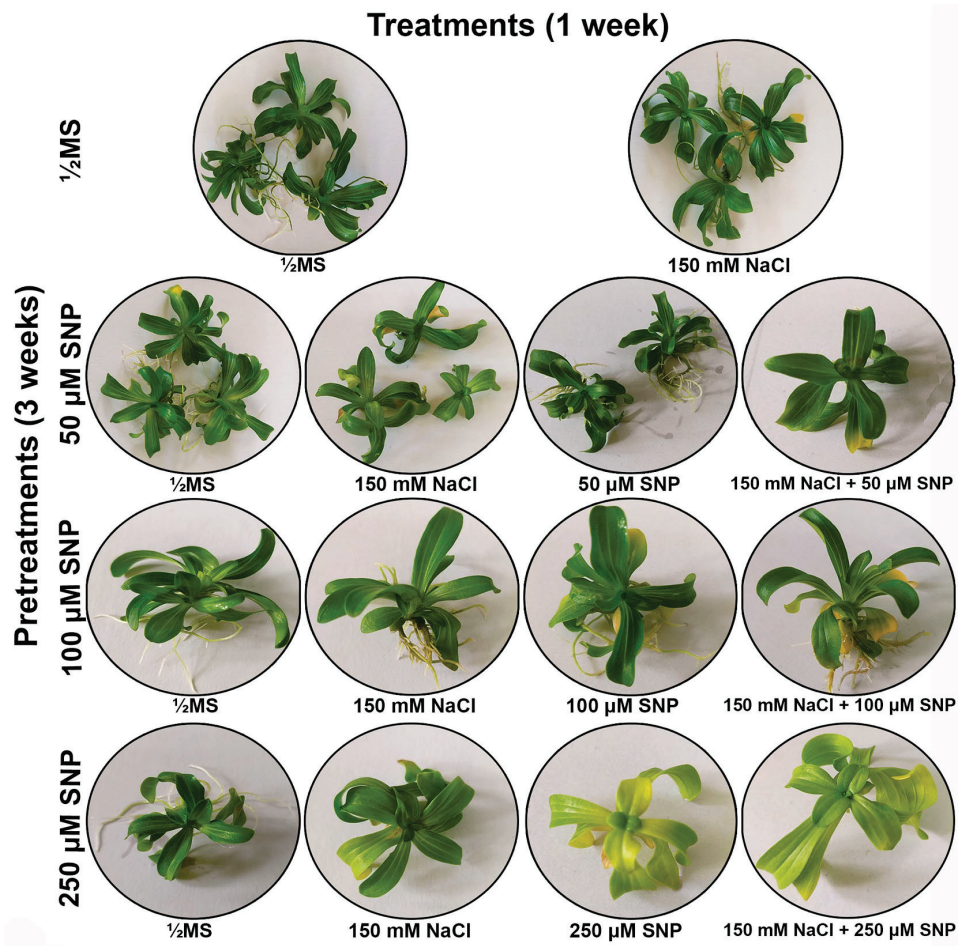


**Figure 2.** *Centaurium erythraea* shoots after four weeks of cultivation on different SNP pretreatments, NaCl and/or SNP treatments.

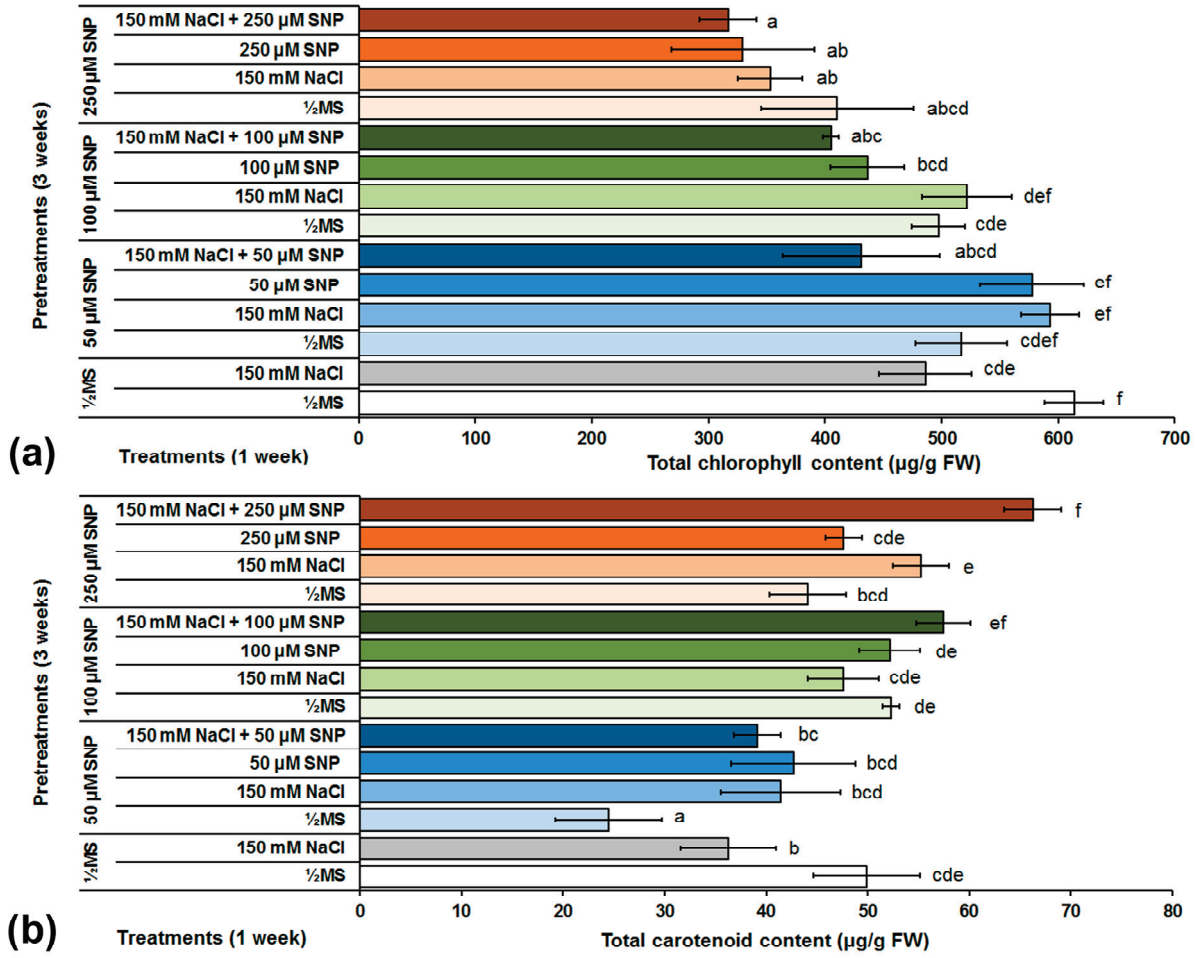


**Figure 3.** The effect of different SNP pretreatments and NaCl and/or SNP treatments on total chlorophyll (**a**) and carotenoid (**b**) content in *C. erythraea* shoots. Data represent mean ± standard error. Bars marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$).

### *3.2. The Effect of SNP on Oxidative Stress Biomarkers during Salt Stress in C. erythreae Shoots*

The effect of different SNP pretreatments and NaCl and/or SNP treatments on level of lipid peroxidation in centaury shoots was determined by monitoring the MDA concentration (Figure 4a). In the control group cultured on medium supplemented with NaCl, a decrease in MDA concentration (15%) was observed in comparison to the control group grown on NaCl-free medium. All SNP pretreatments significantly reduced MDA concentrations in the centaury shoots grown on NaCl-supplemented medium, especially the 50 and 250 µM SNP pretreatments, where the MDA concentrations were reduced to 56 and 52%, respectively, in comparison to the control group grown on NaCl. Treatments with 50 and 250 µM SNP decreased MDA concentration, while 100 µM SNP did not significantly change the MDA concentration in comparison to both control groups. A significant increase in lipid peroxidation was observed after treatments with a combination of 50 or 100 µM SNP with 150 mM NaCl. In addition, the highest degree of lipid peroxidation, compared to all treatments tested, was detected after the treatment using 250 µM SNP and 150 mM NaCl.

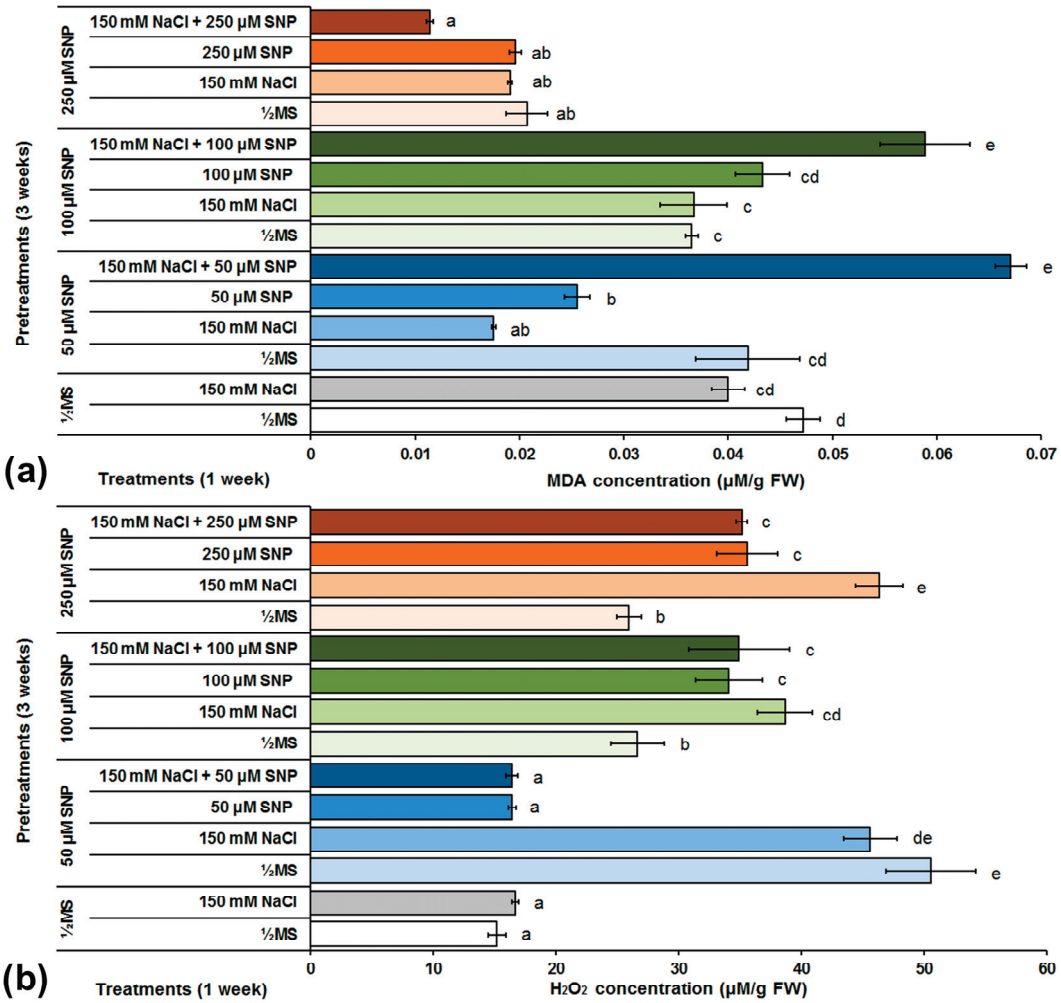


**Figure 4.** The effect of different SNP pretreatments and NaCl and/or SNP treatments on MDA (**a**) and $H_2O_2$ (**b**) concentrations in *C. erythraea* shoots. Data represent mean ± standard error. Bars marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$).

Since lipid peroxidation is one of the consequences of oxidative stress, $H_2O_2$ concentration was also determined as a marker of the degree of plant cell oxidative damage (Figure 4b). The two control groups had approximately the same $H_2O_2$ concentrations. Pretreatments with 50, 100 or 250 SNP concentrations increased $H_2O_2$ in shoots grown on NaCl-free medium by about 233, 75 and 71%, respectively, in comparison to the control centaury shoots grown on NaCl-free medium and by about 173, 131 and 177%, respectively, in comparison to control shoots grown on NaCl medium. Treatment with 50 µM SNP did not significantly change the $H_2O_2$ concentration in comparison to both control groups. Conversely, treatments with 100 and 250 µM SNP significantly increased $H_2O_2$ concentration in comparison to both control groups. The same pattern was also observed in all SNP treatments in combination with NaCl.

### *3.3. The Effect of SNP on Nonenzymatic Antioxidants during Salt Stress in C. erythreae Shoots*

The centaury control shoots grown under unstressed and NaCl-stressed conditions in vitro has similar free proline contents (Figure 5). After pretreatment with 50, 100 or 250 µM SNP, increased proline content (38, 50 and 52%, respectively) was observed in shoots grown on $\frac{1}{2}$MS nutrient medium in comparison to the control group of shoots grown on the same medium. Only pretreatment with 50 µM SNP resulted in a significant increase in proline content (32%) after NaCl treatment in comparison to the control group of centaury shoots grown on medium supplemented with NaCl. Increased SNP concen-

trations, using the same concentration in pretreatments and in following treatments, was positively correlated with increased proline content in comparison to both control groups. However, treatments with all SNP concentrations showed lower levels of proline content in comparison to the corresponding treatments control. On the other hand, pretreatments with 50 and 100 µM SNP followed by treatments with the same SNP concentrations and NaCl together, decreased proline content to the control values of stressed shoots, while the lowest proline content, lower than in both control groups, was detected in centaury shoots grown on treatment with 250 µM SNP and NaCl together.

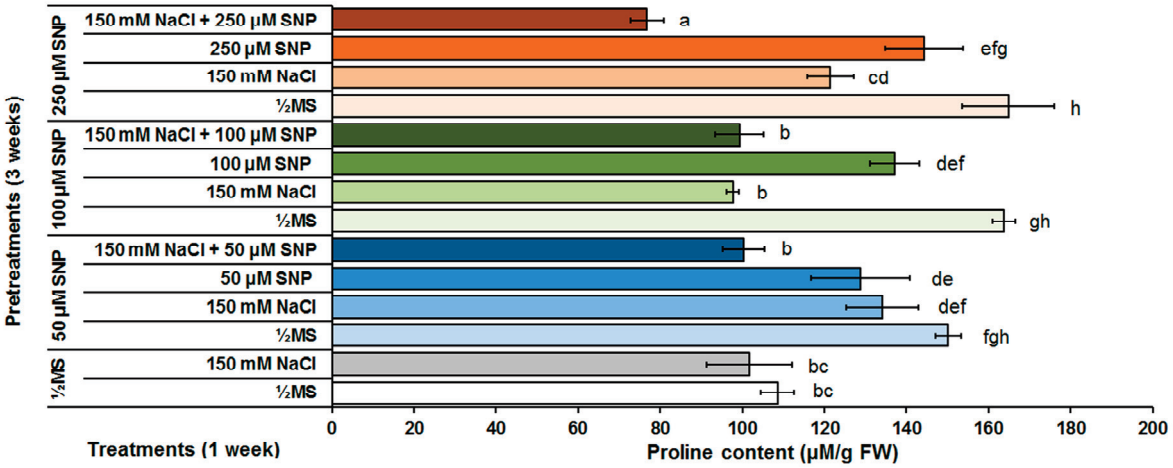


**Figure 5.** The effect of different SNP pretreatments and NaCl and/or SNP treatments on proline content in *C. erythraea* shoots. Data represent mean ± standard error. Bars marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$).

The amount of total phenolic compounds in centaury shoots exposed to different SNP pretreatments and/or treatments was determined (Figure 6a). In the control centaury shoots grown on NaCl-free medium, similar total phenolic content was detected, in comparison to shoots grown on NaCl-supplemented medium. Pretreatments with 50 and 100 µM SNP in NaCl-free medium did not significantly change the amount of total phenolic content in comparison to the corresponding control group, while pretreatment with 250 µM SNP increased the amount of total polyphenols by about 23%. Conversely, all applied SNP pretreatments (50, 100 and 250 µM) caused significant increase in the total phenolic content (29, 69 and 82%, respectively) in shoots grown on medium supplemented with NaCl in comparison to control shoots grown on the same medium. In addition, the application of all SNP concentrations in the pretreatments and treatments, increased the total phenol content in comparison to control shoots grown on NaCl, but these levels still did not exceed the values recorded in control shoots grown on NaCl-free medium. The same pattern was observed after all treatments that included the combinations of 50 or 100 µM SNP and NaCl. The only exception was the combination of 250 µM SNP and NaCl, where an increase of about 26% was observed in comparison to control shoots grown on $\frac{1}{2}$MS medium.

The influence of the different SNP pretreatments and NaCl and/or SNP treatments on the antioxidant capacity of centaury shoots is presented on Figure 6b. In control conditions, the addition of NaCl decreased the DPPH concentration by 28% in comparison to shoots grown on $\frac{1}{2}$MS medium. In comparison to control shoots grown on NaCl-free medium, pretreatments with 50 and 100 µM SNP did not significantly change DPPH concentrations while pretreatment with 250 µM SNP significantly increased DPPH in shoots grown on the same medium. Under the conditions of salt stress caused by NaCl, pretreatments with all SNP concentrations (50, 100 and 250 µM) shown an increase in the degree of DPPH reduction by 11, 17 and 31%, respectively, in comparison to the corresponding control group. Treatments with 50 and 100 µM SNP did not significantly alter DPPH concentrations and both values were similar to control shoots grown on $\frac{1}{2}$MS and NaCl-free

medium, respectively. Only treatment with 250 µM SNP, significantly increased DPPH concentration in comparison to both control groups, but still at the level of control shoots within the same treatment. Using the combination treatments containing NaCl and 50 or 100 µM SNP, an increased DPPH was detected in comparison to control shoots grown on NaCl, but DPPH concentration was not changed in comparison to the second group of shoots grown on $\frac{1}{2}$MS medium. Among all treatments tested, the most significant degree of DPPH reduction, in comparison to both control groups, was recorded in shoots grown on media supplemented with NaCl and 250 µM SNP.

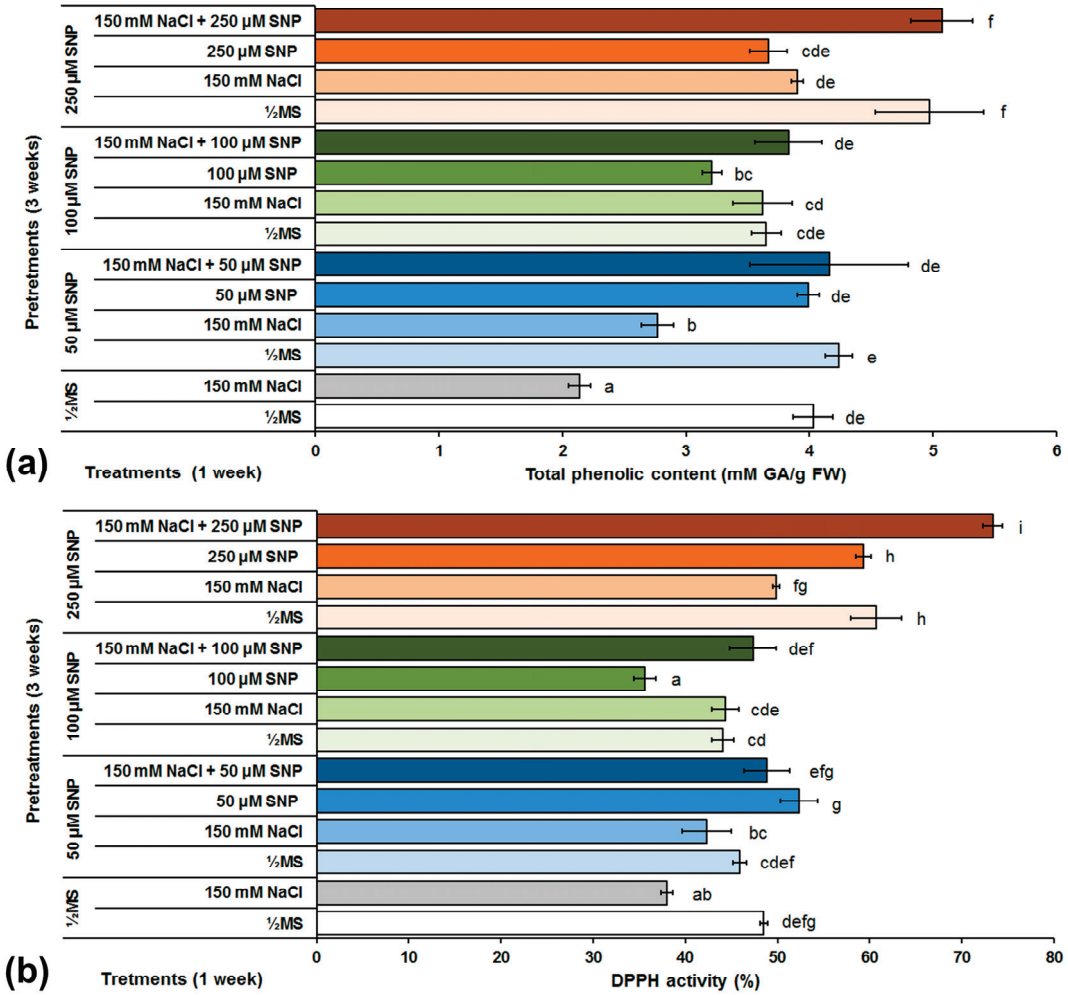


**Figure 6.** The effect of different SNP pretreatments and NaCl and/or SNP treatments on total phenolic content (**a**) and DPPH concentration (**b**) in *C. erythraea* shoots. Data represent mean ± standard error. Bars marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$).

### *3.4. The Effect of SNP on Enzymatic Antioxidants during Salt Stress in C. erythreae Shoots*

In the control groups of shoots grown in the presence of NaCl, SOD activity was decreased by about 18% in comparison to control shoots grown on NaCl-free medium (Figure 7a). In shoots grown on $\frac{1}{2}$MS medium, the 50 and 100 µM SNP pretreatment increased SOD activity by about 34 and 24%, respectively, while pretreatment with 250 µM SNP did not significantly changed SOD activity in comparison to control shoots grown on the same medium. In shoots grown on medium supplemented with NaCl and previously pretreated with 50, 100 and 250 µM SNP, the same pattern was observed. SOD activity was increased by 88 and 71% after the application of 50 and 100 µM SNP, respectively, while after 250 µM SNP treatment, SOD activity was similar to control shoots. The application of

the SNP treatments caused an increase in SOD activity in comparison to both control groups. However, it was interesting to note that the increasing SNP concentrations were inversely correlated with increasing SOD activity. The highest SOD activity among all the treatment combinations was recorded in shoots grown on NaCl and 100 µM SNP. By increasing the SNP concentration to 250 µM along with the NaCl treatment, the SOD activity decreased to the control level of the corresponding treatment and the control shoots grown on $\frac{1}{2}$MS or medium supplemented with NaCl.

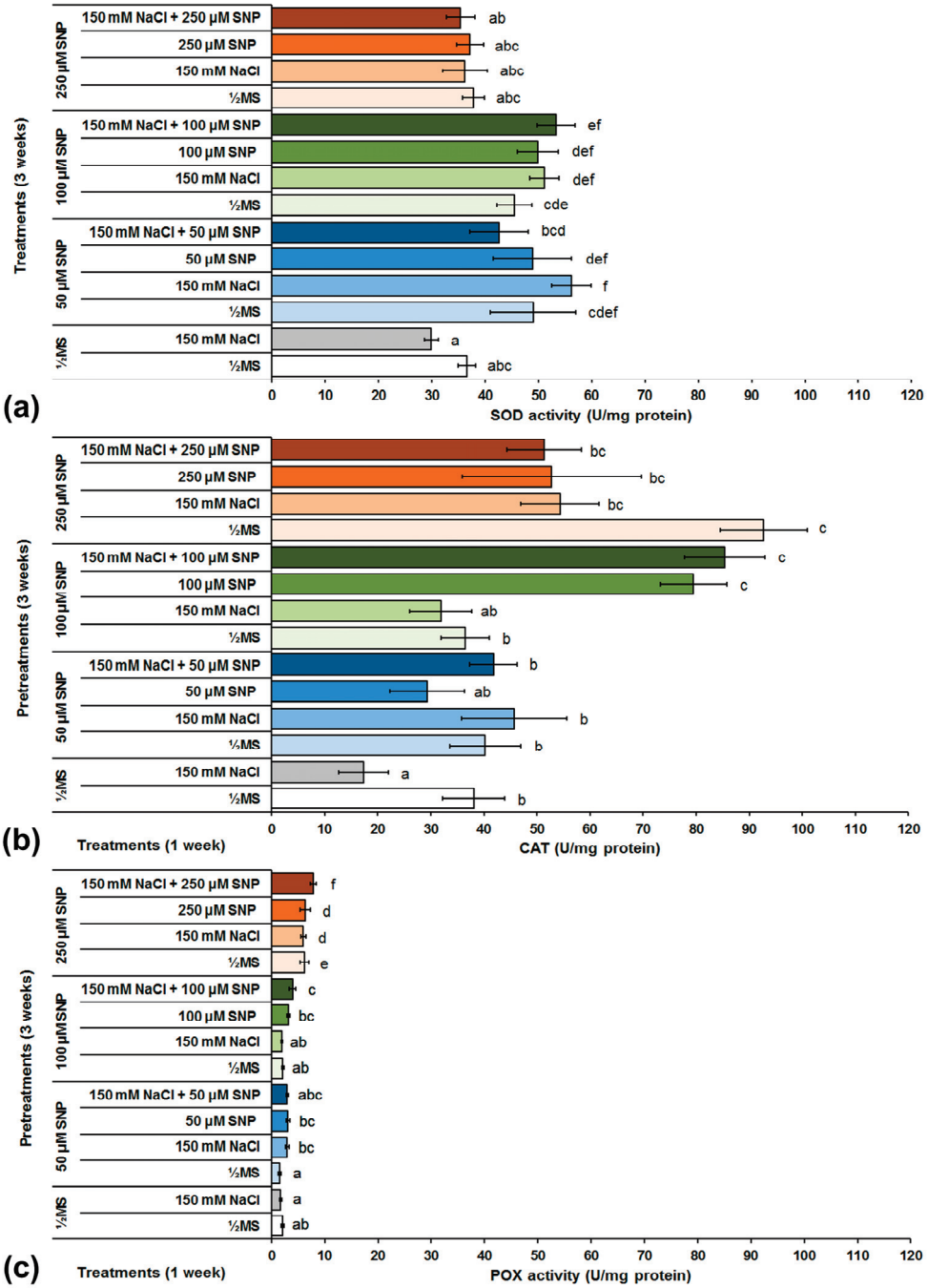


**Figure 7.** The effect of different SNP pretreatments and NaCl and/or SNP treatments on SOD (**a**), CAT (**b**) and POX (**c**) activities in *C. erythraea* shoots. Data represent mean ± standard error. Bars marked with a different letter are significantly different from the control according to the LSD test ($p \leq 0.05$).

Similar to the SOD activity, in control conditions, CAT activity was also decreased (by about 55%) in shoots grown on NaCl medium (Figure 7b). Pretreatment with 50 μM SNP did not significantly change CAT activity in comparison to both control groups. Treatment with 100 μM SNP, individually or together with NaCl, significantly increased CAT activity. In the shoots grown on NaCl-free medium, a significant increase in CAT activity was recorded only after 250 μM SNP pretreatment. At the same time, this is the highest recorded CAT activity in the centaury shoots after all applied treatments, and represents an increase of 143% in comparison to control shoots grown on $\frac{1}{2}$MS medium.

Similar to SOD and CAT activities in the control groups, POX activity was also decreased (by approximately 17%) in shoots grown on NaCl medium (Figure 7c). No significant changes in POX activity were observed in shoots pretreated with 50 or 100 μM SNP and grown on both MS and NaCl-free media. Pretreatments with 50 or 100 μM SNP and furtherr culture on media with the same SNP concentrations and NaCl together, increased POX activity). A significant increase in POX activity was observed after pretreatment with 250 μM SNP and all further treatments. Thus, POX activity was tripled in centaury shoots pretreated with 250 μM SNP and further grown on NaCl-free medium, in comparison to control shoots grown on the same medium. The same pattern was also observed in shoots grown on NaCl and control shoots grown on the same medium but not treated with SNP. Similar POX activity changes were detected in shoots treated only with 250 μM SNP. In comparison to all applied treatments, the highest POX activity was recorded in centaury shoots grown on medium supplemented with 250 μM SNP and NaCl together.

## 4. Discussion

Although in nature, centaury inhabits mountain slopes, dry grasslands, scrublands and saline soils, investigations of centaury's response to stressful conditions in vitro are still at the beginning stages. The role of the widely used NO donor, SNP, on plant tolerance to salt stress conditions is usually demonstrated after foliar treatment or using nanoparticles [53]. In this work, the effect of exogenously applied SNP, alone or in combination with NaCl, on several biochemical parameters of centaury shoots grown in vitro was investigated.

### *4.1. SNP and Photosynthetic Pigments during Salt Stress in C. erythreae*

Due the importance of photosynthesis, as a key physiological process in plants, the effect of different SNP pretreatments and NaCl and/or SNP treatments on the concentration of photosynthetic pigments of centaury was determined. This work demonstrated that total *Chl* content was significantly decreased in control shoots grown on NaCl in comparison to the other control group of shoots grown on $\frac{1}{2}$MS medium (Figure 3a). These results are in accordance with the results previously obtained in centaury shoots grown during NaCl-caused salt stress in vitro [37,39]. The lowest SNP concentration applied at pretreatment (50 μM) shown a positive effect on total *Chl* content in centaury leaves during salt stress. Conversely, the highest SNP concentration (250 μM) decreased total *Chl* content to levels lower than the control group of shoots grown on NaCl. These results could be expected because in addition to oxidative stress, centaury shoots were also exposed to higher intensity of nitrosative stress. The positive effect of SNP on total *Chl* content under stress conditions caused by NaCl was also confirmed in cotton, red raspberry, barley, sunflower and wheat [25,28,54–56]. It was interesting to note that SNP pretreatments did not increase total *Chl* content in comparison to the control group of shoots grown on NaCl-free medium. However, some reports showed that SNP treatment increased total *Chl* content in cotton and raspberry plants grown under salt stress in comparison to control conditions [25,54]. In summary, it can be assumed that a lower SNP concentration had a positive effect on *Chl* preservation by promoting the synthesis, regeneration and/or inhibiting its degradation but also promoting the mechanisms that remove ROS, and the ability of SNP to improve the $K^+/Na^+$ ratio [25,27].

The results presented in this work showed that NaCl had negative effect on total carotenoid content in centaury shoots grown in control conditions (Figure 3b). De-

creased carotenoid content was also recently reported in centaury shoots under salt stress in vitro [39]. In centaury shoots treated with NaCl, the application of SNP pretreatments resulted in increased total carotenoid content in comparison to control group of shoots grown on medium also supplemented with NaCl. The highest total carotenoid content was observed after treatment with 250 μM SNP and NaCl together. These findings, describing the positive effect of SNP on carotenoid content, correspond with published results from cotton, red raspberry and sunflower plants [25,54,56]. It is quite possible that carotenoids, as non-enzymatic antioxidants, prevent or minimize the oxidative damage induced by NaCl. The latest research proposed increased carotenoid content as a marker of salt tolerance [57]. Accordingly, it can be concluded that SNP increased centaury's tolerance to salt stress.

### *4.2. SNP and Oxidative Stress Biomarkers during Salt Stress in C. erythreae*

In control conditions, a decrease in MDA content was observed in centaury shoots during salt stress in comparison to shoots grown on NaCl-free medium (Figure 4a). This result is unexpected because, theoretically, exposure to salt stress should increase the degree of lipid peroxidation. It is possible that the duration and/or level of stress intensity were not sufficient. However, similar results were also recorded in the halophyte species *Prosopis strombulifera* and *Salvadora persica*, as well as in soybean and a salt-tolerant cultivar of date palm, where no significant changes in MDA content under NaCl-induced stress was detected [58–61]. Pretreatments with 50 and 250 μM SNP decreased MDA content in NaCl-treated centaury shoots in comparison to both control groups. The effect of SNP on the reduction of MDA content was also shown in other plant species such as cotton, wheat, apple and lentil [25,27–29]. The most interesting results, in terms of lipid peroxidation, were obtained in centaury shoots treated with SNP and NaCl together. The highest MDA content was obtained with the application of 50 μM SNP and NaCl whereas the lowest recorded rate of lipid peroxidation was obtained with the application of 250 μM SNP and NaCl. According to certain studies, SNP application can reduce the activity of lipoxygenases and thereby reduce the degree of lipid peroxidation. In addition, NO has the ability to remove peroxyl radical and prevent further oxidative damage [62,63]. However, at low concentrations, NO, together with $O_2^{\bullet -}$, forms peroxynitrite, which has the ability to initiate lipid peroxidation [17,53].

In control conditions, salt stress caused a slight increase in $H_2O_2$ content in comparison to shoots grown on NaCl-free medium (Figure 4b). On the same media, pretreatments with all SNP concentrations induced significant $H_2O_2$ production in centaury shoots, with higher $H_2O_2$ content after salt stress. This result can be explained by considering $H_2O_2$ not only as oxidative stress marker, but also as a signaling molecule that is important for the establishment of salinity tolerance [64,65]. The application of all SNP concentrations, alone or in combination with NaCl, reduced $H_2O_2$ content in centaury shoots after all tested treatments. This reduction may be responsible for the induction of antioxidant defense system to scavenge $H_2O_2$. These results are in accordance with SNP application reducing the $H_2O_2$ content in cucumber, lettuce, wheat, brown mustard and lentil [23,28,29,66,67].

### *4.3. SNP and Nonenzymatic Antioxidants during Salt Stress in C. erythreae*

The accumulation of endogenous proline content under salinity conditions can be considered as a marker of plant stress tolerance [14]. Increased proline content during exposure to NaCl-induced stress has been documented in numerous plant species including centaury [38]. The results obtained during this investigation showed that the application of all SNP pretreatments increased the proline content in centaury shoots grown on NaCl, similar to those grown on $\frac{1}{2}$MS medium, in comparison to both control groups (Figure 5). In addition, it was noted that all SNP concentrations in treatments were positively correlated with increased proline content. It is obvious that SNP alone, as a potential stressogenic factor, further induced proline accumulation in centaury shoots, likely with enhanced activity of proline-synthesizing enzymes, together with a reduction in proline catabolism

under stress conditions [68]. On the contrary, pretreatments including combinations of SNP and NaCl together, reduced free proline content in centaury shoots. Many studies indicated that NO is involved in proline metabolism during stress conditions but the detected effects were different. Some reports revealed increased proline content in SNP-treated *Lactuca sativa* [66], *Pisum sativum* [69] and *Brassica chinensis* [70] under saline stress. Conversely, reduced proline content, as a consequence of SNP pretreatment, was detected in cucumber [23] and *Brassica rapa* [71] under salt stress. All of these results imply that enhanced proline content is not always essential for plant stress tolerance response because the accumulation of this osmolyte does not always correlate with better plant responses, as in case of NaCl-treated centaury shoots. In addition, considering that synthesis of different osmolytes is an "energetically expensive" process, it is possible that centaury activates other mechanisms with lower energy demands, for example, efficient ions compartmentalization to achieve salinity tolerance [72].

Phenolic compounds belong to the group of secondary metabolites that participate in numerous physiological processes in plants; one of those roles is ROS scavenging under various environmental stresses [73]. Although in most plant species total phenolic content increased under high salinity, there are reports describing decreased phenol content in *Phaseolus vulgaris* and *Schizonepeta tenuifolia* grown under salt stress conditions [74,75]. The same result was observed in NaCl-treated centaury shoots (Figure 6a). The effect of SNP on the total phenolic content increase under NaCl stress conditions was previously documented in mangrove species *Aegiceras corniculatum*, wheat, sunflower, and apple [27,56,76,77]. A similar result was detected in centaury shoots pretreated with SNP and then grown on $\frac{1}{2}$MS medium or medium supplemented with NaCl. Treatments with all SNP concentrations also increased total phenolic content, while the highest increment among all the treatments was recorded in centaury shoots after combination treatments with all SNP concentrations and NaCl together. During abiotic stress, NO can increase the activity of phenylalanine ammonia-lyase (PAL) and consequently enhance phenolic compounds biosynthesis [17]. The increased activity of the PAL enzyme could be the reason for the increased total phenolic content in centaury shoots after exposure to SNP.

Due to its ability to react with antioxidants, the DPPH radical is a good indicator of the antioxidant capacity of plants [78]. The results obtained in this work showed that, in control conditions, centaury shoots grown on a medium supplemented with NaCl had decreased antioxidant capacity in comparison to shoots grown on a NaCl-free medium (Figure 6b). This result is in accordance with the previous reports where decreased DPPH concentration under NaCl-induced stress in cucumber, sage, spinach, henbane and flax was described [23,79–82]. In order to investigate the changes in centaury antioxidant capacity, the influence of SNP pretreatments on DPPH concentration was tested. The results showed that, in general, all SNP pretreatments increased DPPH concentration in centaury shoots. The largest DPPH concentration was detected in shoots grown on a combination medium supplemented with SNP and NaCl together. These changes in DPPH concentrations, based on their free radical scavenging capacities, positively correlated with total phenolic content in centaury shoots. Furthermore, in several medicinal herbs and selected species of wild vegetables, total phenolic amounts were also significantly correlated with antioxidant capacity [83,84].

### *4.4. SNP and Enzymatic Antioxidants during Salt Stress in C. erythreae*

Various stress conditions can induce ROS production, which leads to a change in enzyme activity in order to maintain homeostasis in plant cells. Antioxidant enzymes that play a significant role in removing ROS forms and protecting plant cell structures from oxidative stress, include SOD, CAT and POX [16]. Increased SOD, CAT and POX activities under NaCl stress have been documented in many species including sunflower and oilseed rape [56,64]. In this work, decreased activities of SOD, CAT and POX were observed in centaury shoots grown under stress conditions caused by NaCl. Although unexpected, the same results were also reported in halophytic species *Salvadora persica*,

date palm and the oil-seed crop *Brassica juncea* [60,61,67]. The positive effect of SNP on the activity of SOD, CAT and POX was previously confirmed in citrus seedlings, wheat and lentil under salinity stress [28,29,85]. The application of SNP increased SOD activity in centaury shoots grown under NaCl, as well as in shoots grown on NaCl-free medium in comparison to the corresponding control groups (Figure 7a). The highest SOD activity was recorded after 50 μM SNP pretreatment while increased SNP concentration decreased SOD activity in centaury shoots. The same trend was also observed in cotton seedlings [25]. The application of SNP pretreatments also increased CAT activity in centaury shoots grown under NaCl-induced stress conditions as well as in shoots grown on nutrient media without NaCl (Figure 7b). The highest CAT activity was determined after the application of 250 μM SNP pretreatment. It can be concluded that SNP stimulated CAT activity in centaury shoots, which has also been observed in tomato and sunflower [22,56]. As in the case of CAT, the same pattern in POX activity was observed. SNP pretreatments increased POX activity, with the highest activity recorded after 250 μM SNP pretreatment (Figure 7c). Similar results were recorded in cotton and sunflower plants grown under salinity stress conditions [25,56]. It is known that the addition of signaling molecules such as NO and hydrogen sulfide ($H_2S$), stimulates the activity of antioxidant enzymes [86]. The role of NO in salt tolerance has been studied in numerous plant species, and there is evidence that the application of NO donors protects plants from salt stress by increasing antioxidant enzyme activity [21]. All the results suggest that NO mitigates the salt-induced oxidative stress by enhancing the activity of enzymatic antioxidants, thus improving centaury's tolerance to salt stress caused by NaCl.

## 5. Conclusions

Centaury shoots grown under NaCl-induced stress decreased the content of photosynthetic pigments, total phenolic compounds and DPPH. The activities of SOD, CAT and POX were also reduced under salt stress conditions. All these results indicate that centaury is a salinity-sensitive plant species. However, the MDA concentration was decreased while $H_2O_2$ concentration did not drastically change under stress conditions caused by NaCl, which indicate that centaury can be also be considered a salinity-tolerant species. Under salt stress conditions, proline content also did not significantly change which is not an attribute of salinity-tolerant species. In addition, it is possible that centaury has a preference for other osmolytes, rather than proline. In salt stress conditions, $Na^+$ and $Cl^-$ ions can act as "cheap osmolytes". In addition, the effective removal of $Na^+$ from the cytosol does not result in excessive ROS generation, eliminating the high activity of antioxidant mechanisms. Therefore, it is necessary to investigate the mechanisms that regulate the transport of ions in centaury in order to reveal if this important medicinal plant is a halophytic species. The results presented in this work also shown that SNP, a widely used NO donor, improved centaury tolerance to salinity (Figure 8). SNP showed a positive effect on total *Chl* and carotenoid content and affected lipid peroxidation, proline and total phenolic content, DPPH concentrations as well as antioxidant enzyme activities in centaury shoots grown under salt stress caused by NaCl. In addition to NO, SNP releases cyanide and iron ions as toxic by-products, and thus limits its potential application in agriculture. Therefore, nanoparticles that release NO, as well as S-nitrosothiols and S-nitrosoglutathione, the natural reservoirs of NO in biological systems, have been suggested as alternatives to SNP application.

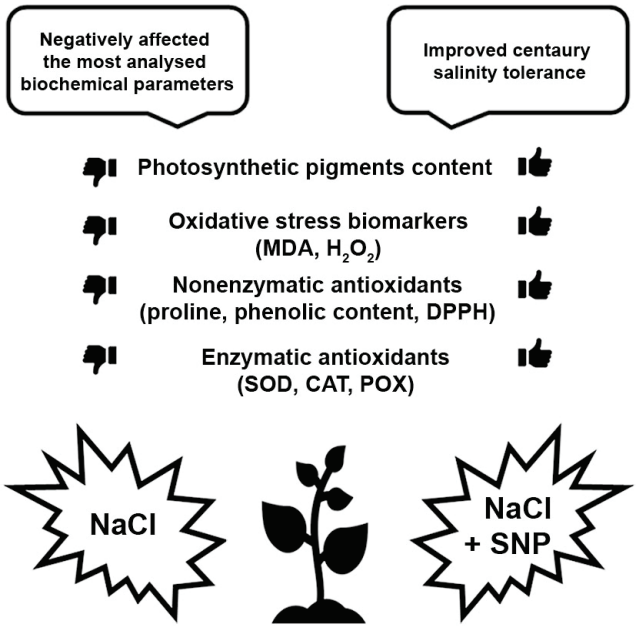


**Figure 8.** Schematic illustration showing how SNP affects centaury shoots during salt stress in vitro.

**Author Contributions:** M.T.-M. and N.S. contributed to all in vitro experiments and manuscript preparation. M.T.-M., N.S., M.Đ., S.M. and M.M. contributed to all experimental work requiring biochemical analyses. Z.G. contributed to data analyses and interpretation of the results. A.S. supervised the whole study and also contributed in preparing the final manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All the data are contained within the manuscript.

**Acknowledgments:** This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, contract numbers 451-03-68/2022-14/200007 and 451-03-68/2022-14/200178.

**Conflicts of Interest:** The authors declare no conflict of interest.

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*Review*

# Applications of Molecular Markers for Developing Abiotic-Stress-Resilient Oilseed Crops

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**Abstract:** Globally, abiotic stresses, such as temperature (heat or cold), water (drought and flooding), and salinity, cause significant losses in crop production and have adverse effects on plant growth and development. A variety of DNA-based molecular markers, such as SSRs, RFLPs, AFLPs, SNPs, etc., have been used to screen germplasms for stress tolerance and the QTL mapping of stress-related genes. Such molecular-marker-assisted selection strategies can quicken the development of tolerant/resistant cultivars to withstand abiotic stresses. Oilseeds such as rapeseed, mustard, peanuts, soybeans, sunflower, safflower, sesame, flaxseed, and castor are the most important source of edible oil worldwide. Although oilseed crops are known for their capacity to withstand abiotic challenges, there is a significant difference between actual and potential yields due to the adaptation and tolerance to severe abiotic pressures. This review summarizes the applications of molecular markers to date to achieve abiotic stress tolerance in major oilseed crops. The molecular markers that have been reported for genetic diversity studies and the mapping and tagging of genes/QTLs for drought, heavy metal stress, salinity, flooding, cold and heat stress, and their application in the MAS are presented.

**Keywords:** molecular markers; MAS; oilseeds; abiotic stress; SSRs; molecular breeding; climate change

**Citation:** Chugh, V.; Kaur, D.; Purwar, S.; Kaushik, P.; Sharma, V.; Kumar, H.; Rai, A.; Singh, C.M.; Kamaluddin; Dubey, R.B. Applications of Molecular Markers for Developing Abiotic-Stress-Resilient Oilseed Crops. *Life* **2023**, *13*, 88. https://doi.org/10.3390/life13010088

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 27 November 2022
Revised: 23 December 2022
Accepted: 25 December 2022
Published: 28 December 2022



## 1. Introduction

All annual oilseed crops have experienced poor growth rates over the previous ten years (negative for area and production), particularly safflower, sunflower, linseed, and niger crops and especially peanut, which has also experienced negative growth for the area [1]. India is the world's largest importer of vegetable oils (15 percent market share), followed by China and the USA, and it relies heavily on imports to meet its edible oil needs [2]. Palm oil accounts for roughly 60% of all imported edible oils, followed by soybean oil (~25%) and sunflower oil (~12%). It is also projected that edible oil demand will be 40.9 Mt by 2026 and that, by 2050, India will need to generate 17.84 Mt of vegetable oils to satisfy the country's estimated 1685 million population [2].

The necessity to scale up oilseed production demands immediate attention, given the rising domestic need for edible oils, alarming shortage, and the expense on the exchequer resulting from imports. Strategies to increase the productivity (and profitability) of oilseed-based production systems include the development of abiotic-stress-tolerant varieties in the

context of changing climatic conditions. Increased frequency of extreme events (floods, cold, droughts, heat, etc.), altered precipitation patterns, and an increase in average temperature (average high night temperature) are all indicators of climate change. As Earth transitioned between ice ages over the last 800,000 years, atmospheric concentration of $CO_2$ fluctuated between 180 ppm (glacial times) and 280 ppm (interglacial eras). The $CO_2$ concentration has steadily increased from pre-industrial levels of 280 ppm to 384 ppm in 2009, while the mean temperature has risen by 0.76 °C over that time. According to projections, atmospheric [$CO_2$] will reach 700 ppm or more by the end of this century, while the global temperature will rise by 1.8–4.0 °C depending on the greenhouse emission scenario [3]. With regard to global climate models, the mean ambient temperature is predicted to further increase by 1.5 °C within the next two decades [4]. The abiotic stresses have been reported to cause moderate to severe yield loss in various oilseeds (Table 1). To develop abiotic stress resistance and thus increase oil yield per unit area, traditional breeding efforts must be amalgamated with biotechnology methods.

**Table 1.** Yield loss reported in oilseeds due to abiotic stresses.

| S.No. | Crop | Abiotic Stress | Yield Reduction | References |
|---|---|---|---|---|
| 1. | Mustard | Moisture stress | 17–94% | [5] |
| 2. | Mustard | Salinity | 50–90% | [6] |
| 3. | Mustard | Heat stress | 34% | [7] |
| 4. | Mustard | Heat stress | >54% | [8] |
| 5. | Soybean | Drought | 73–82% | [9] |
| 6. | Soybean | Drought | 50% | [10] |
| 7. | Soybean | Salinity | Up to 40% | [11] |
| 8. | Soybean | Flooding | Up to 25% | [12] |
| 9. | Soybean | Flooding | 20–39% | [13] |
| 10. | Soybean | Cold stress | 24% | [14] |
| 11. | Sunflower | Drought | Up to 40% | [15,16] |
| 12. | Sesame | Waterlogging | 50–90% | [17,18] |
| 13. | Sesame | Drought | 28% | [19,20] |
| 14. | Safflower | Drought | 17.2% | [21] |
| 15. | Groundnut | Drought | 55–72% | [22] |

Molecular markers such as RFLP (Random Amplified Polymorphic DNA), AFLP (Amplified fragment length polymorphism), SSR (Simple Sequence Repeat), RAPD (Random Amplified Polymorphic DNA), SNP (single nucleotide polymorphisms). etc., are DNA-based oligonucleotide sequences facilitating the detection of variations or polymorphisms in the population for specific regions of DNA. Among the various biotechnological interventions, molecular markers have played a pivotal role in accelerating the crop breeding programs employing marker-assisted selection (MAS). The facts that they are abundant, technically easy to use, and detectable at any stage of plant development have given them an added advantage of not being affected by environmental factors. Rapid advancements have been made in the development of a variety of molecular markers over the past 20 years with refinements on a regular basis depending on the available infrastructure, technical skills required, the importance of the crop, and the trait in question. Figure 1 illustrates how molecular and integrated plant breeding is helpful in developing varieties with abiotic stress tolerance using genomic approaches such as MAS [23]. Success has been achieved in breeding oilseeds, such as canola, mustard, sunflower, soybean, and peanut, through the utilization of molecular marker techniques, mapping traits that control seed quality, and biotic and abiotic stress resistance [24–30]. However, though the techniques and available tools for MAS are well established, there is still a dearth of studies conducted using MAS to achieve abiotic stress tolerance for edible oilseed crops, such as sesame, niger, safflower, and the non-edible oil crops castor and linseed. This article discusses case studies involving the use of molecular markers for developing abiotic-stress-tolerant cultivars/genotypes of various oilseed crops.

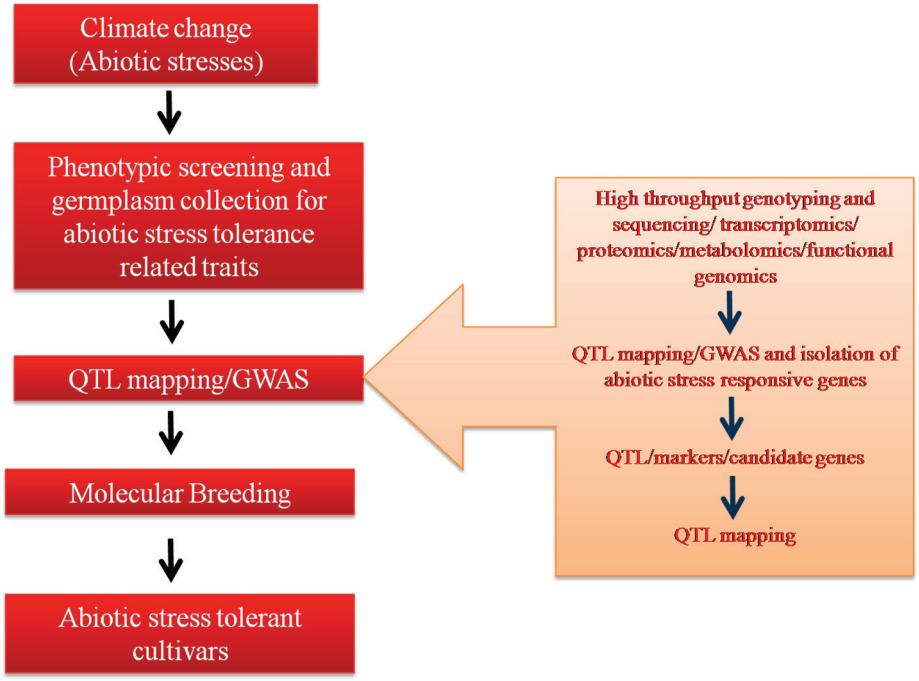


**Figure 1.** A stepwise presentation of molecular breeding and genomics approaches for the development of abiotic-stress-tolerant cultivars.

## 2. Applications of Molecular Markers in Development of Abiotic-Stress-Tolerant Oilseed Crops

### *2.1. Drought*

Fresh water scarcity is an emerging global problem. Since agriculture primarily harnesses freshwater, enhancing agricultural output amid restricted water availability is a major challenge [31]. Although improvements in irrigation and tillage methods can be used to conserve water and increase crop yield, supplementary strategies like genetic modification of crops are required for increasing productivity under moisture deficiency conditions [32,33]. Estimates indicate that adverse environmental factors affect about half of the possible crop production, with water shortage being the most severe stress [34–36]. Tolerance to drought is a quantitative attribute that is influenced by numerous genes via a variety of mechanisms in a plant. Under drought stress, expression patterns in genes that are involved in water transport; osmotic balance; oxidative stress; morphological modifications, including root development and reduced leaf area; and damage repair are altered (Figure 2).

A number of studies have provided deeper insights into understanding the molecular basis of drought tolerance in plants [37–40]. Drought alters the growth, physiology, and metabolic activities of plants, which in turn have an adverse impact on the nutritional quality and yield of important oilseed crops around the world [41,42]. In drought stress, it has been observed that plants' enzymatic activity is reduced, which eventually penalizes yield and quality of oilseeds [43]. Under conditions of water deficiency, a decrease in the oil content of soybean seeds has been reported [44]. Genomic resources created using various methods, such as genotyping-by-sequencing (GBS), genome sequencing, genome-wide association studies (GWAS), etc., have given researchers strong tools for characterizing the genetic diversity of oilseed crops, a solid framework for finding new traits, and next-generation breeding tools to speed up the development of elite cultivars. Comparative genome analysis is one of the significant advantages of the current growth of genomic data.

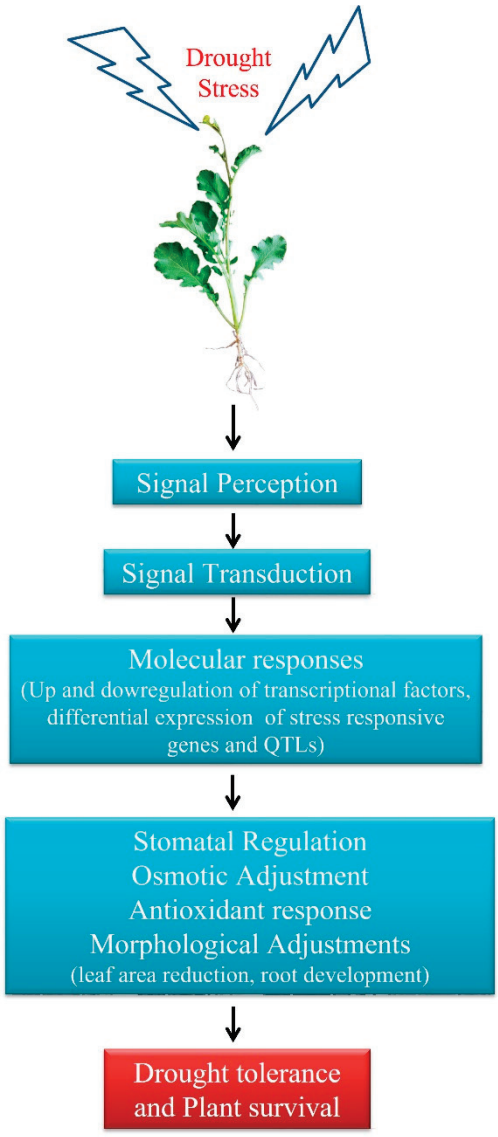


**Figure 2.** Cellular morphological and molecular responses in plants help to combat drought stress.

Numerous quantitative trait loci (QTLs) for traits associated with physiological, agronomic, seed composition, and abiotic and biotic stress parameters have been reported in soybean *(Glycine max)* [24,45]. Only a small number of QTLs have, however, so far been linked to characteristics related to drought resistance. Additionally, reported QTLs account for 10% or less of the phenotypic variance for those traits. To date, the majority of research focusing on the identification of QTLs has used small, single populations. Chen et al. [46] discovered QTLs associated with primary root length on chromosome 16 of soybean, which accounts for 30.25% of the variation in phenotype and will help in the development of markers for root-length selection, which is a crucial trait for drought tolerance. In 1996, a research team led by Mian developed an RFLP map in soybean from a population of 120 $F_4$-derived lines of a cross 'Young × PI416937' that identified the multiple QTLs that are associated with leaf ash and water use efficiency (WUE) [47]. For both attributes, authors reported significant ($p < 0.01$) differences at the phenotypic level among the lines. In total, four and six independent RFLP markers were reported to be linked with the said two traits, respectively, and when added together, each set of markers would be responsible for 38 and 53% of the variance in the corresponding traits. A significant QTL was found at marker position cr497-1 on USDA Linkage Group (LG) J, which accounted for 13.2% of the variability in WUE. The scientists also noted that two QTLs were linked to both WUE

and leaf ash and that leaf ash and WUE had a negative correlation (r = −0.40). One QTL associated with RFLP marker A063E for WUE was also detected in the 'Young × P1416937' population; however, the phenotypic effect was merely <10%, according to authors who tested another soybean population derived from $F_2$ progenies developed from the cross of 'S100 × PI41693 [25]. To date, only WUE and leaf ash QTLs have been documented in soybean under water deficit conditions. More extensive research is required in order to find QTLs that affect shoot turgor maintenance and root architecture. Finding novel QTLs and genes, as well as deciphering the mechanism governing how genes behave during drought, could prove to be hugely instrumental in enhancing soybeans' ability to withstand drought stress.

Numerous genes likely associated with drought tolerance have been identified in sunflower (*Helianthus annuus* L.), including *HaDhn1* (sunflower dehydrin gene), *SunTIP* (sunflower tonoplast intrinsic protein), *HaDhn2*, *Sdi* (sunflower drought induced), *Hahb-4* (sunflower homeobox-leucine zipper gene), and *HAS1* (sunflower, asparagine synthetase) or *HAS1.1*. These genes have been reported to exhibit high levels of expression under drought stress, and it has been speculated that they contribute to the tolerance of sunflower to drought stress [26,48–50]. However, only a handful of studies on sunflower have been conducted to ascertain the development of molecular markers for QTLs linked to drought tolerance [27]. Hervé et al. [28] employed the AFLP linkage map to recognize QTLs for water status (transpiration and leaf water potential), stomatal movements, and net photosynthesis. Using the AFLP linkage map, 19 QTLs were identified, which accounted for 8.8–62.9% of the phenotypic variance for each characteristic. Out of these, two significant QTLs for net photosynthesis were found on linkage group IX [28]. Similar to this, 24 QTLs were discovered in sunflower in well-watered conditions, of which 5 (or around 21%) were also discovered following drought condition. A range of 6% to 29% of phenotypic variance was explained by the QTLs [51].

Safflower (*Carthamus tinctorius* L.) mapping, molecular breeding, and QTL discovery pronouncedly lag behind other oilseed crops due to a lack of genetic data [52]. As a result, there has been very little genetic enhancement of safflower through marker-assisted breeding and linkage of characteristics. In 2010, Tang et al. mapped heat shock protein (HSP) genes by utilizing a cDNA–AFLP linkage study with 192 randomly segregating $F_2$ populations [53]. Genomic and EST-SSR markers, which can be useful for mapping, molecular breeding, and the linkage of desirable QTL traits like drought tolerance, have been developed in safflower by a number of research groups [52,54]. In this direction, an intra-specific $F_2$ population of *Carthamus tinctorius* and an inter-specific $BC_1$ population of *Carthamus tinctorius* × *Carthamus oxyacanthus* were mapped by generating 1142 PCR based markers and 75 RFLP markers to undertake the first major linkage study of the *Carthamus* species. Both of these mapping populations' utilized these EST-SSR markers [55]. Another researcher noted the feasibility of transferring non-genic microsatellite (SSR) markers and gene-based markers from sunflower (*Helianthus annuus* L.) to safflower. These markers comprised resistance gene candidates (RGC)-based markers and intron fragment length polymorphism (IFLP) [56]. In $F_3$ families produced from the hybrid of the tolerant Mex.22-191 (tolerant) and sensitive IL.111 (sensitive) safflower genotypes under drought stress, QTLs linked to seed yield and its attributes were mapped using SSR and ISSR markers [57]. This study discovered 18 QTLs linked to seed yield and its attributes, including four major QTLs and three linkage groups (2, 4, and 6), which were found to be crucial for safflower's ability to withstand drought.

In spite of large morphological variation observed between germplasm accessions, peanut (*Arachis hypogaea* L.) shows very little genetic variation at the molecular level, as detected by markers like isozymes, RFLPs, and RAPDs [58]. Three independent research groups around the world have invested in the development of microsatellite markers for peanut and have reported up to 200 simple sequence repeats (SSRs) [29,59,60]. About 20% of them can detect peanut polymorphism. Moreover, a genetic map of 191 SSR loci was constructed based on a single mapping population (TAG 24 × ICGV 86031) segregating

for drought and surrogate traits [61]. The QTL Cartographer identified 105 significant impact QTLs (M-QTLs) explaining 3.48 to 33.36 percent of the phenotypic variance (PVE), but the QTL Network only identified 65 M-QTLs that explained 1.3 to 15.0 percent of the PVE. Comparing the two programmes together allowed the identification of 53 common M-QTLs. Additionally, genotype matrix mapping (GMM) identified 186 (8.54–44.72% PVE) and 63 (7.11–21.13% PVE) three and two loci interactions, respectively, while only 8 epistatic QTLs (E-QTL) interactions with 1.7–8.34% PVE were identified by the QTL network. This study led the authors to conclude that the discovery of some major and many minor M-QTLs and QTL × QTL interactions underpinned the complex and quantitative nature of drought tolerance in peanut. It was recommended that genomic selection or marker-assisted recurrent selection be used as a breeding strategy for drought tolerance instead of marker-assisted backcrossing [61]. In another related study, a screening of two RIL (recombinant inbred lines) mapping populations, viz., ICGS76 × CSMG84-1 (RIL-2) and ICGS44 × ICGS76 (RIL-3) with 3215 SSR markers, two genetic maps with 119 (RIL-2) and 82 (RIL-3) SSR loci were constructed. Using these aforementioned maps based on two RIL populations and a reference map of 191 SSR loci based on the TAG 24 × ICGV 86,031 RIL population, Gautami et al. [62] constructed a dense consensus map of 293 SSR loci distributed across 20 linkage groups, spanning 2840.8 cm. In addition to a total of 153 M-QTL and 25 E-QTL for drought tolerance, the authors reported the discovery of 16 prospective genomic regions carrying 125 QTL related to biomass, yield, and drought component traits. In summary, this study identified many QTLs with low to moderate phenotypic variance for the complex traits such as biomass, yield, and drought tolerance. These studies potentially provided a direction for additional investigation and exploitation for QTL pyramiding and cloning in the future, though the discovery of major QTL/s for drought tolerance is still awaited.

Sesame is a hardy crop that is well-adapted to drought prone areas. Sesame typically endures drought better than other important food crops [63]. The production of this oil-rich crop is, however, still quite sensitive to droughts that occur during the germination and flowering stages [64,65]. Unfortunately, there are only a few molecular-marker-based studies conducted so far deciphering the genomic regions associated with sesame's tolerance under drought conditions. Dossa et al. [66] conducted a GWAS employing SNP markers for variables interrelated to drought tolerance in 400 different sesame accessions, including landraces and potential modern varieties. This study reported 10 stable QTLs associated with drought-tolerance-linked characteristics located in four linkage groups. Additionally, this study reported two significant pleiotropic QTLs harboring both known as well as unknown genes for drought tolerance, such as *SiTTM3* (*Sesamum indicum* Triphosphate tunnel metalloenzyme 3), *SiABI4* (*Sesamum indicum* ABA insensitive 4), *SiGOLS1* (*Sesamum indicum* Galactinol synthase 1), *SiNIMIN1* (*Sesamum indicum* NIM1-Interacting 1), and *SiSAM* (*Sesamum indicum S*-adenosylmethionine synthetase). In order to identify candidate genes associated with drought tolerance in the whole genome of sesame, researchers conducted a comparative homology search with three relative species, viz., potato, tomato, and *Arabidopsis* [67]. The authors successfully identified 75 candidate genes (42, 22, and 11 from *Arabidopsis*, potato, and tomato, respectively), which were found to be distributed on the 16 sesame linkage groups. Based on their functional classification, authors divided the genes in two groups. One group consisted of genes that protect the plant against drought effects, while the other included signal transduction genes and transcription factors. Several other studies have also employed molecular markers for QTL mapping and GWAS to unravel the genetic basis of drought tolerance in sesame [68–72].

Although we have witnessed remarkable progress in the field of genomics over the last ten years, the availability of precise and high-throughput phenotyping for drought tolerance traits is still a major challenge for QTL mapping studies. Targeting root architecture, photosynthetic efficiency, osmotic adjustment, relocation of stem reserves, and leaf senescence under drought stress are among the phenotypic features that could benefit the most from the application of MAS. Further, the construction of consensus maps integrating

the QTL information provided by different populations needs more attention. It is certain that molecular-assisted breeding has the potential to more effectively address the problems caused by the diminishing availability and rising cost of irrigation water, as well as the escalating demand for food, fiber, and biomass.

### *2.2. Salinity*

One of the key abiotic stress challenges influencing the quality and production of food crops globally is soil salinity, which restricts crop plants' growth and development [73,74]. Furthermore, salinity can pose risks to the production of oilseeds by lowering both the yield and quality of the produce. Globally, salt affects >833 million hectares of land [75], and it is believed that 20% of cultivated and 33% of irrigated land are affected [76]. By preventing cell division, enzyme activity, nucleic acid and protein synthesis, and salinity stress negatively impacts seed germination and seedling growth, height, leaf size, leaf number, reproductive structures, seed quantity, seed content, seed weight, and the quality of seed oil [30–44,77–82]. Figure 3 illustrates how a plant also responds at biochemical, molecular, physiological, and morphological levels to salinity stress in order to sustain its growth and production [83]. However, decades of intensive research have led to the improved comprehension of the mechanisms by which salt stress affects crop development and productivity. Indeed, this information may be used to develop genotypes that are salt-tolerant.

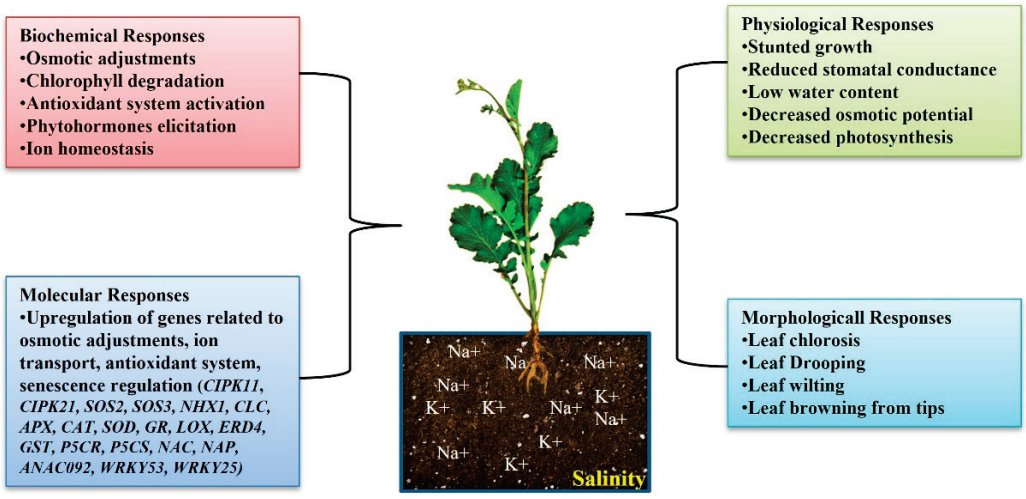


**Figure 3.** Plant responses to salinity stress. [Abbreviations: *CIPK*: CBL-interacting protein kinases; *SOS*: salt overly sensitive; *NHX*: sodium/hydrogen antiporter; *CLC*: chloride channel; *APX*: ascorbate peroxidase; *CAT*: catalase; *SOD*: superoxide dismutase; *GR*: glutathione reductase; *LOX*: lipoxygenase; *ERD*: early responsive to dehydration; *GST*: glutathione S-transferase; *P5CR*: pyrroline-5-carboxylate reductase; *P5CS*: pyrroline-5-carboxylate synthetase; *NAC*: NAC transcription factor; *NAP*: nucleosome assembly protein; *ANAC*: *Arabidopsis* NAC transcription factor; *WRKY*: WRKY transcription factors].

Numerous factors, including soil properties, genotypes, and developmental phases, influence how oilseed crops react to salt stress. Although the majority of oilseed species are prone to damage under salt stress, nevertheless a wide range of diversity in terms of salt sensitivity exists among them. While canola, soybean, sunflower, and safflower exhibit moderate to strong tolerance, peanut and linseed are examples of sensitive species [84]. Likewise, it has been observed that amphitetraploid *Brassica* species, such as *B. juncea, B. carinata,* and *B. napus,* are relatively more tolerant against salt stress compared to their progenitors, such as *B. nigra*, *B. rapa*, and *B. oleracea*. Among all the *Brassica* species, *B. napus* is extremely tolerant to salt stress, whereas *B. rapa* and *B. nigra* are extremely sensitive [85]. Since tolerance to salt stress is a physiologically intricate trait, the development of salt-

tolerant genotypes necessitates a comprehensive approach that involves modifying existing cultivars genetically and biotechnologically.

An essential method for localizing the genomic areas that regulate characters related to salt stress tolerance is QTL mapping. QTLs are identified using powerful DNA marker approaches, such as AFLP, RFLP, RAPD, SSR, and SNPs. Successful breeding for salt stress in oilseeds, notably in *Brassica* species, requires the identification of QTL. It is challenging to detect a genetic basis for salinity tolerance in *Brassica* species, since no significant QTL with relation to salinity tolerance in those species has yet been discovered due to the physiological complexity of the salinity response. However, a limited number of studies have demonstrated the utilization of molecular markers in this field. RFLP markers that were used to characterize each line to find salt-tolerance-related QTL in soybean RILs produced S-100 (tolerant cultivar to salinity) and a Tokyo variation (susceptible cultivar to salinity). After that, a single-factor QTL analysis was performed to discover trait-related genomic areas. To improve mapping accuracy, specific genomic areas were flooded with SSR markers. The study found a QTL related to salt tolerance at SSR marker Sat 091 at LG N. In the field, greenhouse, and mixed environments, this QTL was found to be responsible for 41%, 60%, and 79% of salt tolerance, respectively. In fact, the tolerance-related QTL alleles were found to be derived from S-100 through pedigree tracking [86]. Using two RIL populations resulting from the cross between FT-Abyara C01 and Jindou No. 690197, a similar study discovered a substantial salt-tolerance QTL in soybean's molecular linkage group N. This study employed FT-Abyara C01 and Jindou No. 690,197 RIL populations. This QTL accounted for 44.0% to 47.1% of salt tolerance across the two groups [87]. Using a separate linkage group, Chen et al. [88] found a second significant QTL (qppsN.1) between markers Sat 164 and Sat 358 on linkage group G in a cross of Kefeng No.1 (salt-resistant) and Nannong 1138-2 (salt-sensitive) soybeans.

In similar research, Hamwieh and Xu [89] discovered a QTL related to salt-tolerance in soybean on linkage group N with a substantial dominant impact from 225 lines of $F_2$ population produced from a cross of Jackson (PI548657) (salt-resistant cultivar) × JWS156-1 (salt-sensitive wild soybean). This major QTL explained 68.7% of the variance in the salt tolerance rating scale. The authors concluded that both wild and cultivated soybeans carry the conserved QTL related to salt tolerance, which has a significant dominating effect over salt sensitivity.

Most widely used markers in safflower are ISSRs, AFLPs, and RAPDs because they are ideal for crops with little genetic resources, require no prior knowledge, and perform genome scanning with repetitive sequences [90]. Safflower genetic diversity has been documented in numerous investigations employing a combination of phenotypic variation and molecular polymorphism [91–93].

In 2018, Li and his co-researchers used a diversity panel of 490 accessions of sesame (*Sesamum indicum*) to conduct a genome-wide analysis of stress tolerance indices related to sodium-chloride-induced salt stress and PEG-induced drought stress to understand the resulting genetic variants with respect to drought and salinity tolerance at the germination stage [68]. According to this study, under the stresses of drought and salt, respectively, there were 132 and 120 significant SNPs, which further resolved to be associated with 9 and 15 QTLs. There were just two shared QTLs for the response to salt and drought, which were situated in the linkage groups (LGs) 5 and 7, respectively. Authors also reported a total of 13 and 27 potential candidate genes for drought and salt tolerance indices, respectively, which encode transcription factors, osmoprotectants, and antioxidant enzymes and are associated with signal transduction, hormone biosynthesis, or ion sequestration, which were also reported for the drought and salt tolerance indices, respectively.

In an attempt to elucidate the genesis of wild sunflower hybrid's (*H. annuus* × *H. petiolaris*) adaptation to salt stress, Lexer et al. [94] employed EST markers on 11 genes. One EST was mapped to QTL responsible for salt tolerance, which encodes a Ca-dependent protein kinase (*CDPK*) that originated in stress-induced root tissue of *H. annuus;* hence, a plausible adaptive role for Ca-dependent salt tolerance genes in wild sunflower hybrids was

suggested. Another study by the same author on 172 $BC_2$ hybrids between *Helianthus annuus* and *Helianthus petiolaris* planted in the salt marsh habitat of *Helianthus paradoxus* in New Mexico identified 14 QTLs for mineral ion absorption attributes and three for survivability [95]. The previous results that suggested that salt tolerance in Helianthus is achieved through higher $Ca^+$ absorption, along with stronger exclusion of $Na^+$ and similar mineral ions, were confirmed by mineral ion QTLs mapping to the same place as the survival QTLs (on LG 1, 4, and 17b). In a separate study, researchers evaluated the variability of microsatellites from genomic areas that were neutral in the experimental hybrids with that of microsatellites associated with the three survival QTLs listed above. It was established that populations of the natural hybrid species had significantly less variability according to microsatellites relating to the survival QTLs. However, in parental populations, there was no discernible difference in the levels of diversity between the two microsatellite classes [96].

With the above information in mind, it is evident that considerable effort has been put into identifying the genes or QTLs that contribute to salinity tolerance in oilseed crops; nevertheless, there are presently few reports of cultivars or breeding lines with better salt tolerance that have been successfully developed using molecular markers and MAS technology. The limited use of markers for improving complex traits like salinity tolerance has been attributable to various reasons; however, it is possible to use markers for such complex traits by the identification of reliable QTLs and linked genetic markers. This can be achieved by putting additional efforts, such as conducting mapping experiments in field conditions instead of a greenhouse, so that plants experience actual salt stress in association with other stresses and environmental factors as well, studying the cross-tolerance mechanism that exist in plants against various stresses; the identification of QTLs in multiple environments; splitting complex traits, such as salt tolerance, into individual components; and identifying QTLs and markers for such individual components instead of studying salt tolerance as a whole (such as finding QTLs for salinity tolerance at different developmental stages), and finally the pyramiding of such QTLs may pave the way to develop salt-tolerant oilseed crops. This is a challengeable but achievable strategy to follow in order to develop salt tolerance in plants. Table 2 summarizes some of studies that have used molecular markers in the development of resistance to abiotic stresses in oilseed crops.

**Table 2.** List of studies involving MAS for improvement of abiotic stress resistance in oilseeds.

| Mapping Population/Genotypes | Trait | Marker Used/Markers Linked to QTL | Crop | Reference |
|---|---|---|---|---|
| Hutcheson × PI471938, 140 $F_4$ population | Drought tolerance | SSR (Satt226) | Soybean | [97] |
| Jackson × KS4895, 81 RILs | Drought tolerance | SSR (Sat_044) | Soybean | [98] |
| Minsoy × Noir 1, 236 RILs | Drought tolerance | SSR (Satt205-Satt489) | Soybean | [99] |
| S-100 × Tokyo, 116 $F_2$ population | Drought tolerance | RFLP (A489H) | Soybean | [25] |
| Young×PI416937, 120 $F_4$ population | Drought tolerance | RFLP (B031-1, A089-1, cr497-1, K375-1, A063-1) | Soybean | [47] |
| TAG 24 × ICGV 86031, RILs | Drought tolerance | SSRs | Groundnut | [61] |
| ICGS 76 × CSMG 84-1 and ICGS 44 × ICGS 76, RILs | Drought tolerance | SSRs | Groundnut | [62] |
| Mex.22-191 × IL.111 $F_3$ population | Drought tolerance | SSRs and ISSRs | Safflower | [57] |

**Table 2.** *Cont.*

| Mapping Population/Genotypes | Trait | Marker Used/Markers Linked to QTL | Crop | Reference |
|---|---|---|---|---|
| 400 accessions including landraces and modern cultivars | Drought tolerance | SNPs | Sesame | [66] |
| S-100 × Tokyo RILs | Salt stress | SSRs (Sat_091) | Soybean | [86] |
| FT-Abyara × C01 and Jindou No. 690197, RILs | Salt stress | SSRs (Sat_091) | Soybean | [87] |
| Kefeng No. 1 × Nannong1138-2, RILs | Salt stress | SSRs (Sat_164 and Sat_358) | Soybean | [88] |
| Jackson (PI548657) × JWS156-1, $F_2$ | Salt stress | SSRs | Soybean | [89] |
| 490 accessions | Drought and salt stress | SNPs | Sesame | [68] |
| AC Hime × Westag-97, RILs | Cadmium toxicity | SSRs (SatK147, SacK149, and SattK152) | Soybean | [100] |
| Essex × Forest, RILs | Manganese toxicity | SSRs (Satt291, Satt239, OEO2) | Soybean | [101] |
| Archer × 'Minsoy and Archer × Noir I', RILs | Flooding | SSRs (Sat_064) | Soybean | [102] |
| Misuzudaizu × Moshidou Gong 503, RILs | Flooding | SSRs | Soybean | [103] |
| S99-2281 × PI 408105A, RILs | Flooding | SSRs and SNPs (Sct_033, BARC-024569-04982, BARC-016279-02316) | Soybean | [104] |
| A5403 × Archer (Population 1) × P9641 × Archer (Population 2), $F_{6:11}$ RILs | Flooding | SSRs (Satt385, Satt269, Sat_064) | Soybean | [105,106] |
| Santiago II and Melody | Cold stress | ESTs | Sunflower | [107] |
| HA89 | Cold stress | ESTs | Sunflower | [108] |
| Hayahikari × Toyomusume, RILs | Cold stress | SSRs | Soybean | [109] |
| Hongfeng11 × Harosoy, $BC_2F_3$ | Cold stress | SSRs | Soybean | [110] |
| RILs | Cold stress | SSRs | Soybean | [111] |
| TE5A, $BC_2$ | Heat stress | AFLPs, SCARs | Rapeseed | [112] |
| Jinhuangma (JHM) and Zhushanbai (ZSB), landraces | Drought stress | SNPs | Sesame | [71] |
| RILs (Per × R500) and DH lines (Major × Stellar) | Cold stress | RFLPs, AFLPs | Mustard and canola | [113] |
| Mex.22−191 × Goldasht, $F_9$ RILs | Drought | AFLPs | safflower | [114] |
| K099 × Fendou 16, $F_7$ RILs | Drought | SSR (Sat_165 and Satt621) | Soybean | [46] |
| NTS116 × Danbaekkong, RILs | Flooding | SNPs | Soybean | [115] |

### *2.3. Heavy Metal Stress*

Heavy metal poses a global concern because of its significant technological implications in several industrial processes and applications. Different heavy metals from these sectors that severely contaminate wastewater have numerous long-term ecological and biological harmful impacts [116]. The heavy metals in this wastewater are hazardous, and if this discharged water is used for irrigation purposes, it disturbs the biological balance of the soil and the plants that are grown there. Since heavy metals are naturally prevalent in the earth's crust, they can be found in both polluted and unpolluted soils. Synthetic fertilizers, contaminated sewage/sludge, manure, and mining and industrial operations can all cause heavy metals to emerge in agricultural soil [117]. When sewage water is added to the soil, plant growth may increase, but it may also include toxic substances that

may threaten crops and the food chain. Many heavy metals, including Mo, Fe, Ni, Cu, Mn, and Zn, are advantageous or necessary for plant growth in low concentrations, but in high concentrations, they are all toxic [117]. In particular, a heavy dosage of these metals may cause oxidative stress, inhibit root elongation, displace other essential metals in a plant's enzymes, introduce pigments that cause the function of many metabolic processes to be disrupted, and ultimately compromise the yield and growth [118–120]. Heavy metals are proven to be toxic for oilseed crops in a variety of ways, and the symptoms vary greatly depending on the plant, metal, and its dose [121]. Oilseeds have the ability to reduce toxicity caused by metals optimizing their hemostasis. In oilseeds, heavy metals promote free radicals' generation, compete with metal cofactors of plant enzymes, alter enzyme action through binding sulfhydryl and N-containing groups, and cause the leakage of cellular contents through interactions with phospholipid-containing groups. *Brassica* cultivars have demonstrated decreased plant growth caused by Pb toxicity through altered cellular metabolism and nutrient uptake [122]. In fact, under Cr stress, such negative effects were also seen in shoot growth, leaf area, and length of leaf [123].

In order to facilitate MAS in RIL population ($F_{6:8}$) obtained from a cross of AC Hime (high-Cd accumulation) × Westag-97 (low-Cd accumulation)' soybean, Jegadeesan et al. [100] conducted a study to develop markers for low-Cd accumulation. It was demonstrated by the use of 171 SSR markers that low-Cd accumulation in soybean seeds is regulated by a key gene (*Cda1*), with the low-accumulation allele being dominant. In soybean seeds, *Cda1* was found to be associated with 7 SSR markers, viz. SatK138, SatK139, SatK140, SatK147, SacK149, SaatK150, and SattK152. Each linked marker was assigned the same linkage group K. Markers SatK147, SacK149, and SattK152 distinguished studied genotypes with low and high Cd accumulation. Additionally, a significant QTL linked to a low Cd level in seeds was mapped to the same region at linkage group K as *Cda1*. This QTL was identified as the source of 57.3% of the phenotypic variation [100]. It has been experimentally proven that molecular markers can be used to locate particular loci regulating soybean resistance to Mn toxicity [101]. In a previous study, researchers demonstrated that RAPD markers could identify four QTLs, or hotspots, in an RIL population descended from the "Essex × Forrest" cross that may be responsible for resistance to manganese toxicity [124]. However, a study was conducted by using only high-quality scores generated by 240 microsatellite markers to detect the QTL that underlie tolerance to Mn toxicity in the $F_5$-derived RIL population from "Essex × Forrest" (E × F, $n = 100$). The study was performed in order to rule out the errors occurring in RAPD maps and consequent errors in assigning QTL [101]. The necrosis of the leaves and roots served as markers. The findings showed that root necrosis at 7 days after treatment was strongly linked ($p < 0.005$, R2 = 20%) with the regions on linkage groups I (BARC Satt239), C2 (BARC Satt291), and G (OP OEO2); these three QTLs could explain about 58% of the total variation in root resistance to Mn toxicity. They also affirmed one of the previously identified RAPD-associated root necrosis QTLs, namely, sudden death syndrome QTL on LG (G). However, no QTL for leaf chlorosis were identified ($p < 0.005$), and none of the RAPD-associated with leaf chlorosis QTL could be confirmed [101].

### *2.4. Flooding*

The main barrier to sustainable agriculture is flooding, and the plants exposed to flooding experience significant yield losses. Plants frequently encounter intermittent or persistent floods in their natural habitat. Physio-chemical soil characteristics that are crucial include redox potential, soil pH, and oxygen content, which are altered by flooding in a variety of ways. As a result, plants growing in wet soil endure stressful conditions, such as hypoxia (a lack of oxygen) or anoxia (the absence of $O_2$). These low oxygen environments have a significant negative impact on plant growth, development, and survival. Metabolic changes under oxygen deprivation, including switching to anaerobic respiration and oxidative damage caused by reactive oxygen species (ROS), compromise membrane integrity, as well as damage photosystem II's efficiency, leading to a significant decline in net photosynthetic rates. To combat flooding-induced hypoxia/anoxia and ox-

idative stress, plants that endure waterlogging stress have mechanisms, such as the better availability of soluble sugars, formation of aerenchyma, enhanced glycolysis and fermentation activity, and the contribution of antioxidant defense mechanisms [125,126]. Many flooded plant species, including soybean, have shown evidence of developing adventitious roots [127–129]. Figure 4 illustrates a variety of responses and coping mechanisms used by plants to cope with flooding stress.

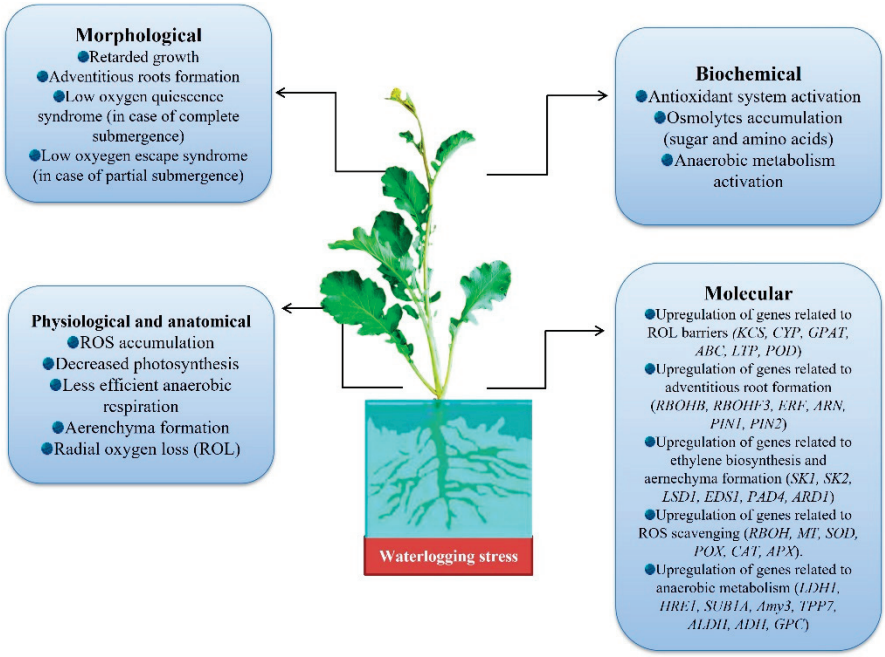


**Figure 4.** Response and the adaptive mechanisms of plants under flooding stress. [Abbreviations: *KCS*: 3-ketoacyl CoA synthase; *CYP*: cytochrome P450; *GPAT*: glycerol-3-phosphate acyltransferase; *ABC*: ATP-binding cassette; *LTP*: lipid transfer protein; *POD*: peroxidase; *RBOHB*: respiratory burst oxidase homolog B; *ERF*: ethylene response factor; *PIN*: PIN formed proteins; *SK1*: SNORKEL1; *SK2*: SNORKEL1; *LSD:* lesion simulating disease; *EDS*: enhanced disease susceptibility; *PAD*: phytoalexin deficient; *ARD:* acireductone dioxygenase; *MT*: metallothionein; *SOD:* superoxide dismutase; *POX*: peroxidise; *CAT*: catalase; *APX*: ascorbate peroxidise; *LDH*: lactate dehydrogenase; *HRE*: hypoxia response element; *SUB1A:* submergence tolerance regulator; *Amy3:* α-amylases; *ADH*: alcohol dehydrogenase.

It has now become simple for scientists to focus on altering or using the key genes that have been linked to flooding tolerance, to eventually develop new flood-tolerant plant varieties. Genomic areas linked to flooding tolerance can be detected using map-based gene cloning and QTL mapping. In the case of rice, it was able to introduce the *Sub1* gene to particular varieties by molecular-assisted backcrossing (MAB) to accommodate a different soil type and farmer preferences, as well as to add new variations through genetic engineering [126]. In an effort to map QTLs conferring flooding tolerance in soybean, two hundred and eight lines of two RIL populations descending from the 'Archer × Minsoy and Archer × Noir I' were placed in two different experimental setups: one under controlled condition (no flooding) and the other under flooding condition (waterlogging). Plants were subjected to 2 weeks of flooding at the early flowering stage in a water-logged setup, in order to identify the QTL linked to soybean flooding tolerance. Authors discovered a single QTL from the Archer parent, associated with marker Sat 064, that was responsible for the increased growth of plants (11 to 18%), as well as seed yield (47 to 180%), in a flood environment. Both RI populations included this highly significant QTL ($p$ = 0.02–0.000001). Authors also reported that Sat_064 QTL on Chromosome 18 was distinctively linked with flooding tolerance and was not linked with normal plant length, maturity, or seed yield. Although the Rps4 gene and Sat 064-QTL are co-localized for resistance to *Phytophthora*

*sojae* resistance, the donor parent Archer lacks the Rps4 resistance allele, proving that Sat 064-QTL is exclusive for flooding-stress tolerance [102]. Further, this QTL was verified in NILs at the $F_6$ generation descended from heterogeneous inbred families [105]. Tolerant NILs produced around 60.9% greater yields under stress-free conditions compared to the yield of sensitive NILs (32.6%) under the same environment. Using bulked segregant analysis (BSA), as well as partial linkage mapping, two more QTLs concerning flooding-tolerance traits were also identified and were found to be linked to markers Satt385 on Chromosome 5 and Satt 269 on Chromosome 13 [106]. The advantageous alleles of these two QTLs came from Archer.

In another investigation, 60 RILs of soybean were derived from cross 'Misuzudaizu (flooding tolerant cultivar) × Moshidou Gong-503 (flooding sensitive cultivar)' in order to study the genetics of tolerance to flooding stress at early vegetative stage. The plants were grown in pots and were subjected to flooding treatment at the two-leaf stage for 3 weeks. Pots were then put back in the greenhouse to mature there. The experiment was conducted for two consecutive years. In 2002, three QTLs for flooding tolerance, *ft1* to *ft3*, were identified, employing 360 genetic markers. Four other QTLs, numbered *ft4* to *ft7*, were discovered in 2003 in addition to the *ft1* (linkage group C2), which was reproducible. In both years, *ft1* possessed a high LOD (logarithm of the odds; relative probability that two loci are linked) score (15.41 and 7.57) and contributed 49.2% and 30.5%, respectively, of the overall variance. At a location identical to *ft1*, a major QTL for days to blooming was seen across all treatments and years [103]. It was further observed that the main QTL caused a prolonged recovery period prior to the reproductive stage by delayed flowering eventually resulting in a higher yield under stress condition. Using $F_7$ RILs originating from cross 'S99-2281 × PI-408105A' at an early reproductive stage, two QTLs were recently identified and mapped on Chromosome 11 (FTS-11), as well as 13 (FTS-13); these QTLs were related to flood injury score and flood yield index. The significant QTL FTS-13 was reported to be linked to partial resistance to *P. sojae*, with an R2 of up to 18.3%, observed at several locations and years [104]. This provides definite evidence of the link between soybean flooding tolerance and *P. sojae* resistance. It implies that adding flooding-tolerance characteristics would boost resistance to rots caused by fungi, such as *P. sojae* [104]. The University of Missouri developed three improved germplasm lines of soybean for flood-tolerance through MAS. Under non-stress conditions, these germplasms have yielded a potential of 90% of commercial checks, and in severe flood condition, they were found to produce higher yield of 0.7–1.0 tonnes/hectare than commercial checks [130]. Dhungana et al. [115] reported QTLs linked with flooding stress at the V1–V2 stage of soybean. In this study, a RIL population derived from crossing a drought-susceptible (NTS116) and drought-tolerant (Danbaekkong) soybean cultivar was investigated. Based on composite interval mapping technique, they identified 10 QTLs associated with flood tolerance at the V1–V2 stage of soybean that possibly explained up to 30.7% phenotypic variations and can eventually be instrumental in soybean improvement programs. To summarize, marker-assisted mapping has been successful to some extent in identifying QTLs associated with flooding tolerance in oilseed crops, but more efforts are required to identify major QTLs explaining big phenotypic variance in large populations.

### *2.5. Cold Stress*

Cold stress, which may include chilling temperatures (below 20 °C) and/or freezing temperatures (below 0 °C), has a severe influence on plant growth and development and greatly impedes plant spatial dispersion and agricultural production. Cold stress may be generated by either chilling (below 20 °C) or freezing (below 0 °C) conditions. Cold stress may also be caused by chilling temperatures (temperatures below 20 degrees Celsius) or freezing temperatures (temperatures below 0 degrees Celsius). It directly inhibits metabolic processes and has indirect effects in the form of cold-induced osmotic (freezing-induced cellular dehydration and chilling-induced reduction of water absorption), oxidative, and other stresses. Cold stress prevents plants from fully expressing their genetic potential by

impeding metabolic activity first hand, while other stressors only do so indirectly. The great majority of plants that can thrive in temperate climates do so due to a process known as cold acclimation, which allows them to gain the capacity to endure temperatures as low as freezing.

The SSR, SNP, and EST markers have been successfully employed in achieving cold tolerance in plants [131,132]. Zhang et al. [133], Shinozaki et al. [134], and Lata and Prasad [135] identified the genes that play critical roles in the process by which plants develop tolerance to cold and osmotic stress. As a consequence of these investigations, as well as the use of molecular markers to build high-density physical and genetic maps of new genes, it is now feasible to enhance genetic diversity for desirable attributes, such as the ability to respond to cold stress. This is made feasible by the fact that molecular markers can now generate high-density maps of new genes [136]. At present, multiple genomics approaches are being employed to create new data through the utilization of genetic maps obtained from diverse *Brassica* and *Arabidopsis* species [137]. The accumulation of expressed sequence tags and single-nucleotide polymorphisms in *Brassica* species is generating critical information on genome polymorphism, as well as sequencing data for all stress-related traits.

Transcriptome adjustments from *Arabidopsis* have been exploited to discover genes related to cold treatment and other forms of stress. According to the findings, thirty percent of transcriptomes indicated sensitivity to regulation to common stress, with the majority clearly responding to particular stimuli [138]. In the first organ-specific cDNA fluorescence microarray investigation to evaluate coordinated transcriptional shifts in response to chilling and salinity stress in cultivated sunflower, Fernandez et al. [108] reported that eighty genes were found to be candidate genes for the early response of sunflowers to low temperature and salt stress. Microarray profiling of chilled and NaCl-treated sunflower leaves by the authors revealed dynamic shifts in the abundance of transcripts, including transcription factors, proteins involved in defense and stress, and effectors of homeostasis, all of which emphasize the complexity of both stress responses.

In a similar study, nylon microarrays with more than 8000 putative unigenes was performed to evaluate the transcriptional profiles of two accessions of sunflower viz. Santiago II and Melody, with differential growth rate ability under low temperatures. The results showed that, between the plants developed at low temperature (15 °C and 7 °C) and the corresponding control plants at two-leaf and four-leaf stages, 108 cDNA clones were found to be differentially expressed across the two genotypes with a $p$ value of $10^{-3}$ [107]. Around 90% of these genes, including those involved in protein biosynthesis, signal transduction, and energy, as well as carbohydrate metabolism and transport, were downregulated. Only four genes were identified as being differentially expressed in both genotypes, which further suggests that the response of sunflower plants to these temperature regimes is driven by identical genetic processes. The authors came to the conclusion that the vulnerability of sunflower to cold stress may be caused by the downregulation and/or non-induction of genes playing a vital role in cold tolerance.

Another research group utilized 104 RILs ($F_6$-derived lines) of soybean resulting from a cross 'Hayahikari (chilling-tolerant cultivar) × Toyomusume (chilling-sensitive cultivar)' in order to identify the QTL linked to freezing tolerance during reproductive stage. This was done to identify the QTL linked with soybean frost resistance during reproductive development. After conducting a genotypic evaluation of the population with 181 markers and correlating genotypic data with seed yield in two different conditions, i.e., chilling and optimal temperature, the researchers were able to identify three QTLs related to freezing tolerance on the basis of seed-yielding ability. These quantitative trait loci (QTLs) were essential for the plant's ability to withstand cold temperatures. Among these, qCTTSW1 and qCTTSW2 were found to be in close proximity to a QTL for flowering time. It was observed that qCTTSW2 interacted epistatically with a marker locus next to a second QTL for flowering time [109]. In fact, no significant QTL for cold tolerance was detected. An $F_2$ generation descended from a cross 'Hayahikari' × 'RIL of

Hayahikari' demonstrated that qCTTSW1 was mostly independent of flowering time. The third quantitative trait locus, qCTTSW3, has been shown to influence chilling tolerance. Another research employed a $BC_2F_3$ population derived from Harosoy (donor parent) × Hongfeng 11 (recurrent parent) to screen soybeans during the germination stage for drought and low-temperature conditions [110]. This population was screened for low-temperature and high-humidity circumstances. The objective of this study was to obtain a deeper knowledge of the genetic overlap between drought and low-temperature-tolerance QTLs in soybean during germination. There is a genetic overlap between drought and low-temperature tolerance during germination, as indicated by the identification of twelve QTLs in soybean that were associated with both drought and low-temperature tolerance. This fact was corroborated by the ability of tolerant soybean to withstand both low temperatures and drought at the same time. On the other side, it was observed that 18 QTLs were associated with drought resistance and that 23 QTLs were associated with cold-temperature resistance. A study was conducted utilizing QTL analysis of seed-yielding capacity at low temperature in soybean, simulated climatic conditions at normal and low temperatures, and RILs obtained from the cross of two cultivars with differing chilling tolerances [111]. The aim of this study was to understand the genetic basis of freezing tolerance and to identify associated genomic regions.

In close proximity to marker Sat 162 at linkage group A2, a quantitative trait locus (QTL) with a substantial effect was identified (LOD more than 15, r2 larger than 0.3). This QTL was shown to be connected with the capacity to generate seeds only at low temperatures. A population of segregating varied inbred families that generated basically identical lineages gave further confirmation of the QTL's significance. This proof of ancestry was provided by the community of inbred families. It was further reported that the genomic region containing the QTL also influenced the node and pod numbers regardless of temperature condition, although this effect was not primarily associated with chilling tolerance. In summary, several studies successfully demonstrated the association of physiological traits with multiple QTLs (including major QTLs) in oilseed crops under chilling stress and can be a great boon for development of tolerant cultivars in the future.

### *2.6. Heat Stress*

One of the major abiotic factors that lower crop productivity is heat stress. More frequent heat waves are predicted to occur and with greater severity as a result of global warming, aggravating the existing conditions. Therefore, it is crucial to understand the molecular processes that increase crop plants' tolerance to heat, especially in their reproductive organs. For the manipulation and exploration of pertinent genes for application in crop development initiatives, precise molecular knowledge will be helpful. This can be accomplished by gaining an in-depth understanding of various plant responses to heat stress, deciphering mechanisms of heat tolerance, and developing potential interventions for improving heat tolerance. Reduced photosynthesis, increased photorespiration, decreased availability of water, loss of cell membrane integrity and function, generation of ROS, and many other detrimental impacts are all driven by heat stress. Plants deploy numerous defense mechanisms to combat heat stress, including the increased expression of different enzymatic and non-enzymatic antioxidants to scavenge ROS, maintaining membrane stability, the production of various compatible solutes and metabolites, and the activation of various signaling cascades (Figure 5). Understanding each of these mechanisms will enable us to develop transgenic, traditional, and molecular breeding methods to enhance plant heat tolerance [139]. Numerous studies have documented adverse impacts of high-temperature stress in oilseed crops, such as decreased pollen germination and pollen tube length, which led to pollen mortality and fruit setting in *Brassica* as a result of heat stress [140]., reduction in soybean yield at temperatures more than 26/20 °C [141], and a reduction in seed weight in soybean due to a rise in temperature from 30/25 °C (day/night) during the seed filling period [142].

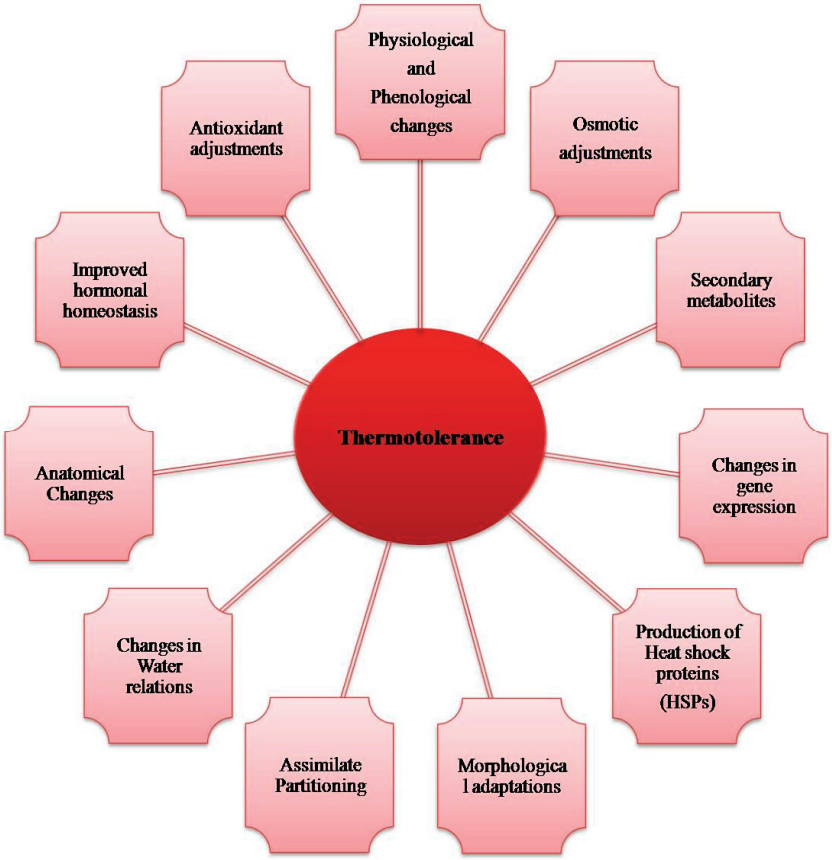


**Figure 5.** Plant responses to heat stress.

Considering the uncontrolled nature of environmental factors and the influence of additional biotic stresses, selection for thermo-tolerance through conventional breeding could be extremely challenging. Better techniques are therefore required for carrying out more precise greenhouse tests. Over the past ten years, scientists have turned to various methods to find the genes and QTLs linked with heat stress tolerance. A foundation for identifying the precise chromosomal position of QTLs responsible for plant heat tolerance is currently being laid by breakthroughs in genotyping assays and marker identification [143].

In oilseed crops, several major or minor QTLs and related markers for heat tolerance have been identified, including in peanuts [144], sesame [145], and soybean [146]. Genome maps and molecular markers for major oilseed crops have been identified by many researchers [145,147–150]. Similar to this, various oilseed crops, including soybean, [151], rapeseed [152,153], cotton [154], sunflower [155], groundnut [156], and sesame [157], are using the genome-wide association mapping technique under heat stress. In a thermo-sensitive dominant genic male sterility (GMS)-based inbred line (TE5A), which originated through the spontaneous mutation of *Brassica napus*, Zeng et al. [112], reported the fine mapping of *BntsMs* (dominant thermo-sensitive GMS gene) using AFLP and intron polymorphism (IP) methodologies. The five AFLP markers associated with the *BntsMs* gene were found by the authors after screening with 1024 primer combinations; two of these markers were then transformed to SCAR markers. Two SCAR markers were found flanking the *BntsMs* gene at a distance of 3.5 and 4.8 cm after studying a sizable $BC_2$ population of 700 recessive-fertility lines. Additionally, seven IP markers were also developed and used on the aforesaid population; two of these markers, IP004 and IP470, were placed at a distance of 0.3 and 0.2 cm, respectively, from the flanking region of the *BntsMs* gene.

## 3. Advent of a New Era for Development of Molecular Markers

With the advent of next-generation DNA sequencing technologies, including whole-genome sequencing (WGS), have opened new avenues for a comprehensive overview of the genetic diversity of oilseed crops and their genetic architecture in last decade. More recent advances involving expressed sequence tags (ESTs) also aided genome annotation and could further boost the molecular breeding program. Decent attempts that have been made in the genome sequencing of major oilseeds, such as sesame [158,159], safflower [160,161], rapeseed [162], mustard [163], sunflower [164,165], castor [166,167], flax seed [168], and peanut [169], have revolutionized the development of advanced co-dominant markers, such as SSR and SNPs, for the molecular mapping of abiotic resilience in oilseed crops. Soybean was sequenced through advanced high throughput technology in 2010 [170], and much progress has been made in this oilseed crop relevant to molecular breeding program for the achievement of abiotic stress tolerance and has been fairly covered in a review by Arya et al. [45]. WGS can provide detailed information about the genes associated with crucial and/or complex traits such as yield, oil content, and abiotic stress resistance, thus allowing for more precise selection of cultivars with desirable traits. Indeed, a novel method for SNP detection and mapping carry a huge potential to overcome the limitations of traditional MAS but are still far from being cost-efficient for the marker-assisted breeding of large populations [171]. Overall, WGS capacitates a system breeding approach with molecular markers that can be coupled with high-throughput phenotypic evaluation; such an approach has a potential to integrate gene function information with the improved field performance of oilseed crops.

## 4. Conclusions

Abiotic stresses have significant effect on the growth parameters of oilseed crops due to global climate change. Breeders of oilseeds should design their breeding programme to account for climate change and breed oilseed cultivars that are resilient to the changing climate. Developing reliable markers, which can be employed for different populations, could further enhance selection efficiency for breeding and could be a great milestone for breeding programs. The strong linkage of molecular markers to the desired attribute necessitates that they allow for preferred genotype selection. Abiotic stress tolerance in oilseed crops has also been established using emerging technologies like high-throughput marker systems and marker-based selection approaches, but their use is still limited. Not much work on MAS in this direction is being conducted. MAS is a highly promising strategy to achieve stress tolerance against abiotic stresses. Before beginning a breeding program, genetic diversity can also be assessed using molecular markers. In fact, several QTLs for economic features have already been reported. The use of a large sample size or the construction of multiple biparental cross populations could be useful to map rare alleles. To increase oilseed productivity, efforts should be made to use molecular breeding techniques, which can be expedited by current advancements in next-generation sequencing. The contemporary trend is to combine QTL mapping with the functional genomics methods (like ESTs and microarray) for gene expression studies that can be used to develop markers from genes itself [172]. This technique, called the "candidate gene approach", holds great potential in identifying the actual gene that controls the trait of interest. These methods can also be used to recognize SNP markers. The development of SNPs and EST-based markers has provided researchers a great tool for QTL mapping and MAS. Moreover, significant progress is being made in QTL mapping between related species through comparative mapping. To reduce the unfavorable effects of various abiotic stresses on oilseeds that are linked to climate change, modern molecular marker technologies must be adopted with traditional breeding techniques to create cultivars resistant to climatic change.

**Author Contributions:** Conceptualization, V.C., D.K., S.P., V.S., P.K. and R.B.D.; literature curation/investigation, V.C., S.P., P.K. and D.K.; validation, D.K., K., H.K. and A.R.; writing—draft preparation, V.C. and D.K.; writing—review and editing, V.C., P.K., V.S., K. and C.M.S.; supervision, R.B.D., P.K. and K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** No new data were created or analyzed in this study. Data sharing is not applicable to this article.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# *DsDBF1*, a Type A-5 DREB Gene, Identified and Characterized in the Moss *Dicranum scoparium*

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**Abstract:** Plant dehydration-responsive element binding (DREB) transcription factors (TFs) play important roles during stress tolerance by regulating the expression of numerous genes involved in stresses. DREB TFs have been extensively studied in a variety of angiosperms and bryophytes. To date, no information on the identification and characterization of DREB TFs in *Dicranum scoparium* has been reported. In this study, a new *DBF1* gene from *D. scoparium* was identified by cloning and sequencing. Analysis of the conserved domain and physicochemical properties revealed that DsDBF1 protein has a classic AP2 domain encoding a 238 amino acid polypeptide with a molecular mass of 26 kDa and a pI of 5.98. Subcellular prediction suggested that DsDBF1 is a nuclear and cytoplasmic protein. Phylogenetic analysis showed that DsDBF1 belongs to group A-5 DREBs. Expression analysis by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) revealed that *DsDBF1* was significantly upregulated in response to abiotic stresses such as desiccation/rehydration, exposure to paraquat, $CdCl_2$, high and freezing temperatures. Taken together, our data suggest that *DsDBF1* could be a promising gene candidate to improve stress tolerance in crop plants, and the characterization of TFs of a stress tolerant moss such as *D. scoparium* provides a better understanding of plant adaptation mechanisms.

**Keywords:** abiotic stress; dehydration-responsive element binding (DREB) transcription factors; gene expression; mosses; stress tolerance

**Citation:** Onele, A.O.; Mazina, A.B.; Leksin, I.Y.; Minibayeva, F.V. *DsDBF1*, a Type A-5 DREB Gene, Identified and Characterized in the Moss *Dicranum scoparium*. *Life* **2023**, *13*, 90. https://doi.org/10.3390/life13010090

Academic Editors: Wajid Zaman and Hakim Manghwar

Received: 5 December 2022
Revised: 23 December 2022
Accepted: 25 December 2022
Published: 28 December 2022



## 1. Introduction

Abiotic stresses, such as drought, salinity, heavy metals, high and low temperatures, can disrupt cellular homeostasis, resulting in redox imbalances and the accumulation of reactive oxygen species (ROS), which can limit plant development and crop productivity [1,2]. To react and adapt to these environmental challenges, plants have developed complex mechanisms including physiological, biochemical, and molecular processes [3–5]. Significant progress has been made over the past two decades regarding the identification and characterization of stress-responsive genes and proteins that directly protect plants against stresses [5,6]. Numerous genes are regulated by transcription factors (TF) in response to various abiotic stimuli [7]. Transcription factors play important roles in controlling the expression of genes in various signaling pathways due to their DNA-binding specificity [8,9].

Dehydration-responsive element binding (DREB) TFs play critical roles in plant tolerance by regulating the expression of stress-inducible genes during abiotic stresses [10]. They have been extensively identified in a wide variety of higher plants (angiosperms), such as *Arabidopsis thaliana* [11], soybean (*Glycine max*) [12], rice (*Oryza sativa*) [13,14], maize (*Zea mays*) [15], barley (*Hordeum vulgare*) [16], and others. The dehydration-responsive element (DRE)-binding factor, also known as DBF1, belongs to the APETALA2/Ethylene-Responsive Factor (AP2/ERF) TF family, which has been demonstrated to be involved in various biological processes in plants, including metabolism, development, and stress

response [12,17,18]. So far, AP2/ERF genes annotated in the mosses *Physcomitrium patens* and *Sphagnum fallax* are the largest TF families found in the plant TF databases (TFDB), although the AP2/ERF gene family has been rarely studied in the moss species [19–21]. Additionally, it has been shown that AP2/ERFs are regulated in response to numerous stresses, such as salinity and UV in *P. patens* [22] and the gene *PpDBF1* played a role in drought, salt, and cold tolerance in transgenic tobacco [23]. Furthermore, in the desiccation tolerant moss *Syntrichia caninervis*, AP2/ERFs were found to be the most abundant TFs [24].

*Dicranum scoparium* is a category "A" moss, one of the most desiccation tolerant moss species [25]. It is a widely distributed Holarctic moss that grows in various habitats and is one of the most polymorphic species in their genus [26]. Our preliminary analysis of class III peroxidase activity in three feather mosses such as *D. scoparium*, *Hylocomium splendens* and *Pleurozium schreberi* growing together in Aisha forest, Tatarstan, Russia revealed that they have high peroxidase activity and diverse peroxidase isoforms [27]. However, *D. scoparium* had the highest activity, approximately double that of *H. splendens* and *P. schreberi*, and this activity was stimulated by the desiccation/rehydration cycle. Therefore, *D. scoparium* was chosen for further investigation of desiccation tolerance mechanisms. Although to date the full genome of *D. scoparium* has not been sequenced and no reports of DREB families are available for this species, we have previously described in *D. scoparium* how temperature and desiccation/rehydration stresses change the expression of genes encoding Class I ascorbate peroxidase (*DsAPX*) and Class III peroxidases (*DsPODs*) [28]. We hypothesized that in Dicranum, abiotic stress will also influence the expression of *DBF1*. In this study, we report the isolation of a cDNA from *D. scoparium* that encodes a new DNA-binding TF, designated as DsDBF1. Furthermore, we analyzed the physico-chemical properties and sub-cellular localization of protein and gene expression patterns after desiccation/rehydration, high and low temperature, paraquat, DCMU, and $CdCl_2$ stresses. Our study showed that *DsDBF1* was significantly upregulated after exposure of *D. scoparium* to abiotic stresses, especially desiccation/rehydration, freezing temperature, paraquat, and $CdCl_2$, suggesting that this TF plays multiple roles in the tolerance of the moss to abiotic stresses.

## 2. Materials and Methods

### *2.1. Plant Material*

*Dicranum scoparium* Hedw. was collected in the Aisha Forest in Tatarstan, Russia (55°53 21.3 N 48°38 14.3 E). Plant material was placed between sheets of paper and left to dry slowly in the open air for 2 days before being stored in the refrigerator at +4 °C in the dark until usage [28].

### *2.2. Identification and Retrieval of DsDBF1protein*

Metatranscriptome data for the moss *D. scoparium* deposited to the Sequence Read Archive in the NCBI under accession numbers: PRJEB21674, ID: 393814 and PRJNA499105, ID: 499105 were extracted from the database [29,30]. The files were downloaded using the SRA Toolkit [31] and then converted to fastq format. FastQC software [32] was used to evaluate library quality control (QC). Adapter removal and trimming was done using Trimmomatic software version 0.39 [33]. After trimming, the reads were reassessed using the FastQC software [32]. Library assembly was performed using Trinity software [34]. All contaminants and foreign fungal and bacterial sequences were removed from the original data [29,30].

BLASTX [35] of representative sequences from mosses and other predicted taxa was used to determine the taxonomic classification of the identified transcripts (E-value < $1 \times 10^{-6}$). To reduce transcript redundancy, moss transcripts containing the top hits were isolated to a separate file and filtered using the EvidentialGene package (https://sourceforge.net/projects/evidentialgene/, accessed on 10 October 2022) with the default parameters. The DBF1 amino acid sequence from *P. patens* was used as a query in a TBLASTN search [36] for a similar protein among the filtered transcripts from *D. scoparium*.

A DBF1 transcript was found after using TBLASTN and open reading frames (ORFs) were detected using the Augustus gene prediction and AssemblyPostProcessor tools in Galaxy version 1.0.3.0 (https://usegalaxy.org/, accessed on 10 October 2022). To confirm the domain-identifying members of the *DBF1* gene family, the predicted sequence was submitted against PFAM [37], NCBI Conserved Domains Database (CDD) [38], InterProScan [39], and HMMER [40].

### *2.3. Cloning and Sequencing of DBF1 Gene*

Total RNA was extracted using the GeneJET Plant RNA Purification Mini Kit (Thermo Scientific, Vilnius, Lithuania). RNA concentration and purity were assessed using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and integrity was confirmed using 1% (*m/v*) agarose gel electrophoresis. First strand and double strand cDNA were synthesized using Evrogen Mint 2 synthesis kit according to manufacturer's protocols.

To verify the *DBF1* from *D. scoparium* identified in silico, the *DBF1* sequence with the highest homology to *DBF1* from *P. patens* was cloned into the pAL2-T vector (Evrogen, Moscow, Russia) using primers: F TGGGTTCACACGATGCGGA; R ACGCTTTGAATCCACTGACGG and then sequenced.

### *2.4. Sequence Analysis*

BLASTN software available online at (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 12 October 2022) was used to perform a homology search to compare our sequenced *DsDBF1* with other genes in the database. Files in Fasta format were downloaded from the NCBI database after BLAST search and then subjected to multiple sequence alignments using Clustal Omega [41] and ClustalW [42] in MEGA X [43]. The Expasy ProtParam tool [44] was used to predict the physico-chemical properties of the DsDBF1 protein, including molecular weight, isoelectric point, instability index, and grand average of hydropathicity (GRAVY). Subcellular localization was predicted by MULocDeep [45].

The homologous sequences of DsDBF1 proteins obtained after BLASTX and other known DREB proteins from the NCBI database were aligned by ClustalW [42] in MEGA X [43]. A phylogenetic tree was constructed in MEGA X [43] using the neighbor joining method for 1500 bootstraps [46]. Evolutionary distances were calculated using the Poisson correction method [47] and all ambiguous positions were removed by pairwise deletion.

The MEME suite (http://meme-suite.org/index.html, accessed on 12 October 2022) was used to analyze DREB protein sequences to find conserved motifs with the following parameters: zero or one site per sequence, number of motifs (1–10), motif width (6–50) [48]. After MEME, the motif map was rebuilt using the TBtools software [49].

### *2.5. Stress Treatments*

For stress treatment in this study, we followed the protocol developed in our early studies [28]. Before the experiment, 2 cm apical stem segments of dry mosses were pre-hydrated at +4 °C for 24 h on wet filter paper. For stress treatments, 0.2 g moss segments were incubated in 20 mL of 100 μM paraquat (1,1-dimethyl-4,4-bipyridylium dichloride), 100 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), or 100 μM $CdCl_2$ for 1 and 12 h. Hydrated apical stem segments were also thermally stressed by their exposure to −20 °C or +30 °C for 1 or 12 h in a dark temperature-controlled chamber (Thermostat LOIP, St. Petersburg, Russia). In all treatments, hydrated mosses kept at room temperature served as controls.

For desiccation stress, three biological replicates per treatment were used, each containing 0.17 g dry mass from 2 cm apical stem segments. Initially, air-dry mosses were fully hydrated by immersing them in a 20 mL volume of distilled water for 1 h while slowly shaking them on an orbital shaker. Then, the hydrated moss was gently blotted with filter paper and placed in the desiccator above silica gel. Here, moss samples were taken at time 0 (after 1 h of hydration), and after 2, 24, and 72 h of desiccation. After 72 h of desiccation,

moss samples were rehydrated for 0.5 and 2 h. The change in relative water content (RWC) was monitored according to the protocol previously described in [28].

### *2.6. RNA Extraction, cDNA Synthesis and RT-qPCR*

Samples exposed to stresses were immersed in liquid nitrogen, then, each sample was ground into a fine powder. For RT-qPCR, 0.1 g of material from each replicate was immediately frozen in liquid nitrogen and stored at −80 °C until use. Extraction of total RNA from *D. scoparium* thalli was performed using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration and purity were measured with NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the integrity was further evaluated by gel electrophoresis in a 1% (*w/v*) agarose gel. First strand cDNA was synthesized using protocols from the Evrogen Mint 2 synthesis kit.

The vector NTI Suite 9 software was used to design RT-qPCR primers with the following parameters: amplicon length from 60 to 300 bp and a Tm range of 55 to 65 °C. RT-qPCR was performed on CFX Connect™ Real-Time System (Bio-Rad Laboratories, Singapore) with qPCRmix-HS SYBR (Evrogen). The templates were amplified three times at 95 °C for 3 min followed by 40 cycles of amplification (94 °C for 10 s and 55/60 °C for 40 s). Melting curve analysis after RT-qPCR and gel electrophoresis examination of the amplified products were used to assess the specificity of the primers. The gene-specific primers used for RT-qPCR are listed in Table S1. Ribosomal RNA (*18S*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH2*) and α-tubulin (*α-TUB1* and *α-TUB2*) were used as internal controls for RT-qPCR normalization [28].

### *2.7. Statistical Analysis*

Three biological and six analytical replicates were used to run all reactions. Gene expression differences were assessed using normalized expression (Cq) in the Bio-Rad CFX Maestro™/Software version 2.3 and were found to be significant for $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) after ANOVA and Shapiro–Wilk Normality tests. The standard errors of the mean are shown as vertical bars (n = 6).

## 3. Results

### *3.1. Characterization and Phylogenetic Analysis of DsDBF1*

A *DBF1* gene was identified based on the metatranscriptome data for the moss *D. scoparium* downloaded from the Sequence Read Archive in the NCBI. To verify the *DBF1* gene identified from *D. scoparium*, specific primers were designed and the PCR product (717 bp) was cloned into the pAL2-T vector (Evrogen, Moscow, Russia) and then sequenced. Blasting the *DBF1* sequence from *D. scoparium* after cloning and sequencing revealed high homology with *ERF/DREBs* of other mosses and vascular plants in the NCBI database. Further analyses of the protein sequence using PFAM [37], NCBI CDD [38], InterProScan [39] and HMMER [40] databases revealed that this protein had a classic AP2 domain structure (Figure 1) and was named DsDBF1. In addition, coding domain sequence (CDS) length (bp), subcellular localization, and physico-chemical properties such as protein length (aa), molecular weight (MW, kDa), isoelectric point (pI), instability index, and GRAVY were predicted (Table 1). The results showed that the cloned *DsDBF1* encoded a 238 amino acid polypeptide (Figure S1) with a predicted molecular weight of 26 kDa and isoelectric point of 5.98. Calculation of the instability index classified the protein as unstable with a value greater than 40. A negative value of GRAVY indicated that DsDBF1 was hydrophilic and subcellular prediction showed that the protein was localized within the nucleus and cytoplasm (Table 1).

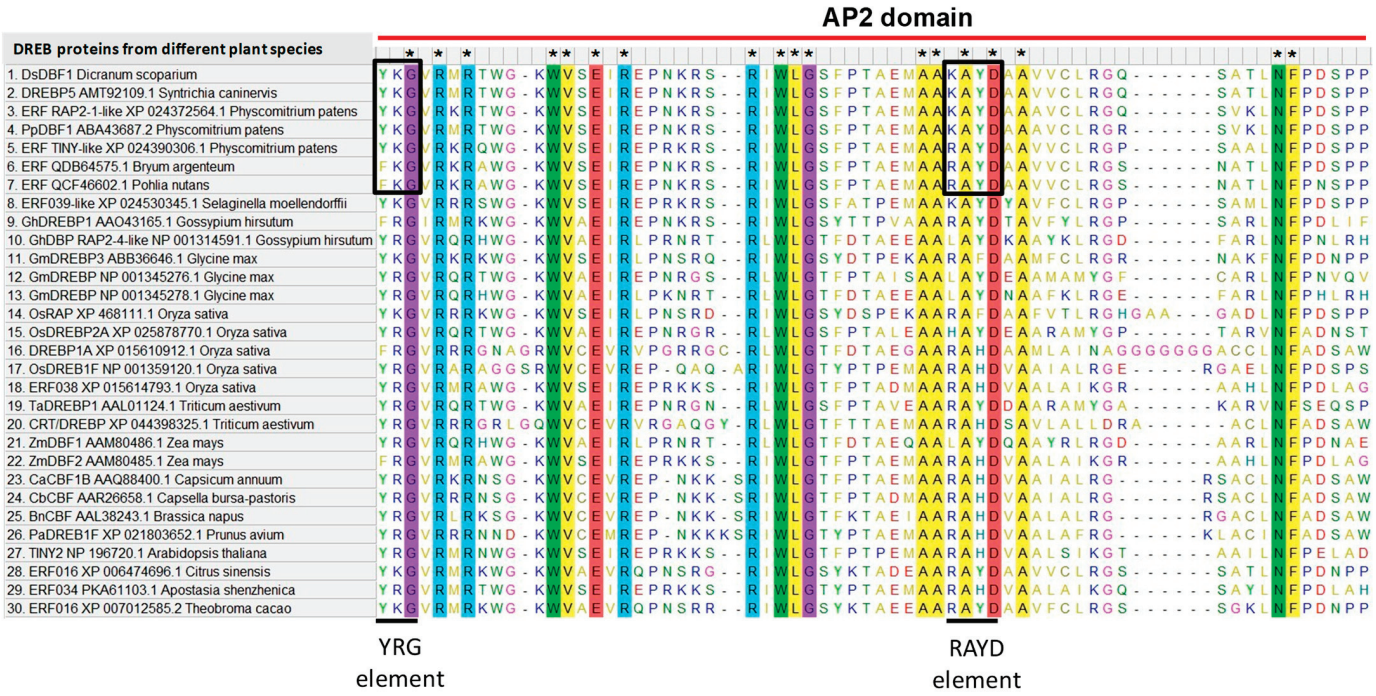


**Figure 1.** Sequence alignments of DsDBF1 and other known ERF/DREB proteins showing the classical AP2 domains from mosses and vascular plants such as *Syntrichia caninervis* (DREBP5_AMT92109.1), *Physcomitrium patens* (ERF RAP2-1-like_XP 024372564.1; PpDBF1 ABA43687.2; ERF_TINY-like_XP 024390306.1), *Gossypium hirsutum* (GhDREBP1_AAO43165.1; GhDBP_RAP2-4-like_NP 001314591.1), *Glycine max* (GmDREBP3_ABB36646.1; GmDREBP_NP 001345276.1; GmDREBP_NP 001345278.1), *Oryza sativa* (OsRAP_XP 468111.1; OsDREBP2A_XP 025878770.1; DREBP1A_XP 015610912.1; OsDREB1F_NP 001359120.1; ERF038_XP 015614793.1), *Triticum aestivum* (TaDREBP1_AAL01124.1; CRT/DREBP_XP 044398325.1), *Zea mays* (ZmDBF1_AAM80486.1; ZmDBF2_AAM80485.1), *Capsicum annuum* (CaCBF1B_AAQ88400.1), *Capsella bursa-pastoris* (CbCBF_AAR26658.1), *Brassica napus* (BnCBF_AAL38243.1), *Prunus avium* (PaDREB1F_XP 021803652.1), *Arabidopsis thaliana* (TINY2_NP 196720.1), *Bryum argenteum* (ERF_QDB64575.1), *Pohlia nutans* (ERF_QCF46602.1), *Selaginella moellendorffii* (ERF039-like_XP 024530345.1), *Citrus sinensis* (ERF016_XP 006474696.1), *Apostasia shenzhenica* (ERF034_PKA61103.1), and *Theobroma cacao* (ERF016_XP 007012585.2). Amino acid sequences are highlighted with different colors. Sequences marked by (*) show conserved amino acid residues. Two conserved elements (YRG and RAYD) are marked by black horizontal lines. The differences in the moss conserved elements are shown in black frames. The red line shows the classical AP2 domain.

**Table 1.** Physico-chemical properties and subcellular localization of DsDBF1.

| Parameters | DsDBF1 |
|---|---|
| CDS length, bp | 717 |
| Number of amino acids | 238 |
| Molecular weight (kDa) | 26 |
| Theoretical pI | 5.98 |
| Instability index | 64.99 |
| Grand average of hydropathicity (GRAVY) | −0.224 |
| Subcellular localization prediction | Nucleus/cytoplasm |

Sequence alignment analysis indicated that DsDBF1 shared high homology and a conserved AP2/ERF domain with other DREBs (Figure 1), but with low similarity in their overall amino acid sequences (Figure S2). Additionally, two conserved elements (YRG and RAYD) were found in the AP2/ERF domain after sequence analysis, although arginine (R) is replaced by lysine (K) in both the first YRG and second RAYD elements in DsDBF1

and some DREBs of other mosses (Figure 1). The homologous protein sequences obtained after BLASTP search of the DsDBF1 sequence and other known ERF/DREB proteins from GenBank were used to construct a phylogenetic tree demonstrating the evolutionary relationship between DsDBF1 and other similar sequences from mosses and vascular plants. The evolutionary tree showed that DsDBF1 belongs to the A-5 group of the DREB subfamily as it shared a common ancestry and homology with other known A-5 DREBs from mosses such as *S. caninervis*, *P. patens*, *Bryum argenteum*, *Pohlia nuntans,* and vascular plants such as *Selaginella moellendorffii*, *G. max*, *O. sativa*, *Citrus sinensis*, *Theobroma cacao,* and *Gossypium hirsutum* (Figure 2). Furthermore, it was found that group A-5 was divided into seven subgroups, with *S. moellendorffii* positioning between the protein subgroups of mosses and the vascular plants. As shown in Figure 2, all other known DREBs from vascular plants were clustered into different DREB subfamilies such as A-1, A-2, A-4, and A-6.

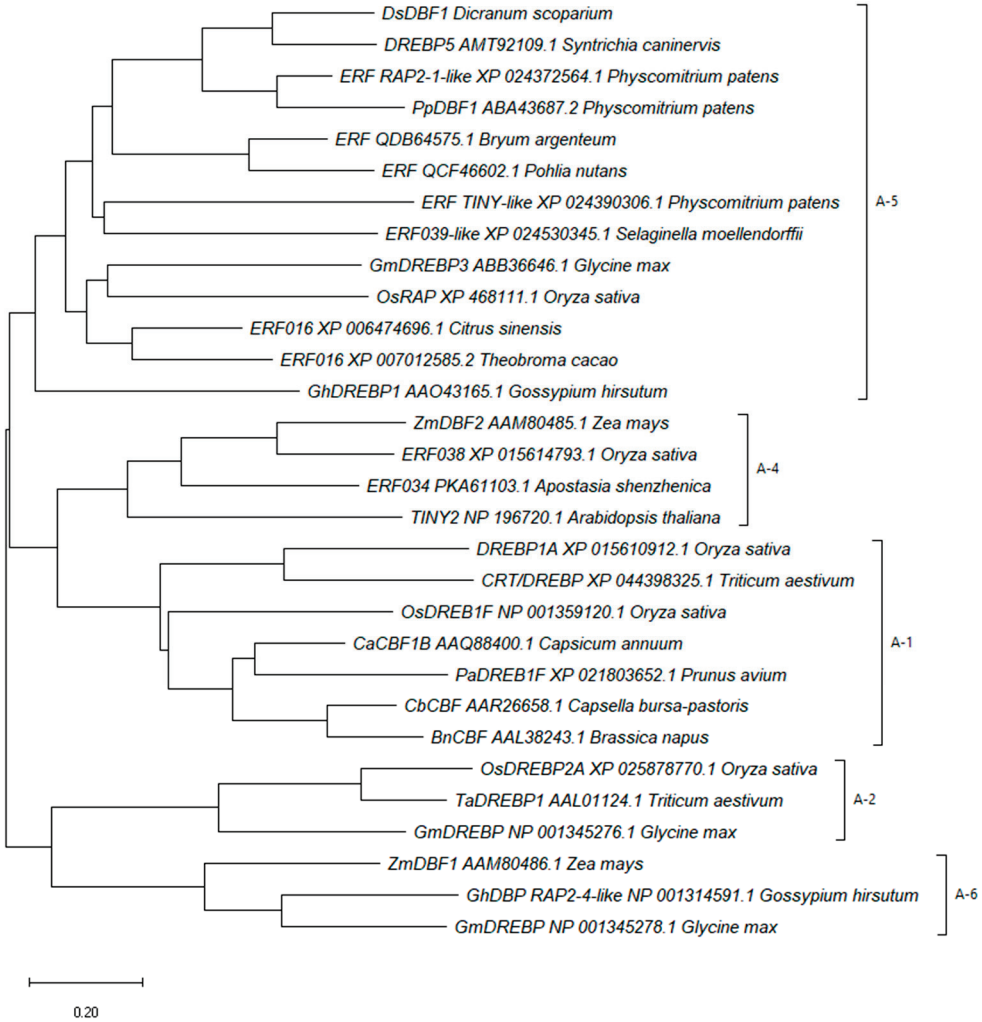


**Figure 2.** Phylogenetic analyses of DsDBF1 and other ERF/DREB proteins from mosses and vascular plant constructed using the neighbor-joining method with 1500 bootstrap test showing the relationship between the amino acid sequences. Evolutionary distances were calculated using the Poisson correction method and all ambiguous positions were removed by pairwise deletion. Amino acid sequences used for phylogenetic tree construction were retrieved, in part, from GenBank and after blast analysis from GenBank: *Syntrichia caninervis* (DREBP5_AMT92109.1), *Physcomitrium patens* (ERF RAP2-1-like_XP 024372564.1; PpDBF1 ABA43687.2; ERF_TINY-like_XP 024390306.1), *Gossypium hirsutum* (GhDREBP1_AAO43165.1; GhDBP_RAP2-4-like_NP 001314591.1), *Glycine max* (GmDREBP3_ABB36646.1; GmDREBP_NP 001345276.1; GmDREBP_NP 001345278.1), *Oryza sativa* (OsRAP_XP 468111.1; OsDREBP2A_XP 025878770.1; DREBP1A_XP 015610912.1; OsDREB1F_NP

001359120.1; ERF038_XP 015614793.1), *Triticum aestivum* (TaDREBP1_AAL01124.1; CRT/DREBP_XP 044398325.1), *Zea mays* (ZmDBF1_AAM80486.1; ZmDBF2_AAM80485.1), *Capsicum annuum* (CaCBF1B_AAQ88400.1), *Capsella bursa-pastoris* (CbCBF_AAR26658.1), *Brassica napus* (BnCBF_AAL38243.1), *Prunus avium* (PaDREB1F_XP 021803652.1), *Arabidopsis thaliana* (TINY2_NP 196720.1), *Bryum argenteum* (ERF_QDB64575.1), *Pohlia nutans* (ERF_QCF46602.1), *Selaginella moellendorffii* (ERF039-like_XP 024530345.1), *Citrus sinensis* (ERF016_XP 006474696.1), *Apostasia shenzhenica* (ERF034_PKA61103.1), and *Theobroma cacao* (ERF016_XP 007012585.2).

Additionally, the results of MEME analyses showed that DsDBF1 contained a total of six motifs, among them, motifs 1–3 represented the basic conserved motifs that made up the AP2 domain (Figure 3). Motif 4 was absent only in the DREB protein of *Triticum aestivum* (TaDREBP1_AAL01124.1), while motif 5 was absent in the DREB proteins of *Gossypium hirsutum* (GhDREBP1 AAO43165.1 and GhDBP RAP2-4-like_NP 001314591.1), *G. max* (GmDREBP3 ABB36646.1 and GmDREBP_NP 001345278.1), *O. sativa* (OsRAP_XP 468111.1 and DREBP1A_XP 015610912.1), *Z. mays* (ZmDBF1_AAM80486.1) including *T. aestivum* (TaDREBP1_AAL01124.1). However, an additional motif 9 was detected in DsDBF1, which was only conserved in *S. caninervis* (DREBP5_AMT92109.1) and two in *P. patens* (ERF RAP2-1-like_XP 024372564.1 and PpDBF1_ABA43687.2) (Figure 3).

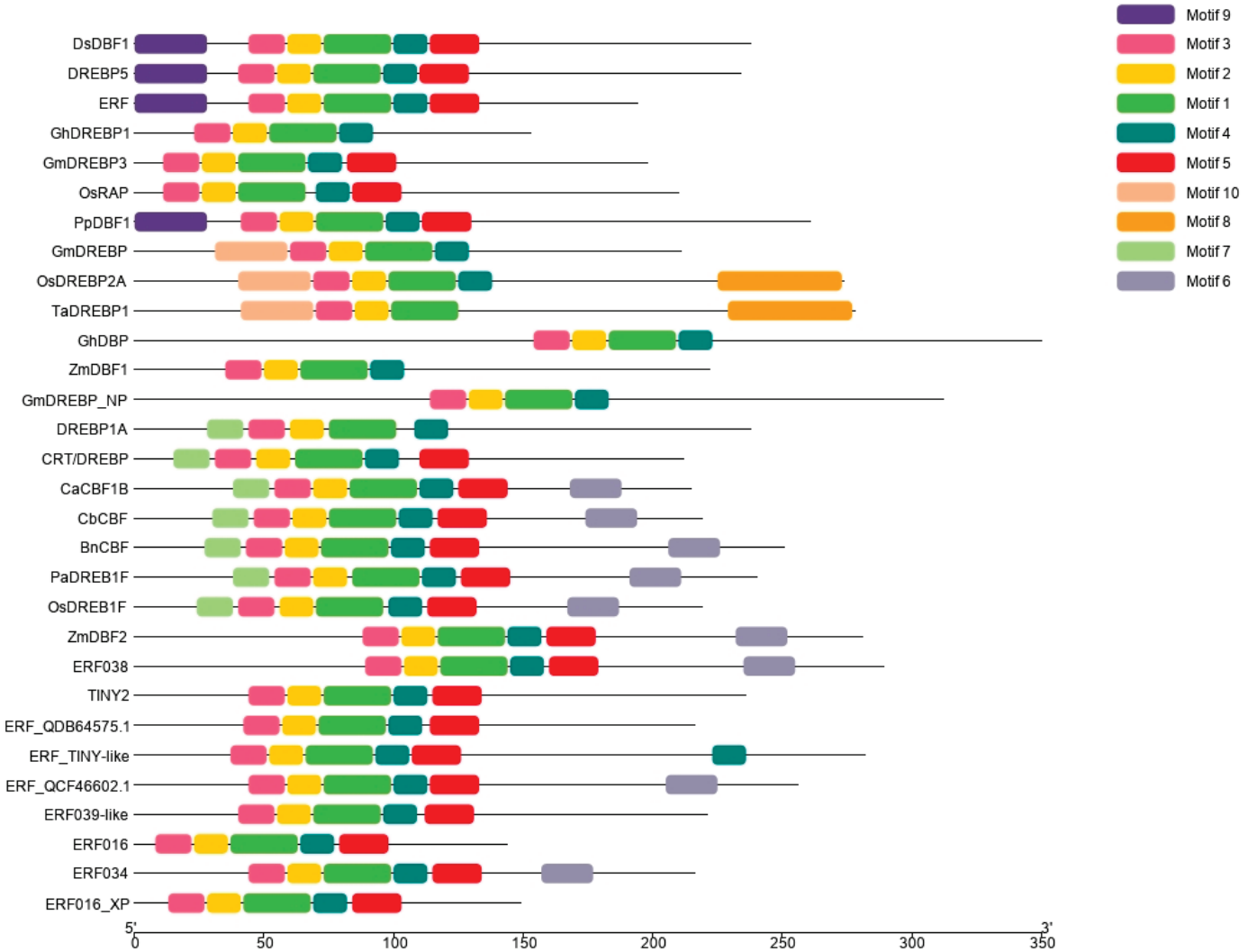


**Figure 3.** Motif analysis of DsDBF1 and other known classic ERF/DREB proteins from mosses and vascular plants: *Syntrichia caninervis* (DREBP5_AMT92109.1), *Physcomitrium patens* (ERF RAP2-1-like

_XP 024372564.1; PpDBF1 ABA43687.2; ERF_TINY-like_XP 024390306.1), *Gossypium hirsutum* (GhDREBP1_AAO43165.1; GhDBP_RAP2-4-like_NP 001314591.1), *Glycine max* (GmDREBP3_ABB36646.1; GmDREBP_NP 001345276.1; GmDREBP_NP 001345278.1), *Oryza sativa* (OsRAP_XP 468111.1; OsDREBP2A_XP 025878770.1; DREBP1A_XP 015610912.1; OsDREB1F_NP 001359120.1; ERF038_XP 015614793.1), *Triticum aestivum* (TaDREBP1_AAL01124.1; CRT/DREBP_XP 044398325.1), *Zea mays* (ZmDBF1_AAM80486.1; ZmDBF2_AAM80485.1), *Capsicum annuum* (CaCBF1B_AAQ88400.1), *Capsella bursa-pastoris* (CbCBF_AAR26658.1), *Brassica napus* (BnCBF_AAL38243.1), *Prunus avium* (PaDREB1F_XP 021803652.1), *Arabidopsis thaliana* (TINY2_NP 196720.1), *Bryum argenteum* (ERF_QDB64575.1), *Pohlia nutans* (ERF_QCF46602.1), *Selaginella moellendorffii* (ERF039-like_XP 024530345.1), *Citrus sinensis* (ERF016_XP 006474696.1), *Apostasia shenzhenica* (ERF034_PKA61103.1), and *Theobroma cacao* (ERF016_XP 007012585.2). Distribution of 10 putative conserved motifs in DREB proteins is shown. Conserved motifs are represented by different colored boxes numbered 1–10.

### *3.2. Expression Patterns of DsDBF1 in Response to Abiotic Stress Treatments*

The expression pattern of *DsDBF1* was studied after application of abiotic stresses such as desiccation/rehydration, exposure to DCMU, $CdCl_2$, paraquat, high and freezing temperatures to moss apical segments. Desiccation of the hydrated moss for 2, 24, and 72 h over silica gel resulted in almost up to 94% loss of RWC in the moss samples, accompanied by downregulation of *DsDBF1*, with the lowest expression observed after 24 h of dehydration (Figure 4A). Rehydration of the mosses after 72 h of desiccation showed a gradual increase in *DsDBF1* expression after 0.5 h and further 2 h with the expression of *DsDBF1* 2-fold higher compared to that in the hydrated mosses before desiccation (Figure 4A). Treatment of moss segments with an inhibitor of photosynthesis DCMU downregulated *DsDBF1* expression after 1 and 12 h (Figure 4B). Subjecting the mosses to heavy metal $CdCl_2$ and prooxidant paraquat significantly increased the expression of *DsDBF1* after 1 h (Figure 4B); however, further treatment for 12 h downregulated *DsDBF1* expression. No significant changes in *DsDBF1* expression were observed after exposing moss to +30 °C for 1 and 12 h. Exposure of mosses to a freezing temperature of −20 °C reduced the level of *DsDBF1* expression after 1 h (Figure 4B); however, further exposure for 12 h at −20 °C upregulated gene expression almost 10-fold compared to a 1 h treatment (Figure 4B).

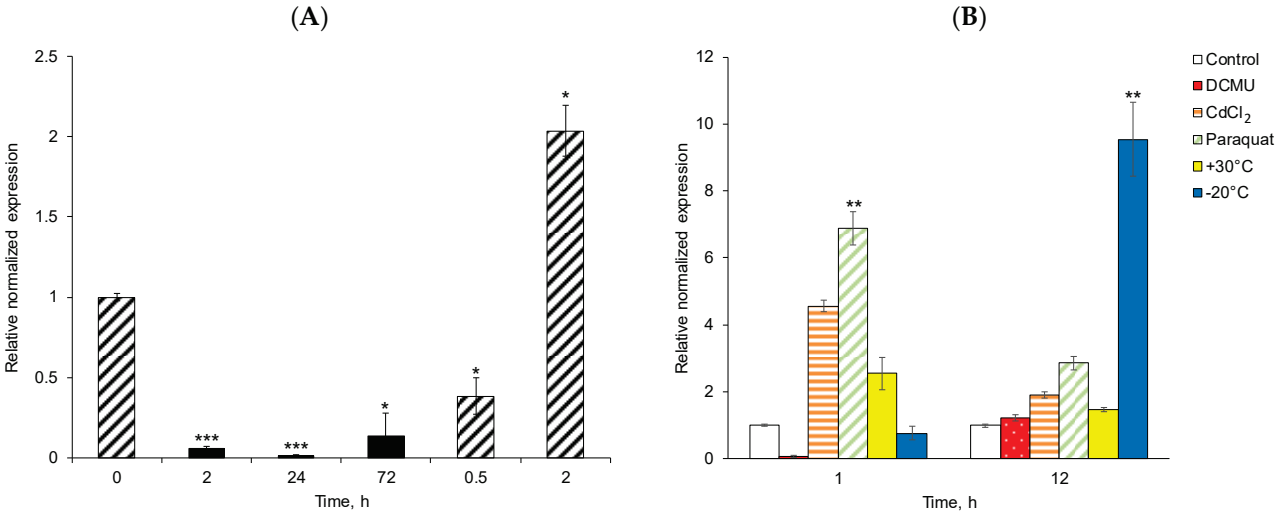


**Figure 4.** Expression patterns of *DsDBF1* under abiotic stress treatments analyzed using RT-qPCR. (**A**) Relative expression of *DsBF1* during desiccation over silica gel and rehydration. Shaded bars

represent the hydrated and rehydrated moss, and solid bars represent the desiccated moss. (**B**) Relative expression of *DsDBF1* exposed to DCMU, $CdCl_2$, paraquat and high/low temperature for 1 and 12 h. Open bars correspond to control samples of mosses kept at room temperature. Red bars with white dots represent mosses treated with 100 µM DCMU, bars with orange horizontal stripes show moss treated with 100 µM $CdCl_2$, bars with green stripes correspond to samples subjected to 100 µM paraquat, yellow and blue bars correspond to mosses exposed to +30 °C and −20 °C, respectively. $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***). The vertical bars indicate the standard errors of the mean (n = 6).

## 4. Discussion

Members of the AP2/ERF family of TFs are among the most important key regulators of genes responsible for stress tolerance and developmental transitions of plants. These TFs regulate transcriptional networks to activate or repress gene expression in response to biotic and abiotic factors through the modulation of several signaling pathways [7,8,17,50]. In the last few years, many DREBs have been identified and characterized in several angiosperms, including *A. thaliana* [11], rice (*O. sativa*) [13,51], soybean (*G. max*) [52], maize (*Z. mays*) [15,53], cotton (*G. hirsutum*) [54,55], barley (*H. vulgare*) [16,56,57], wheat (*T. aestivum*) [58], *Populus euphratica* [59], *Caragana korshinskii* [60], and others. Surprisingly, the AP2/ERF gene family has been rarely studied in stress-tolerant moss species [19–21]. Several recent studies have shown that AP2/ERF TFs play an important role in the developmental processes and stress responses in some moss species, such *P. patens* [22,23,61], *S. caninervis* [24,62–65], *B. argenteum* [21], and *P. nutans* [66]. *Dicranum scoparium* is a desiccation-tolerant moss [25] whose genome has not been fully sequenced, and no TF families of this species have been reported to date. In this present study, we first identified in silico a cDNA of the *DBF1* gene in the moss *D. scoparium*. Then, the identified gene was verified by cloning and sequencing. In addition, we performed molecular characterization of the protein, including analysis of the conserved domain, physico-chemical properties, subcellular localization, phylogenetic relationship, and motif analyses of identified DsDBF1 and DREBs of other plants, and finally, we examined the expression patterns of this gene in response to abiotic stresses. Our results demonstrate that the expression of *DsDBF1* is strongly induced by rehydration after desiccation, and treatments with $CdCl_2$, paraquat, and freezing temperature, providing insights into the roles of *DBF1* in response of *D. scoparium* to abiotic stresses.

Analyses of the physico-chemical properties and the subcellular localization showed that *DsDBF1* encodes a 238-amino acid polypeptide with a molecular weight of 25 kDa and a pI of 5.98 and the protein is localized within the nucleus and cytoplasm (Table 1). While the majority of TFs are nuclear localized, some are not when initially synthesized [67]. Some of these TFs are kept inactive in the cytoplasm when synthesized or expressed as membrane proteins, but when stimulated, they are activated by proteolytic cleavage, releasing the active form, which enters the nucleus and activates target genes [67,68].

Furthermore, the BLASTP search of the NCBI database revealed that DsDBF1 shares high sequence similarities with some DREBs from angiosperms and mosses. In addition, some uncharacterized proteins from mosses such as *Ceratodon purpureus* and *S. fallax* also show very high similarities to DsDBF1. The amino acid composition of the AP2 domain of DsDBF1 revealed that it contains 65 amino acid residues (Figure 1), which approximately corresponds to the conserved 60 amino acids of the AP2/ERF domain found in all DREBs [11]. Amino acid alignments of DREB proteins from different plants show high sequence similarity in the middle of AP2/ERF domain of these proteins (Figure 1), which is a significant feature of plant DREBs [64,69]. However, in general, outside the domain box, low similarity is observed in their overall amino acid sequences (Figure S2).

Analysis of the AP2/ERF domain after multiple sequence alignments revealed the presence of two conserved YRG and RYAD elements (Figure 1), although only glycine (G) is conserved in the YRG element among all the DREBs, while alanine (A) and aspartic acid (D) are conserved in the RYAD elements (Figure 1). Furthermore, in the first YRG

element, tyrosine (Y) and arginine (R) are replaced by phenylalanine (F) and lysine (K), respectively, whereas in the second RYAD element, R is substituted by K, leucine (L), and histidine (H), and the Y is substituted by F and H. The AP2/ERF domain is a type of DNA-binding module that contains two known conserved elements (YRG and RAYD), and these two elements can bind with the promoter sequence or some other interacting proteins [69,70]. Studies have shown that YRG is involved in DNA binding activity and is the basic hydrophilic N-terminal side of the AP2/ERF domain. The N-terminal region is approximately 19 to 22 amino acids in length [50,69,70]. In addition, the second element, RAYD, is located in the acidic C-terminal region of the AP2/EREBP domain with a length of 42 to 43 amino acids. It is suggested that the RAYD element plays a crucial role in mediating protein–protein interactions [50,70]. However, in this study, the substitution of amino acids observed at various positions within the conserved elements in the AP2/ERF domain after multiple sequence alignments (Figure 1) of DREB proteins, may imply their functional divergence within DREB subfamilies.

To understand the evolutionary relationship between DsDBF1 and other well-known DREBs from other plants, a neighbor-joining tree was constructed using the deduced amino acid residues of these DREB proteins (Figure 2). In this analysis, DsDBF1 was found to belong to the A-5 group of the DREB subfamily as it shares a common ancestor with other known A-5 DREBs from mosses such as *S. caninervis*, *P. patens*, *B. argenteum*, *P. nuntans*, a lycophyte, for example, *S. moellendorffii*, and the angiosperms, such as *G. max*, *O. sativa*, *C. sinensis*, *T. cacao,* and *G. hirsutum* (Figure 2). In the A-5 subgroup, *S. moellendorffii* branches from the moss subgroup, positioning itself between the mosses and the angiosperms. This supports the report of early divergence of vascular plants from the ancient non-vascular plants [71]. It has been proposed that *PpDBF1*, an A-5 type DREB from *P. patens*, is an ancestor of DREB proteins and plays a general role in various stresses in non-vascular moss, which has diverged into different subclasses with different functions in the higher plants [23]. Consequently, the grouping of DsDBF1 and some other A-5 DREB proteins from mosses, lycophytes, and angiosperms in one clade suggests that they were established in the early stages of land plant evolution. Additionally, it was found that all other known DREBs from vascular plants diverged into different DREB subfamilies such as A-1, A-2, A-4, and A-6 (Figure 2). The DREB gene subfamily may have evolved and assumed new roles as a result of the divergence of the AP2 genes. The functional diversity and divergence of DREB genes during the adaptive evolution of stress signaling pathways in plants is most likely the result of subsequent duplication and transposition events [23].

Moreover, an investigation of the conserved motifs in DsDBF1 and other selected DREB proteins was carried out using MEME software. From the results, DsDBF1 contains a total of six motifs. Motifs 1–3 represent the conserved motifs of the AP2 domain (Figure 3). Furthermore, an additional motif 9 was detected in DsDBF1. This motif is present in *S. caninervis* (DREBP5_AMT92109.1) and *P. patens* (ERF RAP2-1-like_XP 024372564.1 and PpDBF1_ABA43687.2) (Figure 3), suggesting their common origin. Genome-wide sequence analysis of AP2/ERF family TFs in numerous plants revealed conserved regions and motifs on both sides of the AP2/ERF domain with important roles in transcriptional activity, protein–protein interactions, and nuclear localization. These conserved motifs can serve as an evidence for further classification of subgroups [50,72].

Plant DREB TFs play critical roles in the response to dehydration, salinity, and cold stresses [73,74]. To further understand the role of *DsDBF1* in response to stresses, we examined the expression profile of *DBF1* gene by RT-qPCR in the *D. scoparium* subjected to desiccation/rehydration, exposure to DCMU, $CdCl_2$, paraquat, heat and freezing temperature. Our results indicate that *DsDBF1* gene is upregulated by most of these stresses, suggesting that this gene is involved in *D. scoparium* response to abiotic stresses (Figure 4A,B). Surprisingly, *DsDBF1* gene is downregulated following exposure of the moss to DCMU (Figure 4B). It has been reported that genes assigned to different groups within the same gene family show diverse stress response patterns and stress tolerance [64,75]. To date, most reports on DREB and Cold binding factors (CBFs) have mainly focused on DREBA1

and DREBA2, the largest among the subgroups [69,76]. The A-1 type DREBs (DREB1) are induced by cold and improve plant stress tolerance to low temperatures [73,77], whereas A-2 type DREBs (DREB2) play a major role in response to dehydration and heat stress, and improve drought and salt tolerance in plants [78]. A-5 DREBs have rarely been studied, and the functional and stress response mechanisms are still unclear [63].

Interestingly, desiccation of the hydrated mosses for 2, 24, and 72 h decreased *DsDBF1* expression (Figure 4A). However, rehydrating moss thalli after 72 h of desiccation progressively increased *DsDBF1* expression to 2-fold higher compared to the hydrated mosses before desiccation (Figure 4A). *PpDBF1*, a homolog of *DsDBF1,* was weakly induced by dehydration stress but strongly induced by ABA [23]. Out of ten A-5 type DREBs from *S. caninervis*, *ScDREB5* was downregulated under rapid desiccation stress over silica gel, while *ScDREB3*, *ScDREB9,* and *ScDREB10* were poorly induced by desiccation [63]. However, these four DREBs were significantly induced by cold stress, while *ScDREB3* and *ScDREB5* were upregulated during heat stress [63]. The rapid desiccation used in our experiment, in which moss thalli were dried over silica gel and reached an RWC of 6% [28] after 24 h, never occurs in boreal forests, where drying rates of mosses are much slower [27,79]. Other A-5-type DREBs such as *GhDBP1* and *GmDREB3* have been shown to improve plant stress tolerance [23,55,77]. Moreover, photosynthesis inhibitor DCMU decreased *DsDBF1* expression (Figure 4B). Meanwhile, $CdCl_2$ and paraquat significantly altered the expression of *DsDBF1*, as short-term exposure increased *DsDBF1* expression after 1 h (Figure 4B). Long-term treatment of moss samples with paraquat and $CdCl_2$ resulted in downregulation of *DsDBF1* expression after 12 h. Furthermore, exposure of moss to +30 °C had little effect on *DsDBF1* expression, although a freezing temperature of −20 °C for 12 h upregulated gene expression almost 10-fold compared to a 1 h cold treatment (Figure 4B). It has been reported that *PpDBF1*, *GmDREB2*, *StDREB2, ScDREB1*, *ScDREB2*, *ScDREB4*, *ScDREB6*, *ScDREB7,* and *ScDREB8* responded to drought, salt, and cold treatment among members of the A-5 subgroups [23,63,80,81]. Taken together, the upregulation of *DsDBF1* during rehydration after desiccation, exposure to $CdCl_2$, paraquat, and freezing-temperature stress suggests that *DsDBF1,* like other A-5 DREBs, plays important roles in *D. scoparium* stress tolerance.

## 5. Conclusions

An A-5 type gene, *DsDBF1*, encoding DRE-binding transcription factor TF was identified and cloned in the moss *D. scoparium*. *DsDBF1* protein was predicted to be localized within the nucleus and cytoplasm. Furthermore, RT-qPCR analysis showed that *DsDBF1* expression was significantly induced in response to abiotic stresses such as desiccation/rehydration, exposure to paraquat, $CdCl_2$, high and freezing temperatures. *D. scoparium* is a desiccation tolerant moss species. Based on our results, we believe that *DsDBF1* could be a promising gene candidate to improve stress tolerance in various crop plants, and characterization of transcription factors of a stress-tolerant moss such as *D. scoparium* provides a better understanding of plant response and adaptation mechanisms.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/life13010090/s1, Figure S1: *DsDBF1* coding domain sequence and protein sequence; Figure S2: Sequence alignments of DsDBF1 and other known ERF/DREB proteins from mosses and vascular plants such as *Syntrichia caninervis* (DREBP5_AMT92109.1), *Physcomitrium patens* (ERF RAP2-1-like_XP 024372564.1; PpDBF1 ABA43687.2; ERF_TINY-like_XP 024390306.1), *Gossypium hirsutum* (GhDREBP1_AAO43165.1; GhDBP_RAP2-4-like_NP 001314591.1), *Glycine max* (GmDREBP3_ABB36646.1; GmDREBP_NP 001345276.1; GmDREBP_NP 001345278.1), *Oryza sativa* (OsRAP_XP 468111.1; OsDREBP2A_XP 025878770.1; DREBP1A_XP 015610912.1; OsDREB1F_NP 001359120.1; ERF038_XP 015614793.1), *Triticum aestivum* (TaDREBP1_AAL01124.1; CRT/DREBP_XP 044398325.1), *Zea mays* (ZmDBF1_AAM80486.1; ZmDBF2_AAM80485.1), *Capsicum annuum* (CaCBF1B_AAQ88400.1), *Capsella bursa-pastoris* (CbCBF_AAR26658.1), *Brassica napus* (BnCBF_AAL38243.1), *Prunus avium* (PaDREB1F_XP 021803652.1), *Arabidopsis thaliana* (TINY2_NP 196720.1), *Bryum argenteum* (ERF_QDB64575.1), *Pohlia nutans* (ERF_QCF46602.1), *Selaginella moellendorffii* (ERF039-like_XP 024530345.1), *Citrus sinensis* (ERF016_XP 006474696.1), *Apostasia shenzhenica*

(ERF034_PKA61103.1) and *Theobroma cacao* (ERF016_XP 007012585.2). Multiple alignment was performed using Clustal Omega. Amino acid sequences are highlighted with different colors. Sequences marked by (*) show conserved amino acid residues, Table S1: Primers of RT-qPCR.

**Author Contributions:** Conceptualization, A.B.M. and F.V.M.; methodology, A.B.M. and A.O.O.; software, A.O.O., A.B.M. and I.Y.L.; formal analysis, A.O.O. and I.Y.L.; investigation, A.B.M.; writing—original draft preparation, A.O.O.; writing—review and editing, F.V.M.; supervision, F.V.M.; project administration, F.V.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the state assignment of the FRC KazSC RAS. F.V.M. thanks Russian Foundation for Basic Research [grant number 20-04-00721] for partial financial support (gene cloning and sequencing).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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# Unravelling the Morphological, Physiological, and Phytochemical Responses in *Centella asiatica* L. Urban to Incremental Salinity Stress

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**Abstract:** *Centella asiatica* L. as a traditional medicinal plant is popular in several Asian countries and characterized by the presence of phytochemicals, such as phenolics and flavonoids. Soil salinity can affect the growth and phytochemical composition in this plant species. In this study, the effects of incremental soil salinity (0, 25, 50, 75, and 100 mM NaCl) on growth, physiological characteristics, total phenolic and total flavonoid contents, including the antioxidant activity of *Centella asiatica* L., were evaluated under greenhouse conditions. Salinity stress reduced growth, biomass production, and total chlorophyll contents, while increasing electrolyte leakage, $Na^+$ and $Cl^-$ contents in the shoots and roots. With the increase of salt concentration, total phenolic, total flavonoid content and antioxidant activities were increased. The results showed that centella can tolerate saline conditions up to 100 mM NaCl. $Na^+$ exclusion from the roots, and that increases of phytochemical content in the shoots were related to the salt tolerance of this species.

**Keywords:** total flavonoid; phenolics; antioxidant activity; centella; $Na^+$ content

**Citation:** Hoang, H.L.; Rehman, H. Unravelling the Morphological, Physiological, and Phytochemical Responses in *Centella asiatica* L. Urban to Incremental Salinity Stress. *Life* **2023**, *13*, 61. https://doi.org/10.3390/life13010061

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 6 December 2022
Revised: 20 December 2022
Accepted: 21 December 2022
Published: 25 December 2022



## 1. Introduction

*Centella asiatica* L. Urban (centella) as a medicinal plant of the Apiaceae family that has been used to treat a number of diseases, including varicose veins, certain eczemas, hypertonic scars, and keloids [1]. Centella is also considered a valuable plant in the cosmetics and pharmaceutical industries. As a leafy vegetable, this species is consumed as a juice blend in many Asian countries including Vietnam and Malaysia. The medicinal properties of centella are determined by a variety of phytochemicals such as phenolics, flavonoids, and terpenes [2]. The concentration of these compounds is influenced by several environmental stress factors, including salinity [3].

Salinity as an environmental stress factor affects arable lands worldwide, causing an annual monetary loss of approximately $27.3 billion to the agricultural industry [4]. Excessive salt concentration reduces the water potential, resulting in osmotic stress and an increase in the toxic accumulation of sodium and chloride in plant cells. Ionic toxicity and osmotic stress disrupt photosynthetic functions and reduce growth of plants [5]. This results in the accumulation of reactive oxygen species (ROS), including hydrogen peroxide ($H_2O_2$), superoxide anions ($O2\bullet-$), singlet oxygen ($^1O_2$), and hydroxyl radicals ($OH\bullet$), resulting in protein, DNA, and lipid damages [6]. The extent of the damage depends on the type, variety, and growth stage of the plant [7].

Plants have evolved antioxidant defense systems to reduce oxidative damage from salinity stress. Phenolic compounds play a major role in scavenging free radicals [8] by acting as hydrogen or electron donors that stabilize and delocalize unpaired electrons or chelate metal ions, preventing the generation of ROS [9]. Plants with higher antioxidant levels have an increased tolerance to damages by ROS [10]. Recent studies have shown various changes in antioxidant compounds when plants are subjected to salinity.

Salinity increased the total phenolic content in *Thymus vulgaris* L. [11] and *Brassica oleracea var. acephala* [12]. However, higher salt levels reduced the phenolic content of *Ocimum basilicum* L. [13] and *Nigella sativa* [14]. The significant correlation among phenolic content, antioxidant activity, and salt tolerance is well documented in literature [15]. For example, Sarker et al. [16] reported that salinity stress enhanced total phenolic, total flavonoid, and antioxidant activity of amaranth. Razieh et al. [17] also observed that phenolic content and antioxidant activity were significantly increased by salt stress in wheat. Santander et al. [18] determined that the greatest total phenolic content and antioxidant activity in lettuce prevailed at 50 mM NaCl treatment.

Despite numerous studies reporting the responses of plants to saline stress, there is limited research available on responses of centella to incremental salinity. The present study, therefore, determined the growth and biomass production of centella under saline conditions and their effects on ionic uptake, phytochemical content, and antioxidant activity.

## 2. Materials and Methods

### *2.1. Plant Materials and Experimental Design*

Seeds of *Centella asiatica* L. were collected from the Lucky Seed Company, Vietnam. The seeds were sown in trays containing a mixture of coconut fiber and sand. At the third leaf stage, seedlings were transplanted into garden soil-filled, plastic pots (20 × 18 × 10 cm) containing 2.5 kg soil. The soil had a pH of 5.5, 1.3 ECe, 35% organic matter, 0.5 mg $L^{-1}$ $Na^{+}$, 0.88 mg $L^{-1}$ $Cl^{-}$, 0.65% N, 0.71% $K_2O$, and 0.62% $P_2O_5$. The study was conducted in a greenhouse in Huong Tra Town, Thua Thien Hue Province, Vietnam, from February to November 2021.

The NaCl was applied as: 0, 25, 50, 75 and 100 mM. The experiment design was a completely randomized block (CRB) design with three replicates, and each replicate included 15 plants. One seedling was transplanted per pot. The soil was drenched with 25, 50, 75, and 100 mM NaCl after transplanting. NaCl was stepped up in daily increments of 25 mM until reaching the final concentration of each treatment. No additional nutrients or fertilizers were added. The experiment was terminated 45 days after transplantation. The plants were evaluated for plant growth, as well as phytochemical and ionic analyses.

### *2.2. Growth Measurement*

Plant phenotypes, including the number of leaves, rosette diameter, petiole length, total leaf area, and specific leaf area were recorded. The millimeter graph paper method was used to measure total leaf area per plant [19]. At the end of experiment, shoots and roots were sampled, oven-dried at 60 °C for 60 h, and weighted.

### *2.3. Total Chlorophyll Content Measurement*

Two hundred mg of fresh centella leaves were chopped and ground into fine powder in 5 mL 60% acetone (*v/v*). The extractant was filtered, and then the diluted acetone was added to make up the 20 mL final volume. The supernatant was recorded spectrophotometrically at 663 and 645 nm, and the formula given by Lichtenthaler (1987) was used to calculate the total chlorophyll content:

$$\text{Total chlorophyll} = 7.15A663 + 18.71A645$$

### *2.4. Electrolyte Leakage (EC) Measurement*

The method described by Lutt et al. [20] was used to determine electrolyte leakage. The top 4th leaf was collected and thoroughly rinsed with distilled water to remove contamination. The samples were put into stoppered vials containing 10 mL of distilled water and then incubated at 25 °C on a shaker at 100 rpm for 24 h. After incubation, the electrical conductivity of the bathing solution (EC1) was immediately measured. After this, the same leaf samples were placed in an autoclaved at 120 °C for 20 min, and again, a reading EC2 was measured at room temperature using the portable meter HI993310 (Hanna

Instrument Company, Woonsocket, RI, USA). The electrolyte leakage was measured as a ratio of EC1/EC2 and expressed as a percentage.

### *2.5. Determination of Hydrogen Peroxide ($H_2O_2$)*

Hydrogen peroxide was determined by using the potassium iodide (KI) method. Three mL leaf extract supernatant was mixed with 0.5 mL trichloroacetic acid (TCA) (0.1%), 0.5 mL potassium phosphate buffer (100 mM), and 2 mL reagent 1 mL KI (1 M KI w/v in fresh double-distilled water). A blank probe was made using trichloroacetic acid (0.1%) in the absence of leaf extract. The reaction was developed for 1 h in darkness and absorbance measured at 390 nm. A standard curve was used to estimate the amount of hydrogen peroxide.

### *2.6. Estimation of Ionic Content*

After harvesting, nine plants per treatment were separated into aboveground parts and roots. They were washed with de-ionized water, dried at 80 °C in 48 h, and stored at room temperature for further processing. The $Na^+$ and $Cl^-$ in roots and shoots were determined by using the method described by AcostaMotos et al. [21].

### *2.7. Determination of Total Phenolic Content*

Total phenolic content was determined following Velioglu et al. [22]. Plant extracted solution (0.5 mL) was added to diluted Folin–Ciocalteu reagent (2 N, 5 mL), and then 4 mL of 1 M $Na_2CO_3$ and 1 mL water were added to the mixture. The leaf extracts were left to stand for 90 min at 37 °C, and then the phenolic content was determined by using colorimetry at 765 nm. The results were expressed as gallic acid equivalents per milligram (mg GAE $g^{-1}$ DW). The gallic acid solutions were prepared in methanol: water (50:50, *v/v*) as 0, 50, 100, 150, 200, and 250 mg $mL^{-1}$ for standard curve ($R^2$ = 0.99).

### *2.8. Determination of Total Flavonoid Content*

The flavonoid content was quantified following the method of Zhishen et al. [23]. The 0.5 mL of plant extract solution was added to 1.0 mL methanol, 0.5 mL of aluminum chloride, and 0.5 mL of 1 M potassium acetate and allowed to stand for 30 min. The absorbance of the reaction mixture was detected at 415 nm with a UV/Vis spectrophotometer (Shimazdu UV-2600, Kyoto, Japan). The total flavonoid content was calculated as quercetin from a calibration curve prepared by using quercetin solutions of different concentrations from 12.5 to 100 mg $mL^{-1}$ in methanol.

### *2.9. Determination of Total Antioxidant Activity*

The 1,1-Diphenyl -2-picryl-hydrazyl (DPPH) radical degradation method was used to estimate antioxidant activity [24]. The plant extracts (1 mL) were added at different concentrations with volumes equal to the methanolic solution of 10 mL DPPH (100 μM) in a test tube. The mixture was shaken vigorously and was then allowed to stand in the dark. After 15 min, the absorbance was detected at 517 nm as a lower $IC_{50}$ value corresponding to its higher antioxidant activity. This measurement was repeated three times. The $IC_{50}$ values indicate the concentration of the sample.

### *2.10. Statistical Analysis*

The data was subjected to analysis of variance (ANOVA) using the Statistical Package for the Social Science (SPSS) software version 12. If the F-test was found significant, mean comparison was performed using the least significant difference (LSD) test at 5% level.

## 3. Results

### *3.1. Plant Growth and Biomass Production*

Salinity significantly reduced the centella growth at all NaCl concentrations except 25 mM (Table 1). Plant growth was detrimentally reduced at 100 mM NaCl. High salinity

level (100 mM) reduced the number of leaves, leaf area, and specific leaf area by 45%, 38.4% and 35%, respectively. Low and moderate salinity levels (25 and 50 mM NaCl) had no significant effect on the rosette diameter and petiole length of centella, while high NaCl concentration (100 mM) reduced these up to 18.9% and 33.6%, respectively.

**Table 1.** Morphological parameters of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. The data are presented as treatment mean ± SD. Different letters in the same column represent significant differences at the $p < 0.05$.

| Treatments (mM NaCl) | Number of Leaves | Leaf Area ($cm^2$) | Specific Leaf Area ($cm^2\ g^{-1}$) | Rosette Diameter (cm) | Petiole Length (g) |
|---|---|---|---|---|---|
| 0 | 20.50± 5.20 $^{a}$ | 24.20 ± 2.61 $^{a}$ | 347.50 ± 54.61 $^{a}$ | 17.30 ± 2.53 $^{a}$ | 6.06 ± 1.12 $^{a}$ |
| 25 | 21.62 a ± 6.12 $^{a}$ | 26.00 ± 3.22 $^{a}$ | 358.20 ± 93.41 $^{a}$ | 18.12 ± 3.34 $^{a}$ | 6.37 ± 2.11 $^{a}$ |
| 50 | 18.40 b ± 5.50 $^{b}$ | 22.21 ± 5.81 $^{b}$ | 312.80 ± 32.63 $^{b}$ | 17.95 ± 2.81 $^{a}$ | 5.80 ± 0.92 $^{a}$ |
| 75 | 15.60 c ± 7.01 $^{c}$ | 17.83 ± 3.53 $^{c}$ | 265.20 ± 49.74 $^{c}$ | 15.53 ± 4.21 $^{b}$ | 5.11 ± 1.41 $^{b}$ |
| 100 | 11.21 d ± 3.52 $^{d}$ | 14.92 ± 5.11 $^{d}$ | 225.40 ± 77.52 $^{d}$ | 14.03 ± 3.14 $^{b}$ | 4.02 ± 0.84 $^{b}$ |

Plant dry weight also decreased with incremental salinity, except at 25 mM NaCl (Table 2). The decrease in dry weight ranged from 5.9 to 13.9% with highest reduction of 19.7% at 100 mM NaCl compared with the control.

**Table 2.** Biomass production of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. The data are presented as treatment mean ± SD. Different letters in the same column represent significant differences at the $p < 0.05$.

| Treatments (mM NaCl) | Fresh Weight Leaf (g/plant) | Dry Weight Leaf (g/plant) | Fresh Weight Root (g/plant) | Dry Weight Root (g/plant) | Root/Shoot Ratio Dry Weight (g) |
|---|---|---|---|---|---|
| 0 | 50.33 ± 5.21 $^{c}$ | 10.06 ± 2.15 $^{c}$ | 37.55 ± 6.11 $^{a}$ | 7.51 ± 0.85 $^{a}$ | 0.40 ± 0.03 $^{a}$ |
| 25 | 52.65 ± 3.57 $^{c}$ | 10.53 ± 3.21 $^{c}$ | 33.25 ± 5.40 $^{b}$ | 6.65 ± 0.72 $^{b}$ | 0.42 ± 0.07 $^{a}$ |
| 50 | 61.00 ± 7.54 $^{a}$ | 12.20 ± 2.20 $^{a}$ | 29.65 ± 6.41 $^{c}$ | 5.93 ± 0.63 $^{c}$ | 0.49 ± 0.04 $^{a}$ |
| 75 | 57.15 ± 3.90 $^{b}$ | 11.42 ± 1.50 $^{b}$ | 22.25 ± 8.01 $^{d}$ | 4.45 ± 0.51 $^{d}$ | 0.58 ± 0.02 $^{b}$ |
| 100 | 46.71 ± 6.52 $^{d}$ | 9.34 ± 1.15 $^{d}$ | 20.25 ± 7.24 $^{d}$ | 4.05 ± 0.82 $^{d}$ | 0.80 ± 0.09 $^{a}$ |

### *3.2. Total Chlorophyll Content*

A decrease in the photosynthetic pigment content was observed in centella under salt stress in this study (Figure 1). The total chlorophyll content decreased by 50% at 100 mM NaCl. The highest total chlorophyll content was observed under no salinity followed by of the plants grown under 25 and 50 mM NaCl.

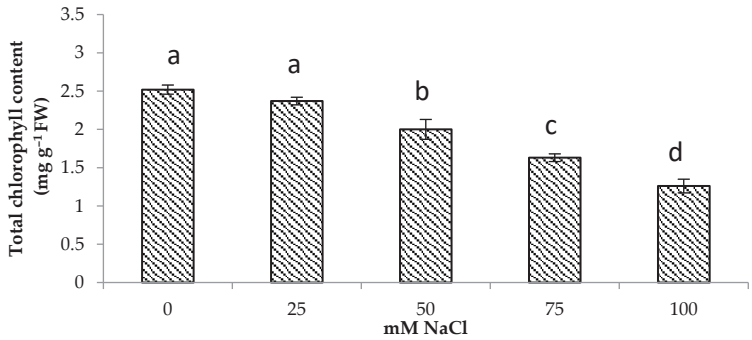


**Figure 1.** Total chlorophyll content of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. Different letters represent significant differences at the $p < 0.05$ (FW: fresh weight).

### 3.3. Electrolyte Leakage

The results showed that electrolyte leakage increased with increasing salt concentrations. Minimum electrolyte leakage was found in control plants followed by 25 mM NaCl salinity. Increasing salinity by 50 and 75 mM NaCl and increased electrolyte leakage by 2.7 and 3.4 times compared to control, respectively, while the highest increase of 4.5 times in electrolyte leakage was found at 100 mM NaCl (Figure 2). There was also a significant and positive relationship of electrolyte leakage with shoot $Na^+$ (r = 0.85, $p < 0.001$) and root $Na^+$ content (r = 0.62, $p < 0.001$) (Table 3).

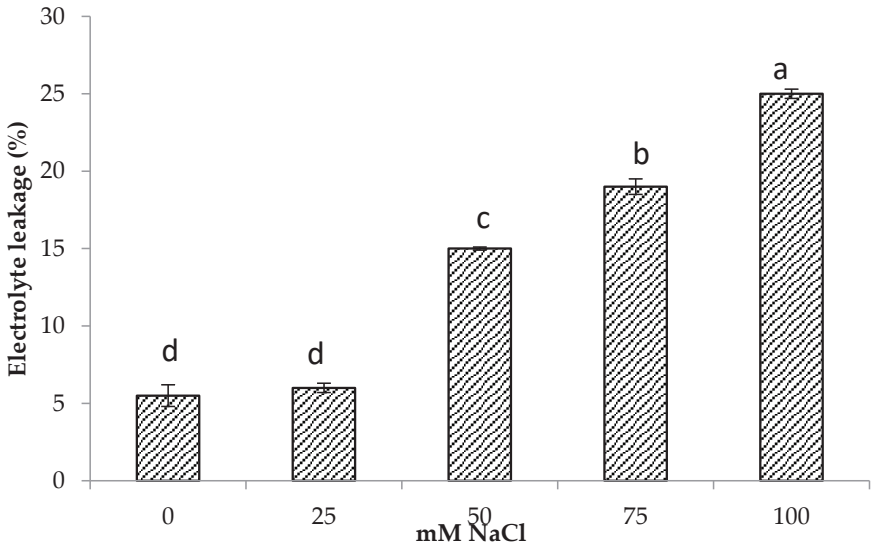


**Figure 2.** Electrolyte leakage of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. Different letters represent significant differences at the $p < 0.05$.

**Table 3.** Correlation coefficients among some morphological and physiological characteristics.

| | Fresh Weight | Dry Weight | Total Chlorophyll Content | Electrolyte Leakage | Hydrogen Peroxide | Shoot $Na^+$ Content | Root $Na^+$ Content | Shoot $Cl^-$ Content | Root $Cl^-$ Content | Total Phenolic Content | Total Flavonoid Content | Antioxidant Activity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fresh weight | 1 | | | | | | | | | | | |
| Dry weight | 0.90 ** | 1 | | | | | | | | | | |
| Total chlorophyll content | 0.85 ** | 0.83 ** | 1 | | | | | | | | | |
| Electrolyte leakage | −0.67 ** | −0.70 ** | −0.67 ** | 1 | | | | | | | | |
| Hydrogen peroxide | −0.56 ** | −0.59 ** | −0.58 ** | 0.81 ** | 1 | | | | | | | |
| Shoot $Na^+$ content | −0.67 ** | −0.62 ** | −0.59 ** | 0.85 ** | 0.80 ** | 1 | | | | | | |
| Root $Na^+$ content | −0.56 ** | −0.58 ** | −0.53 ** | 0.62 ** | 0.65 ** | 0.61 ** | 1 | | | | | |
| Shoot $Cl^-$ content | −0.68 ** | −0.65 ** | −0.60 ** | 0.76 ** | 0.74 ** | 0.67 ** | 0.55 ** | 1 | | | | |
| Root $Cl^-$ content | −0.53 ** | −0.55 ** | −0.52 ** | 0.60 ** | 0.53 ** | 0.45 ** | 0.61 ** | 0.55 ** | 1 | | | |
| Total phenolic content | −0.54 ** | −0.67 ** | −0.51 ** | 0.31 ns | 0.23 ns | 0.65 ** | 0.52 ** | 0.50 ** | 0.52 ** | 1 | | |
| Total flavonoid content | −0.70 ** | −0.64 ** | −0.54 ** | 0.75 ** | 0.62 ** | 0.83 ** | 0.61 ** | 0.65 ** | 0.67 ** | 0.84 ** | 1 | |
| Antioxidant activity | −0.20 ns | −0.31 ns | −0.03 ns | −0.16 ns | −0.21 ns | 0.56 ** | 0.60 ** | 0.58 ** | 0.60 ** | −0.78 ** | −0.56 * | 1 |

* and **: significant difference at 5 and 1%, respectively; ns: not significant.

### 3.4. Hydrogen Peroxide ($H_2O_2$)

Salt stress increased hydrogen peroxide content significantly in centella. The highest hydrogen peroxide was obtained at 100 mM NaCl (5.1 µmol $g^{-1}$ FW) followed by 75 mM NaCl. The lowest hydrogen peroxide content was found in the control and were comparable to 25 mM NaCl content. At 50 mM NaCl, the hydrogen peroxide in the centella leaf was 4.1 µmol $g^{-1}$ FW (Figure 3). There was also a significant relationship between $H_2O_2$ content and $Na^+$ content (Table 3).

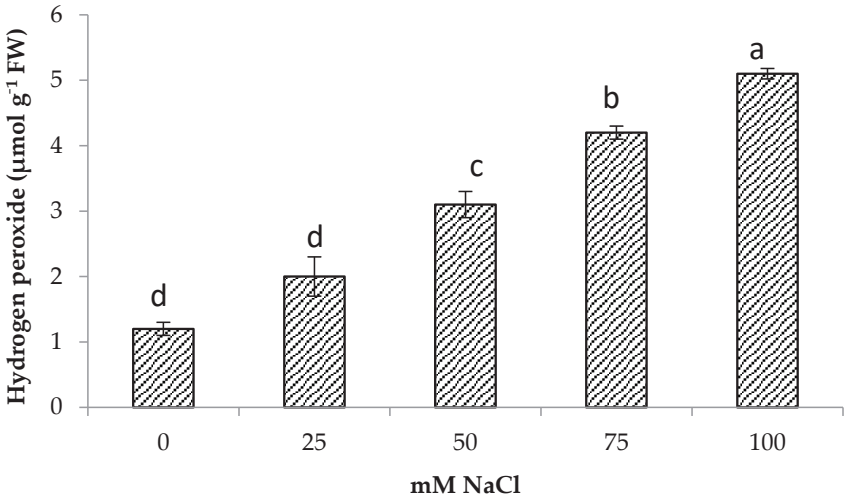


**Figure 3.** Hydrogen peroxide of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. Different letters represent significant differences at the $p < 0.05$.

### *3.5. Shoot and Root $Na^+$ and $Cl^-$ Content*

The presence of NaCl in the soil medium resulted in the accumulation of $Na^+$ and $Cl^-$ in the roots and shoots of centella, with higher accumulation found in the roots than in the shoots (Figures 4 and 5). Incremental salinity increased the accumulation of highest $Na^+$ content with the highest observed in the roots at 100 mM NaCl, which was 5.7 times greater, followed by 3 and 4.4 times at 50 mM and 75 mM NaCl salinity, respectively. A similar trend in $Na^+$ content was observed in the shoots. The highest $Na^+$ in the shoot 3.8 times higher was found at 100 mM NaCl than the control.

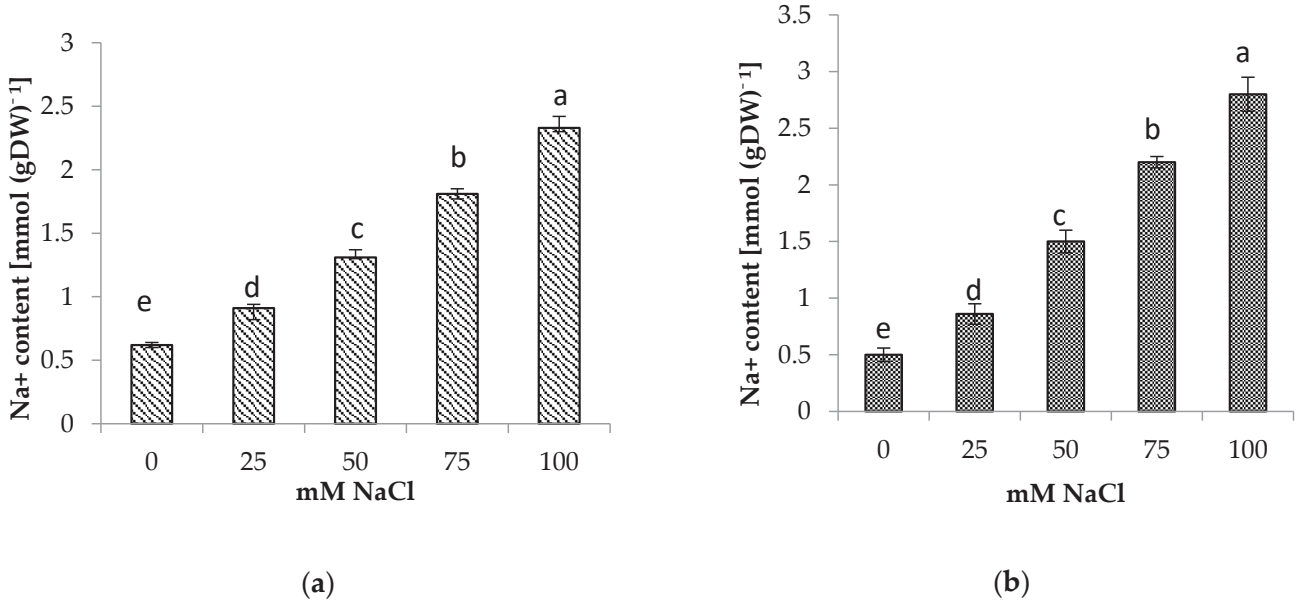


**Figure 4.** (**a**) Shoot and (**b**) root $Na^+$ content of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. Different letters represent significant differences at the $p < 0.05$.

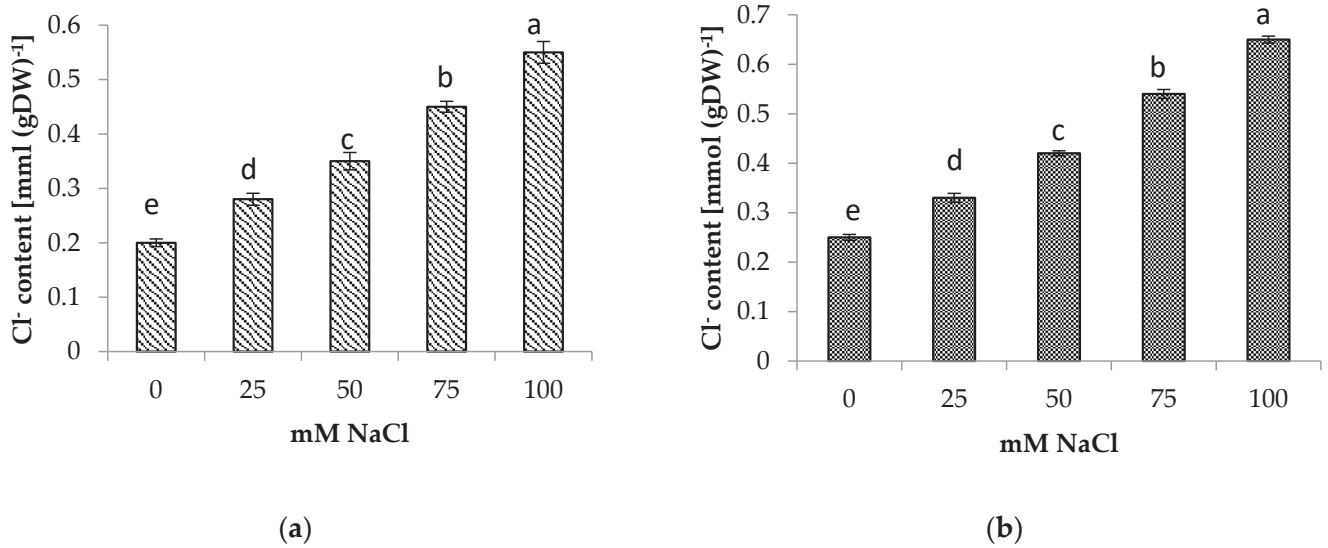


**Figure 5.** (**a**) Shoot and (**b**) root $Cl^-$ content of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. Different letters represent significant differences at the $p < 0.05$.

### *3.6. Phytochemical Content*

Salinity stress significantly increased the total phenolic and total flavonoid contents of the centella (Figure 6). The highest total phenolic and flavonoid contents were found in the plants at 75 mM NaCl. The increase in total phenolic and total flavonoid contents augmented the antioxidant activity up to 34% when compared to the control (Figure 7). At a high salt concentration (100 mM NaCl) their accumulation was reduced; however, it was not significantly different from 75 mM NaCl salinity.

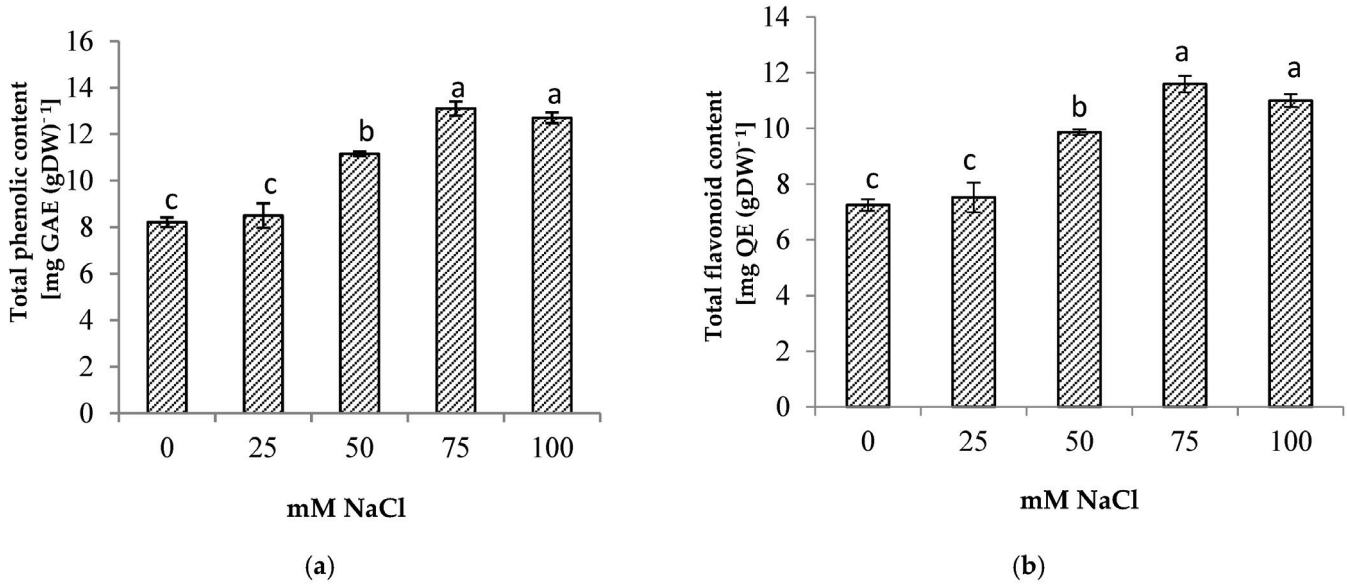


**Figure 6.** (**a**) Total phenolic, (**b**) total flavonoid (**b**) of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. Different letters represent significant differences at the $p < 0.05$. (GAE: gallic acid; QE: quercetin; DW: dry weight).

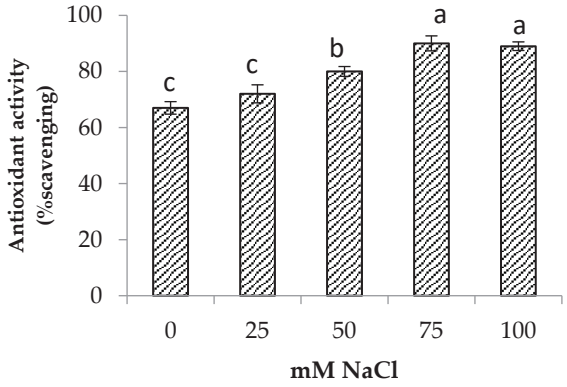


**Figure 7.** Antioxidant activity of *Centella asiatica* L. as affected by different salinity levels. The experiment was carried out in triplicate. Different letters represent significant differences at the $p < 0.05$.

## 4. Discussion

The results of the present study showed that centella growth was decreased by incremental salinity. A similar response in plant growth was reported in *Portulaca oleracea* L. [25], *Prosopis strombulifera* [26], and *Tetragonia decumbens* [27] due to salt stress. High saline concentrations reduced growth by decreasing the uptake of water and nutrients by the plants [28], accumulating toxic ions in the plant cells, and disrupting the metabolic pathways [29]. In this study, specific leaf area decreased with an increase in salinity. Burslem et al. [30] showed that a higher leaf thickness is associated with an increase in the ratio of mesophyll area available for the absorption of $CO_2$ per unit leaf area, thereby enhancing $CO_2$ assimilation and biomass production. However, Omami et al. [31] found that $CO_2$ assimilation decreased with increasing salinity in amaranth. They suggested that the lower specific leaf area in salt stressed plants overloaded the leaves with inorganic and organic solutes, thereby permitting osmotic flow but limiting the efficient use of carbon. Increase in the leaf thickness could be an adaptation of the plant to increase intercellular space and to counteract the decrease of transpiration [32].

The current study indicated that the root biomass decreased under high salt concentration treatments. According to Banaka et al. [33], the main reasons for reduced plant growth and biomass under high salinity were ion toxicity and nutrient imbalance. Moreover, the increase of soluble salts in the soil leads to an increase of osmotic pressure and a reduction of water potential, thus reducing the water uptake by the root [34]. In this study, although salt stress inhibited plant growth and decreased biomass production, the root/shoot dry weight increased. This indicated that salinity affected the aboveground part more severely than the underground part and the plant had the ability to change biomass allocation. It means that the plants had the ability to maintain the root system while salt stress inhibited shoot growth. This response is one of the most popular strategies of plants to adapt to abiotic stress.

Chlorophyll content is an important factor in assessing photosynthetic activity in plants [35]. The results showed a decrease in the total chlorophyll content of the centella under saline conditions. Previous studies showed that the depletion of photosynthetic pigments reducing plant growth and crop yield under saline stress was also evident from a significant relationship between total chlorophyll content and biomass production in the present study ($r = 0.9$, $p < 0.001$) (Table 3). This was observed in *Amaranthus tricolor* [36], *Typha domingensis* [37], and *Lactuca sativa* L. [38].

There was also a negative correlation between the total chlorophyll content and the shoot $Na^+$ content ($r = -0.67$, $p < 0.001$), showing degradation of photosynthetic pigments under the incremental salinity (Table 3). This leads to a reduction in biomass production as indicated by the negative correlation between fresh weight/dry weight with $Na^+$ concentration (Table 3). Depletion of chlorophyll under saline conditions may be

caused by the accumulation of toxic ions, such as $Na^+$ and $Cl^-$ inhibiting the enzymes function responsible for chlorophyll synthesis [39]. Zahra et al. [40] also reported that salt stress could reduce the $CO_2$ supplement through hydrostatic stomata closure or by changing the mesophyll conductance. According to Farhat et al. [41], a high salt concentration may damage the thylakoid membranes and protein modulation by inhibiting photosynthesis. Recent studies showed that the formation of ROS disrupted the chloroplasts and ultimately reduced the total population of *Brassica napus* [42], *Chenopodium quinoa* [43] and *Solanum lycopersicum* [44].

In this study, the centella was able to maintain membrane stability under slight salt stress (Figure 2) as evident from the electrolyte leakage which increased when the plants were subjected to a high salt concentration. A similar response was observed by ElYacoubi et al. [45] in ryegrass and by Behdad et al. [46] in licorice. This was mainly due to the efflux of $K^+$ and the flow of counter ions ($Cl^-$, $HPO_4^{2-}$, $NO_3^-$, citrate$^{3-}$, and malate$^{2-}$) counterbalancing the efflux of $K^+$ [47]. According to Tavakkoli et al. [48], the distribution of $Na^+$ within cells and organs may subsequently cause toxic effects on membrane permeability and increased electrolyte leakage.

In this study, the increase of $Na^+$ and $Cl^-$ concentrations in the tissues was accompanied by salinity stress. High accumulations of $Na^+$ and $Cl^-$ reduced plant growth. High $Na^+$ concentrations interfered with the absorption of $K^+$ and $Ca^{2+}$ ions and disturbed stomatal regulation, thereby inhibiting photosynthesis and growth. High $Cl^-$ concentrations caused the degradation of chlorophyll, leading to a reduction in the photosynthesis rate [48]. However, plants have different coping mechanisms for dealing with $Na^+$ toxicity. Some plants transport $Na^+$ from the roots to the leaves where it is retained in the vacuoles, whereas others store $Na^+$ in the roots [49]. Salt tolerance is associated with the ability to limit the uptake and/or to transport $Na^+$ from the root zone to aerial parts [50]. Based on the distribution of $Na^+$ and $Cl^-$ between shoots and roots, a similar mechanism could occur in centella. The accumulation of $Na^+$ and $Cl^-$ in the roots provided a mechanism for centella to cope with salinity in the rooting medium. This mechanism reduced the transport of $Na^+$ and $Cl^-$ to the leaves, thereby reducing the impact of the toxic ions to the aboveground parts of the plant. The leaves of centella are usually harvested, which is advantageous for growing this plant in saline environments. This mechanism has also been reported in amaranth [36] and rapeseed [51].

One of the effects of salt stress on plants is the overproduction of ROS, which leads to oxidative stress. However, plants have evolved mechanisms to counteract the effects of this process by producing compatible metabolites and different antioxidants [10]. Phenolic compounds are the most abundant secondary metabolites in the plant kingdom which have a pivotal effect in scavenging the excessive ROS. Flavonoids as a group belong to phenolic compounds and are known to have antioxidant properties [10]. The presence of phenolics and flavonoids in plants contributed to the prevention of cell damage by abiotic stress, as demonstrated by several studies on peas [52] and kale [53]. These compounds neutralize the radicals accumulated in lipids or prevent their breakdown into free radicals. Furthermore, they can inhibit lipoxygenase activity, thus preventing lipid peroxidation [54,55]. The result showed that there was a significant increase in the phenolic and flavonoid content in response to salt stress. The increase in phenolic and flavonoid content indicates that they play a significant role in the adaptation of centella to salinity as evident from a positive correlation between total phenolic content and antioxidant activity in the present study (Table 3). The increase in these compounds is related to their function as a non-enzyme antioxidant to counteract the increase of ROS and hence contribute to the plant's health under salt stress. In the present study, antioxidant activity of the centella leaf increased with the salt treatments, and the highest antioxidant activity was observed at 100 mM NaCl. This finding is consistent with the important relationship that exists between antioxidant activity and the total phenolic content in the leaves of *Leucojum aestivum* and *Lactuca sativa* under salt stress conditions [19,56]. Although the centella was also negatively affected by salt stress, as demonstrated by yield decline and increased accumulation of $Na^+$ and

$Cl^-$ ions, the study results showed an increase in phytochemicals content and antioxidant activity in centellas. This opens the way to cultivating this plant in saline soils to boost the production of bioactive compounds used in the pharmaceutical and cosmetics industries. However, studies on extraction techniques for specific bioactive compounds should be carried out to ensure the exclusion of ions and impurities.

## 5. Conclusions

Salinity stress caused a reduction in biomass yield and induced some physiological and phytochemical modification in centella. The results indicated that *Centella asiatica* showed moderate tolerance to severe salt stress, which was attributed to the exclusion of $Na^+$ and $Cl^-$ in the root to protect the aboveground plant tissues from salt toxicity and to increase the total phenolic and flavonoid content of the centella. The centella is an herb with a rich source of phytochemical content. Thus, the response of the centella under salt conditions may be used to improve the production of bioactive compounds to be used in the manufacture of pharmaceuticals, supplements, food, and cosmetics.

**Author Contributions:** Conceptualization, H.L.H.; Methodology, H.L.H.; Software, H.R.; Validation, H.R.; Formal Analysis, H.L.H.; Investigation, H.L.H.; Resources, H.L.H.; Data Curation, H.L.H.; Writing—Original Draft Preparation, H.L.H.; Writing—Review & Editing, H.L.H. and H.R.; Visualization, H.L.H.; Supervision, H.R.; Project Administration, H.L.H. and H.R.; Funding Acquisition, H.L.H. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Ministry of Education and Training (Grant numbers B2020-DHH-03).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data recorded in the current study are available in all tables and figures of the manuscript.

**Acknowledgments:** We are grateful to University of Agriculture and Forestry for the support to conduct this research.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Effects of Vertically Heterogeneous Soil Salinity on Genetic Polymorphism and Productivity of the Widespread Halophyte *Bassia prostrata*

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**Abstract:** Salinity is one of the environmental factors that affects both productivity and genetic diversity in plant species. Within the soil profile, salinity is a dynamic indicator and significantly changes with depth. The present study examined the effects of the vertical heterogeneity of soil salinity chemistry on the plant height, fresh and dry biomass accumulation, water content, level of genetic polymorphism, and observed and expected heterozygosity in seven populations of halophyte *Bassia prostrata* in natural habitats. Soil salinity ranged from slight ($S_{salts}$ = 0.11–0.25%) to extreme ($S_{salts}$ = 1.35–2.57%). The main contributors to salinity were $Na^+$, $Ca^{2+}$, and $Mg^{2+}$. Multivariate analysis revealed that biomass accumulation is positively affected by moderate/high salinity in 20–60 cm soil layers, which may be associated with the salt required for the optimal growth of the halophyte *B. prostrata.* The formation of seed genetic diversity is negatively affected by slight/moderate salinity in the 0–40 cm layers. An increase in divalent ion content can reduce genetic diversity and increase the local adaptation of *B. prostrata* to magnesium–calcium sulfate salinity. The effect of the in-depth distribution of soil salinity on productivity and genetic diversity may be related to seasonal variables during biomass accumulation (summer) and seed formation (autumn).

**Keywords:** plant performance; $C_4$ species; heterozygosity; transient soil salinity; soil layers; desertification; arid regoins

**Citation:** Shuyskaya, E.; Toderich, K.; Kolesnikov, A.; Prokofieva, M.; Lebedeva, M. Effects of Vertically Heterogeneous Soil Salinity on Genetic Polymorphism and Productivity of the Widespread Halophyte *Bassia prostrata*. *Life* **2023**, *13*, 56. https://doi.org/10.3390/life13010056

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 23 November 2022
Revised: 17 December 2022
Accepted: 21 December 2022
Published: 24 December 2022



## 1. Introduction

Salinity is a significant environmental problem that limits plant productivity, especially in arid and semiarid regions that cover approximately 40% of the globe. Semiarid regions are projected to become drier and more saline due to rising global temperatures [1–3]. Vegetation survival and productivity are primarily regulated by the water balance in soil, which affects the water balance and photosynthetic rate in plants [4]. Soils in drylands are usually heterogeneous in space and time due to the presence of biotic and abiotic elements. Spatiotemporal variations in soil salinity and water content are well documented [5]. Salinity amplifies the effects of soil drought on plants by creating additional osmotic pressure. Soil is considered saline when the salt content exceeds 3–5 g salt/L in the soil solution, when electrical conductivity (EC) exceeds 2–4 dS/m, or when the sum of salts exceeds 0.15–0.2%, creating osmotic pressure above 0.2 MPa, which significantly reduces the yield of the most crops [2,6]. Salinity reduces plant growth and prematurely ages mature leaves, which leads to a decrease in the functional leaf area. A decrease in plant biomass is also influenced by $Na^+$ and $Cl^-$ toxicity and the accompanying oxidative stress [2,7]. Halophytes are highly salt-tolerant plants but underutilized resources that occupy naturally saline soil environments in coastal estuaries and inland salt flats in arid and semiarid zones [8]. Nowadays, climate-smart agriculture (CSA) practices increasingly use wild

salt-tolerant species (halophytes) to restore the grazing capacity of degraded pastures, provide forage for livestock and utilize oilseeds and medicinal and aromatic plants [7,9]. Apart from these applications, halophytes play a significant role in the maintenance of ecosystem functions and sustainability [10,11].

Genetic diversity provides plants with the ability to adapt and survive in changing environments, including soil chemistry variability. The genetic architecture of a population plays a fundamental role in the origin and maintenance of local adaptation [12]. The degree of local adaptation is largely determined by the interaction between selection and gene flow along ecological gradients. Different types of selection can operate under natural conditions: (i) conditionally neutral selection occurs when two alleles do not have an advantage in fitness in one environment but differ in fitness in another environment; (ii) environmentally antagonistic selection, when different alleles are locally adapted to different environments, conferring higher fitness there [12]. 'Fitness' is often viewed as the ability to withstand adverse conditions; however, from an evolutionary perspective, fitness is defined as the ability of an individual to spread their genes through offspring. Thus, in plants, fitness depends on the number of seeds that a plant can successfully produce under adverse environmental conditions [13]. A common plant response to environmental stresses is a decrease in fertility, which consists of aborting ovules and/or pollen and redirecting resources from reproductive activity into metabolic reactions for stress tolerance [14]. Plants are reported to control the consumption of maternal resources at several stages of development by regulating the number of flowers, gametophytes, and embryos that develop further [14]. This type of developmental regulation can lead to the favored selection of certain alleles or genotypes, producing genotype–environment associations and/or interactions [15]. The survival rate and adaptation of populations in different and changing environments depend on the genetic diversity of the seed pool. For example, diversity in the genetic composition of seeds allows *Atriplex tatarica* to survive under distinct conditions: heterozygous plants mainly germinate under optimal conditions, and homozygous plants typically germinate under suboptimal conditions [16]. Ecological factors that influence reproduction and seed dispersal are, therefore, particularly important aspects in shaping genetic diversity and population structures. Edaphic conditions, such as soil type, pH, nutrients, moisture, and the depth of soil layers, can significantly affect the level of genetic diversity and local adaptation in plant populations [15,17–19]. For example, *Phragmites australis* populations with high genetic diversity have a high tolerance to soil salinity [20]. The high genetic diversity of populations is fundamental to the long-term survival success of a plant species [21].

In unfavorable environments, such as areas of high soil salinity, plants are forced to seek a 'compromise' between productivity and adaptation, which depends on genetic diversity at the population level. Within the soil profile, salinity is a dynamic indicator; it changes with depth and according to seasons [5]. In turn, plants have a vertical fine-root distribution, which determines the possibility of acquiring resources along the soil profile, since plants rely mainly on their fine roots to acquire belowground water and nutrient resources [22]. To assess and predict the productivity and adaptation of species under changing conditions, it is necessary to understand how the salinization of different soil layers affects biomass and genetic polymorphism formation. A convenient model species for these purposes is the polymorphic widespread halophyte *Bassia prostrata* (L.) A.J. Scott (*Kochia prostrata* (L.) Schrad.) (subfamily Chenopodiaceae), with a significant variety of morphological, biochemical, and ecological–physiological properties; high genetic polymorphism; and wide ecological plasticity [23–27]. Moreover, the effect of soil conditions on both the level of diversity and genetic structure of *B. prostrata* populations has been shown [28].

The present study aims to investigate the effects of the level and chemistry of salinity within different soil layers (including horizontal and vertical variations in the soil characteristics) on the productivity and genetic diversity of the halophyte *B. prostrata* to clarify

the adaptive mechanism it uses to withstand fluctuations of salt accumulation along soil depth profiles.

## 2. Materials and Methods

### *2.1. Study Area*

Studies were carried out in the northwestern Caspian Lowland (Russia) (Figure 1A). The region is a flat marine accumulative plain, which is characterized by the almost complete absence of surface and subsurface runoff. According to climate parameters (Figure 1B), the region is arid, with an average annual temperature of 8.7 °C and precipitation of 291 mm. In Caspian Lowland plain landscapes, solonetzic complexes are widespread; depressions and other negative relief elements (microdepressions, depressions, estuaries) are characterized by dark-colored chernozem-like or meadow–chestnut soils [29]. Seven typical habitats of *B. prostrata* were selected for the study based on differences in the soil salinity levels (No. 1–7 in Figure 1A). Five of them were located near the salt lakes Bulukhta and Elton at different distances from the coastline. The other two habitats were in the plain part between these lakes (Figure 1A). *B. prostrata* habitats were characterized mainly by solonetzic and/or light chestnut solonetzic soils and desert steppe vegetation.

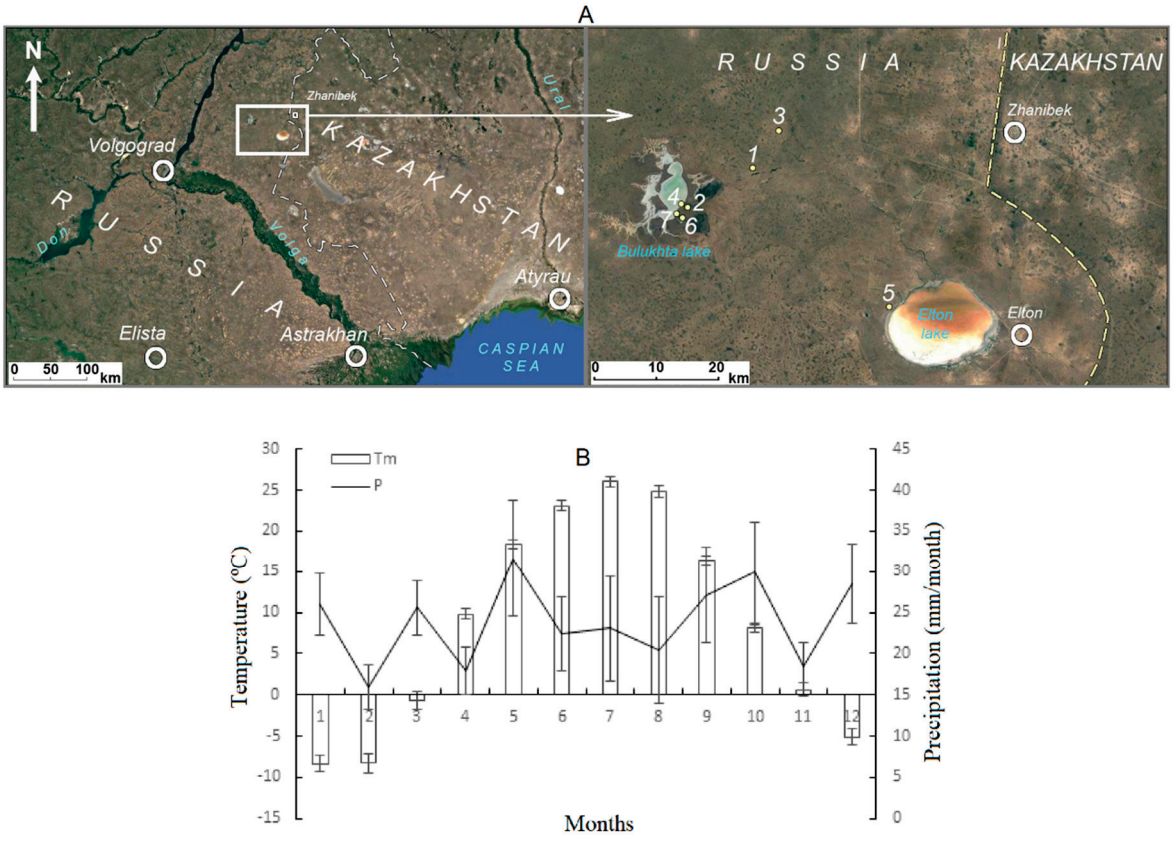


**Figure 1.** The location of seven populations of *Bassia prostrata* (**A**) and the long-term (2007–2018) average atmospheric temperature and precipitation (**B**) of the northwestern Caspian Lowland. 1–7—numbers of populations (habitats); Tm—temperature; P—precipitation.

### *2.2. Plant Sampling*

*Bassia prostrata* (L.) A.J. Scott (*Kochia prostrata* (L.) Schrad.) (Chenopodiaceae) is a typical perennial $C_4$ halophyte native to arid and semiarid rangelands in Central Eurasia and the Western United States. *B. prostrata* naturally occurs in all kinds of soils, such as saline, sandy, rocky, and poor soils [24,30,31]. *B. prostrata* has a thick, woody root system that can penetrate 3–6.5 m depths and lateral roots stretching 130–160 cm that mine for moisture in the upper (up to 60 cm) soil layers [30,31]. This is the reason for studying

the upper soil layers: approximately 0–20 cm, 20–40 cm, and 40–60 cm. Soils, plants, and seeds were sampled in seven typical habitats of *B. prostrata* (No. 1–7 in Figure 1A). The aboveground parts of five plants were harvested in each habitat in the middle of September for biomass analysis. More than 100 seeds from 10 to 15 mother plants from each habitat (population) were collected at the beginning of November and combined to generate a seed pool for population genetic analysis.

### *2.3. Soil Sampling and Analysis*

Seven habitats of *B. prostrata* soil pits (Nos. 14, 11, 15, 10, 18, 7, and 6, corresponding to habitats Nos. 1 to 7 in Figure 1A) were excavated. Profiles were examined to depths of 0 to 60 cm. Three soil samples ($n$ = 3) were used for the analysis of each soil layer of each habitat. Chemical and physicochemical analyses were performed at the Analytical Laboratory of the V.V. Dokuchaev Soil Science Institute using standard methods [32]. Calcium and magnesium concentrations in water extracts (1:5) were determined with the complexometric titration method; sodium and potassium concentrations were determined with the flame photometry method; the total alkalinity was determined using titration with sulfuric acid (with methyl orange indicator); the concentration of chlorine ions was determined with argentometry (according to Mohr); and the concentration of sulfate ions was determined using titration with $BaCl_2$. The content of ions $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$, $Cl^-$, $SO_4{}^{2-}$, and $HCO_3{}^-$ are presented in cmol(eq)/kg. The sum of salts ($S_{salts}$) represents the sum of the mass fraction of ions from the solid soil residue (%) [6].

### *2.4. Plant Biomass and Water Content*

Plant height, fresh (FW) and dry (DW) biomass, and water content (W) were assessed for aboveground parts of *B. prostrata* plants ($n$ = 5) from seven habitats. Biomass was estimated for fresh and dry shoots. Plant samples were dried at 80 °C for two days until reaching a constant mass to quantitatively measure the dry shoot matter. The water content in the shoots was calculated according to the following formula:

$$W = (FW - DW)/DW. \quad (1)$$

### *2.5. Population Genetic Analysis*

Genetic diversity can be studied using neutral markers (based on differences in DNA sequences) and partially selective markers (isozymes), which can reflect changes in environmental conditions [33,34]. In this study, we used isozymes (alternative forms of the enzymes encoded by different alleles of the same gene) to assess the genetic diversity of the populations.

For each population of *B. prostrata*, 50 seeds from the seed pool (more than 100 seeds from 10 to 15 mother plants) were germinated, and all good germinated seeds ($n$ = 25–35 per population) were analyzed for genetic polymorphism. Population genetic analysis was performed on embryos using starch gel electrophoresis of the following enzymatic systems: glutamate oxaloacetate transaminase (GOT (AAT), E.C. 2.6.1.1), diaphorase (DIA, E.C. 1.6.99), glutamate dehydrogenase (GDH, E.C. 1.4.1.2), superoxide dismutase (SOD, E.C. 1.15.1.1), glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49), 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44), malate dehydrogenase (MDH, E.C. 1.1.1.37), and malic enzyme (Me, E.C. 1.1.1.40). The seeds were cleaned of their wings and soaked in water for 12 h and homogenized in 80 µL of Tris-HCl buffer with KCl, $MgCl_2$, EDTA, Triton X-100, and PVP. Enzymes were separated in 10% starch gel using two buffer systems. In system 1, the electrode buffer was 160 mM Tris–50 mM citric acid, pH 8.0; the gel buffer was prepared by diluting 10 mL of the electrode buffer with 90 mL $H_2O$. In system 2, the electrode buffer was 300 mM boric acid–60 mM NaOH, pH 8.2; the gel buffer was 80 mM Tris–9 mM citric acid, pH 8.7. Electrophoresis was performed at 90 V, 40–50 mA in buffer system 1 or at 210 V, and 70–80 mA in buffer system 2 for 4–6 h at 5 °C. Staining of particular enzymes and genetic interpretation of the results followed standard techniques according to Soltis and Soltis [35] and Spooner et al. [33]. The level of genetic polymorphism was estimated

by calculating observed ($H_o$) and expected ($H_e$) heterozygosity for each polymorphic loci and by calculating the proportion of polymorphic loci ($P_{99}$) and the average (for all loci) observed ($H_o$) and expected ($H_e$) heterozygosity in POPGEN 1.32.

### *2.6. Statistical Analysis*

Principal component analysis (PCA) was carried out using R software (version 3.6.1). Table 1 and Figure 2 show the means of the obtained values and their standard errors ($n$ = 3 for soil samples and $n$ = 5 for plant samples).

## 3. Results

### *3.1. Soil Characteristics*

The soils in *B. prostrata* habitats (populations) differed significantly in the degree and vertical changes in salinity chemistry. In each of the seven habitats, the soil salinity the 0–20 cm, 20–40 cm, and 40–60 cm in layers was studied. Two habitats (Nos. 1 and 2) had non-saline or slightly saline soils (Table 1). The soils in the other five habitats (Nos. 3–7) were much more saline: the upper 0–20 cm layers were non-saline or slightly saline ($S_{salts}$ = 0.11–0.25%); the 20–40 cm layers were moderately or highly saline ($S_{salts}$ = 0.5–1.17%); and the 40–60 cm layers were highly or extremely saline ($S_{salts}$ = 1.35–2.57%) (Table 1). In all habitats, except for No. 4, $Na^+$ was the dominant cation: 0.03–2.27 cmol(eq)/kg, 0–20 cm layer; 0.32–8.95 cmol(eq)/kg, 20–40 cm layer; and 3.15–23.25 cmol(eq)/kg, 40–60 cm layer. The $Ca^{2+}$ ion predominated in habitat No. 4. In other soils, $Ca^{2+}$ and $Mg^{2+}$ contents also significantly contributed to salinity at 0.28–12.9 and 0.26–8.32 cmol(eq)/kg, respectively. Chlorides and sulfates were the dominant anions (Table 1).

**Table 1.** Contents of anions and cations in soils of the seven *Bassia prostrata* habitats in the northwestern Caspian Lowland.

| Habitats, No | Soil Layers | Anions, cmol(eq)/kg | | | Cations, cmol(eq)/kg | | | | Ssalt, % | Salinity Level |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $HCO^{3-}$ | $Cl^-$ | $SO_4^{2-}$ | $Ca^{2+}$ | $Mg^{2+}$ | $Na^+$ | $K^+$ | | |
| 1 | 0–20 | 1.04 ± 0.08 | 0.26 ± 0.02 | 0.78 ± 0.04 | 0.78 ± 0.02 | 0.52 ± 0.04 | 0.56 ± 0.01 | 0.22 ± 0.04 | 0.15 | non-saline |
| | 20–40 | 1.25 ± 0.11 | 0.17 ± 0.01 | 0.52 ± 0.03 | 0.52 ± 0.01 | 0.26 ± 0.03 | 1.45 ± 0.12 | 0.02 ± 0.01 | 0.16 | non-saline |
| | 40–60 | 2.18 ± 0.17 | 0.26 ± 0.03 | 0.52 ± 0.03 | 0.52 ± 0.02 | 0.78 ± 0.08 | 1.74 ± 019 | 0.03 ± 0.01 | 0.23 | slight |
| 2 | 0–20 | 0.10 ± 0.02 | 0.17 ± 0.01 | 0.52 ± 0.03 | 0.26 ± 0.01 | 0.26 ± 0.01 | 0.24 ± 0.01 | 0.03 ± 0.01 | 0.05 | non-saline |
| | 20–40 | 0.31 ± 0.02 | 0.34 ± 0.04 | 0.26 ± 0.01 | 0.26 ± 0.02 | 0.26 ± 0.01 | 0.32 ± 0.04 | 0.07 ± 0.01 | 0.06 | non-saline |
| | 40–60 | 0.94 ± 0.13 | 1.89 ± 0.14 | 1.82 ± 0.23 | 0.78 ± 0.66 | 0.78 ± 0.32 | 3.15 ± 0.07 | 0.04 ± 0.01 | 0.31 | slight |
| 3 | 0–20 | 1.35 ± 0.08 | 0.34 ± 0.07 | 0.52 ± 0.01 | 0.52 ± 0.02 | 0.26 ± 0.01 | 1.31 ± 0.04 | 0.12 ± 0.01 | 0.17 | slight |
| | 20–40 | 1.77 ± 0.21 | 5.41 ± 0.43 | 1.04 ± 0.08 | 0.52 ± 0.01 | 0.78 ± 0.03 | 7.01 ± 0.16 | 0.01 ± 0.01 | 0.50 | moderate |
| | 40–60 | 0.52 ± 0.02 | 10.22 ± 0.93 | 18.98 ± 0.21 | 9.88 ± 0.10 | 8.32 ± 0.72 | 11.48 ± 0.81 | 0.04 ± 0.01 | 1.87 | extreme |
| 4 | 0–20 | 0.94 ± 0.10 | 0.26 ± 0.03 | 1.56 ± 0.33 | 2.08 ± 0.50 | 0.26 ± 0.09 | 0.14 ± 0.07 | 0.27 ± 0.05 | 0.20 | slight |
| | 20–40 | 0.57 ± 0.04 | 0.17 ± 0.01 | 15.60 ± 0.42 | 12.09 ± 0.74 | 3.12 ± 0.41 | 1.84 ± 0.09 | 0.15 ± 0.01 | 1.11 | moderate |
| | 40–60 | 0.52 ± 0.03 | 0.69 ± 0.08 | 18.72 ± 1.53 | 9.88 ± 0.51 | 3.64 ± 0.33 | 6.27 ± 0.71 | 0.14 ± 0.02 | 1.35 | high |
| 5 | 0–20 | 0.73 ± 0.17 | 0.49 ± 0.0.1 | 0.35 ± 0.07 | 0.43 ± 0.08 | 0.43 ± 0.11 | 0.74 ± 0.26 | 0.06 ± 0.02 | 0.11 | non-saline |
| | 20–40 | 1.25 ± 0.76 | 7.90 ± 0.06 | 2.60 ± 0.32 | 1.04 ± 0.09 | 0.52 ± 0.07 | 10.28 ± 0.93 | 0.01 ± 0.01 | 0.75 | moderate |
| | 40–60 | 0.31 ± 0.02 | 7.39 ± 0.07 | 23.92 ± 1.30 | 10.14 ± 0.12 | 6.76 ± 0.54 | 14.77 ± 1.10 | 0.05 ± 0.01 | 2.06 | extreme |
| 6 | 0–20 | 0.78 ± 0.13 | 0.39 ± 0.04 | 0.91 ± 0.11 | 0.52 ± 0.01 | 0.52 ± 0.01 | 0.78 ± 0.07 | 0.26 ± 0.09 | 0.15 | non-saline |
| | 20–40 | 1.77 ± 0.17 | 3.18 ± 1.05 | 4.03 ± 0.98 | 1.04 ± 0.21 | 0.39 ± 0.11 | 7.50 ± 1.94 | 0.04 ± 0.01 | 0.76 | moderate |
| | 40–60 | 0.42 ± 0.03 | 7.65 ± 1.11 | 22.36 ± 1.74 | 11.70 ± 1.03 | 8.06 ± 0.92 | 10.67 ± 1.05 | 0.10 ± 0.03 | 1.95 | extreme |
| 7 | 0–20 | 1.51 ± 0.04 | 1.42 ± 0.74 | 1.69 ± 0.32 | 0.52 ± 0.01 | 0.39 ± 0.11 | 3.51 ± 1.01 | 0.20 ± 0.11 | 0.25 | slight |
| | 20–40 | 0.73 ± 0.04 | 9.02 ± 1.08 | 8.58 ± 0.91 | 3.12 ± 0.07 | 2.08 ± 0.34 | 13.14 ± 1.11 | 0.09 ± 0.01 | 1.17 | high |
| | 40–60 | 0.62 ± 0.03 | 16.06 ± 1.77 | 23.66 ± 1.73 | 10.66 ± 0.85 | 6.50 ± 0.77 | 23.25 ± 2.76 | 0.04 ± 0.01 | 2.57 | extreme |

Ssalt—the sum of salts represents the sum of the mass fraction of ions from the solid soil residue (%). Values are means ± standard errors ($n$ = 3).

### *3.2. Plant Growth and Water Content*

*B. prostrata* plants varied significantly in growth parameters between populations (Figure 2). The greatest plant heights were in populations Nos. 2, 4, and 7 (Figure 2A), while the highest fresh (FW) and dry (DW) biomass aboveground parts of plants were observed in populations No. 4 and No. 6 (Figure 2B,C). At the same time, the water content (W) in the plants was the highest in population No. 7 (Figure 2D).

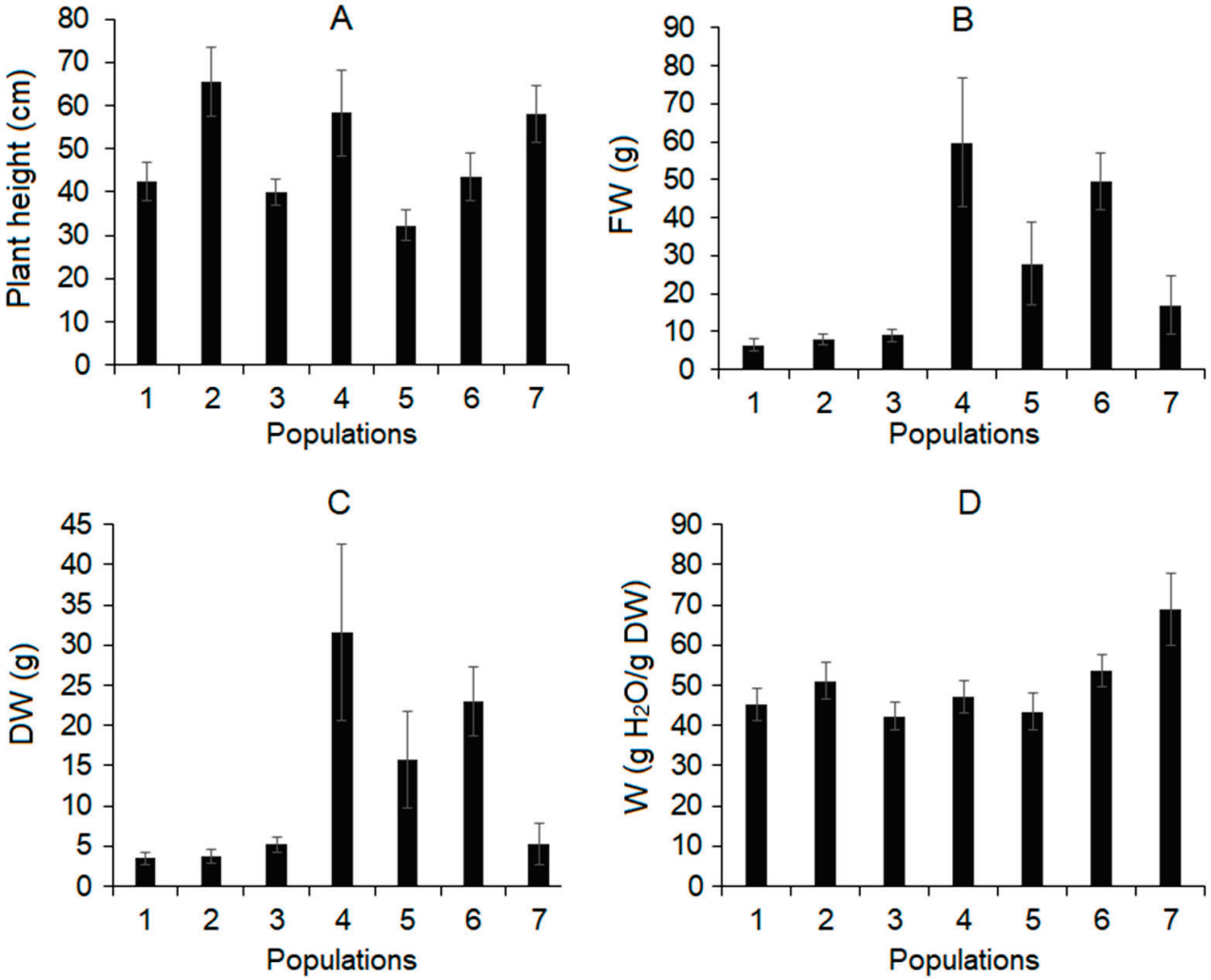


**Figure 2.** Growth parameters and water content of *Bassia prostrata* plants from seven populations. 1–7—populations; (**A**)—plant height; (**B**)—fresh biomass (FW) aboveground part of plants; (**C**)—dry biomass (DW) aboveground part of plants; (**D**)—water content (W) of plants. Values are means ± standard errors ($n$ = 5).

### *3.3. Population Genetic Diversity*

An analysis of eight enzyme systems in seven *B. prostrata* populations revealed ten loci; one of them (*Sod*) was monomorphic in all populations. The other nine loci were polymorphic: *G6pd* in all populations; *Me* in six populations; *Gdh* in four populations; *Got, 6pgd*, and *Mdh-A* in 3 populations; and *Dia-A, Dia-B*, and *Mdh-B* in one population. Values of observed heterozygosity ($H_o$) varied from 5 to 47% among polymorphic loci and populations (Figure 3A), whereas expected heterozygosity ($H_e$) varied from 5% to 59% (Figure 3B). The average (for all loci) observed heterozygosity varied from 5.5% to 11.1%, and expected heterozygosity varied from 6.2% to 15.9% in populations of *B. prostrata* (Figure 3C). The polymorphic loci proportion ($P$) among the populations was 20–70% (Figure 3C). On average, populations Nos. 2, 3, 5, and 6 were more polymorphic than populations Nos. 1, 4, and 7 (Figure 3).

### *3.4. Plant–Soil Interaction*

Principal component analysis (PCA) did not reveal significant correlations between *B. prostrata* fresh and dry biomass and soil properties in 0–20 cm soil layers (Figure 4A). There were significant positive correlations between *B. prostrata* fresh and dry biomass

and the sum of salts and the sum of the contents of anions $Ca^{2+}$, $Mg^{2+}$, and $SO_4^{2-}$ in the 20–40 cm and, to a lesser degree, 40–60 cm soil layers (Figure 4B), as well as with $K^+$ content in 40–60 cm layers (Figure 4C).

PCA revealed the negative dependencies of genetic polymorphism parameters (*P*, $H_e$, and $H_o$) on $K^+$, $Ca^{2+}$, and sulfate ions contents and, to a lesser degree, on the sum of salts and the sum of anions in the 0–20 cm soil layers (Figure 4D). In addition, a negative correlation was observed between *P*, $H_e$, and $H_o$ from one side and $Mg^{2+}$, $K^+$, $Ca^{2+}$, and $SO_4^{2-}$ contents in 20–40 cm soil layers from the other side (Figure 4E). There were no correlations between genetic polymorphism parameters and soil properties in the 40–60 cm layers (Figure 4F).

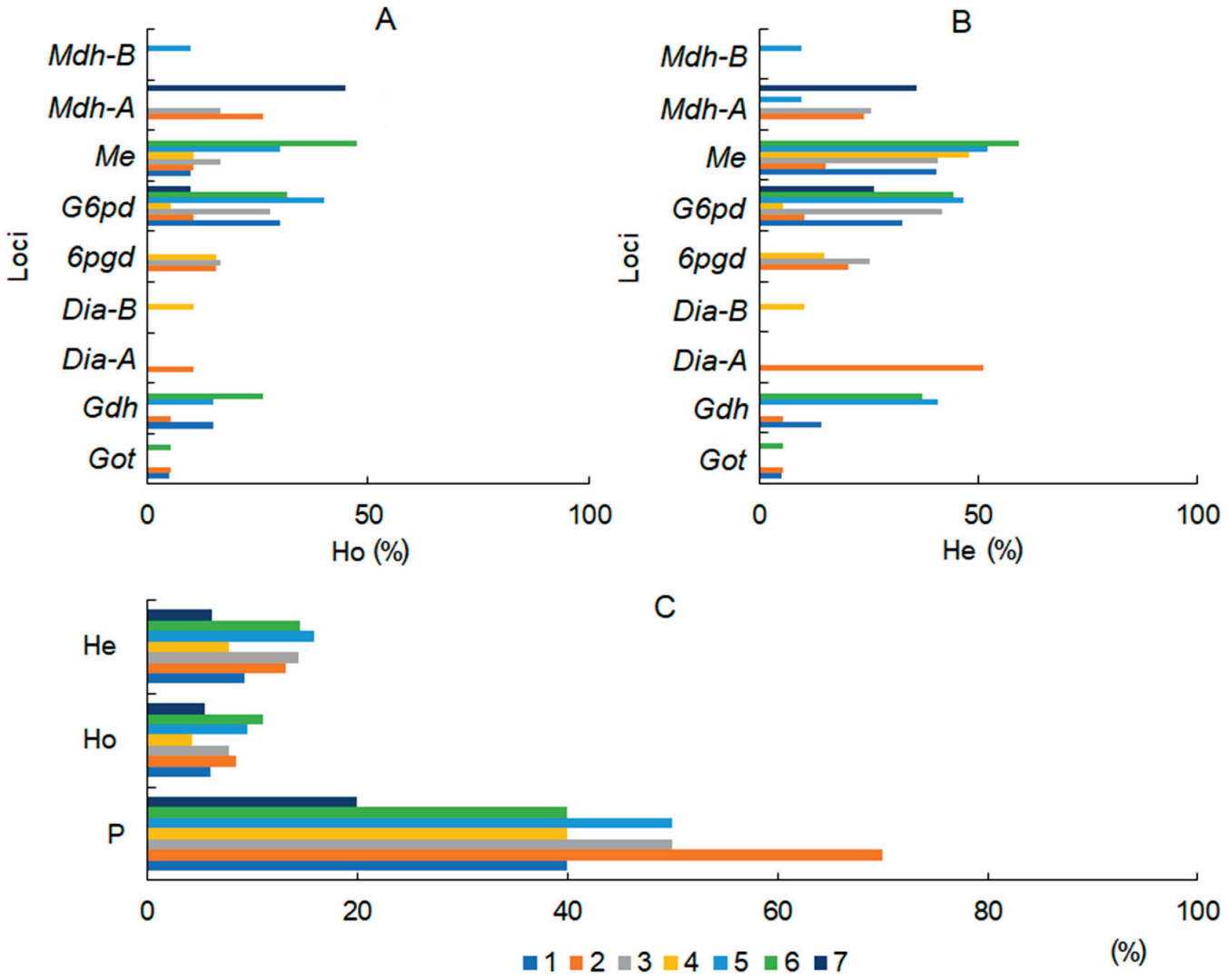


**Figure 3.** Genetic polymorphism in seven populations of *Bassia prostrata*. (**A**)—observed heterozygosity ($H_o$) of polymorphic loci; (**B**)—expected heterozygosity ($H_e$) of polymorphic loci; (**C**)—polymorphic loci proportion of population (*P*), average (for all loci) observed heterozygosity ($H_o$), and average (for all loci) heterozygosity ($H_e$) of seven populations of *Bassia prostrata*.

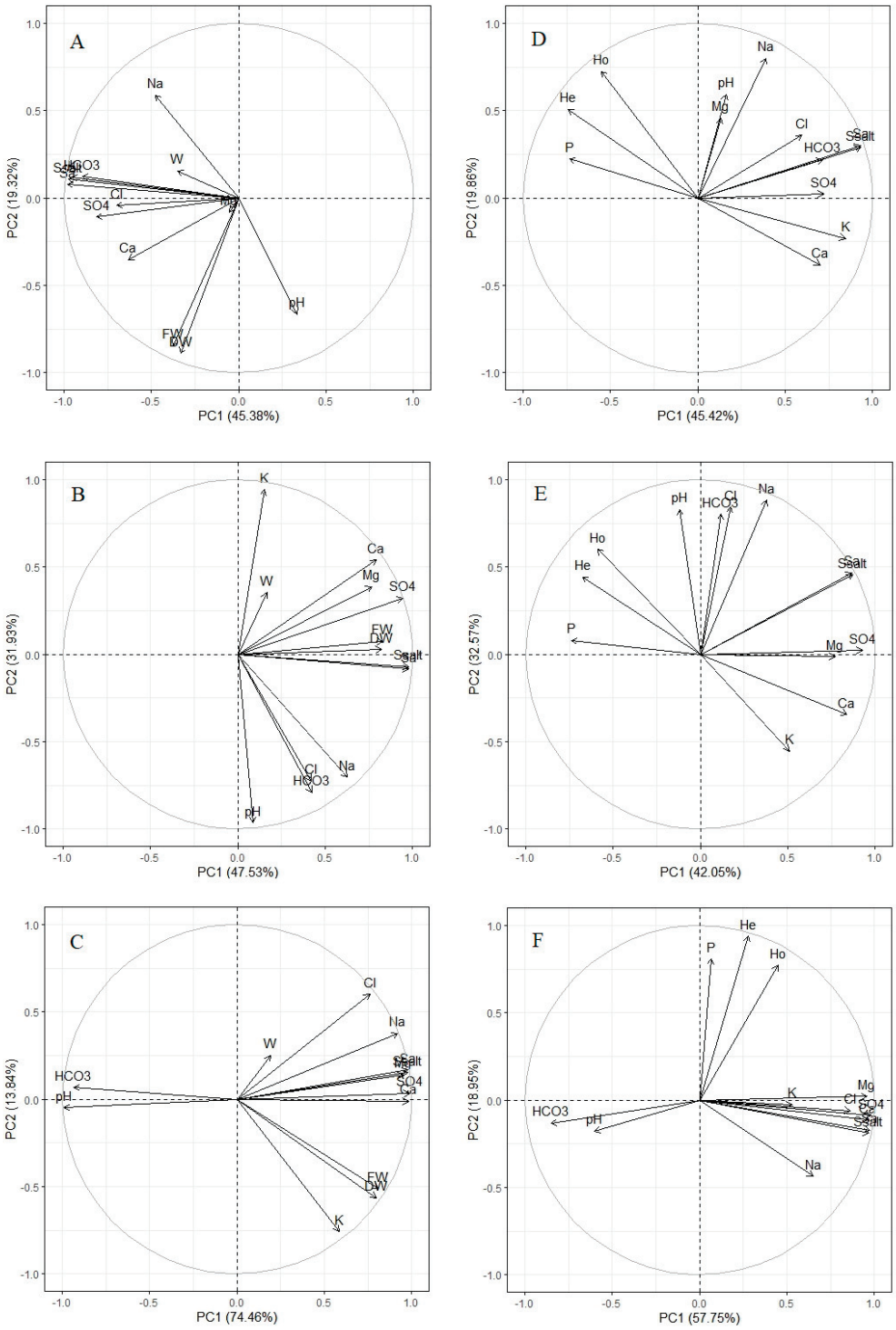


**Figure 4.** Principle component analysis (PCA) of growth (**A**–**C**), genetic diversity (**D**–**F**) parameters of *Bassia prostrata*, and salinity of 0–20 cm (**A**,**D**), 20–40 cm (**B**,**E**), and 40–60 cm (**C**,**F**) soil layers. $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$, $Cl^-$, $SO_4{}^{2-}$, $HCO_3{}^-$—ions content in soil; Ss—sum of salts; Sa—sum of anions in soil; FW—fresh plant biomass; DW—dry plant biomass; W—water content in leaves; *P*—proportion of polymorphic loci; $H_o$—observed heterozygosity; and $H_e$—expected heterozygosity of *B. prostrata*.

## 4. Discussion

The habitats of *Bassia prostrata* in this study were characterized by significant diversity in the degree and chemistry of soil salinity; high salinity occurred at different soil depths (Table 1). *B. prostrata* has wide edaphic plasticity and can grow on various soil genesis,

e.g., chestnut, light-chestnut alkaline soils, and solonetz, as well as on soil-forming rocks of different compositions, from light sandy to heavy loamy, stony, and gypsum [30,36].

Our results revealed differences in correlations between *B. prostrata* aboveground biomass accumulation and seed genetic polymorphism and the chemistry and degree of salinity of different soil layers. The genetic diversity level was affected by the salinity degree and the chemistry of the uppermost soil layers (0–20 cm, 20–40 cm), and biomass accumulation was mainly affected by the salinity of the 20–40 cm and 40–60 cm soil layers. Such differences may be associated with different seasons of aboveground biomass and seed pool formation. *B. prostrata* biomass accumulation (before flowering) occurs mainly in the summer, the hottest and driest season: 23–26 °C, 40–43% humidity, and 65.7 mm precipitation (Figure 1B). In the summer, the drying of the uppermost soil layers can be observed, and plants receive water and dissolved salt ions from lower soil layers, affecting biomass formation. Our study showed a positive dependence of *B. prostrata* productivity on the degree of salinity in 20–40 cm soil layers (Figure 4B). *B. prostrata*, as a halophyte, requires a certain amount of salt in the substrate for optimal growth [37] and has high productivity in soils with 20 dS/m (EC) salinity [31]. The content of the main plant nutrient $K^+$ in seven soil habitats decreased from the upper to lower layers, whereas the $Na^+$ concentration increased (means of $K^+/Na^+$ were 0.45 and 0.01 in the 0–20 cm and 40–60 cm soil layers, respectively; Table 1). Despite the fact that plants growing in saline habitats have acquired mechanisms that allow for selective uptake of $K^+$ when $Na^+$ dominates in the substrate [37], in *B. prostrata* plants, $K^+$ content in tissues decreased when $Na^+$ exceeded 100–200 mM NaCl [38]. Thus, the selective absorption of $K^+$ from the 40–60 cm soil layer under conditions of increased competition with $Na^+$ affects *B. prostrata* biomass accumulation in natural habitats (Figure 4C).

$Ca^{2+}$ and $Mg^{2+}$ ions are also essential mineral nutrients. $Ca^{2+}$ is a universal signal in all eukaryotic cells and participates in many other cellular processes, for example, in the maintenance of cell membrane integrity, cation–anion balance, and osmoregulation [39,40]. $Mg^{2+}$ is an activator of more than 300 enzymes, in particular, photosynthetic and respiratory ones, which are also needed for DNA and RNA synthesis [41,42]. It is well known that $Ca^{2+}$ plays a protective role in a plant's response to salinity. Much less is known about the role of $Mg^{2+}$ in the salt tolerance of plants [39]. However, it was shown that low concentrations of mixed salts with $CaCl_2$, and $MgSO_4$ are necessary for the successful seed germination of *B. prostrata* [43]. Our study showed that $Ca^{2+}$ and $Mg^{2+}$ contents contributed significantly to soil salinity in *B.prostrata* habitats (Table 1). Positive correlations between biomass accumulation and $Ca^{2+}$ and $Mg^{2+}$ contents in the 20–40 cm soil layer (Figure 4B) indicate their necessity for *B. prostrata* growth. The influence of magnesium in this soil layer can be associated with the optimal $K^+/Mg^{2+}$ ratio. The $K^+/Mg^{2+}$ ratio for soils and plant tissues is critical to maintaining optimal plant nutrition and, hence, plant productivity [42]. The $K^+/Mg^{2+}$ ratio ($0.09 \pm 0.03$) in the 20–40 cm soil layer in *B. prostrata* habitats was less than that of the 0–20 cm soil layer ($0.56 \pm 0.17$) but higher than that of the 40–60 cm soil layer ($0.02 \pm 0.01$).

*B. prostrata* seeds are formed in autumn, during a cooler and rainier period (1–16 °C, 49–81% humidity, 77.1 mm precipitation; Figure 1B) when the upper soil layers are moist and plants receive water and dissolved salt ions from them. At the same time, the need for water decreases due to lower air temperatures and higher humidity. Therefore, the formation of seed genetic diversity in *B. prostrata*, upon which the future stability of populations in changing environments depends [12,44,45], is affected by the salinity level and ionic composition of the 0–40 cm soil layers. In heterogeneous environments, the processes of gene flow, mutation, and sexual reproduction generate local genetic variation, providing material for local adaptation [45]. The influence of soil factors such as soil type, pH, moisture, and soil layer depth on population genetic diversity has been demonstrated in different plant species [15,17–19]. A nine-year experiment on the influence of soil moisture and nitrogen, phosphorus, and potassium content in soil on allozyme frequency revealed an allele–habitat association in *Festuca ovina* [15]. It was found that in natural populations the *Pgi*-2-2 allele is

significantly associated with soil moisture and is affected by nutrient/water treatments [15]. Negative correlations between *B. prostrata* genetic diversity with inorganic ion content (except for $Na^+$ and $Cl^-$) and the sum of salts in the 0–40 cm soil layers (Figure 4D,E) indicate selection in favor of homozygotes. Since isozymes (allozymes) were also used in our study, a question arises regarding the functional significance of enzymes under selection. Loci *G6pd* and *Me* were the most polymorphic among the *B. prostrata* populations (Figure 3A). They encode the enzymes glucose-6-phosphate dehydrogenase (G6PD) and malik-enzyme (NADP-Me), respectively, which are associated with the regulatory nodes of dark respiration and photosynthesis. G6PD is a key enzyme in the alternative apotomous oxidative pentose phosphate pathway (OPPP), whose role is enhanced under stress [46]. Malik-enzyme is involved in photosynthesis and is especially active in $C_4$ species, and it plays a vital role in the tolerance to salt stress [47]. The adaptive–compensatory reactions of plants under stress are always associated with additional energy costs, which leads to a change in the balance between photosynthesis and respiration [46]. Any shifts in this balance are reflected in the total plant productivity. Selection leads to local adaptation, and the strength of local adaptation depends on the strength of selection. Strong selection leads to strong local adaptation, which is significantly affected by landscape heterogeneity [48]. The negative influence of $Ca^{2+}$, $Mg^{2+}$, and $SO_4{}^{2-}$ contents in the 0–40 cm soil layer on heterozygosity indicates the formation of the local adaptation of *B. prostrata* to magnesium–calcium sulfate soil salinity. The detected level of sodium chloride salinity did not negatively impact seed genetic polymorphism (Figure 4D,E). This is probably due to the necessity of these ions in maintaining water balance in the aboveground organs of *B. prostrata* (Figure 4C).

## 5. Conclusions

Our study demonstrates that in natural habitats the productivity and seed genetic polymorphism of halophytes may be affected by the salinity of different soil layers. These differential plant responses to vertically heterogeneous soil salinity could be attributed to seasonal variables during biomass accumulation (summer) and seed formation (autumn). An excess of some ions in the uppermost soil layers can lead to increased local adaptation to a certain type of salinity and the appearance of genotype-environment associations. Genotype–environment association analyses may allow us to develop adaptive measures for natural resource management, pasture improvement, and the phytoremediation and restoration of lands with different salinity chemistries.

**Author Contributions:** Conceptualization, E.S., M.L., and K.T.; methodology, E.S. and M.L.; software, M.P.; validation, E.S., K.T. and M.L.; formal analysis, A.K.; investigation, E.S., A.K. and M.L.; resources, E.S. and K.T.; data curation, A.K.; writing—original draft preparation, E.S.; writing—review and editing, E.S., K.T. and M.L.; visualization, M.P.; supervision, K.T.; project administration, E.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by the Science and Technology Research Partnership for Sustainable Development (SATREPS), in collaboration with the Japan Science and Technology Agency (JST, JPMJSA2001) and the Japan International Cooperation Agency (JICA), and by the Ministry of Science and Higher Education of the Russian Federation (theme No. 122042700044-6) and FGUR-2022-001- 1021060307664-8-4.1.4.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data are available from the authors upon request.

**Acknowledgments:** We are grateful to the administration of the Elton Regional Nature Park for the opportunity to conduct research in the park.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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# Influence of Mineral Treatment, Plant Growth Regulators and Artificial Light on the Growth of Jewel Sweet Potato (*Ipomoea batatas* Lam. cv. Jewel) In Vitro

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**Abstract:** Sweet potato (*Ipomoea batatas* (L.) Lam), a member of the bindweed family (*Convolvulaceae* Juss.), is well known for its food, medicinal, and industrial values. It is estimated that more than 7000 sweet potato cultivars have been bred to date. Jewel sweet potato (*I. batatas* Lam cv. Jewel) is one of the most popular cultivars of sweet potato grown today because of its high nutritional value, delicious taste, and is suitable for all processing methods. However, little is known about the micropropagation of jewel sweet potato. The purpose of this paper was to study the effect of three important factors, including culture medium, plant growth regulators (PGRs), and artificial light sources, on the induction, proliferation, and growth of in vitro *I. batatas* 'Jewel' shoots obtained from the axillary bud and shoot tip explants. The different Murashige and Skoog (MS) salt levels (33%, 50%, 100%, and 150%) were used to study the influence of mineral treatment. To assess the influence of PGRs, we used 0.5 mg/L indole-3-acetic acid (IAA) combined with various cytokinins, including 0.5–2.0 mg/L 6-benzylaminopurine (BAP), 0.5–2.0 mg/L kinetin (Kn), and 0.1–1.0 mg/L thidiazuron (TDZ). On the other hand, the in vitro shoots were cultivated in a light room with different lighting conditions. Three lighting treatments (differences in the ratio between the red (R) and blue (B) spectra) were used. Research results have shown that the medium containing 50% MS salt concentration supplemented with 0.5 mg/L BAP or 0.5 mg/L Kn combined with 0.5 mg/L IAA was the most suitable for induction, proliferation, and growth of in vitro jewel sweet potato shoots. On the other hand, stem pieces bearing the axillary buds' explants were determined to be suitable for the shoot induction. Using artificial light with different blue/red ratios also had a significant effect on the growth of explants and stimulates shoot or root formation.

**Keywords:** jewel sweet potato; shoot tip; axillary bud; different MS salts concentration; plant growth regulators; artificial light; micropropagation

**Citation:** Kirakosyan, R.N.; Kalashnikova, E.A.; Abubakarov, H.G.; Sleptsov, N.N.; Dudina, Y.A.; Temirbekova, S.K.; Khuat, Q.V.; Trukhachev, V.I.; Sumin, A.V. Influence of Mineral Treatment, Plant Growth Regulators and Artificial Light on the Growth of Jewel Sweet Potato (*Ipomoea batatas* Lam. cv. Jewel) In Vitro. *Life* **2023**, *13*, 52. https://doi.org/10.3390/life13010052

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 10 November 2022
Revised: 6 December 2022
Accepted: 21 December 2022
Published: 24 December 2022



## 1. Introduction

Sweet potato (*Ipomoea batatas* (L.) Lam), a member of the bindweed family (*Convolvulaceae* Juss.), is well known for its food, medicinal, and industrial value [1–3]. More than 5000 years ago, this species was first domesticated in the Americas. It is estimated that more than 7000 sweet potato cultivars have been bred to date. Sweet potatoes are considered one

of the most important root crops after potatoes and cassava [4], especially in developing countries in Latin America, Southeast Asia, and Africa [5]. In these countries, sweet potato is a main food crop for the people because of its richness with healthy proteins, vitamins, antioxidants, and minerals [6,7]. Studies have shown that this species possesses a number of pharmaceutical properties, such as antibacterial, antioxidant, anticancer, anti-inflammatory, and antiulcer activities [8–10]. In traditional medicine in many countries, sweet potato is used to treat diabetes, anemia, hypertension, stomach cancer, cardiovascular disease, allergies, constipation, eye disease, arthritis, dengue fever, and nausea [6,10–15]. On the other hand, sweet potato can be used in cooking, as well as in different industrial foods production [16–18].

Traditionally, sweet potato cultivars have been propagated by vegetative propagation using stem cuttings or tubers [7,19]. However, this method has certain limitations, such as slow propagation speed, being time-consuming, and being season-dependent. On the other hand, stem cuttings or tubers used for this method often accumulate pathogens (such as nematodes, insect pests, and pathogens that cause black rot, scurf, and stem rot) and can be spread from one generation to the next, causing great losses in yield and the production of poor-quality tubers [3,7,19]. Therefore, if large-scale, uniform, and disease-free plant material is required for production, this method is not always suitable [3,7]. On the other hand, the seeds of sweet potato are only used to breed and develop new cultivars of sweet potatoes because of their highly heterozygous nature [20]. In order to overcome the limitations, biotechnological methods using plant tissue culture techniques have been used for the commercial scale-up of many cultivars of sweet potato. In previous reports, several types of explants, e.g., nodal segment, axillary shoot, and shoot tip from mature or in vitro plants, have been used as explants for in vitro shoot proliferation of sweet potato cultivars [21–39]. In there, nodal segments have been recognized as the best or most commonly used explants for the micropropagation of various sweet potato cultivars, e.g., 'Carmen Rubin' and 'White Triumph' [32], 'Gaozi No.1' [27], 'Naruto Kintoki' [40], 'KSP 36' and 'KEMB 36' [41], and 'Abees' [4]. However, little is known about the micropropagation of jewel sweet potato, which is dubbed the 'sweet potato queen' due to its high nutritional value and delicious taste with all processing methods. This sweet potato cultivar was bred in 1970 at North Carolina State University. To date, jewel has become one of the most commonly grown sweet potato cultivars, its tubers are characterized by tan skin and orange flesh and that is considered a rich source of provitamin A carotenoid [42,43].

In fact, the efficacy of micropropagation techniques depends on a variety of factors, of which three important factors are culture medium, plant growth regulators (PGRs), and artificial light sources (type and intensity). In most reports, Murashige and Skoog (MS) [44] base medium was reported to be the most suitable medium for shoot initiation, shoot proliferation, and rooting in sweet potato cultivars [21–39]. However, in some sweet potato cultivars, 1/2MS medium has been found to be more suitable for shoot proliferation and rooting, e.g., 'purple flesh sweet potato' [37] and 'red-peeled sweet potato' [4]. Most of the authors have shown that the medium without any PGRs is not suitable for in vitro shoot regeneration. In sweet potato, nutrient media supplemented with cytokinins in combination with auxins were reported to be the best for in vitro shoot proliferation [21–23,26,37]. Besides the above two factors, the type and intensity of artificial light also affect the micropropagation of sweet potatoes. Yang et al. [45] found that the different ratios of photosynthetic photon flux (PPF) of red LED (R) and PPF of blue LED (B) had different effects on the growth of sweet potato plantlets in vitro.

The aim of the present study was to determine the effects of different PGRs, different MS salt concentrations, and different artificial light conditions on the induction, proliferation, and growth of in vitro *Ipomoea batatas* 'Jewel' shoots obtained from the axillary bud and shoot tip explants.

## 2. Materials and Methods

### *2.1. Plant Material Preparation*

The work was carried out at the Department of Biotechnology of the Russian State Agrarian University—Moscow Agricultural Academy named after K. A. Timiryazev (Moscow, Russia). All work on sterilization, introduction into culture in vitro, and further work on the study of callogenesis and morphogenesis were carried out in aseptic conditions of laminar hood flow (Biokom). The object of the study was explants of sweet potato (*Ipomoea batatas* Lam.), cultivar Jewel (USA), provided by the Federal Research Center for Potato named after A. G. Lorch.

Before being placed in vitro, the jewel sweet potato tubers were placed in water to activate the dormant meristem. On the 7th day, the shoots began to form, and on the 21st day, they reached 10–12 cm. The shoot tips of 1.0 cm in length and stem pieces bearing the axillary buds of 1.5 cm in length from plantlets sprouting from tubers were used as explants for experiments. The explants' surface was sterilized with a 0.1% solution of mercuric chloride ($HgCl_2$). They were soaked in the solution for 5 min and then rinsed with sterile distilled water [4].

### *2.2. Experiment 1: Evaluate the Influence of Different MS Salt Concentrations on Induction and Growth of In Vitro Jewel Sweet Potato Shoots*

The shoot tips of 1.0 cm in length and stem pieces bearing the axillary buds of 1.5 cm in length from plantlets sprouting from tubers were used as explants for this experiment. Nutrient media containing different MS salts (MS: DUCHEFA, Haarlem, The Netherlands) were created. The addition of 4.4 g/L MS was determined as 100%. The ratios of MS salts used in nutrient media: 33% (1.45 g/L-MS1), 50% (2.2 g/L-MS2), 100% (4.4 g/L-MS), 150% (6.6 g/L-MS3) [46]. Sucrose was present at a concentration of 2% and agar of 0.8% in all variants of nutrient media. PGRs were not added to the nutrient medium in all treatments. The pH of the nutrient medium in all treatments was 5.8. The plants were cultivated in a well-lit growth chamber at 21–23 °C under a 16 h photoperiod provided by 3–3.5 klx white fluorescent lamps (OSRAM AG, Munich, Germany).

In vitro shoot growth indices (including shoot length and root length) in the treatments were measured after one and four weeks of culture.

### *2.3. Experiment 2: Evaluate the Influence of Different PGRs on Proliferation and Growth of In Vitro Jewel Sweet Potato Shoots*

Shoots 1.5–2.0 cm in length from the first experiment were used as explants for this experiment. The MS2 semisolid media supplemented with a combination of 0.5 mg/L indole-3-acetic acid (IAA) with various cytokinins, including 0.5–2.0 mg/L 6-benzylaminopurine (BAP) (Sigma, Schnelldorf, Germany), 0.5–2.0 mg/L kinetin (Kn) (Merck, Darmstadt, Germany), and 0.1–1.0 mg/L thidiazuron (TDZ) (Russia), were used to culture the in vitro shoots. There were 2% sucrose and 0.8% agar in all media. In vitro shoots were subcultured to a fresh medium every 6 weeks. Visual observations were made after 45 days. The following indicators were taken into account: explants' survival rate, number of adventitious shoots per explant, shoot length, number of leaves per shoot, number of roots, and root length. The in vitro shoots were cultivated in a well-lit growth chamber at 21–23 °C under a 16 h photoperiod provided by 3–3.5 klx white fluorescent lamps (OSRAM AG, Munich, Germany).

### *2.4. Experiment 3: Evaluate the Influence of Different Artificial Light Conditions on Induction and Growth of In Vitro Jewel Sweet Potato Shoots*

The shoots 1.5–2.0 cm in length from the first experiment were cultivated on two different types of media:

(1) PGR-free MS medium containing sucrose 2% and agar 0.8%;
(2) Medium containing only distilled water and agar 0.8%, without mineral salts and PGRs.

The in vitro shoots were cultivated in a light room with different lighting conditions. Three lighting treatments (differences in the ratio between the red (R) and blue (B) spectra) were used:

Treatment 1 (control): illuminators based on white LEDs with a color temperature of 3500 K and 6000 K and a monochromatic red LED with a peak of 660 nm (OSRAM AG brand, made in Germany).

Treatment 2a: multichannel illuminator based on white LEDs with a color temperature of 3500 K and 6000 K and monochromatic red (R) and blue (B) LEDs with peaks of 660 nm and 460 nm, respectively. The channel power of monochromatic LEDs was set in the ratio R: 70%/B: 30%.

Treatment 3a: multichannel illuminator based on white LEDs with a color temperature of 3500 K and 6000 K and monochromatic red and blue LEDs with peaks of 660 nm and 460 nm, respectively. The power of monochromatic LED channels was set in the ratio R: 30%/B: 70% [47].

In vitro shoot growth indices (including shoot length, root number, and root length) in the treatments were measured after 45 days of culture.

### *2.5. Statistical Analysis of Experimental Data*

The experiments were arranged completely randomly and repeated three times. Mean values of all data were calculated using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). Analysis of variance (ANOVA) was performed in AGROS software (version 2.11, Russia) and means were compared using Fisher's least significant difference (LSD) test at a significance level of $p \leq 0.05$.

## 3. Results

### *3.1. Influence of Different MS Salt Concentrations on Induction and Growth of In Vitro Jewel Sweet Potato Shoots*

The induction and growth of in vitro shoots from explants depend on the amount of salts dissolved in the medium. In this experiment, the standard MS salt composition was changed to 33% (MS1), 50% (MS2), 100% (MS), and 150% (MS3) of the normal concentration (100% MS).

After 7 days of culture, the shoot tip explants showed no response to the culture medium (Figure 1a), while the stem pieces bearing the axillary buds showed to form the axillary shoots and roots (Figure 1b). Observations in the following weeks did not record the response of shoot tip explants.

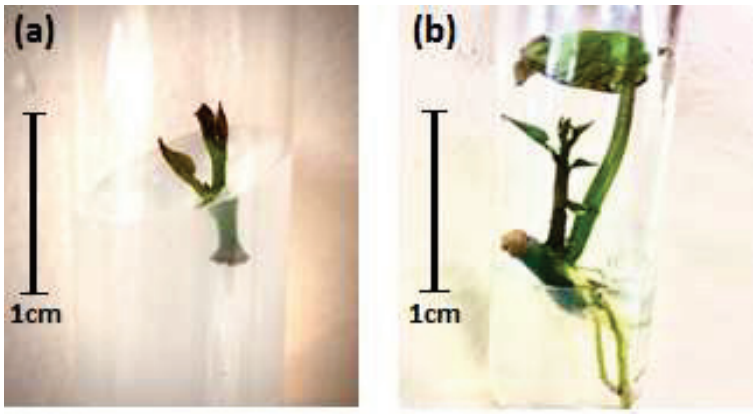


**Figure 1.** Response of the explants after 7 days of culture: (**a**) shoot tip; (**b**) stem pieces bearing the axillary bud.

On the other hand, the results also showed that the medium containing different concentrations of MS salts had a significant effect on the growth of axillary shoots and

roots of sweet potato after four weeks of culture. The reduction in salt content compared with basal MS medium showed good results for the sprouting of axillary buds, as well as axillary shoots' subsequent growth. The best growth indices (including axillary shoot length and root length) were obtained from explants on the MS2 medium (Figure 2). High concentrations of mineral salts in the culture medium (MS3) showed a negative effect on axillary shoots' and roots' growth. Based on these obtained results, MS2 medium (containing 50% salt content of the salt concentration in basal MS medium) was used for the following experiments.

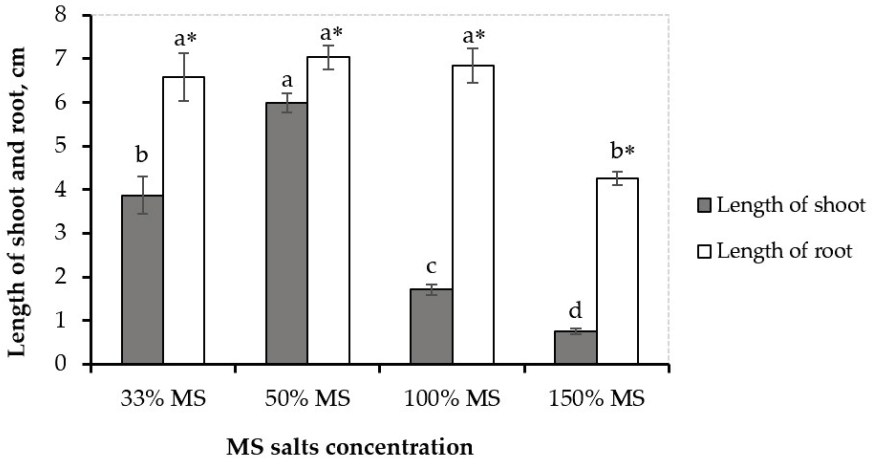


**Figure 2.** Influence of different MS salt concentrations on in vitro shoots' growth of jewel sweet potato after four weeks of culture. (*) indicate a significant interaction between the evaluated parameter at 0.05 probability levels. Means with different letter(s) within the bars differ significantly at a 0.05 probability level using LSD.

### *3.2. Influence of Different PGRs on Proliferation and Growth of In Vitro Jewel Sweet Potato Shoots*

The MS2 medium (containing 50% salt content of the salt concentration in basal MS medium) supplemented with a combination of 0.5 mg/L IAA with various cytokinins such as 0.5–2.0 mg/L BAP, 0.5–2.0 mg/L Kn, and 0.1–1.0 mg/L TDZ was used for the rapid proliferation of in vitro jewel sweet potato shoots (Table 1, Figure 3).

The obtained results demonstrate that increased cytokinin concentration in the nutrient medium reduced the explants' ability to form axillary and adventitious shoots, whose growth was also reduced due to higher hormonal concentrations. It was found that the best results were obtained in the medium containing 0.5 mg/L BAP and 0.5 mg/L Kn combined with 0.5 mg/L IAA, and the worst in the medium containing TDZ combined with 0.5 mg/L IAA. The media with BAP combined with 0.5 mg/L IAA exhibited average results (Table 1).

Differences were also observed for such parameters as shoot length and the number of leaves per shoot (Table 1). The experiments demonstrated that the MS2 medium supplemented with 0.5 mg/L Kn combined with 0.5 mg/L IAA provided the highest mean shoot length value (6.1 cm) and the number of leaves per shoot (9.2). The second effective medium was one containing 0.5 mg/L BAP combined with 0.5 mg/L IAA; its shoot length was 5.2 cm, and the number of leaves was 8.0. In the other medium, the in vitro shoots grew slowly and formed very short internodes, which was especially evident for the medium containing 1.0 mg/L TDZ combined with 0.5 mg/L IAA.

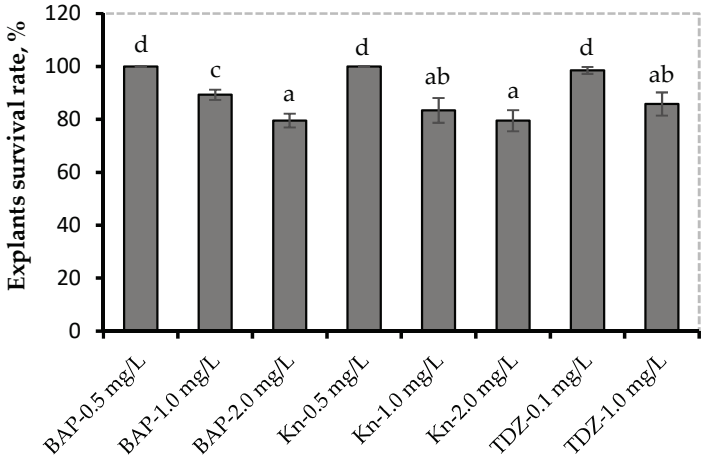


**Figure 3.** Combined effect of auxin and cytokinin on explants survival rate (%) after 45 days of culture. Values of germination percentage were arcsine $\sqrt{X}$ transformed prior to statistical analysis. Means with a different letter(s) within the bars differ significantly at a 0.05 probability level using LSD.

**Table 1.** Combined effect of auxin and cytokinin on proliferation and growth of in vitro jewel sweet potato shoots after 45 days of culture.

| Plant Growth Regulator, mg/L | | Number of Shoots per Explant | Shoot Length | Number of Leaves per Shoot | Number of Roots per Shoot | Root Length |
|---|---|---|---|---|---|---|
| **Cytokinin** | **Auxin** | | **(cm)** | | | **(cm)** |
| BAP, 0.5 mg/L | IAA, 0.5 mg/L | 3.2 ± 0.2 [1] d | 5.2 ± 0.2 b | 8.0 ± 0.3 b | 2.02 ± 0.12 a | 8.95 ± 0.75 a |
| BAP, 1.0 mg/L | | 1.8 ± 0.5 b | 4.0 ± 0.2 c | 6.6 ± 0.3 c | 1.51 ± 0.51 ab | 8.06 ± 1.02 abc |
| BAP, 2.0 mg/L | | 1.6 ± 0.3 b | 3.0 ± 0.1 d | 3.0 ± 0.1 d | 0.86 ± 0.26 bc | 7.68 ± 0.65 bc |
| Kn, 0.5 mg/L | | 3.9 ± 0.1 d | 6.1 ± 0.3 a | 9.2 ± 0.4 a | 1.98 ± 0.07 a | 8.52 ± 0.38 ab |
| Kn, 1.0 mg/L | | 2.6 ± 0.1 c | 4.3 ± 0.2 c | 5.1 ± 0.2 c | 1.35 ± 0.36 abc | 8.01 ± 0.52 abc |
| Kn, 2.0 mg/L | | 2.0 ± 0.4 bc | 3.3 ± 0.2 d | 2.9 ± 0.1 d | 1.01 ± 0.65 bc | 7.51 ± 0.36 bc |
| TDZ, 0.1 mg/L | | 1.5 ± 0.2 b | 2.6 ± 0.1 de | 3.0 ± 0.1 de | 1.25 ± 0.14 bc | 7.23 ± 0.45 c |
| TDZ, 1.0 mg/L | | 0.8 ± 0.1 a | 0.7 ± 0.1 f | 2.5 ± 0.1 f | 0.65 ± 0.43 c | 5.86 ± 0.14 d |

[1] Mean ± standard error (SE), means followed by the same letter are not significantly different at $p \leq 0.05$ according to the Fisher's least significant difference (LSD) test.

In addition, the combined addition of cytokinin and auxin (IAA) showed the in vitro rooting effect. The results showed that the MS media supplemented with 0.5 mg/L BAP and 0.5 Kn combined with 0.5 mg/L IAA gave the highest number of roots and mean root length (0.5 mg/L BAP: 2.02 roots/shoot, 8.95 cm; 0.5 mg/L Kn: 1.98 roots/shoot, 8.52 cm).

### *3.3. Influence of Different Artificial Light Conditions on Induction and Growth of In Vitro Jewel Sweet Potato Shoots*

In this experiment, we studied the effect of the spectral composition of the light (red and blue spectrum) on the in vitro shoots' growth of jewel sweet potato. The main research results are shown in Table 2. Studies have shown that the addition of red and blue spectra in different proportions to normal illumination did not lead to an increase in the growth of cultivated explants. As a rule, the specific growth rate of the main shoot from axillary buds was about 2–2.5 times less than in the control variant.

**Table 2.** Influence of the ratio of red and blue spectra on growth of in vitro jewel sweet potato shoots after 45 days of culture.

| Medium Type | Light Treatment | Number of Shoots per Explant | Shoot Length, (cm) | Number of Roots | Root Length, (cm) |
|---|---|---|---|---|---|
| PGRs-free MS medium | Control | 1 | 4.06 ± 1.32 [1] e | 3.75 ± 0.15 c | 10.25 ± 0.69 c |
| | R 70%: B 30% | 1 | 2.95 ± 0.54 d | 3.25 ± 0.16 b | 9.75 ± 0.60 c |
| | R 30%: B 70% | 1 | 1.80 ± 0.12 d | 5.25 ± 0.25 d | 9.87 ± 0.63 c |
| Distilled water and agar 0.8% | Control | 1 | 0.72 ± 0.10 a | 2.25 ± 0.11 a | 7.62 ± 0.38 b |
| | R 70%: B 30% | 1 | 1.33 ± 0.10 c | 2.33 ± 0.15 a | 7.33 ± 0.33 ab |
| | R 30%: B 70% | 1 | 0.95 ± 0.10 b | 5.67 ± 0.38 d | 6.83 ± 0.30 a |

[1] Mean ± standard error (SE), means followed by the same letter are not significantly different at $p \leq 0.05$ according to Fisher's least significant difference (LSD) test.

According to the obtained results, studied treatments had an ambiguous influence on the growth indices of jewel sweet potato shoots. Within the nutrient medium containing MS salts, the studied indicators were less or equal to the control variant. The exception was observed at the R = 30%: B = 70% treatment. In this treatment, the mean number of roots per shoot was 5.25, which is about 1.75 times higher than in the two other treatments.

As regards cultivation of in vitro shoots on medium free of mineral salts with only water, sucrose, and agar, clearer dependences were observed. Under these conditions, the mean shoot length was maximal at the R = 70%: B = 30% treatment. At the R = 30%: B = 70% treatment, the mean number of roots was 5.67, which was nearly 2 times more than in other treatments within the used medium.

When growing in vitro shoots of jewel sweet potato on a nutrient medium without mineral compounds, an inverse relationship was observed between the mean number of roots and the proportion of red and blue spectra. There was an increase in root formation as a result of the increase in the blue spectrum proportion. Shoot growth was observed with the predominance of the red spectrum. It can be seen that, by changing the composition of light, it is possible to regulate the morphogenetic potential of jewel sweet potato.

## 4. Discussion

Like most European countries, the sweet potato cultivation area in the Russian Federation is limited, concentrated mainly in southern provinces. In recent years, one of the popular trends in the food industry is the manufacturing of functional and dietary food products. Only in Russia, about 1400 tons of such products are consumed annually, and most of these are imported. The practical requirement is to expand the area of material plants for this industry and sweet potato is one of them. In the present study, we selected the 'Jewel' sweet potato cultivar, which has high nutritional value and is popularly grown in many countries around the world, to study the factors affecting their in vitro propagation ability, thereby creating a premise to expand large-scale production in the direction of gradually replacing imported raw materials for the food industry.

Investigations in the field of plant cell engineering start from a well-grown sterile culture. Many publications have demonstrated that the proper selection of a sterilizing agent, its concentration, and its effect on an explant are vital parts of a study that in many ways determine the success of an experiment [48]. To obtain a sterile sweet potato culture, many authors applied 0.1% $HgCl_2$ solution to soak the explants for 14–15 min [49–51]. However, such a long exposure may cause necrotic lesions in the young and actively growing plant tissues, leading to their premature death. Our experiment, performed in plants of different taxonomic groups, showed that the best sterile explants were obtained from the plant tissues socked in 0.1% $HgCl_2$ solution for 5 min. Similarly, Dewir et al. [4] also obtained good surface sterilization of red-peeled sweet potato explants using 0.1% $HgCl_2$ solution for 5 min.

The success of clonal micropropagation depends on the balanced composition of the nutrient medium, both in terms of mineral and PGR composition. Several reports on other plant species have demonstrated that different MS salt concentrations affect the growth development or regeneration values of plants, such as *Mentha spicata* L. [52], *Bacopa monnieri* L. [53], *Lophophora williamsii* Coult. [54], and *Staurogyne repens* (Nees) Kuntze [46]. In sweet potato, the MS base medium was reported to be the most suitable medium for shoot initiation, shoot proliferation, and rooting in most reports [21–39]. However, in some sweet potato cultivars, 1/2MS medium has been found to be more suitable for shoot proliferation and rooting, e.g., 'purple flesh sweet potato' [37] and 'red-peeled sweet potato' [4]. The results of our study on the sweet potato jewel cultivar also showed that the medium containing 50% MS salt was the most suitable for shoot initiation, shoot proliferation, and rooting. On the other hand, most of the reports also showed that the addition of BAP or Kn combined with IAA resulted in good shoot regeneration and rooting effects [21–23,26,37]. Similar to these reports, our results suggest that a nutrient medium supplemented with 0.5 mg/L BAP or 0.5 mg/L Kn combined with 0.5 mg/L IAA was best for shoot initiation, shoot proliferation, and rooting in sweet potato jewel cultivar.

It is known that the spectral composition of light is an important physical factor influencing morphogenetic processes. It was shown that different light spectra affect the proliferation and differentiation of plant cells in different ways. For example, violet and blue spectra increase the process of photosynthesis, which leads to the rapid formation of more powerful plants [55]. Plant photomorphogenesis depends on the intensity of the red and blue spectrum of light, as well as their ratio. It has been experimentally shown that the spectrum of red light is quite wide. Its different parts are responsible for the regulation of various physiological processes. This may affect the production process as a whole [55]. In addition, the synthesis of auxins depends on red light. Auxins are responsible for root differentiation in an intact plant. The blue spectrum is responsible for the differentiation of buds and the formation of the aboveground biomass. The green spectrum leads to an increase in the effectiveness of the action of various spectra on the morphophysiological processes of the studied objects [56,57]. The results of our study have shown that an increase in the proportion of the blue spectrum stimulates an increase in root formation. The predominance of the red spectrum stimulates the activation of shoot growth. Due to the fact that very few similar studies have been reported previously, present and future studies in this direction are of interest.

## 5. Conclusions

This study is one of the first to report the effects of culture conditions on the micropropagation of *Ipomoea batatas* 'Jewel' cultivar. Effects of three important factors (including MS salt concentration, PGRs, and artificial light) on the initiation, proliferation, and growth of in vitro jewel sweet potato shoots obtained from the axillary bud and shoot tip explants were studied. Research results have shown that the medium containing 50% MS salt concentration supplemented with 0.5 mg/L BAP or 0.5 mg/L Kn combined with 0.5 mg/L IAA was the most suitable for induction, proliferation, and growth of in vitro jewel sweet potato shoots. This result will pave the way for further studies on the jewel sweet potato cultivar towards determining the biochemical composition, especially inulin content, biological activity, and adaptability to field conditions in the Russian Federation of plantlets. Thereby creating a premise to expand large-scale production in the direction of replacing imported raw materials for the food industry to meet practical needs.

**Author Contributions:** Conceptualization, E.A.K. and R.N.K.; methodology, E.A.K., R.N.K., H.G.A., A.V.S., S.K.T. and Q.V.K.; software, Q.V.K.; validation, R.N.K. and A.V.S.; formal analysis, R.N.K., H.G.A., A.V.S., Y.A.D. and A.V.S.; investigation, E.A.K. and R.N.K.; resources, V.I.T., E.A.K. and R.N.K.; data curation, Q.V.K.; writing—original draft preparation, E.A.K.; writing—review and editing, E.A.K., R.N.K., A.V.S., S.K.T. and N.N.S.; visualization, E.A.K., R.N.K., A.V.S., S.K.T. and N.N.S.; supervision, E.A.K. and R.N.K.; project administration, V.I.T., E.A.K. and R.N.K.; funding acquisition, R.N.K. and V.I.T. All authors have read and agreed to the published version of the manuscript.

**Funding:** The article was completed within the framework of the thematic plan task for the implementation of research work by the federal state budgetary educational institution of higher education 'Russian State Agricultural University-Moscow Agricultural Academy named after K.A. Timiryazev' by order of the Ministry of Agriculture of Russia at the expense of the federal budget in 2022. Additionally, Sections 2.1 and 2.4 were supported by the Ministry of Science and Higher Education of the Russian Federation in accordance with agreement № 075-15-2020-905, date 16 November 2020, on providing a grant in the form of subsidies from the federal budget of the Russian Federation. The grant was provided for state support for the creation and development of a World-Class Scientific Center 'Agrotechnologies for the Future'.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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*Review*

# Microbial Inoculants as Plant Biostimulants: A Review on Risk Status

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**Abstract:** Modern agriculture systems are copiously dependent on agrochemicals such as chemical fertilizers and pesticides intended to increase crop production and yield. The indiscriminate use of these chemicals not only affects the growth of plants due to the accumulation of toxic compounds, but also degrades the quality and life-supporting properties of soil. There is a dire need to develop some green approach that can resolve these issues and restore soil fertility and sustainability. The use of plant biostimulants has emerged as an environmentally friendly and acceptable method to increase crop productivity. Biostimulants contain biological substances which may be capable of increasing or stimulating plant growth in an eco-friendly manner. They are mostly biofertilizers that provide nutrients and protect plants from environmental stresses such as drought and salinity. In contrast to the protection of crop products, biostimulants not only act on the plant's vigor but also do not respond to direct actions against pests or diseases. Plant biostimulants improve nutrient mobilization and uptake, tolerance to stress, and thus crop quality when applied to plants directly or in the rhizospheric region. They foster plant growth and development by positively affecting the crop life-cycle starting from seed germination to plant maturity. Legalized application of biostimulants causes no hazardous effects on the environment and primarily provides nutrition to plants. It nurtures the growth of soil microorganisms, which leads to enhanced soil fertility and also improves plant metabolism. Additionally, it may positively influence the exogenous microbes and alter the equilibrium of the microfloral composition of the soil milieu. This review frequently cites the characterization of microbial plant biostimulants that belong to either a high-risk group or are closely related to human pathogens such as *Pueudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, etc. These related pathogens cause ailments including septicemia, gastroenteritis, wound infections, inflammation in the respiratory system, meningitis, etc., of varied severity under different conditions of health status such as immunocompromized and comorbidity. Thus it may attract the related concern to review the risk status of biostimulants for their legalized applications in agriculture. This study mainly emphasizes microbial plant biostimulants and their safe application concerns.

**Keywords:** biostimulants; biofertilizers; rhizosphere; soil microorganisms; phytostimulator

**Citation:** Kumari, M.; Swarupa, P.; Kesari, K.K.; Kumar, A. Microbial Inoculants as Plant Biostimulants: A Review on Risk Status. *Life* **2023**, *13*, 12. https://doi.org/10.3390/life13010012

Academic Editors: Hakim Manghwar and Wajid Zaman

Received: 1 November 2022
Revised: 12 December 2022
Accepted: 13 December 2022
Published: 21 December 2022



## 1. Introduction

Plant biostimulants, as the name implies, are substances or microorganisms, which stimulate plant growth. Horticulturists coined the word biostimulant as a substance promoting plant growth that does not belong to the group of nutrients, soil improvers, or pesticides. It includes a diverse collection of compounds, substances, and microorganisms that are applied to plants to improve the crop yield, quality, and tolerance to biotic and abiotic stress [1]. According to the European Biostimulants Industry Council (EBIC) [2] "Plant biostimulants contain substance(s) and/or microorganisms whose function, when applied

to plants or the rhizosphere, is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides" [2]. Other terminologies can also be used to define biostimulants such as biogenic stimulators [3], organic biostimulants [4], plant strengtheners [5], phytostimulators [6], and agricultural biostimulants [7]. It has been reported that the global market for biostimulants reached $2241 million by 2018 and Europe was the largest market for biostimulants in 2012 [8]. Biostimulants were later elaborated by EBIC as "Biostimulants foster plant growth and development throughout the crop life cycle from seed germination to plant maturity in several demonstrated ways, including but not limited to: improving the efficiency of the plant's metabolism to induce yield increases and enhanced crop quality; increasing plant tolerance to and recovery from abiotic stresses; facilitating nutrient assimilation, translocation, and use; enhancing quality attributes of produce, including sugar content, color, fruit seeding, etc.; rendering water use more efficiently; enhancing certain physicochemical properties of the soil and nurturing the development of complementary soil micro-organisms" [9]. Thus, biostimulants are organic-based plant growth promoters and regulators. They provide nutrients to plants and enhance crop productivity in an eco-friendly manner. The word 'biostimulant' was first defined by Kauffman et al. [10] as materials, other than fertilizers that promote plant growth when applied in low quantities. They are mostly organic products and comprise amino acids, plant hormones, polysaccharides, and humic substances and are easily available for uptake by plants. They may not only deliver nutrients to the plant but also stimulates plant metabolism and alleviate biotic and abiotic stresses [11]. Parrado et al. [12] reported various mechanisms of biostimulant action through which they enhance crop yields, such as stimulation of soil microbial activity, intensification of vital soil-enzyme activities, or phytohormone production. In November 2012, the first world congress was held on the topic of the use of biostimulants in agriculture in Strasburg, France, which was participated in by 30 countries. The main intention of this congress was to bring together people working on the features of biostimulants in academia, industry, and regulatory agencies. Therefore, the uses of these biological substances became commercialized [8].

In this review, we are mainly emphasizing plant biostimulants and their role in agriculture. Besides these, we also discuss microbial inoculants as plant biostimulants, associated risk, and their biosafety regulations when applied in the agricultural field to promote plant growth.

## 2. History of Classification of Biostimulants

Classification of biostimulants is widely documented by many scientists, stakeholders, and regulators [8,13,14]. In 2012, du Jardin classified biostimulants into eight classes which were based on bibliographic analysis of plant biostimulants and microbial inoculants, and they were not included in these categories [13]. Calvo et al. [8] reviewed five different categories of biostimulants based on a critical review of selected scientific publications related to biostimulants. Furthermore, based on practical and theoretical knowledge of agricultural and horticultural biostimulant products used, du Jardin categorized them into seven categories containing substances as well as microbes [1]. According to the Agriculture and Horticulture Development Board (2017), they were further classified into two main groups, non-microbial and microbial [15]. Recently, Pascale et al. [16] classified plant biostimulants based on enhancing plant nutrition into five categories including microorganisms. The chronological order of classification of plant biostimulants and the basis of their categorization is summarized in Table 1.

**Table 1.** Chronological order of classification of biostimulants.

| **Year** | **2012** [13] | **2014** [8] | **2015** [1] | **2017** [15] | **2018** [16] |
|---|---|---|---|---|---|
| **Basis of categorization** | Bibliographic analysis | A critical review of selected scientific publications | Substances and microorganisms | Type of products | Plant nutrition |
| **Role of microbes** | No | Yes | Yes | Yes | Yes |
| **Categories** | 1. HS<br>2. Complex organic materials<br>3. Beneficial chemical elements<br>4. Inorganic salts<br>5. SWE<br>6. Chitin and chitosan derivatives<br>7. Antitranspirants<br>8. Free amino acids and other N-containing substances | 1. Humic acids<br>2. Fulvic acids<br>3. Microbial inoculants<br>4. PHs and amino acids<br>5. SWE | 1. Humic and fulvic acids<br>2. Protein hydrolysates and other N-containing compounds<br>3. Seaweed extracts and botanicals<br>4. Chitosan and other biopolymers<br>5. Inorganic compounds<br>6. Beneficial fungi<br>7. Beneficial bacteria | 1. Non-microbial<br>(i) SWE<br>(ii) HS<br>(iii) Phosphite and other inorganic salts<br>(iv) Chitin and chitosan derivatives<br>(v) Antitranspirants<br>(vi) PHs and free amino acids<br>(vii) Complex organic materials<br>2. Microbial<br>(i) PGPR<br>(ii) Non-pathogenic fungi<br>(iii) AMF<br>(iv) Protozoa and nematodes | 1. HS<br>2. PHs<br>3. SWE<br>4. PGPR<br>5. AMF |

HS—Humic substances, PHs—Protein hydrolysates, SWE—Seaweed extract, PGPR—Plant Growth Promoting Rhizobacteria, AMF—Arbuscular Mycorrhizae Fungi.

## 3. Properties of Plant Biostimulants

A new category of agricultural chemicals derived from biological sources and applied as foliar applications or as seed treatments to improve the productivity of crops and overall growth and yield is known as plant biostimulants [17]. They mostly act as biofertilizers in agriculture and horticulture. There are various properties reported by many scientists of plant biostimulants which are mentioned below:

- Improving plant metabolism which induces crop yield and increases the quality of crops [18].
- Plant biostimulants protect plants against environmental stresses such as water deficiency, exposure to sub-optimal growth temperatures, and soil salinization [1].
- They are also known to promote plant growth through better nutrient uptake.
- Increasing soil enzymatic as well as microbial activities [16].
- Alteration of the architecture of roots along with enhancement of solubility and mobility of micronutrients [19–21].
- Enhancing fertility of the soil, predominantly by nurturing the development of complementary soil microbes [18].

## 4. Plant Biostimulants and Their Mechanism

### *4.1. Humic Substances (HS)*

Humic substances (HS) are diverse organic molecules that are formed during microbial and chemical degradation of organic matter in soils and are found most abundantly in nature [21–23]. They also contain a total of 60% organic molecules in the world's soils [23,24]. HS was earlier called "gelbstoff", a yellow-colored organic compound generally found

in marine water, freshwater, and soil, and made of linked polymers of amino acids, fatty acids, and carbohydrates which are resistant to further degradation by microbes [24,25]. But today, it is believed that HS is composed of small organic molecules linked together by hydrogen bonds and hydrophobic interactions [23,26,27].

Aiken et al. [28] defined HS as "a general category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in color, of high molecular weight (MW), and refractory". Humic substances obtained from freshwater and terrestrial ecosystems contain lignin, but it is absent in marine ecosystems [25]. Many scientists have reported that humic substances play an imperative role in the function of soil and plants [29]; for instance, by exchange of carbon and oxygen between the soil and atmosphere, and regulating the availability of nutrients [8], they enhance the physical and chemical properties of soil [16] and transform toxic materials and transport them [29,30]. In addition to this, humic substances also affect the chemical and functional properties of rhizospheric microorganisms [31]. One important feature of humic substances is the formation of complexes, although the solubility is a function of pH and the molar ratio of the complex, with micronutrients (iron) avoiding leaching, and in turn, making them available in the soil for plant nutrition [32,33].

Based on their solubility and molecular weight, humic substances can be divided into three groups: humic acids; humins; and fulvic acids. Humic acid is a relatively high-molecular-weight compound and is soluble in alkaline media. It can be easily extracted from soil by treating with dilute alkali and gets precipitated in acidic media, whereas fulvic acid is a low-molecular-weight compound. It is soluble in acidic and alkaline media and cannot be easily extracted [29,34]. Humin is a humic-containing substance instead of a humic substance because it is made up of humic and non-humic materials [35,36]. HS is known to increase the fertility of soil and also alleviate heavy-metal stress. Heavy metals can bind with the carboxyl and phenolic groups (binding site for heavy metal) of the humic substance, resulting in heavy-metal-deficient areas and plants which are unable to take up these metals [37]. Lead toxicity can be minimized with the application of humic and fulvic acid, which reduces the transfer and contamination of $Pb^{2+}$ in the food chain [38,39]. Thus, humic substances have multiple roles and can be applied to stimulate the physical, chemical, and biological activity of soil and plants.

### *4.2. Protein Hydrolysates and Amino Acids*

Stimulation of plant growth and tolerance to abiotic and biotic stress can also be reported using a variety of protein-based products which are different from nitrogen sources. These protein-based products can be categorized into two main groups—protein hydrolysates and amino acids. A combination of peptides and amino acids of either plant or animal origin that are manufactured from partial hydrolysis of a protein source are known as protein hydrolysates [8,16]. Some specific amino acids also function as plant stimulators such as glutamine, glycine betaine, proline, and glutamate. Protein hydrolysates are commercially available in different formulations in the form of powder, granules, and liquid and they may be applied to plants near the root system or as foliar sprays [40]. Various processes are involved in the preparation of protein hydrolysates, e.g., chemical, enzymatic, or thermal hydrolysis of plant residues (carob-germ protein, alfalfa residues, algal proteins, and wheat-condensed distiller solubles) and animal residues (connective or epithelial tissues, collagen, and elastin of animals) [8]. Protein hydrolysates are commercially available in the market of various countries with diverse names such as Aminoplant or Siapton (Italy), Macro Sorb foliar (Spain), and ILSATOP (Italy). The concentration of free amino acids and protein/peptides generally present is 2–18% and 1–85%, respectively, in protein hydrolysates preparation. Free amino acids such as arginine, alanine, valine, leucine, glutamate, proline, and alanine are the major components usually present in protein hydrolysates. In addition to protein/peptide and free amino acids of hydrolysates, some non-protein components also influence plant-growth stimulation. For instance, carob-germ extract, a plant-based product comprising carbohydrates, fats,

macronutrients, and micronutrients and also containing phytohormones [8]. Another group of protein-based products is individual amino acids which include non-protein amino acids that are found extensively in a few plant species, such as glycine betaine, glutamate, histidine, and proline, render anti-stress properties [8,41]. There are many stimulatory effects of protein hydrolysates on plants such as improvement of soil respiration, increase in biomass, and activity of microorganisms because plants and microorganisms can easily utilize these amino acids and peptides as a source of nitrogen and carbon [42]. They also provide macronutrients (Ca, Mg and K) and micronutrients (Fe, Mn, Zn, and Cu) to the plants because they can chelate these metal nutrients present in soil and make them available to plant roots [1,40]. Some industries use this strategy for making biofertilizers having high nutrient efficacy. Moreover, protein hydrolysates are also known to induce the defense mechanism of plants and also increase tolerance to a range of abiotic stresses such as drought, salinity, oxidative conditions, and temperature [43–47]. Several higher plants that include soybean, alfalfa, rice, barley, and maize can tolerate a wide variety of abiotic stress due to the exogenous supply of these compounds [44,48,49]. Corte et al. [50], in their study, found that there was the absence of any kind of genotoxic effects shown by animal-based protein hydrolysates on soil microflora and fauna, yeast, and plant bioassay systems [49,50].

### *4.3. Seaweed Extracts and Botanicals*

Seaweeds are also known as large marine algae including multicellular, macroscopic, and benthic organisms that inhabit the world's oceans and provide shelter and food for oceanic animals and also offer a valuable product as single cell protein for mankind [51]. In Asian countries, fresh seaweed is mainly used for food along with traditional remedies [52]. It contains a variety of constituents, i.e., polysaccharides, proteins, polyunsaturated fatty acids (PUFA), polyphenols, pigments, and plant growth hormones [53]. In coastal regions, seaweed has been used as a fertilizer to enhance the growth of plants [54].

These liquid extracts are commercially available for horticulture and agriculture [54,55]. The extracts act by adding chelators and phytohormones and by improving soil structure and aeration [56]. Seaweed extracts are commercially made from brown algae such as *Ralfsia*, *Ascophyllum nodosum*, *Padina*, *Turbinaria*, *Sargassum*, *Laminaria*, *Fucus* spp., and others [57–60]. The seaweed extract is formulated in liquid or dried form and can be blended with micronutrients and fertilizers for field application [8]. The biostimulation effects of seaweed extracts include increasing plant growth, fruit and flower production, and crop yield, helping to develop resistance against abiotic and biotic stresses, enhancing shelf life after harvest, and increasing chlorophyll levels [8,60].

Botanicals are substances that are extracted from plants and are used in cosmetic products and pharmaceuticals, food ingredients, and plant protection products [61]. The biostimulatory effects of botanicals, except seaweed extracts, are not well understood and need to be explored. In ecosystems, plant-active compounds known as allelochemicals, which are known to mediate plant interactions, receive more attention regarding sustainable crop management [1]. Recent studies exploring biostimulatory effects of higher plant botanicals on white hat cabbage and radish have led to valuable additions for the vegetable plant under study [62,63]. Further study is required to establish the biostimulatory effects of these botanicals.

### *4.4. Chitin and Chitosan Derivatives*

After cellulose, chitin is the second most copious biodegradable polysaccharide in nature and is composed of N- acetyl-d-glucosamine groups linked by β (1–4) glycosidic bonds through the activity of chitin synthases to form a linear chain [64]. It is mostly obtained from the exoskeleton of shrimp, insects, and crabs along with the cell walls of fungi and algae [65]. Chitin is mostly insoluble in water, ia a high-molecular-weight biopolymer, and has a porous structure favoring high water absorption. Chitosan is a derivative of chitin and is produced after the deacetylation of chitin which influences its chemical and biological

properties and is also responsible for antimicrobial activity due to the protonation of its amino groups in solution [66]. Another derivative of chitin is oligochitosan (chitooligosaccharides), formed during the chemical and enzymatic hydrolysis of chitin and composed of mainly 3–10 saccharide residues of N- acetylglucosamine or glucosamine [67]. Biostimulatory effects of chitin and their derivatives have been reported by many researchers, and include the protection of plants from pests and diseases, enhancing the antagonistic action of microorganisms, improving the beneficial plant-microbe interactions, and regulating plant growth and development [65,68]. Shahrajabian et al. [69] reported various beneficial effects of chitin and its derivatives on vegetable crops such as increased photosynthetic activity, tolerance to abiotic stressors (salinity, drought, temperature), expression of defensive genes, increased antioxidant-enzyme activity, activation of plant innate immunity, induction of secondary metabolite synthesis, etc.

### *4.5. Antitranspirants*

Chemical compounds which favor a reduction in the rate of transpiration from plant leaves are known as antitranspirants and alleviate drought stress by reducing the size and number of stomata [70]. Many chemicals reported as antitranspirants, such as chitosan, kaolin, calcium carbonate, salicylic acid, etc., are eco-friendly and increase the water-holding capacity of soil as well as reduce the rate of transpiration. Thus, the use of antitranspirants in plants increase crop yield in water- and high-temperature-stress conditions [71].

### *4.6. Microbial Inoculants*

The agriculture system is heavily dependent on chemical inputs such as pesticides, herbicides, and fertilizers to enhance yield [72,73]. Due to the thrilling use of these chemicals, the quality of soil and the health of plants are being deteriorated, ultimately affecting human health. Therefore, there is a need to develop a sustainable approach to minimize the harmful effects of these chemicals and promote plant growth, and also improve soil quality without disturbing the natural ecosystem. The use of microbial inoculants is an alternative to these chemical inputs, which can act as biofertilizers, bioherbicides, biopesticides, and biocontrol agents [73]. Microbes are also one of the important categories of biostimulants for plants.

The formulations of beneficial microorganisms, which play an affirmative role in the soil biome in an eco-friendly manner, are called microbial inoculants [74]. Natural soil contains a variety of agriculturally important microorganisms that have a beneficial effect on soil and plants by providing nutrients and also protecting the plant from pests and diseases. Generally, there are two groups of microbial inoculants (i.e., biofertilizers and biopesticides), but those that function as biofertilizers are grouped under biostimulants [8,75]. They are also known as bioinoculants, which contain living organisms and promote plant growth through a variety of mechanisms, such as, increasing root growth and biomass, suppling nutrients, and also enhancing the capacity of nutrient uptake when applied to seeds, plants, or soil [76]. Microorganisms acting as biostimulants mainly belong to beneficial fungi groups including arbuscular mycorrhizal fungi and free-living bacteria [76–78]. Kloepper et al. [79] reported that plant growth-promoting bacteria (PGPB) and plant growth-promoting rhizobacteria (PGPR) are free-living bacteria, isolated from the rhizosphere of plants that can act as biofertilizers and stimulate plant growth. Many factors are responsible for the development of microbial inoculants as biofertilizers, such as the variety of plants [80,81], compatibility with chemical fertilizers, types of soil, and environmental conditions [8]. The activity of microbial inoculants is mostly influenced by root exudates (extracellular secretions by plants) and also serves as a substrate for the formation of biologically active substances [82]. Based on their biostimulatory effect, microbial inoculants (bioinoculants) can be divided into two groups, which are discussed below:

#### 4.6.1. Plant Growth Promoting Bacteria

Plant growth-promoting bacteria (PGPB) are beneficial, free-living, rhizoplanic, rhizospheric, and phylospheric bacteria and play a dynamic role in plant growth [83]. They belong to diverse genera such as *Bacillus* sp., *Pseudomonas* sp., *Azotobacter* sp., *Enterobacter* sp., *Azospirillum* sp., etc. Bashan and de Bashan suggested various positive effects of plant growth that include improved plant nutrition, abiotic and biotic stress [84,85] tolerance, increased growth and yield [85–87], and also control of plant pathogens. They are either applied either directly to the seed and plant or mixed with a carrier material such as compost, peat, sawdust, vermiculite, or compost, which provides a suitable environment for their growth [88,89]. A lot of studies have been conducted by many researchers to demonstrate the role of PGPB on plants. They promote plant growth by various mechanisms and provide nutrients to plants. These mechanisms include: (i) biological nitrogen fixation [90,91]; (ii) solubilization of inorganic P [92]; (iii) production of iron chelating compounds (iv); and phytohormones production, which is discussed in detail below:

##### Biological Nitrogen Fixation

Nitrogen is an essential component of all living organisms including plants. It is an important constituent of amino acids, nucleic acids, proteins, and energy currency (ATP, GTP, ADP), etc. Therefore, it is known as a building block of cells. Although $N_2$ is present in about 78% of the atmosphere, it is unavailable for plants and animals due to its complex structure, i.e., the triple bond between two nitrogen atoms [93]. PGPB can convert this gaseous form of nitrogen into a usable form, i.e., ammonia, by the use of an enzyme system, nitrogenase, and make it available to plants. Microorganisms that fix nitrogen belong to diverse genera such as *Azotobacter* spp. [94], *Bacillus polymyxa* [95], *Gluconoacebacter diazotrophicus* [96], and *Burkholderia* spp. [97]. There is also a report that inoculation of mixed inoculants of *Gluconacetobacter diazotrophicus, Burkholderia tropica, Azospirillum amazonense, Herbaspirillum rubrisubalbicans,* and *Herbaspirillum seropedicae* has been very effective in promoting N fixation in sugar cane [98]. *Azospirillum* is the most studied nitrogen fixer among these bioinoculants as reported by Calvo et al. [8]. It has been reported that there is a major increase in nitrogen content in plants when some plant species are inoculated with *Azospirillum* strains. For instance, *A. lipoferum* and *A. brasilense* show 7–12%, and *A. diazotrophicus* gives a 60–80% increase in wheat and sugarcane, respectively.

##### Solubilisation of Phosphate

Phosphorus is also a chief nutrient for plants next to nitrogen. In agricultural soils, the total concentration of phosphorus generally varies between 400 and 1200 $mg/kg^{-1}$ but only 1 $mg/kg^{-1}$ is available in the forms of dihydrogen phosphate ($H_2O_4P^-$) and hydrogen phosphate ($HO_4P^{-2}$) [14]. Soil contains P in inorganic and organic forms which are insoluble [14]. The inorganic form of P contains about 20–50% of total P [99] and is generally available in the form of $PO_{4-}$ ions, which are sparingly soluble due to the adsorption of positively charged ingredients of soil and also are precipitated with some metals such as Al, Fe and Ca [99]. Insoluble organic P is also available in the form of inositol phosphates, phosphate esters, and uncharacterized large organic molecules and contains 50–80% of total P [99]. In agriculture systems, the low availability of P in soil is a significant problem [8]. PGPB can increase the nutrition of plants through the process of solubilization of P [14]. To solubilize insoluble inorganic and organic P, bacteria use several approaches. There are two mechanisms for the solubilization of P by bacteria that are predominant, i.e., through the production of organic acids or through the production of phosphatase and phytase enzymes [100,101]. Due to organic acid production, insoluble inorganic P gets transformed into soluble form due to the presence of carboxyl and hydroxyl groups that chelate the cations bound to phosphates [102] and transform them into soluble forms. The pH of immediate soil is also decreased by the production of organic acids and the solubility of P is improved by releasing phosphate ions [103]. PGPB are also known to solubilize

organic P by the production of phosphatase and phytase enzymes and converting them into soluble forms [104] which plants can easily uptake from the soil.

The predominant rhizospheric bacteria and others that have the ability to solubilize P belong to the genera *Burkholderia*, *Pseudomonas*, *Bacillus*, *Rhizobium*, *Agrobacterium*, *Achromobacter*, *Streptomyces*, *Micrococcus*, *Erwinia*, etc. Most phosphate-solubilizing bacteria that show good results under laboratory conditions, may not work well in soil conditions [99]. Therefore, rigorous field studies are ongoing to successfully characterize field-compatible phosphate-solubilizing potent bacteria.

Production of an Iron Chelating Compound

In the biosphere, iron is the fourth most abundant micronutrient. In aerobic conditions, iron is mostly found in ferric ions or $Fe^{+3}$ which is insoluble and not easily accessible to plants and microorganisms [105]. In calcareous soil, Fe is not available for plants due to alkaline conditions making it less soluble [106]. Microorganisms, especially PGPB have a mechanism for producing low-molecular-weight iron-chelating compounds known as siderophore [93]. These compounds help in transporting iron into the bacterial cells and also make it available to plants. Siderophores also act as a biocontrol agent as they can create iron-deficient areas near the plant roots by inhibiting plant pathogens [105]. There is a significant increase in Fe uptake in some plants such as sunflower and maize in nonsterile calcareous soils as compared to sterile soil. This occurs due to the action of soil microorganisms that help in the uptake of Fe to plants [106]. It has been shown by Sharma et al. [107] that there is a significant increase in iron content in rice when inoculated with the strain of *Pseudomonas* because of the production of siderophores and also enhanced nutritive value of rice grains due to the increased levels of iron. Thus, siderophore production is an important trait of PGPB and enhances the iron uptake of plants.

Phytohormone Production

Microbial inoculants such as PGPB are also known to produce a number of plant hormones or plant-growth regulators that alter the architecture of roots and the growth of plants [108–110]. These plant hormones are gibberellins, auxins, ethylene, cytokinins, and abscisic acid [111]. A number of physiological processes can be regulated by these hormones, including root elongation, formation of root hairs, and root initiation [8]. Indole-3-acetic acid (IAA) has been widely reported as a natural auxin produced by microbial inoculants [112]. Many plant functions are influenced by IAA such as root initiation, differentiation of vascular tissue, expression of many plant genes, and mediation of tropic responses [8]. Cytokinins also play an important role in plants, including delaying leaf senescence and promoting mitotic cell division in roots and shoots [16]. Flower and fruit production, seed germination, and dormancy of vegetative organs are affected by gibberellin hormones [113]. Furthermore, abscisic acid is mainly involved in responses to environmental stresses such as high salinity, and drought along with plant development [114]. In addition, ethylene is well known as a ripening hormone, but there are other roles that have been reported such as cell expansion, flower and leaf senescence, and seed germination. Ethylene is also known as a stress hormone because it is produced under abiotic as well as biotic stress [115]. An inhibitory effect on root growth has also been reported due to the production of high concentrations of ethylene, which ultimately reduces plant growth. To overcome this problem, PGPB are also known to produce a vital enzyme, L-aminocyclopropane—1-carboxylate deaminase (ACC deaminase), which catalyzes the formation of the intermediate precursor of ethylene, ACC (1-amino cyclopropane-1-carboxylic acid), into α ketobutyrate and ammonia and regulates the biosynthesis of ethylene [8]. Moreover, PGPB also produced some low-molecular-weight volatile organic compounds (VOCs) such as ketones, alcohols, hydrocarbons, and aldehydes, which have generally high vapor pressure and enter into the atmosphere [116]. These compounds are collectively termed microbial volatile organic compounds (mVOCs) [117]. Initially, Fernando et al. [118] and Vespermann et al. [119] reported some biocontrol activity of these

VOCs of some rhizospheric microorganisms, but later promotion of plant growth also reported the role of VOCs, for instance, the growth promotion in *Arabidopsis thaliana* by VOCs of PGPB strains containing acetoin and 2,3 butanediol [120]. PGPB also affects the morphology of roots and provides nutrition to plants.

#### 4.6.2. Arbuscular Mycorrhizal Fungi (AMF)

Fungi are also found in soil and are associated with plant roots in the following two ways: through mutualistic symbiosis and parasitism. In mutualistic symbiosis, both organisms live together and establish beneficial relationships, whereas, in parasitism association, one partner benefits and the other is harmed [121]. A beneficial and heterogeneous group of fungi that establishes symbiotic relationships with more than 90% of plant species is known as mycorrhizal fungi [1]. Mycorrhizal fungi can be categorized into different groups, but arbuscular mycorrhizal fungi are a prevalent type of endomycorrhiza and are commonly associated with horticultural and crop plants. Early in the history of land plants [122], arbuscular mycorrhizal fungi (phylum Glomeromycota) appeared first and were associated with diverse plant taxa [123]. A special branched structure formed during the penetration of fungal hyphae of Glomeromycota species in root cortical cells of plants is called arbuscules [121,124]. Today, there is great interest in the use of these mycorrhizal fungi in sustainable agriculture, which have been established to provide enhanced nutrients (macro and micronutrients) and water uptake and also help plants survive biotic and abiotic stress [125–130]. There is a recent report that not only is there interconnection between fungi and plants established by the hyphal network, but also connecting individual species of plants within a community and helping in signaling among interplant species [131,132]. AMF plays an important role in stimulating plant growth through several mechanisms [133]: (i) enhancing the uptake of water; AMF increases the surface area of the root through which plants can easily take up water; (ii) availability of nutrients, especially phosphorus, under nutrient-deficiency condition; (iv) modifications of root architecture; (v) changes in enzymatic and physiological activities, especially for plants that are involved in antioxidative responses; and (vi) induction of ABA plant hormones, which are mainly involved in stress conditions [134]. Auge, Brundertt, and Begum [125,135,136] reported some ameliorating effects of drought due to mycorrhizal symbiosis in some plant species, including wheat, onion, soybean, lettuce, and corn. This occurs due to increased root growth resulting in enhanced tolerance to drought. It also maintains high water efficiency and increased growth when plants are colonized by AMF [8]. Furthermore, the water potential of plants may also be affected by the changes in the structure of soil by the production of a soil-binding material such as glomalin, a glue-like substance that is insoluble in nature by the hyphae of AMF [125]. Protection of plant roots from the toxicity of heavy metals by the use of AMF has also been reported by Leyval et al. [137]. There are also some reports available and reporting that drought tolerance of plants is augmented by the application of co-inoculation with AMF and PGPR. For example, improved plant growth, stomatal conductance, the efficiency of water use, as well as increased photosynthetic rate, being reported in lettuce plants when co-inoculated with AMF *Glomus mosseae* and *G. intraradices* and *Bacillus* spp (PGPR). A better result was obtained in co-inoculation with AMF and *Bacillus* spp. as compared to individual organisms. This occurred due to PGPR, i.e., *Bacilus* spp., enhancing the growth and colonization of AMF [138]. But there are some limitations on the use of AMFs as biostimulants, which may result from their biotrophic character; they have difficulty for propagation on a large scale, and researchers have been unsuccessful in understanding the determinants of host specificities and other population dynamics of mycorrhizal fungi in agricultural ecosystems [139]. Some fungi which are distinct from mycorrhizal species are also reported such as *Trichoderma* spp and *Sebacinales*, which are able to colonize roots and provide nutrients to their hosts, but the mechanisms are not well studied [121]. However, these fungi can be used as bioinoculants to improve the nutritional status of plants. *Trichoderma* spp. is well known for its biocontrol and biopesticidal activities, but Colla et al. [40] and Shoresh et al. [140] also reported some

stimulatory effects on plants such as enhanced efficiency of nutrients, morphogenesis, and organ growth along with increased tolerance to abiotic stress. These fungal endophytes may be considered biostimulants as well as biopesticides based on these effects on plants as reported by researchers [40,140]. Therefore, microbial inoculants including beneficial bacteria as well as fungi are a promising tool in sustainable agriculture. They not only enhance plant nutrition but also assist plants in tolerating a number of environmental stresses. They improve our agriculture system without any deleterious effects. The overall mechanisms of action of different plant biostimulants in the plant are represented below (Figure 1):

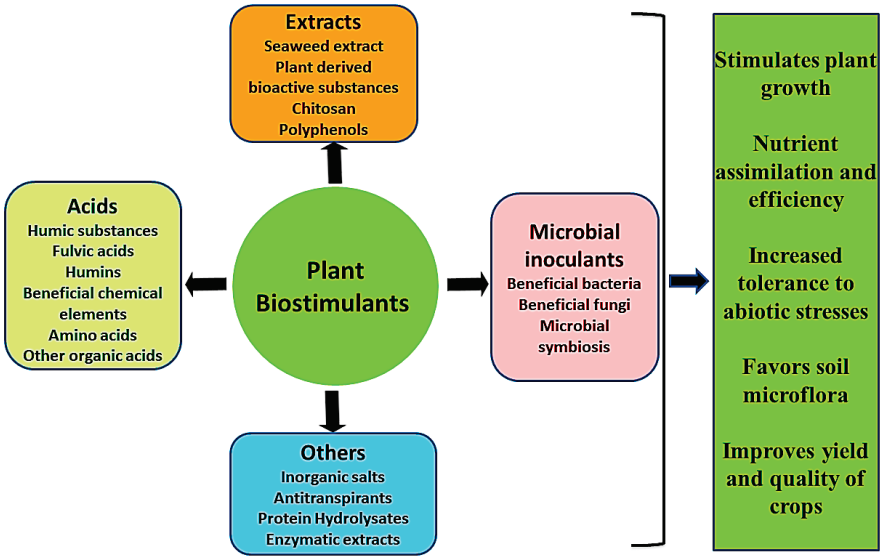


**Figure 1.** Schematic representation of the different types of plant biostimulants and their beneficial mechanisms in plants [1,8].

## 5. Risk Status of Microbial Inoculants (Plant Growth Promoting Bacteria)

PGPB, which are considered potent candidates for plant growth, should be safe for mammals. Some of the microbial inoculants commonly used as biostimulants, and their risk groups are listed in Table 2. Despite their array of beneficial effects on plants (Table 2), they may pose a risk to other living organisms, especially human beings. Although most PGPB do not have a negative effect, some genera are involved in causing infections in animals and humans. Bacteria belonging to the genera *Serratia, Acinetobacter, Bacillius cereus*, *Stenotrophomona, Enterobacter, Herbaspirillum, Ochrobactrum,* and *Pseudomonas* are not only powerful candidates for plant-growth promotion but may also cause disease in humans [141,142]. *Pseudomonas,* besides being a potential candidate as PGPR, is also responsible for many types of opportunistic infections in humans who are aged, immunocompromised, or suffering from conditions such as cancer, severe burns, or cystic fibrosis. Some common pathogenic species of *Pseudomonas* are *P. cepacia, P. aeruginosa, P. putida, P. fluorescens,* etc [143]. Although *Bacillus* sp. is commonly known for its wide variety of applicability in agriculture, industry, and the pharmaceutical sector, it still is associated with many types of illness in humans and animals. It can cause disease in immunocompromised as well as in healthy individuals. Some species may cause minor infections, but some species may be associated with severe or lethal infections. *B pumilus, B licheniformis, B coagulans,* and *B thuringiensis* are examples of *Bacillus* species that are associated with various infections [144]. *Aeromonas* sp. *is* used as PGPR but also causes diseases in immunocompetent and immunocompromised people such as septicemia, gastroenteritis, and wound infections [145]. Another potent PGPR, *Comamonas* spp., is also associated with many life-threatening illnesses such as endocarditis, and septicemia in immunocompetent individuals [146]. *Streptomyes* sp. can cause changes in tissue structure in humans leading to diseases such as cancers, mycetomas, and actinomycetomas [147]. In spite of such im-

mense positive impact of *Trichoderma* sp. on plant health, it is now emerging as a human pathogen causing diseases such as peritonitis, subcutaneous infections, and hematologic disorders [148]. Although *Enterobacter* sp. has a variety of uses as a plant growth stimulator, is also known to cause nosocomial infections and is involved in an array of ailments such as skin infections, inflammation in the respiratory system, and meningitis in neonates, immunocompromised individuals and hospitalized patients [149]. But there are no national or international rules or regulations to assess the risk associated with the commercial use of these plant-beneficial microbes [150]. Even commercial biofertilizers such as Biosubtilin, Nitrofix, and Bioderma. (Table 2) do not mention risks associated with the respective inoculants in their packets.

Risk groups and biosafety levels (BSL) are two terminologies used to describe and categorize microbes as per the level of hazards they can cause [93]. According to the World Health Organization (WHO 2015), microorganisms that are categorized under various risk groups (RG) are based on certain criteria such as their pathogenicity and virulence, host range, mode of transmission, availability of vaccines for effective prevention, availability of medications, etc. Thus, the classification, e.g., from RG-1 to RG-4 articulate the level of hazard a particular microorganism causes. RG-1 refers to a group of microbes that do not cause or are not associated with any type of illness in healthy animals (including humans). Microbes under RG-2 group are associated with a disease that is generally mild and there are medications readily available to treat the disease. RG-3 microbes are concomitantly associated with a serious and lethal disease that may or may not be treatable. Microbes belonging to RG-4 category have the ability to cause fatal and deadly diseases for which treatment is rarely found. Biosafety level (BSL), e.g., from BSL-1 to BSL-4, is a precautionary procedure and protocol used to avoid or prevent risks associated with these risk groups while handling them. Organisms belonging to BSL-1 are nonpathogenic in nature and can be easily handled in the laboratory through general laboratory guidelines. Microorganisms under BSL-2 have the ability to cause disease in a healthy individual, but there are ample medications and vaccines available to easily cure such diseases. Proper laboratory guidelines and special training are required to handle BSL 2 organisms. Specialized safety measures and containment facilities are required to handle microbes that come under the BSL-3 group because such microbes can cause fatal infections but do have effective remedies and anticipatory treatments available. BSL-4 encompasses high-risk-associated organisms, which have aerosol-transmission ability and for which effective treatment is not available. Laboratory personnel handling such organisms must have special training and should know the primary and secondary containment of BSL-4 organisms. The literature suggests that many bacteria isolated from the rhizosphere, soil, and water, besides having PGP activity, are also involved in causing diseases in immunocompromised and healthy individuals [151–153]. Hence there is an immense need to develop a systemic and polyphasic approach through which we can check the disease-causing ability of microbes isolated from an environmental niche in addition to checking their PGP activity and bioinoculant development [154]. A study by Vílchez et al. stabilized a polyphasic protocol called EHSI (environmental and human safety index) to check the biosafety level of plant-growth-promoting bacteria. EHSI articulates the overall effect of PGPB on soil microflora, beneficial macroflora and fauna, and animal and human health. In this study, according to EHSI, both being potent PGPB, *Pseudomonas putida* KT2440, is relatively safe as compared to *Burkholderia cepacia* CC-Al74. In another study by Kim et al. [155], it was suggested to assess or check for the presence of genes involved in the virulence or pathogenicity of novel bacteria isolates to determine their safety level concerning humans and plants. Keswani et al. [150] suggested that whole-genome sequencing of a bacterial isolate is the best way to obtain a complete understanding of its phylogenetic categorization and pathogenic behavior. Hence, research organizations and institutions which are involved in isolating novel microbial isolates and bioinoculant development, after thorough polyphasic characterization, should use isolates that belong to BSL-1 and Risk group-1 for bioformulation because they will pose minimum risk to the environment and human health [150].

**Table 2.** Microbial inoculants in agriculture and horticulture systems and their indicative risk status in the risk group databases.

| **Microbial Inoculants in Research and Commercial Biofertilizers (Risk Group by TRBA/ATCC/ZKBS)** | **Commercial Status/Formulation (Brand Name and Manufacturer)** | **Plants** | **Effects on Plants** |
|---|---|---|---|
| *Pseudomonas putida* [107]<br>(RG2$^{G}$/BSL1/RG2) [156–158]<br>#BSL 1- *P. putida* (Trevisan) *migula* | Yes/Powder<br>(Pseudomonas putida, Organoponix private Limited, Orissa [159] | Rice | Increased iron uptake |
| *Pseudomonas fluorescens* [160–162]<br>(RG1/BSL1/- [156,158,163]<br>#BSL-1- *P. fluorescens migula* | Yes/Powder and Liquid<br>(PSEUDOMONAS FLUORESCENS Bacterial biocontrol agent, Manidharma Biotech Private Limited, Tamil Nadu, India) [164] | Rapeseed, sweet potato, rice | Increased plant height, biomass, grain yield |
| *Streptomyces strain* [165,166]<br>(RG1/BSL 1/RG1) [156,158,167]<br>#BSL 1- *Streptomyces azureus* Kelley et al. | No/- | Tomato and rice | Plant growth |
| *Azospirillum brasilense* Sp245 [115]<br>(RG 1/BSL 1/RG1) [115,156,158,168]<br>#BSL1- *A. brasilense* | Yes/Liquid<br>(Sardar Liquid Biofertilizers-*Azospirillum* culture, Gujrat State Fertilizers, and Chemicals, India) species and strain not specified) [169] | Spring wheat | The increased dry weight of the shoot and leaf length |
| *Aeromonas* spp [170]<br>(RG 1/BSL 2/RG2) [156,158,171]<br>#BSL 2- *Aeromonas hydrophila* (Chester) Stanier | No/- | Rice | Increased root area |
| *Comamonas acidovorans* [172]<br>(RG1$^{G}$/BSL1/RG2) [156,158,173]<br>#BSL1- *Comamonas* sp. | No/- | Lettuce | Plant growth promotion such as IAA production |
| *Bacillus subtilis* [174]<br>(RG1/BSL1/RG1) [156,158,175]<br>#BSL1- *B. subtilis* (Ehrenberg) Cohn | Yes/Aqueous suspension and wettable powder<br>(Biosubtilin, Biotech International Limited, New Delhi, India) [176] | Lettuce | Increased cytokinin content in roots and shoots |
| *Bacillus licheniformis* [177]<br>(RG1/BSL1/RG1) [156,158,178]<br>#BSL1- *B. licheniformis* (Weigmann) Chester | No/- | Cucumber | Increased fresh weight, higher chlorophyll content, and enhanced cell division |
| *Azospirillum lipoferum* [179]<br>(RG1/BSL1/RG1) [156,158,180]<br>#BSL1- *A. lipoferum* (Beijerinck) | Yes/Carrier powder, soluble powder, and soluble liquid<br>(Nitrofix, Agri Life, Andhra Pradesh, India) [181] | Maize seedlings | Increased root hair density |
| *Azospirillum lipoferum* [182]<br>(RG1/BSL1/RG1) [156,158,180]<br>#BSL1- *A. lipoferum* (Beijerinck) Tarrand et al. | Yes/Carrier powder, soluble powder, and soluble liquid<br>(Nitrofix, Agri Life, Andhra Pradesh, India) [181] | Wheat | Increased tolerance to salinity conditions |
| *P. putida* [183]<br>(RG2$^{G}$/BSL1/RG2) [156–158]<br>#BSL1- *P. putida* (Trevisan) *migula* | Yes/Powder<br>(Pseudomonas putida, Organoponix private Limited, Orissa) [159] | White clover | Increased root and shoot biomass and water content |
| *B. megaterium* [183]<br>(RG1/BSL1/RG1) [156,158,184]<br>#BSL1- *B. megaterium* de Bary | Yes/Carrier powder, soluble powder, and soluble liquid<br>(P Sol B®, Agri Life, Andhra Pradesh, India) [185] | White clover | Increased root and shoot biomass and water content |
| *Alternaria* sp. [186,187]<br>(-/BSL1/RG1/2) [158,188] | No/- | Wheat | Stimulate drought tolerance |
| *Trichoderma* sp. [40,140,186,187]<br>(-/ BSL1/RG1) [158,189]<br>#BSL1- *T. harzianum* Rifai | Yes/Wettable powder and Aqueous suspension<br>(Bioderma, Biotech International Limited, New Delhi) [190];<br>Ecosom®- TV, [191];<br>Ecosom®-TH [192]<br>(Agri Life, Andhra Pradesh, India) | Barley | Increased drought tolerance |

**Table 2.** *Cont.*

| Microbial Inoculants in Research and Commercial Biofertilizers (Risk Group by TRBA/ATCC/ZKBS) | Commercial Status/Formulation (Brand Name and Manufacturer) | Plants | Effects on Plants |
|---|---|---|---|
| *Azoarcus* sp. [193] (RG1/BSL1/RG1) [156,158,194] #BSL1- *A. oleivorans* | No/- | Wheat | Enhanced plant nitrogen nutrition and root growth and alleviate the nutrient deficiency |
| *Azorhizobium* sp. [195] (RG1/BSL1/-) [156,196] #BSL1- *A. caulinodans* Dreyfus et al. | No/- | Wheat | Enhanced plant nitrogen nutrition and root growth and alleviate the nutrient deficiency |
| *Azospirillum* sp. [193] (RG1/BSL1/RG1) [156,158,168,180] #BSL1- *A. lipoferum* (Beijerinck) Tarrand et al., *A. brasilense* Tarrand et al. | Yes/Liquid (Sardar Liquid Biofertilizers- *Azospirillum* culture, Gujrat State Fertilizers, and Chemicals, India) [169] | Wheat | Enhanced plant nitrogen nutrition and root growth and alleviate the nutrient deficiency |
| *Bradyrhizobium* sp. (RG1/BSL1/RG1) [156,158,197–199] #BSL1- *Bradyrhizobium* sp. | No/- | Mungbeans | Increases growth parameters and seed yield |
| *Rhizobium meliloti* [200,201] (RG1/BSL1/RG1) [156,158,202] #BSL1- *Rhizobium* sp. | Yes/Aqueous suspension and wettable powder (Biobium Biofertilizers, Biotech International Limited, New Delhi), Species not specified [203] | Peanuts | Increases plant growth, quality of pods enhanced, and efficiency in the use of nitrogen |
| *R. leguminosarum* [204] (RG1/BSL1/RG1) [156,158,205] #BSL1- *R. leguminosarum* jordan | Yes/Aqueous suspension and wettable powder (Biobium) Species not specified [203] | Soybean | Increases growth and yield performance under drought stress |
| *Bacillus* spp. [206,207] (RG1/BSL 1/ RG1/2/3) [156,158,208] | Yes/Carrier powder, soluble powder, and soluble liquid (Si-Sol B TM, Agri Life, Andhra Pradesh, India) [209] | Strawberry | Increases fresh and dry weight parameters, increases yield |
| *Azotobacter chroococcum* [210] (RG1/BSL1/RG1) [156,158,211] #BSL1- *A. chroococcum Beijerinck* | Yes/Liquid (Reap®-N1, NCS Green Earth Private Limited, Maharashtra) [212] | Maize | Increased shoot and root length, leaf and root number, chlorophyll content |
| *Azotobacter vinelandii* [210] (RG1/BSL1/RG1) [156,158,213] #BSL1- *A. vinelandii* Lipman | Yes/Carrier-based powder (Nitrofix ®, Agri Life, Andhra Pradesh, India) [181] | Maize | Increased shoot and root length, leaf and root number, chlorophyll content |
| *Bacillus halotolerans* [204,214] (RG1/-/-) [156] | No/- | Wheat and soybean | Improved germination, growth, and yield, better draught resistance, improved nitrogen, potassium, and Zn uptake |
| *Enterobacter hormaechei* [204,214] (RG2/BSL2/-) [156,215] #BSL 2- *E. cloacae* (Jordan) Hormaeche and Edwards | No/- | Wheat and soybean | Improved germination, growth, and yield, better draught resistance, improved nitrogen, potassium, and Zn uptake |
| *Pseudomonas frederiksbergensis* $RG2^{G}$ * [204,214] (RG1/BSL1/-) [156,216] #BSL1- *P. frederiksbergensis* Andersen et al. | No/- | Wheat and soybean | Improved germination, growth, and yield, better draught resistance, improved nitrogen, potassium, and Zn uptake |

Risk group * (classification of prokaryotes into risk groups under Biological Agents Ordinance: RG 1 refers to prokaryotes that generally do not cause infectious disease in humans; RG 2 refers to those microbial groups which do not pose a significant risk to laboratory workers but may cause disease if there is exposure and for which there are therapeutic interventions available), RG—Risk group, BSL—Biosafety level as per ATCC; #—The exact name of the organisms in the original concerned database of risk group; (-) indicates that it has not been commercially formulated.

In addition to posing health risks to animals, unprecedented use of PGPR also affects other biotic communities of an ecosystem, especially soil resident flora. As it is already known that newly introduced microorganisms change the microenvironment of soil, creating their niche which can have an immense effect on the structure and composition of resident microbes [217]. The interaction of PGPR with soil flora may be negative, positive,

or neutral depending upon the nature of the PGPR introduced into the soil [218]. The main concern is the introduction of antimicrobial-producing PGPR in the soil milieu [219]. A study by Walsh et al. [220] revealed that there was a reduction in the diversity of the rhizobacterial population due to the introduction of 2,4-diacetylphoroglucino (an antibiotic substance) producing bacteria in the rhizosphere. Some type of perturbance in the resident-flora population is also possible as found in the study by Albright et al. [221].

## 6. Safety Determination of Microbial Inoculants

Several microbes belonging to *Pseudomonas, Bacillus, Acinetobacter, Burkholderia, Staphylococcus, and Stenotrophomonas* have been used as inoculants for plant-growth promotion and biocontrol of plant pathogens; however, these also include microbes identified as opportunistic pathogens and that cause human pathogenesis [150]. It has also been reported that the invasion and colonization mechanisms involved in the pathogenesis of PGPR on plant and human tissues are similar [151,222,223]. Therefore, the safe application of PGPR to protect human health and the environment is needed, which involves collaborative efforts of different expertise, and technological advancements. Microbial inoculants need to be identified and well characterized to unveil their hidden risks to humans and the environment. Several physiological and molecular approaches are now used to check the virulence and pathogenicity of infectious microbes. These methods can also be employed to detect the pathogenicity level of PGPB. The following are some important detection methods that can be taken into consideration.

### *6.1. Morphological and Biochemical Methods*

To detect the pathogenicity level of bacteria, it is necessary to identify the species of bacteria which can be done through cultural studies and fast biochemical tests. For example, growth on blood agar will indicate that the bacteria are pathogenic in nature. The use of differential and selective media will enhance the probability of isolating microbes that have a pathogenic nature. Various biochemical tests such as tests for enzyme detection of catalase, urease, deaminase, decarboxylase, deaminase, β galactosidase, hydrolase, etc., are helpful in the polyphasic characterization of bacteria. These enzymes can also be detected using chromogenic media that contains specific chromogenic substrates which are hydrolyzed and produce a particular color in the media indicating the presence of enzymatic activity in bacteria. Nowadays various biochemical kits and their detecting instruments are available commercially, which enables the rapid detection of microbes [224–226].

### *6.2. Antibiotic Sensitivity Method*

Sensitivity to various antibiotics will indicate whether the given bacterial isolate is safe for release into the environment or not because multiple drug-resistant PGPB bacteria that somehow cause disease in humans and animals will be difficult to treat or cure such disease through prevalent antibiotics. In addition, antibiotic resistance is generally plasmid-borne and most of the plasmid can be transferred from one bacterium to another, thereby spreading the antibiotic-resistant character in the soil microbiome [227].

### *6.3. Protein Profiling Method*

Every genus has a particular set of proteins, and protein profiling will help in identifying the bacterial genus. Even various species in one genus can be differentiated through protein profiling as they have a particular set of proteins, i.e., they contain enzymes involved in a unique biochemical pathway. In addition, it may be possible that these unique biochemical pathways enable a particular microbe to thrive in a harsh climate making them a more favorable candidate for bioinoculant production [227].

### *6.4. Molecular Level Detection Techniques*

Studying at the genetic level is the most precise, rapid, and sensitive technique in today's era to help in the proper understanding and identification of microbial species.

Detection of ubiquitous and universal sequences (containing conserved and variable regions) such as 16s rDNA/18s rDNA is the most prominent and simple way to identify microbes at the species level. Techniques based on the hybridization process are used to detect genes of interest through the use of probes tagged with fluorescent dyes. For example, the fluorescence in situ hybridization (FISH) method uses universal probes to detect a particular microbe [228,229].

Amplification of genes conferring the virulence property of a particular microbe is also an effective way to check the pathogenic nature of bacteria. Quantitative polymerase chain reaction (qPCR) and reverse transcriptase real-time PCR (RT-qPCR) are employed as amplification techniques. One such example is an *invA* gene, which is a virulence gene found in *Salmonella* sp. that is detected through PCR using compatible primers [230].

Gene chip technology or DNA microarray is yet another efficient technique that can not only identify and differentiate among various species of microbes through a variety of probes and universal or consensus primers, but can also give information regarding different resistant measures adopted by a specific microbe [231]. With the advent of the Sanger method of sequencing, a first-generation sequencing technique, it is now possible to sequence the whole genome of a particular microorganism in a very rapid and efficient way. The sequence of the whole genome will not only identify the bacterium but also disclose its pathogenic nature and resistant profile. Whole-genome sequencing also helps in the rapid designing of primers [232,233]. Nowadays, NGS (next-generation sequencing) has proven to be a powerful method for the detection of virulence factors of infectious microorganisms within a few hours. In clinical microbiology, there are numerous methods available for the detection of human pathogens, which are compiled in Table 3. These technologies, in combination with the routine characterization and evaluation of potential microbial biostimulants, can be used to guide as per Figure 2 for the safe development and enrichment of microbial stimulants for use in agriculture.

**Table 3.** Comparative table of different technological approaches for the detection of pathogenic organisms.

| Technological Approach | Major Targets for Pathogens Detection | Advantage/Limitations | References |
|---|---|---|---|
| **Phenotypic methods** | | | |
| (i) Morphological and biochemical methods | Metabolic potential and specific enzymes such as catalase, oxidase, phosphatase, hydrolase enzymes, etc. | Traditional low-cost, easy-to-operate, standardized methods cannot differentiate between target and non-target endogenous microorganisms, time and labor-consuming procedures, and also unable to detect viable unculturable organisms | [227] |
| (ii) Antibiotic-sensitivity testing | Resistant markers transmission | | |
| **Protein profiling method (Proteomics)** MALDI-TOF MS | Specific proteins of particular bacteria to identify specific genera and species. | Qualitative and quantitative determination of proteins in most clinical laboratories. Low concentration of proteins leads to errors in the data interpretation (resistant mechanisms). Unable to differentiate taxonomically related bacteria | [227,234,235] |
| **Molecular methods (genomics)** (i) Amplification methods: Quantitative real-time polymerase chain reaction (qPCR), reverse transcriptase real-time PCR (RT-qPCR), and Loop-Mediated Isothermal Amplification (LAMP) | hybridization between the target nucleic acid and the pathogen-specific probe | More sensitive methods for the identification of pathogens at the molecular level suffer in case of low concentrations of pathogens. | [228,231,236,237] |
| (ii) Hybridization-based methods | hybridization between the target nucleic acid and the pathogen-specific marker gene panels. | Identification of pathogens, profiling of resistant genes, recognition of outbreaks, and immediate design of PCR probes based on the generated genetic data in the outbreaks. | |
| (iii) DNA microarrays (gene chip technology) | | | |
| (iv) Whole-genome sequencing (v) Next-generation sequencing | whole genome sequence | | |
| **Microfluidics based methods** | It is a multidisciplinary strategy and utilizes pathogen markers | extraction and identification of pathogens from clinical/environmental samples. | [238,239] |

MALDI-TOF MS- Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight mass spectrometry, MS-Mass spectrometry.

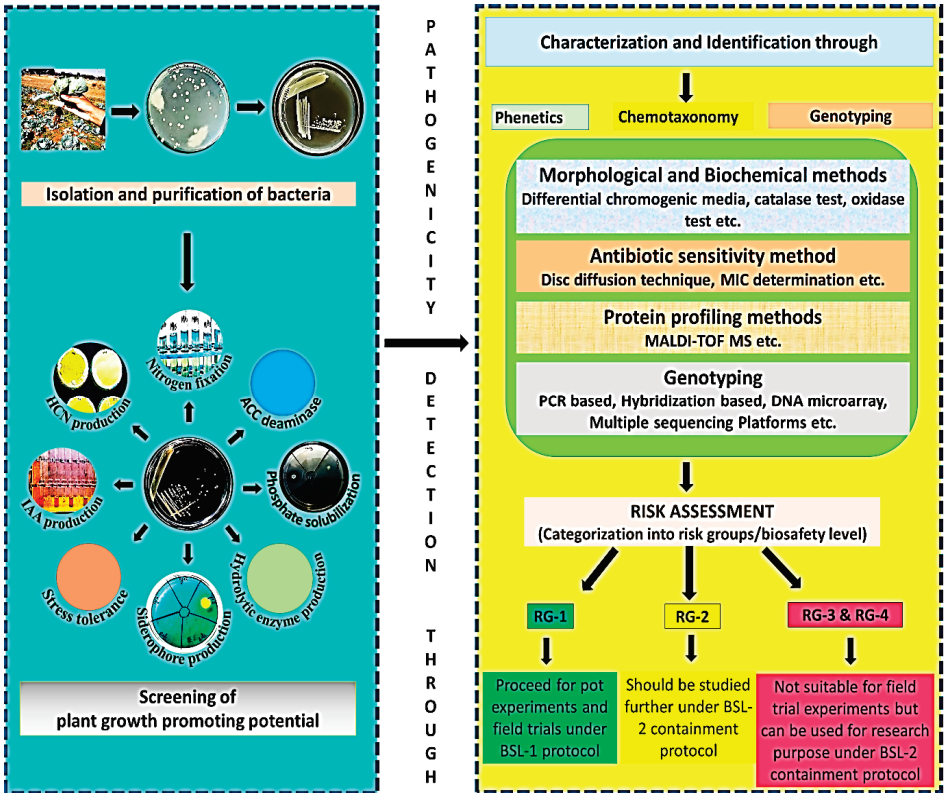


**Figure 2.** A graphical abstract of isolation, characterization, and identification of microbial inoculants and their commercialization as per their respective categorization into different risk groups (compiled from) [150,227,237,238].

## 7. Legal Framework of Biofertilizer Implementation in Different Countries

In spite of having so many advantages over chemical pesticides, the biofertilizer industry faces too many legal obstacles to overcome before entering into commercial production. Earlier the legal regulations regarding biofertilizer use were very inadequate and weak. But in today's era, as researchers have shown the great potential of biofertilizers, many countries amended and developed strong policies and legal regulations to increase the usage of biofertilizers [240,241].

Having an appropriate legal definition is a crucial part of making biofertilizers an appealing commercial product to the producers. In the USA and European Union (EU), there is no proper definition of biofertilizers that can define their actual characteristics. In the EU, biofertilizer comes under e EU Commission Regulation n. 889/2008 on organic production, which states that biofertilizers can only be used as plant protectants against pests and diseases. Hence, biofertilizer comes under the legal agenda of plant protection products. The same outline is followed by the US National Organic Program which categorizes biofertilizers as biological organisms that can only be used as plant protectants [242].

Compared to other countries, India has the most comprehensive and defined legal regulation and framework for biofertilizer implementation. In India, biofertilizer comes under the Essential Commodities Act of 1955, Ministry of Agriculture, and can be defined as "the product containing carrier based (solid or liquid) living microorganisms which are agriculturally useful in terms of nitrogen fixation, phosphorus solubilization or nutrient mobilization, to increase the productivity of the soil and/or crop". Seven standard criteria have been set to formulate a biofertilizer that includes viable inoculum density, the physical form, level of contamination, pH, moisture content, the particle size of carrier-based products, and efficacy level. Four groups of microbes are mainly included under the biofertilizer category i.e., *Azotobacter, Rhizobium, Azospirillum*, mycorrizal fungi, and phosphate-solubilizing bacteria [242,243].

In Poland, Polish Law on Fertilizers and Fertilization 2007 includes biofertilizers under "growth stimulators" and groups them under plant conditioners. This law defines biofertilizer as "a positive impact on plant growth or other metabolic processes of plants in other ways than plant nutrients" and shall "pose no threat to [the] health of humans or animals or to the environment after their use and storage instructions" [242].

Spain, which is one of the leading countries in organic farming, does not have a separate category and definition of biofertilizer in its legal structure. It includes microorganisms as one of the components of compost and organic amendments under Real Decreto 506/2013 [242].

China has a strict and defined legal framework for biofertilizer implementation. It has set various parameters through which it can access the quality of biofertilizer including inoculum density, water, and carbon content, outer appearance, granule size, contamination, viability, and validity. Chinese standards mostly rely on the amount of inoculum to access the quality of biofertilizer, which should range between >1.5 × 109 CFU $mL^{-1}$ or >0.2 × 109 CFU $g^{-1}$ and >0.5 × 109 CFU $mL^{-1}$ or >0.1 × 109 CFU $g^{-1}$, for solid and liquid products, respectively. Seven categories of microorganisms are included in biofertilizers, i.e., fast- and slow-growing species of rhizobia, organic and inorganic phosphate-solubilizing bacteria, nitrogen-fixing bacteria, silicon-solubilizing bacteria, and various consortia containing multiple microorganisms [244].

## 8. Conclusions

Plant biostimulants prove beneficial to plants by improving their growth. Microbial inoculants, single or consortia, naturally improve plant growth and performance without using any agrochemicals in the field. They can act as biofertilizers, soil improvers, growth regulators, stress relievers, and biocontrol agents. However, more research needs to explore and establish their biocontrol properties. Much research has been conducted to understand their properties and functions followed by their commercialization to promote eco-friendly and safe agriculture practices for the fortification of plants with nutrients. The global markets of biostimulants also need to be expanded in the near future so that farmers can easily buy these products at affordable prices. Furthermore, extensive characterization research emphasizing the safety issues of the inoculant microbes becomes inevitable to address recent reports of many inoculants belonging to either higher-risk groups or potential pathogens of human beings, such as *Pueudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter,* etc., which may cause various kind of suffering, for example, septicemia, gastroenteritis, wound infections, inflammation in the respiratory system, meningitis, etc., of varied severity under different conditions of human-health status, such as immunocompromized and comorbidity with other diseases, etc. Advances in technologies including biochemical, immunological, proteomics, and genomics approach unraveling the characters and identification of microbes have enabled the research community to rapidly and accurately address safety concerns, such as pathogenicity, of biostimulant microbes following a suitable strategic plan before releasing the inoculant for field application.

**Author Contributions:** Conceptualization, M.K., P.S. and A.K.; resources, A.K.; writing—original draft preparation, M.K., P.S. and A.K.; writing—review and editing, K.K.K. and A.K.; visualization, K.K.K. and A.K.; supervision, K.K.K. and A.K.; project administration, A.K.; funding acquisition, K.K.K. and A.K. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Hydropriming and Osmotic Priming Induce Resistance against *Aspergillus niger* in Wheat (*Triticum aestivum* L.) by Activating *β-1, 3-glucanase, Chitinase,* and *Thaumatin-like Protein* Genes

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**Citation:** Gul, S.; Hussain, A.; Ali, Q.; Alam, I.; Alshegaihi, R.M.; Meng, Q.; Zaman, W.; Manghwar, H.; Munis, M.F.H. Hydropriming and Osmotic Priming Induce Resistance against *Aspergillus niger* in Wheat (*Triticum aestivum* L.) by Activating *β-1, 3-glucanase, Chitinase,* and *Thaumatin-like Protein* Genes. *Life* **2022**, *12*, 2061. https://doi.org/10.3390/life12122061

Academic Editor: Balazs Barna

Received: 11 November 2022
Accepted: 6 December 2022
Published: 8 December 2022



**Abstract:** Priming is used as a method to improve plant growth and alleviate the detrimental effects of pathogens. The present study was conducted to evaluate the effects of different priming methods in the context of resistance to *Aspergillus niger* in wheat (*Triticum aestivum* L.). Here, we show that different priming treatments—viz., hydropriming, osmotic priming, halopriming, and hormonal priming techniques can induce disease resistance by improving the biochemical contents of wheat, including chlorophyll, protein, proline, and sugar. In addition, physiological parameters—such as root length, shoot length, fresh and dry root/shoot ratios, and relative water content were positively affected by these priming methods. In essence, hydropriming and osmotic priming treatments were found to be more potent for enhancing wheat biochemical contents, along with all the physiological parameters, and for reducing disease severity. Hydropriming and osmotic priming significantly decreased disease severity, by 70.59–75.00% and 64.71–88.33%, respectively. RT-PCR and quantitative real-time PCR analyses of potentially important pathogenesis-related (PR)-protein genes (*Thaumatin-like protein* (*TLP*), *chitinase*, and *β-1,3-glucanase*) in primed plants were evaluated: *β-1,3-glucanase* was most highly expressed in all primed plants; *Chitinase* and *TLP* exhibited higher expression in hormonal-, halo-, osmotic-, and hydro-primed plants, respectively. These results suggest that the higher expression of *β-1,3-glucanase, TLP*, and *chitinase* after hydropriming and osmotic priming may increase disease resistance in wheat. Our study demonstrates the greater potential of hydropriming and osmotic priming for alleviating stress caused by *A. niger* inoculation, and enhancing resistance to it, in addition to significantly improving plant growth. Thus, these priming methods could be beneficial for better plant growth and disease resistance in other plants.

**Keywords:** wheat; priming; *Aspergillus niger*; qRT-PCR; wilting; *TLP*; *chitinase*; *β-1,3-glucanase*

## 1. Introduction

Priming is a seed treatment in which seeds are first soaked and then dried to their original weight, during which time germination continues, but radicle protrusion does not occur [1]. Seed priming offers the following advantages: improved, uniform, and fast

emergence of seedlings; healthier grain; excessive vigor; and better straw yield, tillering, allometry, and harvest index in floriculture [2], vegetables [3,4] and field crops [5–9]. Various seed treatment techniques have been established, such as osmotic priming, hydropriming, halopriming, thermopriming, and hormonal priming. In hydropriming, pre-germination starts, but actual germination does not occur. Hydro-primed plants tolerate dryness, and the negative effects of pests are decreased by the faster emergence of seedlings [10,11]. Hydropriming enhances seedling growth in rice (*Oriza sativa* L.), corn (*Zea mays* L.), chickpea (*Cicer arietinum*), and mung bean (*Vigna radiata*) seeds [12–14]. Hydropriming can be a cheap and easy seed invigoration treatment for wheat, especially in salinity and drought stresses.

In osmotic priming, the seeds are soaked in a low osmotic potential solution, having chemicals like polyethylene glycol (PEG), menthol, chemical fertilizers, sugar, glycerol, and sorbitol [15]. Osmotic priming has been known to improve seed dormancy, and to enhance vigor in soybean (*Glycine max* L.) [16] and tomato (*Solanum lycopersicum*) [17]. PEG solution enhances the emergence percentage and the homogeneity of germination, and increases water absorbance by the seeds, and the development of the shoot and radicle [15,18]. In abiotic stress conditions, cellular stability is maintained by metabolic osmo-regulators, such as glycerol, mannitol, and trehalose, which are well-known osmo-conditioners [19]. Few reports have concluded that osmotic priming agents play a key role in activating crop disease resistance [19]. In wheat, powdery mildew caused by *Blumeria graminis* is controlled by trehalose, which induces systemic acquired resistance [20].

During host–pathogen interactions, pathogenesis-related (PR) proteins are produced: these proteins are encoded by the host plant, but they are induced specifically in pathological or related situations [21]. PR proteins are of paramount importance, as they increase plant resistance to pathogens. Thaumatin-like proteins (TLPs) are important PR-proteins (PR-5), consisting of 200 amino acid residues [22–24]. TLPs are produced in plants: they protect the plants from the harmful effects of phytopathogens, stresses, and elicitors, and are also involved in a wide range of developmental signals. The antifungal property of TLPs renders them useful in genetic engineering to produce disease-resistant plants [25–28]. The role of TLPs in resistance to several basidiomycete fungi—including *Rhizoctonia solani*, *Lentinula edodes* (Berk.), and *Irpex lacteus* (Fr.)—has been reported [29]. Despite exhibiting resistance to biotic stresses, TLPs also confer resistance to abiotic stress conditions [30].

Chitinase is another PR protein (PR-3) expressed in response to a variety of stresses [31]. Chitinase has antifungal activities against plant pathogenic fungi, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Rhizoctonia solani*, *F. udum*, *Alternaria* sp., *Bipolaris oryzae*, *Curvularia lunata*, and *Mycosphaerella arachidicola* [32–34]. The mode of action of PR-3 proteins is relatively simple, e.g., chitinases cleave the chitin polymers of the cell wall in situ, leading to a compromised cell wall that renders fungal cells osmotically sensitive [35]. Another highly complex gene family is the plant β-1,3-glucanase (β-1, 3-G); β-1,3-glucanases play a role in developmental processes and pathogen defense responses [36]. The expression of these genes is triggered by plant hormones, which also affect germination [37]. β-1,3-glucanases are well-recognized PR proteins, which belong to the PR-2 protein family. These PR proteins are strongly induced in response to wounds or infection by viral, bacterial, and fungal pathogens [38,39]. This study aimed to ascertain whether improvement in plant growth and disease resistance could be induced by using different priming techniques. For that purpose, the present study was designed to investigate the role of hydropriming, osmotic priming, halopriming, and hormonal priming in response to *A. niger* inoculation in wheat (*T. aestivum* L.). We found that, of all the priming methods, hydropriming and osmotic priming had the most significant effect on growth and development, decreasing disease severity, and increasing resistance to *A. niger* in wheat: this is most probably due to the higher expression of genes (in hydropriming and osmotic priming) involved in plant defense mechanisms, and their role in disease resistance.

## 2. Materials and Methods

### *2.1. Seed Collection and Preparation*

#### 2.1.1. Seed Sterilization

Healthy seeds of the susceptible wheat cultivar "Sahar" were obtained from the National Seed Corporation, Fatteh Jhang, and Rawalpindi, Pakistan. The seeds were surface-sterilized, by being soaked in 70% ethanol for 3 min, washed thoroughly with sterilized distilled water many times, and then dried.

#### 2.1.2. Seed Priming

Four priming methods were used for comparative analysis. In each treatment, 20 g (g) of wheat seeds was used. The osmotic priming technique employed 30 g of polyethylene glycol (PEG 6000), which was dissolved in 100 mL of distilled water. The wheat seeds were soaked in PEG solution for 2–3 days at room temperature, dried to their original weight under shade, and used for sowing [40]. For the hydropriming, the seeds were soaked in distilled water for 24 h at room temperature: these seeds were re-dried to their original weight under a shade with continuously passing air [40]. For the hormonal priming, the wheat seeds were soaked in 200 mL of hormonal solution (100 ppm solution of Indole acetic acid (IAA)) for 12 h at room temperature; then, the seeds were re-dried to their original weight, under shade, and used for sowing. For the halopriming, the seeds were primed in 100 mL of NaCl solution (100 mM) for 12 h, and allowed to air-dry for 12 h at room temperature before sowing.

#### 2.1.3. Seed Sowing and Germination

After priming, the seeds were sown in plastic pots containing sterilized soil, and were kept under controlled conditions in a growth chamber at 20–25 °C day/night temperature, 60% relative humidity, and 14/10 hrs light-and-dark periods. Ten to fifteen seeds were sown in each pot. Non-treated seeds were used as the control.

### *2.2. Fungus Inoculum Preparation*

A fresh culture of *A. niger* was obtained from the National Agricultural Research Centre (NARC), Islamabad, and observed under a microscope for confirmation. Using a sterilized spatula, the fungus was transferred to Czpeck media. The flasks were incubated in a shaker incubater (200 rpm) at 30 °C. After 3 days, the number of spores was calculated by hemocytometer, and adjusted to $10^6$ spores/mL concentration. The spore suspension was filtered using a muslin cloth, and the filtrate was used for further foliar and systemic inoculations.

#### Fungus Inoculation

Two methods were used for fungus inoculation. In the foliar (surface) inoculation method, spore suspension ($10^6$ spores/mL) was sprayed on 8–10-day-old plants, with the help of a spray bottle. For one week post-inoculation, the symptoms were observed every 24 h. For systemic inoculation, sorghum seeds were used to completely disperse the fungus in the soil. The sorghum (*Sorghum bicolor*) seeds were sterilized in 70% ethanol, washed three times with distilled water, and soaked overnight in distilled water. The seeds were then dried, autoclaved, and soaked in spore suspensions for 5–7 days [41]. The inoculated sorghum seeds were isolated from the spore suspension, re-dried under shade, and 2 g of sorghum seeds was added to 1 kg of soil, which was used to grow the primed wheat seeds. In addition, non-treated sterilized sorghum seeds were used as a negative control.

### 2.3. Disease Severity Analysis

Disease symptoms were evaluated and defined by two different methods. In the first method, total leaf area and infected part were measured, and disease severity was calculated in percentage, using the following formula [42]:

$$\text{Disease severity} = \frac{\text{Area of plant tissue affected by disease}}{\text{Total area}} \times 100$$

In the second method, a visual assessment of wilting was performed after foliar and systemic inoculations, by following standard scaling [43–45].

### 2.4. Determination of Biochemical Contents

Different biochemical contents were investigated in the primed plants in response to fungal inoculation. The sugar contents of the leaves were determined by following the method of [46]. The protein, proline, and chlorophyll contents were determined by following the methods of [47–49], respectively.

### 2.5. Analysis of Physiological Parameters

Various physiological parameters were measured to evaluate the effectiveness of different priming techniques in response to fungal inoculation: in this respect, the lengths of freshly harvested shoots and roots were measured with measuring tape, and the root/shoot ratio was calculated. The fresh plant samples were kept in an oven at 70 °C for 72 h, in order to analyze the dry root/shoot ratio [50]. The relative water content of the leaves was measured after the different priming methods and induction of biotic stress by the method of [51].

### 2.6. RNA Extraction, Quantification, and cDNA Synthesis

The total RNA from the leaves was extracted by using a ThermoFisher scientific® Gene JET plant RNA purification kit, according to the manufacturer's protocol. The RNA concentration was calculated by Nanodrop, and was utilized for cDNA synthesis, using a ThermoFisher scientific® cDNA synthesis kit.

### 2.7. Primer Designing and RT-PCR

RT-PCR (BIO-RAD) was performed, to examine the expressions of thaumatin-like protein, β-1,3-glucanase and chitinase genes. Total cDNA was used as a template. The primers used in this experiment are given in Table 1. PCR was carried out in a 25 µL reaction mixture comprising 16 µL of water, 2.5 µL of buffer, 1.5 µL of $MgCl_2$, 1.5 µL of dNTPs, 0.5 µL of Taq, 1 µL of template, and 1 µL of both forward and reverse primers. The thermal profile was as follows: 5 min at 94 °C, 25 cycles of 40 s at 94 °C, 1 min at 49 °C, 1 min at 72 °C, and a one-step final extension of 5 min at 72 °C.

**Table 1.** Primers used in this experiment.

| S. No. | Protein | Primers |
|---|---|---|
| 1 | Thaumatin-like protein (TLP) | Forward 5′ GCAGTCAAGGCAGTTGGTGGTA 3′, Reverse 5′ GCAGTCAAGGCAGTTGGTGGTA 3′ |
| 2 | Chitinase | Forward 5′ CGCAGTCACCTAAACCTTCG 3′ Reverse 5′ GCAGTAGCGCTTGTAGAACC 3′ |
| 3 | β 1,3-glucanase | Forward 5′ CTACAGGTCCAAGGGCATCA 3′ Reverse 5′ CCGGACATTGTTCTGAACCC 3′ |
| 4 | Actin | Forward 5′ CAAAGAGATCACGGCCCTTG 3′ Reverse 5′ ACTTCATGTGGACAATGCCG 3′ |

### *2.8. Real-Time PCR Analysis*

Quantitative real-time PCR was carried out, using the Applied Biosystems 7300 Real-Time PCR System. The PCR was performed using 3 µL of first strand cDNAs and SYBR Green PCR Master Mix (ThermoScientific®, Waltham, MA, USA) under the following conditions: initial denaturation at 95 °C for 1 min; 40 cycles of denaturation at 95 °C for 15 s; annealing at 49 °C for 15 s; and extension at 72 °C for 45 s. Data were normalized to the housekeeping Actin gene.

### *2.9. Statistical Analysis*

All the experiments were carried out in triplicates ($n = 3$). Microsoft Excel 365 software was used for compiling the experimental data, to form a database for further analysis. All the data were evaluated by one-way ANOVA, and for the graphical illustrations and Tukey's HSD test to examine the difference among treatment means ($p \leq 0.05$), the Origin software (Version 2022, OriginLab Corporation, Northamptom, MA, USA) was used.

## 3. Results

### *3.1. Biochemical Content Analysis of Primed Plants in Response to Fungal Stress*

#### 3.1.1. Proline

The seed priming exhibited a positive effect, by stimulating all the biochemical parameters under both the priming as well as the systemic inoculation conditions. Proline performs its function as a beneficial solute under normal conditions, and as stress tolerance in non-healthy conditions [52]. In our experiment, the total proline content was significantly increased at the seedling stage after each priming treatment, as compared to the control: this significant increase was more pronounced in osmotic and hydro-primed plants—71.56% and 70.09%, respectively—followed by halo-primed and hormonal-primed plants—57.88 and 52.44%, respectively, compared to the control. After systemic inoculation of *A. niger*, the highest increase in proline content was observed in osmotic and halopriming—51.26% and 49.50%, respectively—followed by hydropriming and hormonal priming—47.30% and 4.11%, respectively, compared to the control (Figure 1A).

#### 3.1.2. Protein Content

Production of protein in stress conditions is mainly associated with plant defense responses against fungi [53]. In each priming treatment, protein content was observed to be significantly more increased than in the control. The hydro and osmotic-primed plants had no significant differences in protein content, but when compared to the halo-primed, hormonal-primed, and control plants, a significant change was observed. However, osmotic priming, hydropriming, halopriming, and hormonal priming enhanced protein content by 31.09%, 28.05, 17.00%, and 13.25%, respectively. Moreover, in systemic inoculation of *A. niger* also, a significant increase in protein content was recorded, as compared to the control in all groups. Overall, 32.93% and 22.27% increases in the protein content were observed in osmotic priming and hydropriming, followed by hormonal priming (16.29%) and halopriming (14.00%), compared to the control (Figure 1B).

#### 3.1.3. Sugar Content

Sugar is considered a primary source of energy, which acts as a building block for providing defense-responsive material in plants [54]. Our results showed that the primed plants contained more soluble sugar in their leaves than non-primed and inoculated primed plants. In essence, the osmotic-primed and hydro-primed plants showed a significant increase in sugar content, increasing by 24.60% and 24.75%, respectively, while the halo-primed and hormonal-primed plants exhibited 12.56% and 12.87% increases in sugar contents, respectively, compared to the control plants. By contrast, the systemic inoculation of *A. niger* resulted in a significant drop in sugar content in all primed plants as compared to the control, where 14.32%, 23.07%, 23.92%, and 27.45% greater reductions in sugar content

were observed for osmotic-, halo-, hormonal-, and hydro-primed plants, respectively, than in the control (Figure 1C).

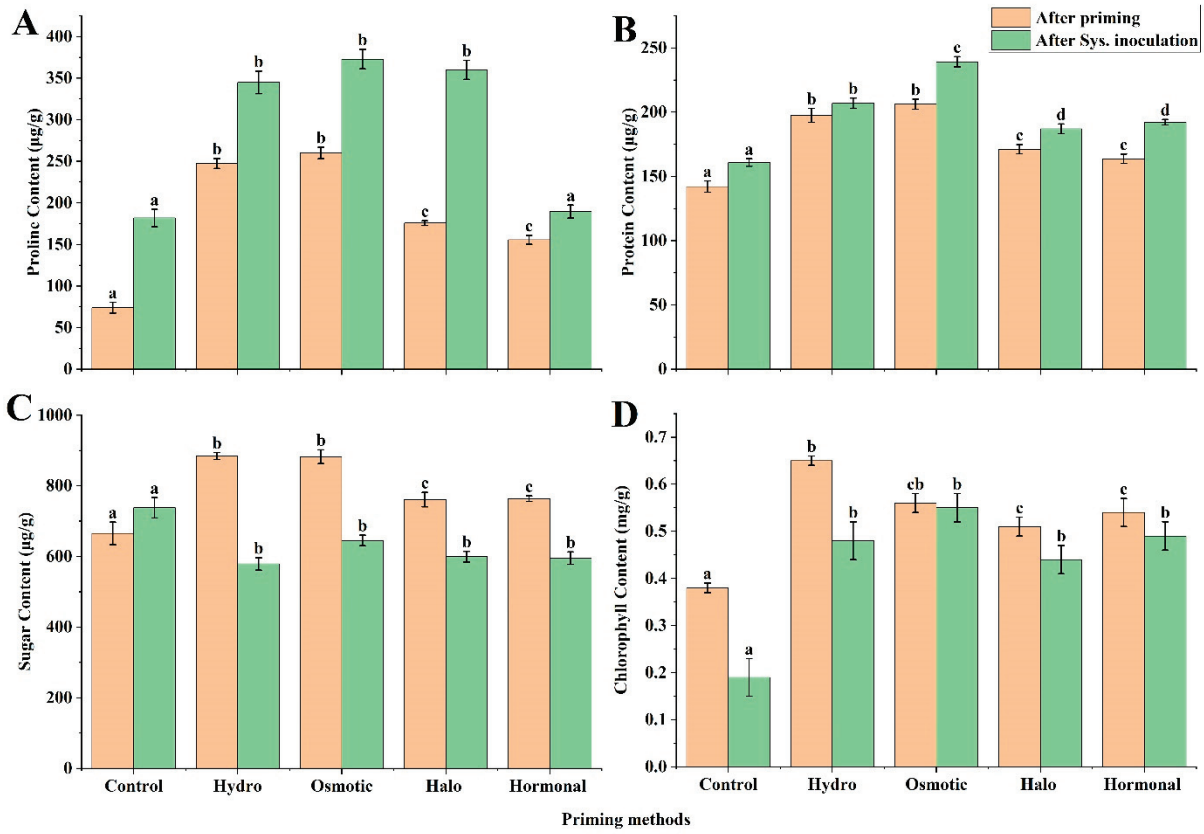


**Figure 1.** Biochemical contents of wheat under hydropriming, osmotic priming, halopriming, and hormonal priming: (**A**) proline content, (**B**) protein content, (**C**) sugar content, (**D**) chlorophyll content. The mean values with different letter(s) indicate significant differences at $p \leq 0.05$. Vertical bars represent standard deviation of means ($n = 3$). Sys. inoculation: Systemic inoculation.

#### 3.1.4. Chlorophyll Content

The photosynthetic capacity of plants is determined by their leaf chlorophyll content and measurement [55]. The results of the present study revealed that seed priming exerted a positive effect on the chlorophyll content. The hydro-primed plants showed the highest increase in chlorophyll content, of 41.54%, followed by the osmotic-, hormonal-, and halo-primed plants, which enhanced chlorophyll content by 32.14%, 29.63%, and 25.49%, respectively, as compared to the control. Similarly, under systemic inoculation, all the primed plants showed a significant increase in chlorophyll content, as compared to the control (Figure 1D). However, osmotic priming exhibited the highest chlorophyll contents (65.45%), followed by hormonal priming (51.22%), hydropriming (60.42%), and halopriming (56.82%), compared to the control.

### *3.2. Response of Wheat Physiological Parameters to Different Priming Treatments*

#### 3.2.1. Relative Water Content (RWC)

In the context of RWC, all the primed plants showed a significant increase in RWC, compared to the non-primed plants; however, the greatest increases in RWC—of 30.74%, 28.09%, 17.08%, and 14.98%, for hydropriming, osmotic priming, halopriming, and hormonal priming, respectively—were observed in comparison to the control. A similar trend of increased RWC was also observed in systemic inoculation of *A. niger* in wheat plants, wherein a significantly greater increase in RWC was observed in all priming treatments than in the control (Figure 2A): the halopriming showed the highest RWC (55.85%), while

55.00%, 52.14%, and 43.52% increases were noted for osmotic priming, hydropriming, and hormonal priming, respectively.

#### 3.2.2. Shoot Length

The application of different priming techniques stimulated shoot growth. An increase in shoot length was significant in plants subjected to all priming treatments, except halopriming, as compared to the control plants (Figure 3A). In principle, the osmotic priming exerted the highest shoot length (45%) compared to the control, while the hormonal priming exhibited a 43.41% increase, the hydropriming a 41.13% increase, and the halo priming a 34.23% increase in shoot length (Figure 2B). Similarly, the shoot length was significantly increased in all primed plants, in comparison to non-primed plants, after systemic inoculation of *A. niger*, where the maximum increases in shoot length—i.e., 38.24%, 35.98%, 30.46, and 19.23%—were recorded for osmotic priming, halopriming, hydropriming, and hormonal priming, respectively.

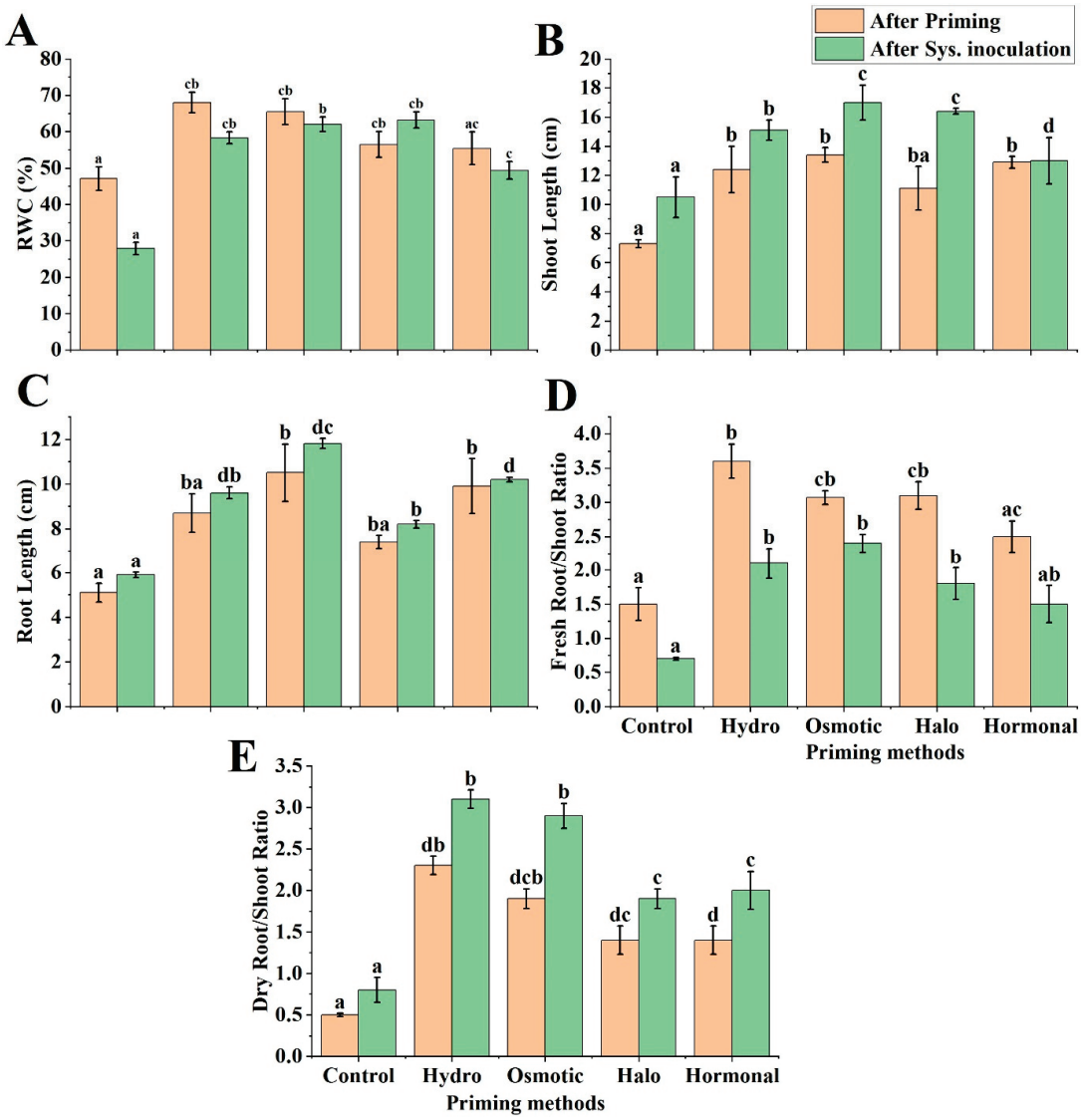


**Figure 2.** Physiological parameters of wheat under hydropriming, osmotic priming, halopriming, and hormonal priming: (**A**) relative water content (RWC); (**B**) shoot length; (**C**) root length; (**D**) fresh root/shoot ratio; (**E**) dry root/shoot ratio. The mean values with different letter(s) indicate significant differences at $p \leq 0.05$. Vertical bars represent standard deviation of means ($n = 3$). Sys. inoculation: Systemic inoculation.

### 3.2.3. Root Length

All the priming treatments exhibited a pattern of increase in root length similar to that of shoot length. The highest increases—of 51.43% and 48.48%, respectively—were observed in the root length of plants subjected to osmotic priming and hormonal priming, followed by hydropriming and halopriming, with increases of 41.38% and 31.08%, respectively. Similarly, all primed plants revealed a significant increase in root length, in comparison to the control, after systemic inoculation, where the maximum root length was recorded for osmotic-primed (50.00%) and hormonal-primed plants (42.16%) (Figure 2C).

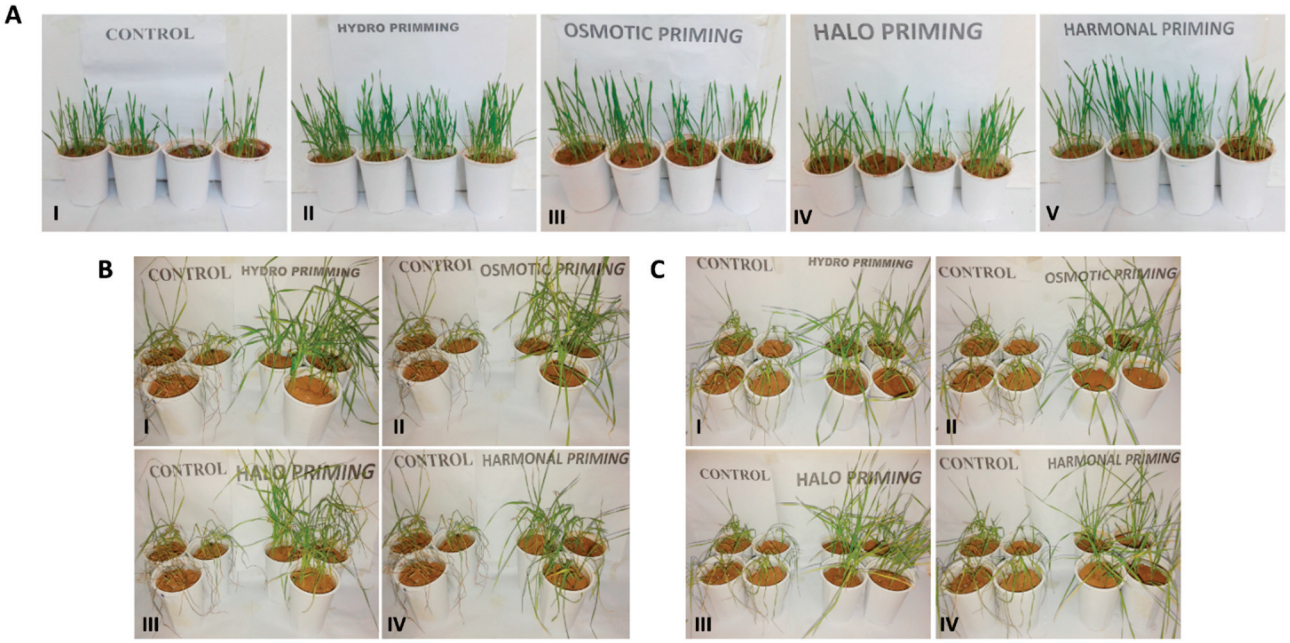


**Figure 3.** Effects of different priming methods on the growth of wheat plants. (**A**) (**I**): control; (**II**): hydropriming; (**III**): osmotic priming; (**IV**): halopriming, (**V**): hormonal priming. (**B**) Disease severity after foliar inoculation. (**I**): control vs. hydropriming; (**II**): control vs. osmotic priming; (**III**): control vs. halopriming; (**IV**): control vs. hormonal priming. (**C**) Disease severity after systemic inoculation. (**I**): control vs. hydropriming; (**II**): control vs. osmotic priming; (**III**): control vs. halopriming; (**IV**): control vs. hormonal priming.

### 3.2.4. Fresh and Dry Root/Shoot Ratio

The fresh plant root/shoot ratio was significantly increased in all primed plants, while a non-significant increase was observed in hormonal priming, as compared to the control. With respect to the fresh root/shoot ratio, hydropriming presented the highest increase—of 58.33%—while 51.61%, 51.14%, and 40.00% increases were recorded for halo-, osmotic-, and hormonal-primed plants. The same tendency of increase in the fresh root/shoot ratio was observed after systemic inoculation, where 70.83-enhanced, 66.67%-enhanced, 61.11%-enhanced, and 53.33%-enhanced fresh root/shoot ratios were observed for osmotic priming, halopriming, hydropriming, and hormonal priming (Figure 2D).

In addition, the results exhibited a similar trend of increase in dry root/shoot ratio in all the primed plants: however, this increase was more significant in the hydro-primed plants, whose dry root/shoot ratio increased by 78.26%, while the dry root/shoot ratio of the osmotic-, halo-, and hormonal-primed plants showed 73.68%, 64.29%, and 64.29% increases, respectively, compared to the control plants. Furthermore, in the case of systemic inoculation of *A. niger*, hydropriming and osmotic priming showed the highest increase in dry root/shoot ratio (74.19 and 72.41%, respectively), followed by halopriming and hormonal priming, with enhanced dry root/shoot ratios of 57.89% and 60.00%, respectively, as compared to the control (Figure 2E).

### *3.3. Disease Severity Analysis*

#### 3.3.1. Foliar Inoculation

Our results revealed that the foliar inoculation of *A. niger* induced a drastic disease severity in non-primed (control) plants; however, it was observed that the priming treatments significantly reduced disease severity, by alleviating the stress caused by *A. niger* inoculation. Among the priming treatments, hydropriming and osmotic priming showed the maximum decreases in disease severity, of 70.59% and 64.71%, respectively. Halo- and hormonal-primed plants also showed pronounced reductions in disease severity, of 58.82% and 47.06%, respectively, in comparison to the control plants. In general, hydropriming and osmotic priming were observed to be more effective in reducing disease severity, in comparison to halopriming and hormonal priming (Figures 3B and 4A).

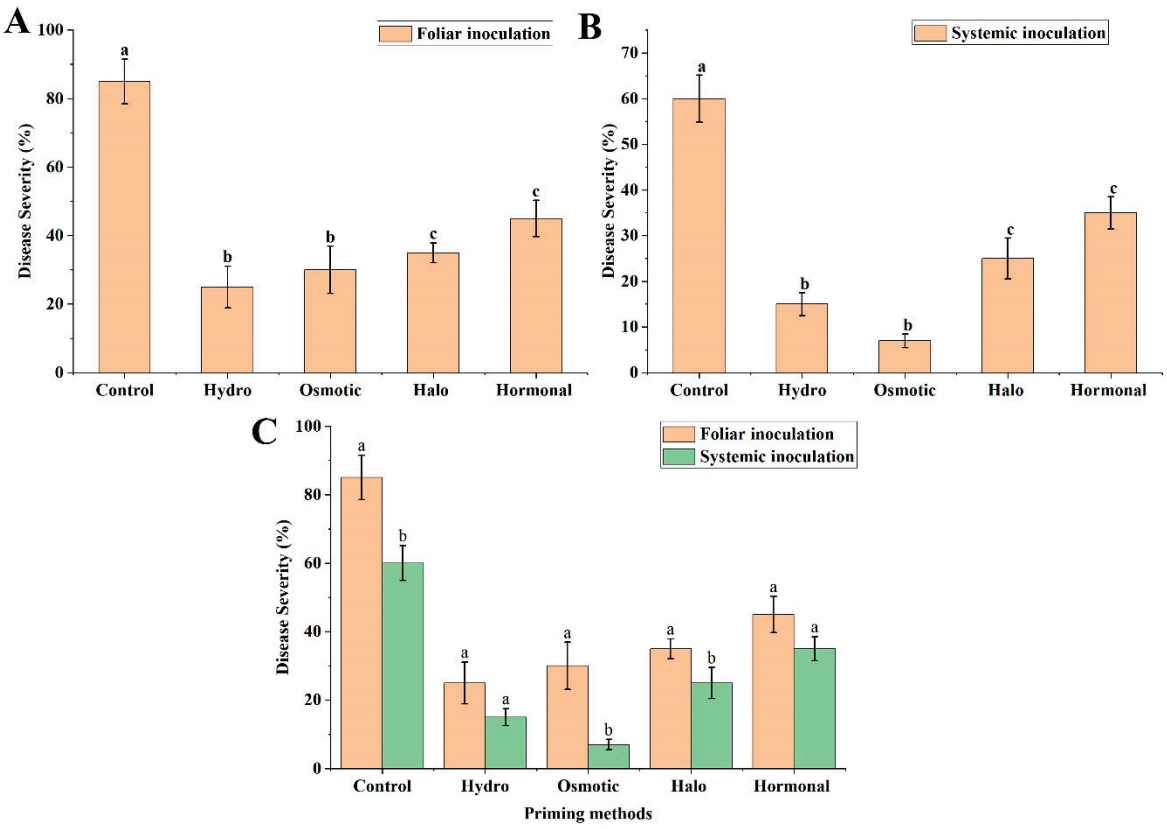


**Figure 4.** Disease severity analysis of wheat plants in response to hydropriming, osmotic priming, halopriming, and hormonal priming. (**A**) Disease severity analysis after foliar inoculation of *A. niger*. (**B**) Disease severity analysis after systemic inoculation of *A. niger*. (**C**) Disease severity comparison between foliar and systemic inoculation. The mean values with different letter(s) indicate significant differences at $p \leq 0.05$. Vertical bars represent standard deviation of means (n = 3).

#### 3.3.2. Systemic Inoculation

Similarly, the non-primed (control) wheat plants subjected to systemic inoculation showed acute disease severity, with drastically reduced growth. In the case of the primed plants, however, the hydro- and osmotic-primed plants were found to be the most resistant, significantly reducing disease severity by 75.00% and 88.33%, respectively, as compared to the control, while halopriming and hormonal priming showed comparatively less resistance than osmotic priming and hydropriming (Figures 3C and 4B). However, both halopriming and hormonal priming also induced considerable reduction in disease severity, i.e., 58.33% and 41.67%, respectively, compared to non-primed plants.

#### 3.3.3. Comparison of Foliar and Systemic Inoculation

In this study, we obtained promising results with respect to disease severity reduction for the systemic inoculation method, in comparison to the foliar spray method. Both methods were applied for the same length of time, i.e., 2 weeks, and disease symptoms appeared more rapidly in the foliar spray method than in the systemic method. The results revealed that the plants treated with systemic fungus inoculation exhibited more resistance to disease in comparison to the foliar spray technique. In particular, osmotic priming and halopriming in systemic inoculation presented significant differences in reducing disease severity—by 76.67% and 40.00%, respectively—compared to foliar-sprayed plants of the same group. In addition, halopriming and hormonal priming also revealed a considerable decrease in disease severity reduction—of 28.57% and 22.22%, respectively—when compared to foliar-sprayed plants of the same treatment (Figure 4C).

#### 3.3.4. Visual Assessment of Wilting

Visual assessment of wilting also revealed the same pattern as described above for the disease severity percentage. After foliar inoculation of *A. niger*, the control plants were found to be nearly dead, while the hydro- and osmotic-primed plants were normal, but slightly wilted. The halo-primed plants showed wilting (W), while the hormonal-primed plants were wilted severely (Figures 3B and 5). Likewise, the same pattern of visual assessment of wilting was observed with systemic inoculation, where the control plants were found to be severely wilted, while the hydro- and hormonal-primed plants were wilted slightly; however, the osmotic- and halo-primed plants seemed to be normal (Figures 3C and 5).

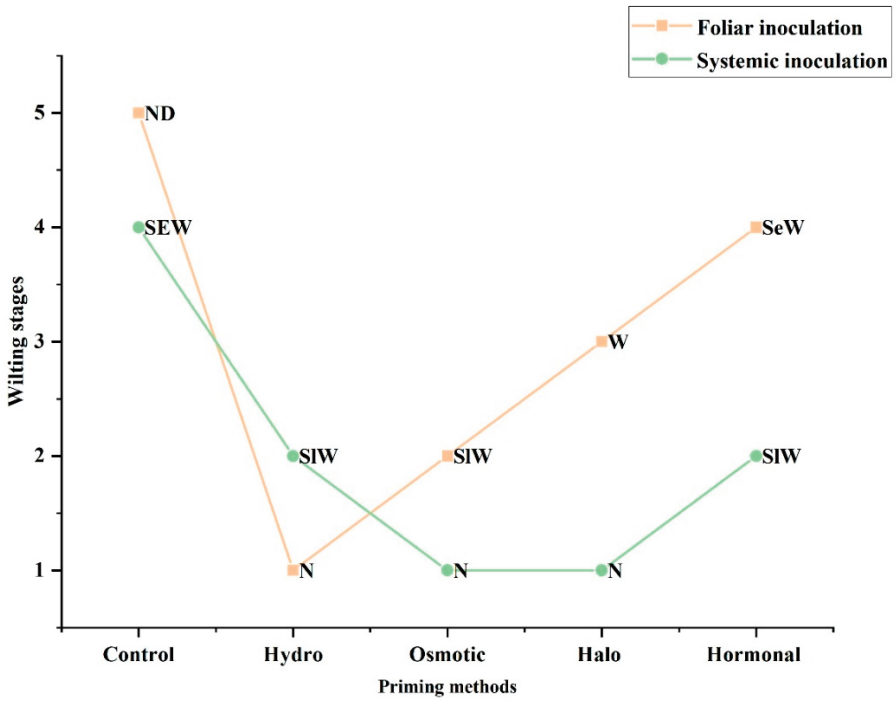


**Figure 5.** Measurement of disease severity after foliar and systemic inoculation, by visual assessment of wilting. Different wilting conditions are described as normal (N), slightly wilted (SlW), wilted (W), severely wilted (SeW), nearly dead (ND), and dead (D).

### *3.4. Expression Profiling of TLP, Chitinase, and β-1,3-glucanase Genes*

TLP gene expression was down-regulated in halo-primed plants compared to the control, while osmotic- and hydro-primed plants showed significantly higher expression of *TLP*. In halo-primed plants, almost no detectable expression of the *TLP* gene was seen. The expression profile of *TLP* in RT-PCR and qRT-PCR was comparable (Figures 6 and 7).

Both RT-PCR and qPCR showed that *chitinase* gene expression was significantly increased in hydropriming compared to the plants treated with osmotic priming, halo priming, and hormonal priming (Figures 6 and 7). RT-PCR and qPCR results also confirmed that *β-1,3-glucanase* was highly expressed in hydro- and osmotic-primed plants compared to non-primed plants, while halo- and hormonal-primed plants also showed a considerably increased expression of *β-1,3-glucanase*; however, the change was not as significant as compared to the control (Figures 6 and 7). Overall, the analysis of the relative gene expression indicated that *β-1,3-glucanase* presented a significant role in inducing resistance to *A. niger* under each priming treatment, followed by *chitinase* and *TLP*, which played a considerable role in resistance to *A. niger* under halopriming and hormonal priming, and under hydropriming and osmotic priming, respectively.

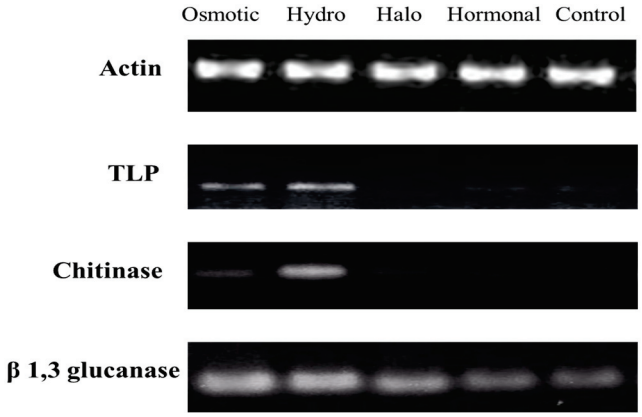


**Figure 6.** Expression profiling of *TLP, Chitinase, and β-1, 3-glucanase* by RT-PCR.

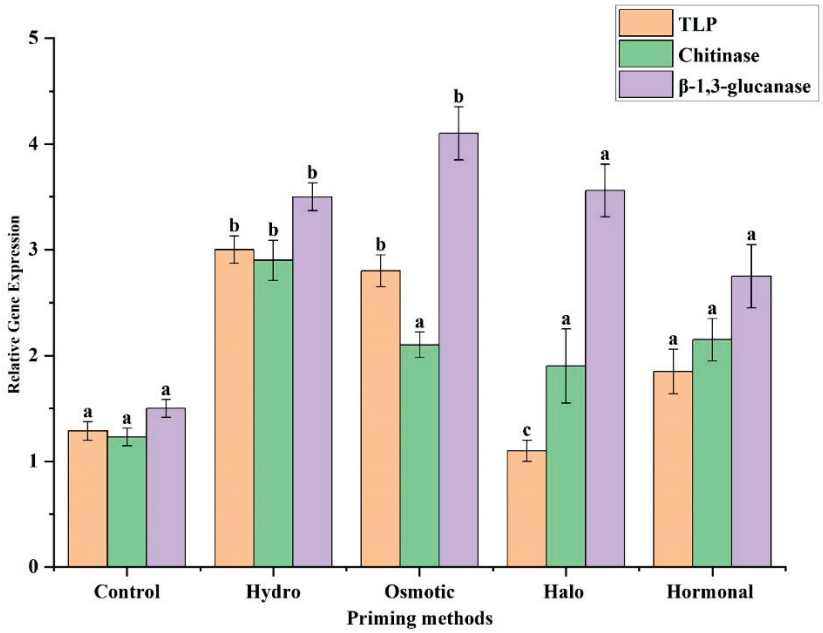


**Figure 7.** Relative expression of *TLP, Chitinase*, and *β-1,3-glucanase*, obtained through quantitative real-time PCR analysis. The mean values with different letter(s) indicate significant differences at $p \leq 0.05$. Vertical bars represent standard deviation of means ($n = 3$).

## 4. Discussion

Seed priming has been extensively used for the improvement of seed quality yield, and to lower seedling protrusion time. Different priming techniques are being used in this regard, all of which have their own advantages [56]. This study was conducted to evaluate the potential of different priming techniques—i.e., hydropriming, osmotic priming,

halopriming, and hormonal priming—to not only contribute to gain in seed growth and health, but also confer resistance against a pathogenic fungus, *A. niger*. To evaluate disease severity and resistance, we conducted disease severity analysis, as described above, and measured the expression level of the genes—namely *chitinase*, *TLP*, and *β-1,3-glucanase*—which mainly contribute to the host resistance to pathogens.

We evaluated biochemical and physiological parameters after treatment with different priming techniques. In the present study, higher proline content was observed in all priming treatments, but this effect was more pronounced in hydropriming and osmotic priming, which enhanced proline content by 70.09% and 71.56% more than non-primed plants (control) (Figure 1A). It has been shown that under various stress conditions—e.g., high salinity, drought, and biotic stress—proline accumulates in high concentration [57–59]. Previous studies on coriander (*Coriandrum sativum*) [60] and sorghum [61] have also described the increased synthesis of proline due to priming. In the case of systemic inoculation, the hydro-, osmotic-, and halo-primed plants showed, by increased proline content, better disease resistance to fungus inoculation (47.30%, 51.26%, and 49.50%, respectively) (Figure 4). Similarly, a significant increase in proline was noted in *Brassica napus* during osmotic priming [62]. Manghwar et al. [28] also observed enhanced proline content in wheat under *Fusarium equiseti* stress. Proline is a compatible solute, usually accumulated under stress in plants, and acts in osmotic adjustment [57,63]. The results of the present study showed significantly increased protein content with all priming treatments compared to the control. Comparatively, all the primed plants inoculated with *A. niger* resulted in higher protein production than non-inoculated primed plants. The findings of [64] also showed the positive effect of priming on the protein contents of the common bean: fungal inoculation led to an overall increase in protein content and a decrease in sugar contents, which is a sign of the stimulation of osmotic material synthesis under stress conditions [65].

Moreover, an increase in sugar content after priming may be because leaves synthesize more soluble sugars after seed priming. The same beneficial effect was found in safflower (*Carthamus tinctorius*) [66], wheat [67], pepper (*Capsicum annuum* L. var Chargui) [68], and barley (*Hordeum vulgare* L.) [69]: this increase may be due to increased α-amylase activity [70]. Sugar content in our study was slightly decreased in response to fungal stress in all the pre-treated plants. Other studies have also confirmed the decrease in sugar content of primed plants after stress conditions [71]. Generally, some pathogenic infections bring changes to the photosynthetic rate and respiratory pathway, and cause fluctuation in sugar content [72–74]. The priming treatments in our study also led to increased chlorophyll content (Figure 1D). A significant increase in chlorophyll contents has been observed after osmotic priming and hydropriming. The study reported 43% and 100% increases in chlorophyll a and b contents, respectively, after priming [75]. Another study, of water, auxin, and gibberellins priming, has been reported to uplift chlorophyll content in soybean [76]. Related results after different priming methods have been observed in rice [77] and coriander [78]. An increase in the chlorophyll content of inoculated primed plants indicates the possible role of priming in disease resistance. The decrease in chlorophyll content of non-treated control plants after systemic inoculation of *A. niger* suggests the positive role of seed priming in maintaining chlorophyll content and disease resistance.

In the present study, higher RWCs were observed after seed treatments. Of all the treatments, the hydro- and osmotic-primed plants showed the highest accumulation of RWCs (Figure 2A). The same results were reported by Namdari and Baghbani [79] and by Mahboob et al. [80], who reported higher water content in *Vicia dasycarpa* and *Zea mays* with hydropriming and osmotic priming, respectively. Our findings revealed an increase in shoot and root length after priming compared to the control, which is supported by the findings of Dessalew et al. [4] and Kumar and Rajalekshmi [81]. Anwar et al. [62] observed an increase in root length in primed seeds in comparison to their control, and suggested that it could be because of embryo cell wall extensibility. In addition, it has been reported that after priming, cell division increases in the apical meristem in roots, leading to an increase in plant growth [82].

The present study showed the beneficial effects of hydropriming and osmotic priming on shoot length, root length, and fresh and dry root/shoot ratios, in response to fungal attack (Figure 2B–E). The hydro- and halo-primed China aster (*Callistephus chinensis*) plants showed significantly enhanced seed germination percentage, seedling survival, and root/shoot ratio [83]. Bourioug et al. [75] reported that hydropriming and osmotic priming in sunflower (*Helianthus annuus*) promoted overall plant growth and increased grain number and grain yield per plant by 2.5-fold and 3.3-fold, respectively. It has been suggested that seed priming enhances plant growth by decreasing the effect of oxidative reactions triggered by reactive oxygen species (ROS) in plant cells [84,85]. According to Al-Abdalall [86], laboratory treatment of both wheat and barley crops by fungi reduces root and shoot lengths and yield significantly. We also observed a decrease in all these parameters in the control (non-primed) plants after *A. niger* inoculation, in comparison to the primed plants, which could be a reason for providing resistance to the pathogen.

Zida et al. [87] reported that seed priming of sorghum plants exhibited significant increase in crop yield, of 19.6% to 51.7%. In addition, the study described threefold to fivefold decreases in the fungal species, *Curvularia* and *Epicoccum*, respectively. Similarly, Rashid et al. [88] demonstrated that, due to hydropriming, mung bean appeared to be more disease-resistant, by having fewer disease symptoms after being infected with Mung bean Yellow Mosaic Virus (MYMV). Rashid et al. [89] also reported an increase in biomass and grain weight due to priming. Likewise, our results also represent that primed plants have a considerable decrease in disease severity, by having improved biochemical (proline, protein, sugar, and chlorophyll contents) and physiological parameters (fresh root, shoot length, dry root/shoot ratio, and RWC). Foliar inoculation of *A. niger* showed a higher percentage of disease or leaf necrosis in the control (>80% leaf area) (Figure 4). The plants were found to be nearly dead, by visual assessment of wilting, as shown in Figure 5. At the same time, a considerable decrease was observed in disease severity, especially in hydro- and osmotic-primed plants—70.59% and 64.71%, respectively—compared to the control, which could be effective in increasing the yield of the wheat crop. Systemic inoculation also had the same pattern of disease severity, but the capacity of disease accumulation was much less (about 60% in the control, Figure 4B,C) as compared to foliar inoculation, which gives an indication that systemic inoculation might be a vigorous method of pathogen inoculation, to show a more robust response.

Results from RT-PCR and qPCR suggest a possible role of *TLP, chitinase*, and *β-1, 3- glucanase* genes in inducing disease resistance in hydro- and osmotic-primed plants (Figure 7). Higher expression of these genes may increase resistance to *A. niger*. The higher expression of *TLP* genes in plants has been shown to provide enhanced tolerance to fungal pathogens [90,91]. Constitutive expression of *TLP*s is typically absent in healthy plants, but is induced exclusively in response to wounding or pathogenic attack [23,26]. After infecting potato plants with *Phytophthora infestan*s, the *TLP* gene was observed to be up-regulated [92]. We also recorded a significant up-regulation of the *TLP* gene in both hydro- and osmotic-primed plants—suggesting its positive role in disease resistance. *Chitinase* has been reported to have a prominent role in plant defense against fungi [27,28]: this gene is thought to play a dual role in fungal growth inhibition, both by cell wall digestion and by releasing pathogen-borne elicitors that induce further defense reactions in the host [93]. Plants subjected to hydropriming also have higher expression of chitinase, which could possibly be considered highly resistant to disease. It has been shown that due to pathogenic attack, the activity and expression of chitinase are elevated [94]. The best-known examples of protection conferred by transgenic expression of plant antifungal genes are represented by overexpression of *chitinases* and *β-1,3-glucanase*s [28,95]. Importantly, gene expression analysis of the current study revealed that *β-1,3-glucanases* showed the highest expression in each priming treatment, as compared to *TLP* and chitinase: their highest expression was observed in osmotic-primed plants, which resulted in the greatest disease resistance with the lowest disease severity in inoculated wheat plants. These results indicate the possible involvement of β-1,3-glucanase in disease resistance, by inducing its high expression in

both hydro- and osmotic-primed plants. In our previous study, we also observed higher expression of β-1,3-glucanase, TLP, and chitinase2, which increased the resistance of wheat plants to *F. equiseti* [28].

## 5. Conclusions

The present study observed the roles of different priming methods—including hydropriming, osmotic priming, halopriming, and hormonal priming—in enhancing resistance to *A. niger* in wheat. All the priming methods used in our study exerted positive effects on plant growth and development, and on resistance to *A. niger*: however, hydropriming and osmotic priming proved to be the best, by significantly improving biochemical (proline, protein, sugar, and chlorophyll contents) and physiological parameters (RWC, root length, shoot length, and fresh and dry root/shoot ratio). In addition, we observed that hydropriming and osmotic priming induced the highest expression of different stress-related genes, such as *TLP, chitinase*, and *β-1, 3-glucanase*: this may be why wheat plants under hydropriming and osmotic priming exhibited the least disease severity, and higher resistance to *A. niger*. Thus, we conclude that hydropriming and osmotic priming may play an important role in reducing the severity of, and resistance to, disease in plants, which could eventually lead to improved crop yield.

**Author Contributions:** S.G., H.M. and M.F.H.M. planned and designed the research; S.G. and A.H., performed the research and contributed to the methodology, writing, and editing; Q.A., I.A., R.M.A. and Q.M., assisted in the analysis, and compiled the data and results; W.Z., H.M. and M.F.H.M. critically revised and improved the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** This article does not contain any studies performed by any of the authors with human participants or animals.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** We confirm that all the authors in our manuscript have no conflict of interest.

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ISBN 978-3-0365-8537-6