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Crop resistance mechanisms to alleviate climate change-related stress

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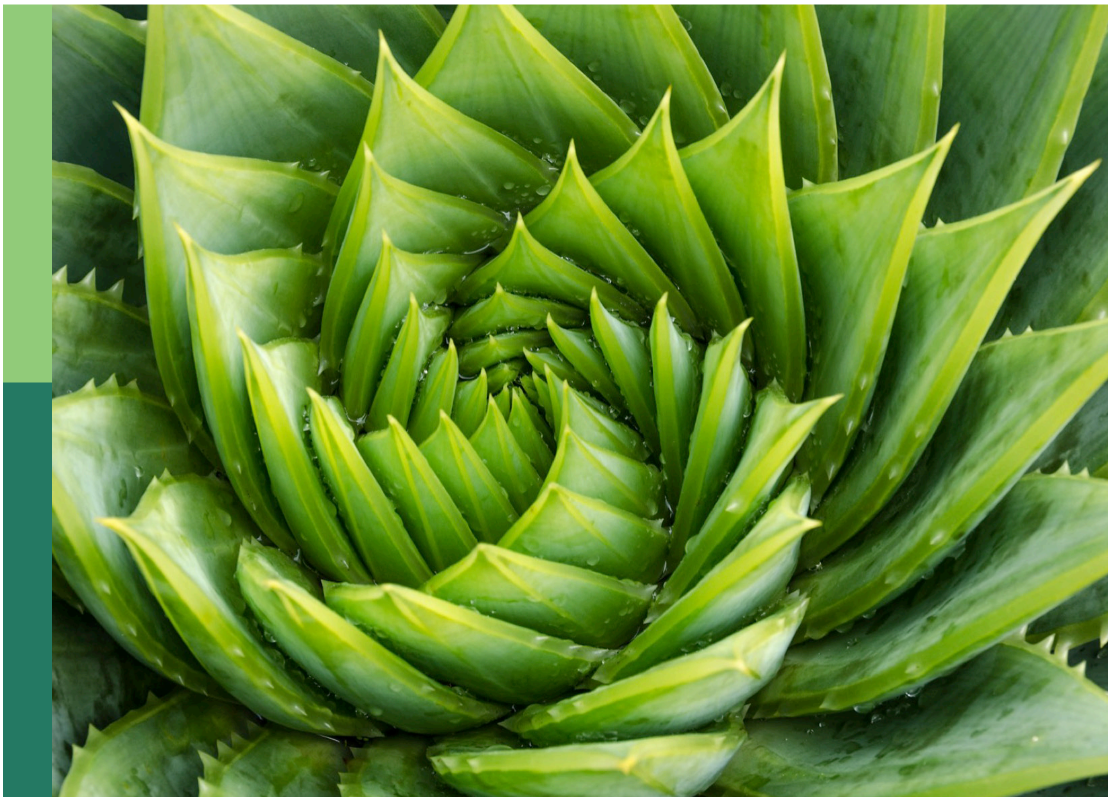

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Crop resistance mechanisms to alleviate climate change-related stress

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Editorial: Crop resistance mechanisms to alleviate climate change-related stress

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Editorial on the Research Topic
Crop resistance mechanisms to alleviate climate change-related stress

Anthropogenic activities have aggravated the effects of global climate change on ecosystems (IPCC, 2018). Plants are sessile organisms unable to escape from an adverse environment and for this reason they suffer to a great extent from stresses, which can negatively impact their growth and development (Aroca, 2012; Gull et al., 2019). Global warming is increasingly causing extreme climatic situations such as very high or low temperatures, drought and flooding events, hailstorms, wildfires, extreme precipitation events, and the reduction of fertile soil through desertification and salinization (Comas et al., 2013; FAO, 2016; Acosta-Motos et al., 2017; Franco-Navarro et al., 2021). In addition to this, warmer temperatures and higher humidity related to climate change can also increase pest and disease pressure on plants by altering the geographic range, population size, and timing of pest and disease outbreaks. Taken together abiotic stress related with climate change, such as drought or extreme temperature, may exacerbate the spread and severity of various diseases associated with biotic stress, increasing the vulnerability of plants to pathogens (some examples include insects, fungi, bacteria or viruses) (IPPC Secretariat, 2021).

Biotic and abiotic stresses affect plant growth and development. Since the development of a plant includes its vegetative and reproductive development, any stress that affects these processes is important to consider as it could cause irreversible damage to the plant and could ultimately lead to its death (Gull et al., 2019). The most relevant issue of climate change's related-stresses in plants is the decrease in crop production (Lobell & Gourdji, 2012). Considering that the growing world population is predicted to reach 9.7 billion in 2050, global efforts are being made to increase food resources, improving crop or agronomic practices, especially in developing countries (Tilman et al., 2002; Godfray et al., 2010; Jurado et al., 2024). However, there are other issues promoted by climate change, affecting native and ornamental plants (Álvarez et al., 2019), floricultural production (Kumar et al., 2013), among others.

This editorial introduces a Research Topic which collects publications that study in detail the resilience mechanisms (tolerance and/or resistance) developed by plants to successfully cope with different biotic and abiotic stresses related to climate change at morphological, physiological, biochemical, and molecular levels (Figure 1). By promoting discussions on innovative farming practices for environmental mitigation and sustainable food production, it aims to provide a guide for resilient ecosystems and empower researchers, farmers and policymakers to manage climate change challenges.

Each publication compiled in this Research Topic is focused on a specific sort of climate change's related-stress (or a combination of two, i.e., drought and salinity), and all of them are presented below.

Drought or water scarcity is one of the major constraints limiting crop production worldwide. Water deficit applied to different plant species of the family Gramineae (*Trichloris crinita*, Dominguez et al.; *Rice - Oryza* sp., Hassan et al.; *Wheat - Triticum aestivum*, Shamloo-Dashtpagerdi et al.), *or* Solanaceae *(Capsicum annuum*, Padilla et al.), revealed significant variation in morphological, physiological, biochemical, and molecular levels. Tolerant variants and specific markers were screened supporting their integration into breeding programs to develop new plant varieties tolerant to drought stress (*Trichloris crinita*, Dominguez et al.; and Rice, Hassan et al.). Besides, Hassan et al. underscores the role of Molecular Assisted Selection (MAS) in advancing varietal development, emphasising the need for further exploration of genes and Quantitative Trait Loci (QTLs). Other works study the effect of changes in phytohormone modulation to enhance drought tolerance in pepper plants (Padilla et al.,2023); reveal a drought-responsive miRNA-target modules (miR1119-MYC2), which emerges as a potential biomarker for assessing wheat genotypes' drought tolerance levels (Shamloo-Dashtpagerdi et al.); or review the critical role of protein degradation as a marker for the selection of drought-tolerant genotypes (Moloi and Ngara).

Water deficit and salt stress were explored in the Mediterranean Anacardiaceae *Pistacia lentiscus* by Álvarez et al. employing moderate and severe deficit irrigation, along with using saline water (around 4 dS m^{-1} salinity). Under these conditions, *Pistacia* exhibits favourable behaviour, making it suitable for landscaping in arid and saline regions.

A review about the effect of water deficit in combination with heat in plants highlights the changes of protein markers in both treatments, and emphasises the decrease of protein abundance, and its structure and stability, in addition to changes in the expression pattern of post-translational modifications (PTMs) and differentially expressed proteins (DEPs), which are also available markers for plant breeding (Xu et al.).

Salinity and salt stress is one of the most important abiotic stresses. Vives-Peris et al. focused on the selection of morphological, physiological, and molecular markers to form the basis for future breeding programs, guiding the selection of optimal rootstock-scion combinations tailored to specific conditions.

Fgaier et al. assayed salinity combined with seed *priming* effect (seeds exposed to UV-C light) as a mitigation mechanism for the effects of salt stress on Lettuce seeds (*Lactuca sativa*). Increased concentrations of Salicylic acid (SA) and cytokinins are likely to contribute to positive effects under high salinity.

The most important soil-associated abiotic stresses is due to the excessive accumulation of herbicides along with the increase of salinity in soils. Pascual et al. observed in tomato plants subjected to salt-stress conditions and in the presence of the herbicide Paraquat, a reduction in the oxidative damage, promoted by the protective effect of the exogenous application of spermine.

Among the abiotic environmental factors, abrupt change in temperature (heat and cold stress) is the most important factor which significantly affects life processes of all organisms. Alcantud et al. assayed, for 2 years, with the Veronicaceae *Antirrhinum majus* under

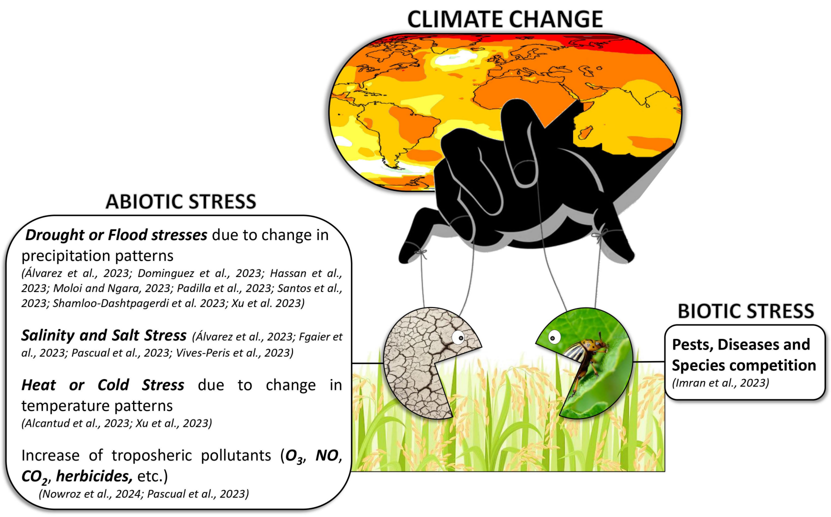

FIGURE 1
This figure represents a brief outline/summary of the Research Topic including its references.

three different temperature regimes: control (22/16°C), cold (15/5°C), and hot (30/23°C), suggesting that short-term heat or cold exposure induces changes in plant gene expression, affecting crucial plant life processes (seed production, flower development, flower colour, etc.). It emphasises ecological and economic implications of temperature-induced changes in floriculture.

Anthropogenic activities of the last century have changed the concentration of pollutants in the Troposphere (NO, CO_2, O_3, etc.). All those contaminant gases are in contact with animals, plants, soil, etc. Nowroz et al.(2024) gives light to the knowledge on plant defensive responses against contamination of tropospheric Ozone (O_3), which levels are increasing mainly due to human activities. A reduction in the stomatal conductance and in the carbon fixation are the first symptoms of the interaction with O_3, reducing the net photosynthetic rate and plant growth.

Another of the great challenges that crops face is biotic stress. Imran et al. highlight Trichoderma culture filtrates' potent antifungal effect on *Alternalia solani* mycelial growth, demonstrating strong inhibitory potential. In greenhouses and fields, these filtrates not only decrease early blight infection, but also promote plant growth and fruit production, serving as effective plant growth promoters. This practice contributes to sustainable agricultural production by mitigating the risk of fungicide-resistant early blight pathogens.

Finally, Santos et al. review the recurrent pressing issue of fruit cracking and highlights its potential for molecular breeding research, driven by genetic factors. Omics technologies offer molecular-level insights into this disorder. While direct evidence through mutations is lacking, the study identifies exocarp-specific transcripts crucial for cracking development, involving cuticular membrane, cell wall mechanisms, and wax biosynthesis. With climate change on the horizon, understanding plant responses at the transcriptomic level is deemed crucial for effective molecular breeding and enhancing crop resilience

This Research Topic delves into the intricate challenges posed by global warming and environmental shifts. Acknowledging the contributions of each article, we extend our gratitude to the authors for their valuable insights. Their exploration of diverse plant responses, spanning molecular mechanisms, breeding strategies, and ecological implications, paves the way for sustainable agriculture. Special thanks to the dedicated reviewers for ensuring the quality and depth of these contributions.

Author contributions

JA-M: Writing – original draft, Writing – review & editing. JF-N: Writing – original draft, Writing – review & editing. MG-B: Writing – original draft, Writing – review & editing. SÁ: Writing – original draft, Writing – review & editing.

Conflict of interest

Author JF-N was employed by the company CLECE S.A.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Flower transcriptional response to long term hot and cold environments in *Antirrhinum majus*

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Short term experiments have identified heat shock and cold response elements in many biological systems. However, the effect of long-term low or high temperatures is not well documented. To address this gap, we grew *Antirrhinum majus* plants from two-weeks old until maturity under control (normal) (22/16°C), cold (15/5°C), and hot (30/23°C) conditions for a period of two years. Flower size, petal anthocyanin content and pollen viability obtained higher values in cold conditions, decreasing in middle and high temperatures. Leaf chlorophyll content was higher in cold conditions and stable in control and hot temperatures, while pedicel length increased under hot conditions. The control conditions were optimal for scent emission and seed production. Scent complexity was low in cold temperatures. The transcriptomic analysis of mature flowers, followed by gene enrichment analysis and CNET plot visualization, showed two groups of genes. One group comprised genes controlling the affected traits, and a second group appeared as long-term adaptation to non-optimal temperatures. These included hypoxia, unsaturated fatty acid metabolism, ribosomal proteins, carboxylic acid, sugar and organic ion transport, or protein folding. We found a differential expression of floral organ identity functions, supporting the flower size data. Pollinator-related traits such as scent and color followed opposite trends, indicating an equilibrium for rendering the organs for pollination attractive under changing climate conditions. Prolonged heat or cold cause structural adaptations in protein synthesis and folding, membrane composition, and transport. Thus, adaptations to cope with non-optimal temperatures occur in basic cellular processes.

KEYWORDS

cold stress, heat stress, adaptation, transcriptome, flower development, ribosomal genes, floral scent, phenylpropanoid metabolism

1 Introduction

The response of organisms to high and low temperatures field of study has very solid knowledge based on short-term lab and field experiments. Many laboratory experiments have been performed with short exposures to changing temperatures, from minutes to hours, and up to several days. These experimental approaches, beginning in the mid-seventies, have shown that rapid responses to heat are a universal feature of bacteria, archeobacteria, fungi, plants, and animals. This rapid response to increased temperature raised the concept of heat shock, which coined the name of a complete set of genes involved in response to high temperatures, known as heat shock genes, which code for heat shock proteins (Lindquist and Craig, 1988). Experiments using cold stress in plants have identified the so-called cold acclimation pathways, activated by short exposures to low temperatures. After an initial period of heat or cold, plants adapt to non-optimal temperatures (Thomashow, 1998). However, climate change conditions, represented as hot or cold periods lasting longer than a few days, are poorly represented by such short-term experiments that explore the early players in adaptation processes, but not the long-term effect on biological systems.

Plant vegetative growth is coupled to reproductive development and success, and flower development is a highly conserved process controlled by flower organ identity genes (Egea-Cortines et al., 1999; Theissen and Saedler, 2001). A high expression of floral organ identity genes is required to achieve completely functional flowers in terms of size, color and scent emission (Manchado-Rojo et al., 2012). These characters are known as floral traits (Ramos and Schiestl, 2019). Flower color is one of the main floral traits used by pollinators to locate flowers. Aside from pigment concentration within the petal cells, petal color appearance relies on light reflectance by conical cells typical of the petal surface (Dyer et al., 2006; Glover, 2011; Brockington et al., 2013).

Together with color, floral volatiles function both as flower attractants to pollinators, as well as deterrents of parasites (Muhlemann et al., 2006). Floral volatile synthesis and emission is coordinated by several factors. These include flower age, pollination status, circadian regulation, and environmental conditions (Schuurink et al., 2006; Cna'ani et al., 2014; Weiss et al., 2016a).

The orchestration of a response against non-optimal temperatures occurs in several steps. The first genes identified as heat response genes code for the so-called heat shock proteins (HSP), and share the molecular function of dealing with protein misfolding and aggregation (Wang et al., 2004). As previously mentioned, short heat treatments lasting minutes in Arabidopsis, have an important impact on plant survival, thus coining the concept of adaptation to heat. The down regulation of HSP101 by interfering RNA decreases heat adaptation, whereas its overexpression enhances performance at high temperatures (Queitsch et al., 2000). The fast activation of heat responsive genes is downstream of several transcription factors such as HsfA2, DREB2A or bZIP28 (Sakuma et al., 2006; Schramm et al., 2006). Heat and cold stress share some signaling and transcriptional pathways, yet partially diverge in the output, as they activate differing gene networks. Cold responses occur *via* DREB2 and other transcription factors such as CBFs (Cook et al., 2004; Alonso-Blanco et al., 2005; Maruyama et al., 2009). The studies mentioned above share short periods of exposure to high or low temperatures, with the period of time under stress varying between one hour to a maximum of four days (Sakuma et al., 2006; Schramm et al., 2006; Gao et al., 2008; Gutha and Reddy, 2008; Maruyama et al., 2009; Bihani et al., 2011). Studies of cold acclimation usually comprise a period of one to two weeks at a mild temperature, followed by a period of freezing temperatures of up to six hours (Cook et al., 2004; Alonso-Blanco et al., 2005).

In the present study, we performed a long-term experiment using snapdragon as a model, as it is a semi-perennial ornamental plant (Stubbe, 1966). The aim of the experiments described was to identify the impact of long-term, non-optimal temperature, i.e. mild heat and cold in flower development. We discuss our work considering the current knowledge in the field of adaptation and survival under long-term non-optimal temperatures.

2 Materials and methods

2.1 Plant material and growth conditions

We used seeds of *Antirrhinum majus* inbred line 165E (Schwarz-Sommer et al., 2010). The seeds were germinated on fine vermiculite. The seedlings were transplanted to Nursery Plastic Pots (650 ml volume) after two weeks. During the entire treatment period, plants were watered as required. Plants were kept in Sanyo MRL350 growth chambers with day/night temperatures of 22/16 °C at a regime of 16 h fluorescent light at a photosynthetically active photon flux density of 250 μE s^{-1} m^{-2}, and 8 h darkness.

Traditionally, Antirrhinum plants have been grown in greenhouses with temperatures ranging between 10°C at night and 28°C during the day. However, temperature effects have not been recorded for this plant. We established the initial conditions to grow *Antirrhinum* plants under long-term high and low temperatures. Traditionally, Antirrhinum plants have been grown in greenhouses with temperatures ranging between 10°C at night and 28°C during the day. Plants were grown for two weeks at a thermoperiod of 22/16°C and a photoperiod of 16/8 light/dark corresponding to long days. These long day conditions induce flowering in *Antirrhinum* (Bradley et al., 1996). After two weeks, the plants were transferred to their final temperature growing regimes for the rest of their development. Initially, the high temperatures were set at 34/28°C, but the plants did not survive, and we could not obtain flowers for further work. Thus, we decreased the temperature to 30/23°C where most plants survived producing a few flowers. Plants grown in the cold were kept at 15/5°C, and they developed slowly. The plants were kept under control, heat, or cold conditions for a minimum of four months, with some of them for over two years, as flowering was strongly delayed (data not shown). During this period, we sampled flowers from the different treatments.

Flower developmental stages were categorized as shown in Figure 1: Flowering stage 0 coincided with day 0 or anthesis. The sampling of flowers was performed on days 0, 3, and 5 days after anthesis (DAA). Flower organ parameters were selected as described previously (Weiss et al., 2016b) and were measured in flowers on day 3 (Figure 1).

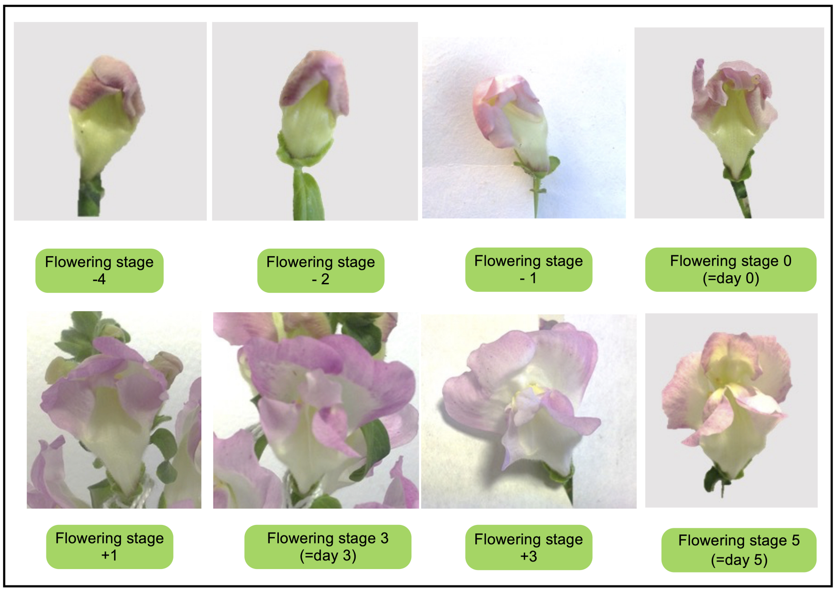


FIGURE 1
Flowering stages of *Antirrhinum majus* line 165E. Flowering stage range from -IV (before flower opening) until stage V (day 5 after flower opening).

2.2 Pigment measurements

We extracted total anthocyanins in flowers from 0.1 g of petal tissue at day 3 after anthesis using a methanol-HCl method, and spectrophotometric absorption measurement at 530 and 657 nm (Zhang et al., 2019), using a UV-1600 spectrophotometer (SHIMADZU, Kyoto, Japan). The extraction was performed on nine flowers from three different plants per temperature regime. Chlorophyll was measured in apical, median, and basal leaves with a CM-500 Chlorophyll Meter, which determines the relative chlorophyll content by measuring the light penetration coefficient in a 2-wavelength range corresponding to red light (660nm) and IR light (850 nm).

2.3 Scanning electron microscopy analysis

We sampled adaxial (upper) petal regions encompassing areas 6, 7 and 8 as described previously (Delgado-Benarroch et al., 2009b) with a scalpel blade. Petal sections of approximately 0.75 cm^2 were prepared for scanning electron microscopy. The cell size of flowers from different temperature regimes were measured based on the cell area of 40 cells per preparation using the program ImageJ (https://imagej.nih.gov/ij).

2.4 VOC analysis

We sampled flowers at day 0, day 3 and day 5 from the control, low and high temperature treatments. This resulted in nine experimental groups.

We collected volatiles with polyvinyl siloxane coated bars (Twister® Gerstel), as previously described (Ruiz-Hernández et al., 2018). Briefly, every snapdragon flower was cut, weighted, and placed in a glass beaker with 4 mL of a 4% sugar solution. We attached a Twister to each beaker, after which it was placed in a glass desiccator. We removed the Twister after 24 hours. The control consisted in a beaker with the sugar solution without snapdragon flowers, which was also placed in a desiccator. Once the Twisters were removed, they were stored at 4°C until further analysis. For each flower stage and temperature conditions, we sampled 8 flowers, except for the control group and 5 DAA, which consisted of 7 samples. In total, we collected volatiles from 71 snapdragon flowers.

The VOCs adsorbed by the Twisters were separated using a GC-MS HP-6890N coupled to a 5975 mass-spectrometer (Agilent Technologies, Palo Alto, CA, USA), combined with a TDU and cooling injector system (CIS4) (Gerstel).

The Twisters were desorbed by heating, starting from an initial temperature of 40°C to 250°C, at a ramping speed of 100°C min^{-1}, with 5 min hold time on a splitless mode. The desorbed compounds were captured in a cool trap at – 100°C. The process was automated by using an MPS2XL multipurpose sampler (Gerstel).

The chromatograms were obtained with a HP5MS-UI column (Agilent Technologies), with helium as the gas carrier, in constant pressure mode and a 1:50 split ratio. The initial temperature was 50°C, increasing at a ratio of 5°C min^{-1} until 70°C, after which the temperature was held for one min. In the following step, the temperature was increased to 240°C at a rate of 10°C min^{-1}, and held for 15 min.

The mass spectrometer operated at a 70 eV ionization voltage. The source and quadrupole temperatures were 230 and 150°C, respectively. The mass range was 30.0 to 450.0 uma at 4 scan/s. The MSD transfer line was maintained at 280°C.

We used the ChemStation software (version E.02.02 SP1, Agilent Technologies) to acquire chromatograms. The compounds were qualitatively identified by comparison with the Wiley10th-NIST11b mass spectral database (Agilent Technologies, Wilmington, DE, USA).

Once we integrated all the chromatograms, we used the R package gcProfileMakeR to obtain the scent profile (Perez-Sanz et al., 2021). We determined the VOCs that were present in all the samples of a given treatment. We will refer to this first profile as "constitutive scent profile". Additionally, we evaluated the volatiles present in more than 70% of the samples. To obtain these profiles, we set the gcProfileMakeR parameters as follows: pFreqCuttof = 1 (constitutive profile or volatiles detected in all samples). In addition, we removed contaminating VOCs such as PDMS-derived volatiles as hexamethylcyclotrisiloxane, with the cas2rm function, and setting the minQuality to 80.

The volatile amounts, expressed as integrated peak area divided by flower fresh weigh, were compared among snapdragon groups and flower stages using Dunn test, implemented in the R package FSA.

2.5 Pollen viability analysis

Pollen viability was evaluated with pollen from seven to ten flowers at day 3 after flower opening, grown under standard conditions as well as high and low temperature regimes. The pollen was tested for the presence of cytoplasm by aniline blue staining with 0.1% aniline blue in 0.1N K_3PO_4 at pH 8.5 (Kho and Baër, 1968).

2.6 Capsule development and seed germinability

Flowers developing under the different temperature regimes were hand self-pollinated, and total seed weight and seed number of 10 capsules from 3 plants were recorded. From each capsule, we performed a germinability assay by placing 20 seeds on a filter paper moistened with distilled water in a petri dish, which was kept under darkness in a growth chamber at 22°C. The germinated seeds were counted after two weeks.

2.7 Statistical analysis

To test differences in flower parameters, chlorophyll, and anthocyanin content between the different temperature groups, we used the non-parametric Wilcoxon test, implemented in R. Their respective graphs were plotted by using the R package ggplot2 (Wickham, 2011, 2). A Principal Component Analysis (PCA) of the VOCs was performed using log-transformed data and expressed as a proportion of the total volatile amount (Tang et al., 2016).

2.8 Transcriptomic analysis

Total RNA was isolated from petal tissue using the NucleoSpin RNA plant kit (MACHEREY-NAGEL, https://www.mn-net.com/) which includes DNase. The quality and concentration of the RNA was determined spectrophotometrically with a NanoDrop ONE (Thermo-Fisher). First strand cDNA was synthesized using 500 ng of total RNA with Maxima kits (Thermo-Fisher), according to the user's manual. Due to differences in flower size resulting from the treatments, we took five flowers per sample of cold, control and heat treatment for a total of 15 samples. We used five samples per treatment corresponding to five biological replicas to perform the transcriptomic analysis.

The quality and concentration of total RNA samples were analyzed before the experiment to ensure sufficient integrity and quantity. To achieve this, the RNA integrity number (RIN) of each sample was investigated. The Kapa Stranded mRNA Library Preparation Kit was used for the library construction of cDNA molecules. Furthermore, the generated DNA fragments were sequenced using the Illumina Hiseq 4000 platform with 150 bp paired-end sequencing reads (Stabvida, Portugal). We obtained a total of 992 million reads comprising 149 Gbp of DNA. The raw data can be downloaded from the European Nucleotide Archive (ENA) https://www.ebi.ac.uk/ena/browser/home (Study: PRJEB54068, Samples: ERS12336218-ERS12336232, Experiments: ERX9450224-ERX9450238, Runs: ERR9907396-ERR9907410)

HiSat2 (version 2.1.0) (Kim et al., 2019) was used to align the RNA-seq reads to the genome assembly version 2 of *Antirrhinum majus* available at http://bioinfo.sibs.ac.cn/Am (snapdragon.chr.IGDBV1.fasta). StringTie (Kovaka et al., 2019) (version 2.0) was used for assembling the transcripts and estimating abundances. We also used the gene annotation of this assembly of the genome (snapdragon_IGDBV1.chr.gene.gff). The NOISeq (Tarazona et al., 2011) R package (version 2.31.0) was used for the differential expression tests, accepting results with probability of differential expression ≥ 0.95. The data analysis and the graphical representations were done using an in-house R script.

The enrichment analysis and the CNET plots were performed by using the ClusterProfiler R package (version 3.16.1) (Yu et al., 2012), using 0.05 as thresholds for the p-value and q-value. An in-house R script was used for generating an annotation package for *Antirrhinum majus* compatible with ClusterProfiler. The GO annotation for biological processes and molecular functions for the genome assembly version 2 of *Antirrhinum majus* was obtained from http://bioinfo.sibs.ac.cn/Am. However, as the number of annotated genes was 20,820, we annotated further for a total of 26,109 genes (out of 37,234). The description of this additional annotation process and the resulting files are available at https://github.com/jesualdotomasfernandezbreis/snapdragon-annotation.

3 Results

3.1 Changes in flower size as a response to temperature are organ specific

Flower size was significantly affected by temperature. Flowers from plants grown under standard temperature conditions had an average weight of 255 mg. Development at cooler temperatures resulted in 15% heavier flowers, while higher temperatures produced 32% lighter flowers (Figure 2, Table S1). We measured eleven flower parameters: P1: petal tube length; P2: lower petal length; P3: petal height; P4: sepal length; P5: tube width; P6: upper petal length; P7: lower petal expansion; P8: upper petal expansion; P9: stamen length; P10: gynoecium length; P11: palate expansion. All

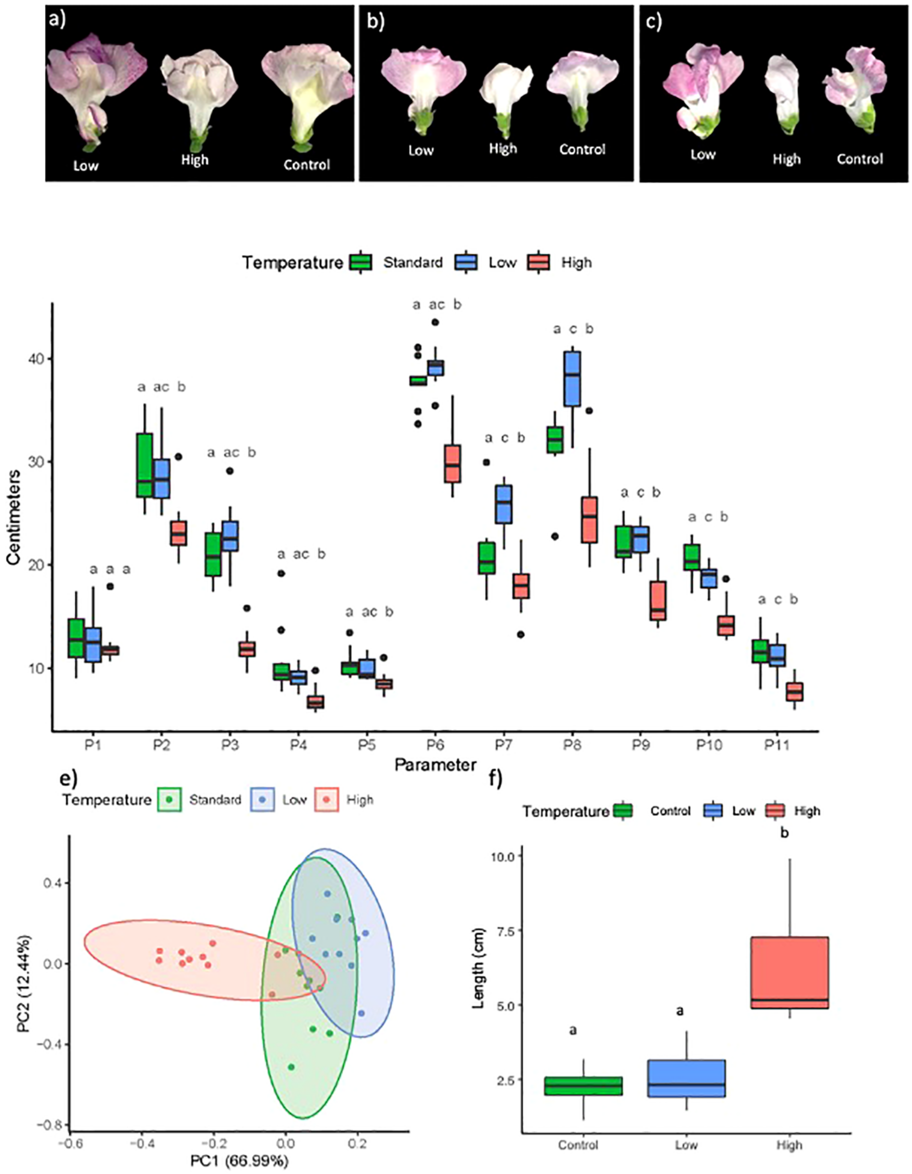


FIGURE 2
Images of **(A)** front, **(B)** back and **(C)** lateral view of flowers of *Antirrhinum majus* kept during inflorescence development at low, high and standard temperatures. **(D)** Flower parameters **(P)** under standard, high and low temperatures. P1: petal tube length: P2: lower petal length; P3: petal height: P4: sepal length; P5: tube width; P6: upper petal length: P7: lower petal expansion; P8: upper petal expansion: P9: stamen length: P10: gynoecium length: P11: pallate expansion. Different letters for each parameter indicate significant differences according to Fisher's F test or Wilcoxon test (see Supporting Information Table S1) (Sepal length and upper petal expansion). **(E)** PCA of floral weight and flower parameters. Each point represents one of the flowers analyzed. **(F)** Pedicel length (see Supporting Information Table S2).

flower parameters were significantly reduced at high temperatures as compared to control conditions, except for petal tube length (P1), which remained stable (Figure 2D, Supplementary Table S2). Lower temperatures resulted in significantly increased values of the lower and upper petal expansion (P7 and P8, Figure 2D). Gynoecium length (P10) was significantly reduced, while other parameters were not significantly different as compared to control conditions (Figure 2D).

To identify overall changes in flower parameters in plants under the different conditions, we performed a PCA analysis (Figure 2E). The first principal component explained 66.99% of the variance, corresponding to upper petal length, and the second principal component explained 12.44% of the variance the data, corresponding to sepal length. The data showed that the flower structure was similar under low temperatures as compared to control conditions, while heat drastically affected petal growth in most parameters. Pedicel length doubled in flowers grown under high temperature conditions, as compared to control conditions, while flowers from control and low temperature conditions had a similar pedicel length (Figure 2F, Supplementary Table S3).

As petal size showed significant differences under different temperatures, we analyzed cell size by SEM. Cell area in the adaxial petal region, encompassing regions of conical and flat cells, were not

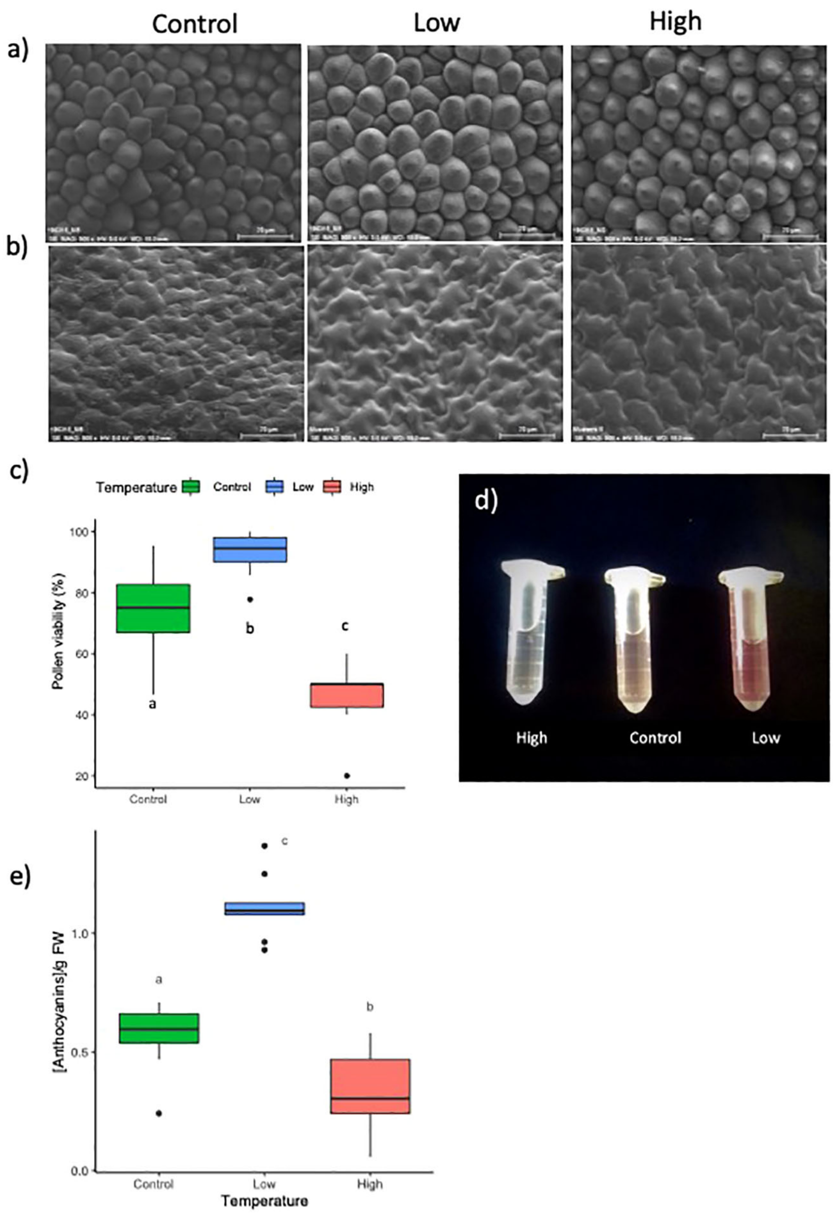


FIGURE 3
(A) From left to right, conical cells and **(B)** flat cells at control, low and, high temperatures. **(C)** Pollen viability at control (green) low (blue) and high temperature (red), expressed as percentage. **(D, E)** Total anthocyanin content in petals of *Antirrhinum majus* flowers grown under control, low, and high temperatures (see Supporting Information Table S3). Letters show significant differences between samples.

significantly different between temperature regimes for either cell type (Figures 3A, B; Table 1). This indicates that the temperature effects on flower size were the result of changes in cell division that translated into larger or smaller flowers, while cell expansion was probably not affected. We did not observe changes in flower opening, a process requiring local cell expansion (Van Doorn and Kamdee, 2014; Sun et al., 2016).

3.2 High and low temperatures significantly influence pollen performance and seed set

The different temperature regimes also affected the quality of the pollen (Figure 3C). While a higher temperature significantly reduced pollen viability from 74.5% under standard conditions to 45%, lower temperatures increased viability to 93%. We hand-pollinated autogamous *A. majus* flowers under the different temperature regimes to evaluate the possible effects on fertility, capsule development, and seed set. High and low temperatures did not affect seed germinability significantly. However, while the average percentage of seed germination was comparable between control and low temperatures, this factor increased 1.5 times at higher temperatures (Table 2). On the contrary, the number of seeds was significantly reduced to 46% at a low temperature and was also reduced by 16% at a high temperature, although this change was not significant. No significant differences were observed concerning seed weight. We can conclude that cold temperatures increased pollen germination, while heat has a strong negative effect. However, seed

TABLE 1 Cell area in flowers of *A.majus* under control, cold and hot conditions.

Temperature	Conical cells	Flat cells
Control	849.09 ± 131.89	1401.00 ± 144.38
Cold	878.04 ± 10.73	1397.53 ± 98.79
Hot	770.85 ± 106.52	1591.45 ± 177.25

Total number of cells per treatment n= 40. Values represent average area (μm^2) and standard deviation. Statistical differences with T-test show no differences between cell types.

number appeared to be at an optimum at control and high temperatures, as cold treatments decreased seed numbers.

3.3 High and low temperature regimes affect flower pigmentation but not petal cell structure

Temperature had a significant effect on petal pigmentation (Figures 2A-C; Figure 3D). As compared with the control temperature condition, cooler temperatures resulted in visually darker pink to red pigmentations. In contrast, higher temperatures produced flowers with a paler color, sometimes appearing completely white. Petal color appearance depends both on anthocyanin concentration and conical cell structure due to light reflectance (Noda et al., 1994). We analyzed total anthocyanin content and found that the apparent color change coincided with a significantly higher total anthocyanin content in petal tissue under cold temperatures, and a significantly lower total anthocyanin content in petal tissue under higher temperatures, as compared to standard conditions. (Figure 3E, Supplementary Table S4). As conical cells that form the petal epidermis play a key role in light reflectance, we studied the floral epidermal structure by SEM. We did not observe any apparent difference in the structure of conical or flat cells in petals from the different treatments (Figure 3A).

We also analyzed the effect of temperature regime on leaf pigmentation (Supplementary Figure S1, Table S5). Just as the petal anthocyanins, leaves kept under lower temperatures showed a significantly higher relative chlorophyll content in apical, median, and basal leaves. However, under high temperatures, we did not observe significant differences in chlorophyll as compared to control leaves.

Altogether, we can conclude that low and high temperatures have opposite effects on flower anthocyanin concentrations, although the temperatures used did not interfere with conical or flat cell morphogenesis in petal epidermis. Chlorophyll concentrations are affected by cold but not by heat.

TABLE 2 Effect of temperature on seed germination and size.

Temperature	% Germination	N° of seeds/10 capsules	Seed weight (gr)
Standard	29a	1404.00 ± 59.34a	0.025 ± 0.029a
Cold	25a	755 ± 26,18b*	0.012 ± 0.003a
Hot	46b**	1175.53 ± 39.81c*	0.007 ± 0.002b*

The asterisks refer to p values of 0.05 (*) and 0.01 (**).
Statistical differences with T-test are marked by letters.

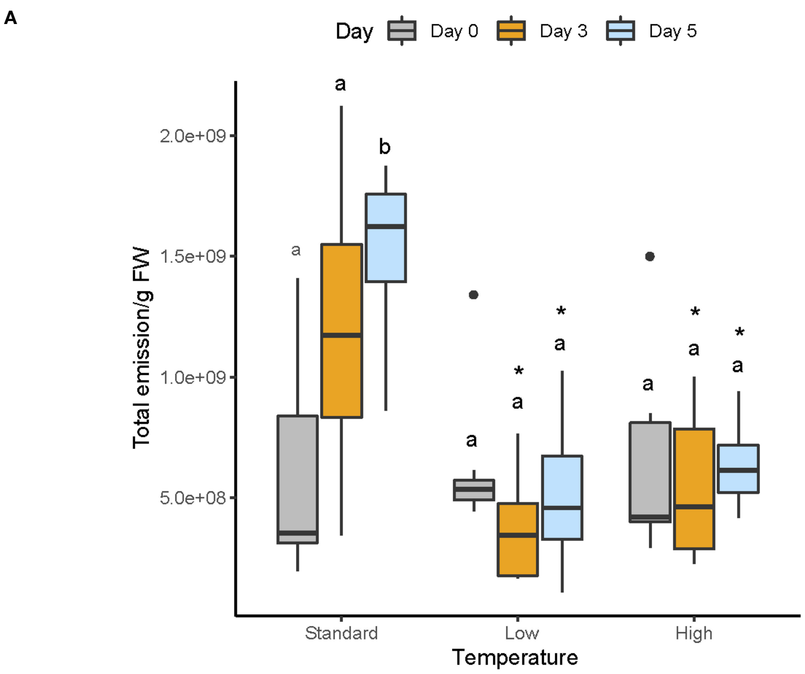
3.4 Temperature affects quantity and quality of volatile compounds

We analyzed the effect of temperature on floral scent emission. We observed significant differences in 1) total emission 2) changes in total emission during aging, and 3) changes in the VOC profile during aging.

Flowers grown under control conditions showed increased VOC emission after flower opening, which was significantly higher at day 5 as compared to day 0 or day 3 (Figure 4A). In contrast, flowers grown at low or high temperatures showed a stable VOC quantitative emission throughout development, and did not display increased VOC emission late in development (Figure 4A). When we compared the effect of temperature on total emission within treatments, we found that flowers grown under hot conditions emitted similar amounts as those grown under control conditions at 0 DAA (Figure 4A, Supplementary Table S6). However, emission was significantly lower at 3 and 5 DAA, 53% and 68% as compared to control flowers (Figure 4A, Supplementary Table S7). Under cold conditions, VOC emission again started at similar levels as control plants, but the reduction in emission was more pronounced. VOC emission was significantly lower, amounting to 25% and 29% at day 3, and 5 DAA, respectively, of the VOCs emitted under control conditions.

We analyzed the VOC profile of the different temperature regimes at days 0, 3 and 5. We identified a total of 111 different volatiles comprising alcohols, aldehydes, phenylpropanoids, fatty acid derivatives, and mono- and sesquiterpenes (Supplementary Figure 2). We defined the volatile profile into two overlapping datasets. The constitutive scent profile, i.e. the volatiles present in all flowers of a given treatment at a given age (Figure 4B), and the non-constitutive (Supplementary Figure 2), found in 70% of a given temperature and age group. The constitutive profile consisted in a set of 14 volatiles (Figure 4B). We found phenethyl alcohol, acetophenone, 2'-hydroxyacetophenone, and myrcene present in all flowers analyzed irrespective of age or temperature treatment. Thus, these VOCs can be considered the basic or core volatilome of this snapdragon line. We observed that the complexity, i.e. the number of detected volatiles, varied across temperature and flower stages. Plants grown under control conditions emitted a total of 11 constitutive compounds. The number of VOCs increased from six up to ten between day 0 and day 5. Under high temperature conditions, the profile was more complex, with the emission of a total of 13 different compounds. It started with 11 VOCS, and decreasing to eight and ten. In contrast, the VOC profile under cold conditions was the poorest, with a total emission of ten volatiles, starting with eight and decreasing to only six. When we analyzed the non-constitutive profile, it included 111 different compounds (Supplementary Figure 2). We observed that at DAA 0, the control group had the highest number of VOCs (52), decreasing progressively to 49 at day three and 31 at day five. The complexity of cold grown flowers was smaller, with 27 VOCs at day 0, 24 at day three and 16 at day five. Under hot conditions, the VOCs showed an overall higher complexity, starting with 44 at day 0, 51 at day three, and 45 at day five. Overall, cold-grown plants produced a subset of the complete volatilome found in normal conditions, while heat-grown plants maintained a somewhat more complex volatilome at day five.

In addition, some volatiles were not affected by temperature, and were emitted at the same flower stage. Thus, at 0 DAA, we identified



B

Class	VOC	CAS	Standard Day 0	Standard Day 3	Standard Day 5	Low temperature Day 0	Low temperature Day 3	Low temperature Day 5	High temperature Day 0	High temperature Day 3	High temperature Day 5
Aldehyde	Decanal	000112-31-2	□	■	■	■	□	□	■	■	■
	Nonanal	000124-19-6	□	□	□	□	□	□	■	□	□
Alkane	Tetradecane	000629-59-4	■	□	□	■	□	□	■	■	□
Benzenoid/ phenylpropanoid	Methyl benzoate	000093-58-3	□	□	■	□	■	■	■	□	■
	Phenethyl alcohol	000098-85-1	■	■	■	■	■	■	■	■	■
	Ethyl benzoate	000093-89-0	□	□	■	□	□	□	□	□	□
	Acetophenone	000098-86-2	■	■	■	■	■	■	■	■	■
	Styrene	000100-42-5	□	□	□	■	□	□	■	■	■
	Methyl cinnamate	000103-26-4	□	□	■	□	□	□	□	□	■
	2'-Hydroxyacetophenone	000118-93-4	■	■	■	■	■	■	■	□	□
Ester	α-Phenylethyl acetate	000093-92-5	□	□	□	■	□	□	□	■	■
Monoterpene	β-Myrcene	000123-35-3	■	■	■	■	■	■	■	■	■
	β-Ocimene	003779-61-1	■	■	■	□	■	■	■	□	■
Sesquiterpene	α-Farnesene	000502-61-4	□	■	■	□	□	□	■	■	■
		Total	6	7	10	8	6	6	11	8	10

■ Detected
□ Non-detected

FIGURE 4
(A) Total floral scent emitted by flowers. Letters over bars indicate differences across each group (see Supporting Information, Table S5) whereas asterisks denote differences across flower stages (see Supporting Information, Table S6), **(B)** Constitutive scent profile of snapdragon flowers under three different conditions (standard, low and high temperatures) and at three development stages, expressed as days after anthesis. This profile comprises those volatiles that were present in all samples from each group. Black and white cells indicate constitutive and non-constitutive compounds, respectively.

2'-hydroxyacetophenone and tetradecane. However, at 5 DAA, all snapdragon flowers emitted methyl benzoate and ocimene.

Some volatiles were detected in 70% of the samples of a given treatment and age (Supplementary Figure 2). This dataset showed that some VOCs were produced at control and high temperatures, but not at cold temperature. These included volatiles of different chemical types, indicating a general inhibitory effect of cold temperatures on floral scent complexity.

3.5 Transcriptomic signature of long-term growth under low and high temperature

We used petals at day 3-5 to obtain a comprehensive transcriptomic profile. We obtained a set of 2202 genes that were differentially expressed between the cold and control conditions, 3994 between hot and control conditions, and 5384 when we compared hot vs cold conditions.

We performed a Gene Enrichment Analysis followed by CNET plots depicting the GO terms and KEGG pathways. We found several gene networks confirming the temperature phenotypes observed in the control and treatment flowers. There were several categories in biological processes found in hot vs cold (Supplementary Figure S3). These included phenylpropanoid synthesis, sulfur compound metabolic process, response to toxic processes, and response to heat. A set of genes showed GO terms related to changes in unsaturated fatty acid metabolism, and a second set to hypoxia (see below). Cold showed two meaningful but unrelated CNET networks, one was pollination, including terms such as unidimensional cell growth or cell tip growth, and again, a second network with an increased number of genes involved in response to hypoxia. Additional related terms included response to oxygen levels or cellular response to decreased oxygen levels. When we analyzed hot vs cold, we found basically the sum of terms, plus additional ones including response to chitin, response to salicylic acid, or antibiotics.

Pollen viability followed a continuum from cold to heat conditions. Amongst the 345 genes enriched with the GO term pollen development, *Am06g26270* and *Am04g06020* are two MADS-box transcription factors orthologous to *AGL65* and *AGL66*. The AGL55 and AGL66 proteins interact *in vitro* and *in vivo* with the ARID-HMG DNA-binding protein AtHMGB15 (Xia et al., 2014). The ortholog of *AtHMG15*, *Am01g14540*, was also differentially expressed, and this transcriptional complex is required for pollen tip growth in Arabidopsis. Indeed, other genes such as *Am05g01300*, a WD40 transducin involved in pollen growth (Wang et al., 2008), *Am03g02130*, an SRK lectin of the S-locus (Goubet et al., 2012), several lectin receptors, and protein kinases, were differentially expressed when comparing hot vs cold. Additional genes involved in pollen development included *Am01g51790*, a calcium-dependent protein kinase known to play a role in cell polarity required for pollen growth in petunia (Yoon et al., 2006). Several Rho-encoding genes involved in microtubule growth, and *Am06g24120*, coding for an Armadillo protein, were differentially expressed. Armadillo proteins confine Rho to pollen tips, where they promote growth (Kulich et al., 2020). This indicates that the impact of temperature on pollen viability is the result of a major disruption of several independent pathways.

An important question we had was what components are found with GO terms related to stress under long-term temperature changes. The largest sets of biological process (BP) genes were related to response to toxic substances (322 genes), response to antibiotics (311 genes), hypoxia (210), and response to salicylic acid (SA) (188 genes). These four sets were largely overlapping. In fact, all the SA response genes were found within the response to antibiotics category and toxic substances. Furthermore, from the 245 transcripts related to heat, all of them except 24 were found within the toxic substance GO term. This indicates that long term non-optimal temperatures cause a steady transcriptional signature related to stress. We identified 245 transcripts with a heat enrichment term. Amongst them, we found several bona fide heat shock genes. These included 20 HSP20, 38 HSP70, four HSP90, 14 DNAJ type chaperones, and 13 FKBP-type peptidyl-prolyl cis-trans isomerases with chaperone activity. This revealed that the protein folding machinery was activated by heat at early stages and maintained throughout development.

While hypoxia occurs in plant roots under water logging (Huang and Johnson, 1995), it also appears to play a role in the maintenance of the stem cell niche in the shoot apical meristem (Weits et al., 2019). Several transcription factors found in the transcriptional network, which were activated during hypoxia, were differentially expressed. These included AP2 transcription factors such as ATRAV1-EDF4, a gene involved in response to hypoxia, activated by cold in a circadian fashion and transported between cells (Thieme et al., 2015), *ERF9*, *DREB2* (*Am01g12910*, *Am03g34650*, *Am01g54280*) and *DREB2A* (*Am07g22160*) (Mustroph et al., 2009). Additional AP2 members differentially expressed included *Am07g28230 and Am07g28230* (RAP2.2) *Am42820* corresponding to *DREB26* and *Am08680* (*ERF114*). A second set of genes included four WRKY genes, three with high homology to *WRKY70*, and one more closely related to *WRKY22*, a gene sharing a hairpin sequence acting as a thermo-switch (Chung et al., 2020). At least ten independent genes coding for alpha crystallin/HSP20 were enriched, coinciding with reports in a variety of plants (Lasanthi-Kudahettige et al., 2007; Ji et al., 2019). Amongst the enzymes responsive to hypoxia, we found an ortholog of ADH1 involved in alcohol metabolism (Loreti et al., 2005).

The molecular functions corresponding to the previous GO biological function terms included several independent gene networks that were enriched in heat vs control (Supplementary Figure S4; Figure 6). These included terms related to stress such as ribosome structure, glutathione transferase, unfolded protein binding, and others related to metabolic processes including unsaturated fatty acid and phenylpropanoid metabolism.

Protein synthesis is affected by cold in many plants (Cheong et al., 2021), and indeed we found a large dataset of 284 genes coding for ribosomal components that were differentially affected by cold (Figures 5, Figure 6). These included genes coding for ribosomal proteins S2, S3, S4, S9, L7, L11, L24, L27, or L32. The major changes observed in ribosomal proteins were also accompanied by changes in gene expression of genes involved in ribosomal biogenesis, such as Am01g11680, orthologous to *SMOG4*. This homolog of yeast NOP53 is involved in maturation of 5.8rRNA, and has a strong effect in cell proliferation in Arabidopsis (Micol-Ponce et al., 2020). The down regulation of the genes coding for ribosomal genes appears to affect most paralogs present in the genome. Indeed, there were four paralogs of the S4/S9 terminal domain, three out of four S12/S23, and three out of four S7p/S5e paralogs were down regulated, suggesting a major reprogramming of the ribosomal structure.

We found that the molecular function (MF) enriched for unsaturated fatty acid metabolism comprised 33 fatty acid desaturases, three SnrK2-like proteins, and 13 fatty acid desaturases involved in changes of fatty acids in the endoplasmic reticulum. Changes in the membrane's biophysical properties are associated to modified fatty acid composition, indicating an adaptation to differing temperatures (see discussion). Together with changes in unsaturated fatty acid metabolism, we found MF related to transport of carboxylic acids, amino acids, inorganic anions, and glutamine.

The transcriptional structure found in long-term cold and hot growing conditions, thus showed a large set of enriched fractions corresponding to the different phenotypes identified, such as pollen growth, anthocyanin metabolism, or VOC synthesis. This apparent coincidence is only partial, as we sampled petals, not pollen (see discussion). In addition, we found hypoxia and a major reprograming of the ribosomal machinery. Our results indicate that a long-term exposure to non-optimal temperatures has a different transcriptional signature, compared to the classic short-term heat/cold shocks.

3.6 Response of developmental and signaling processes to temperature changes

While GEA/CNET identifies major changes in biological pathways, some genes involved in development and signaling do not appear in this type of analysis. Phytochrome B is a major gene involved in both light and temperature signaling (Legris et al., 2016). The *Antirrhinum* ortholog of PHYB Am01g64810.T01, was differentially expressed between cold and heat. However, PIF4

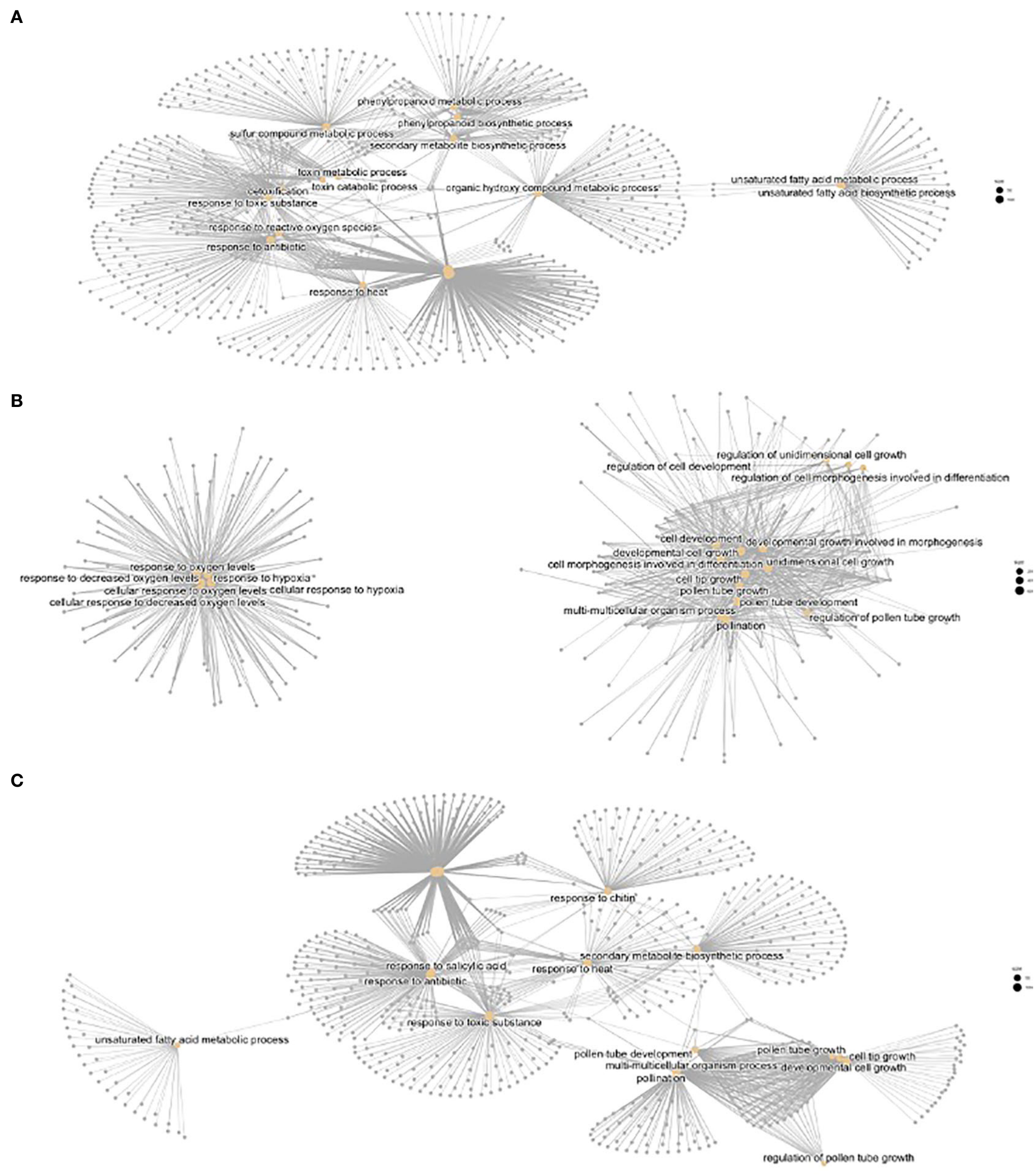


FIGURE 5
CNET plots of Biological Functions with differential regulation. **(A)** Heat vs control, **(B)** cold vs control, **(C)** heat vs cold.

(Am02g50260) did not significantly changed in expression. It is involved in the activation of auxin synthesis and cell expansion at high temperature in Arabidopsis (Franklin et al., 2011) and we did not find cell expansion changes. The ortholog of FT (Am01g05660), was not differentially expressed. However, a phosphatidyl glycerol phosphatase 1, controlling FT movement (Susila et al., 2021), and its activator CO (Am03g06610), were differentially expressed, indicating that the apparent differences in flowering time observed in the different temperature treatments (unpublished results) were caused *via* the conserved FT-CO signaling pathway.

Temperature had a stronger effect on perianth organs, i.e. sepals and petals, than in stamens and carpels. We found that both B function genes *DEFICIENS* (Am01g03890) and *GLOBOSA* (Am04g30510) were differentially expressed, together with *SQUAMOSA* and *DEFH200* (Am06g13650). These results support the phenotypes found after treatment with non-optimal temperatures, as genes involved in perianth development were affected.

The so-called temperature compensation is thought to be a mechanism where plants modify their metabolism to maintain an internal pace irrespective of temperature. Circadian clock genes are involved in temperature compensation (Gould et al., 2006). Although our transcriptomic sampling was not performed in a circadian fashion, we observed significant changes in expression of the core clock genes *AmLHY* and *AmTOC1*. The genes coding for the evening complex *AmELF3* and *AmGIGANTEA*, involved in stabilization of ZTL (Kim et al., 2007), were also differentially expressed in cold versus heat, and cold versus control, suggesting a modified circadian transcriptome resulting from long-term temperature changes.

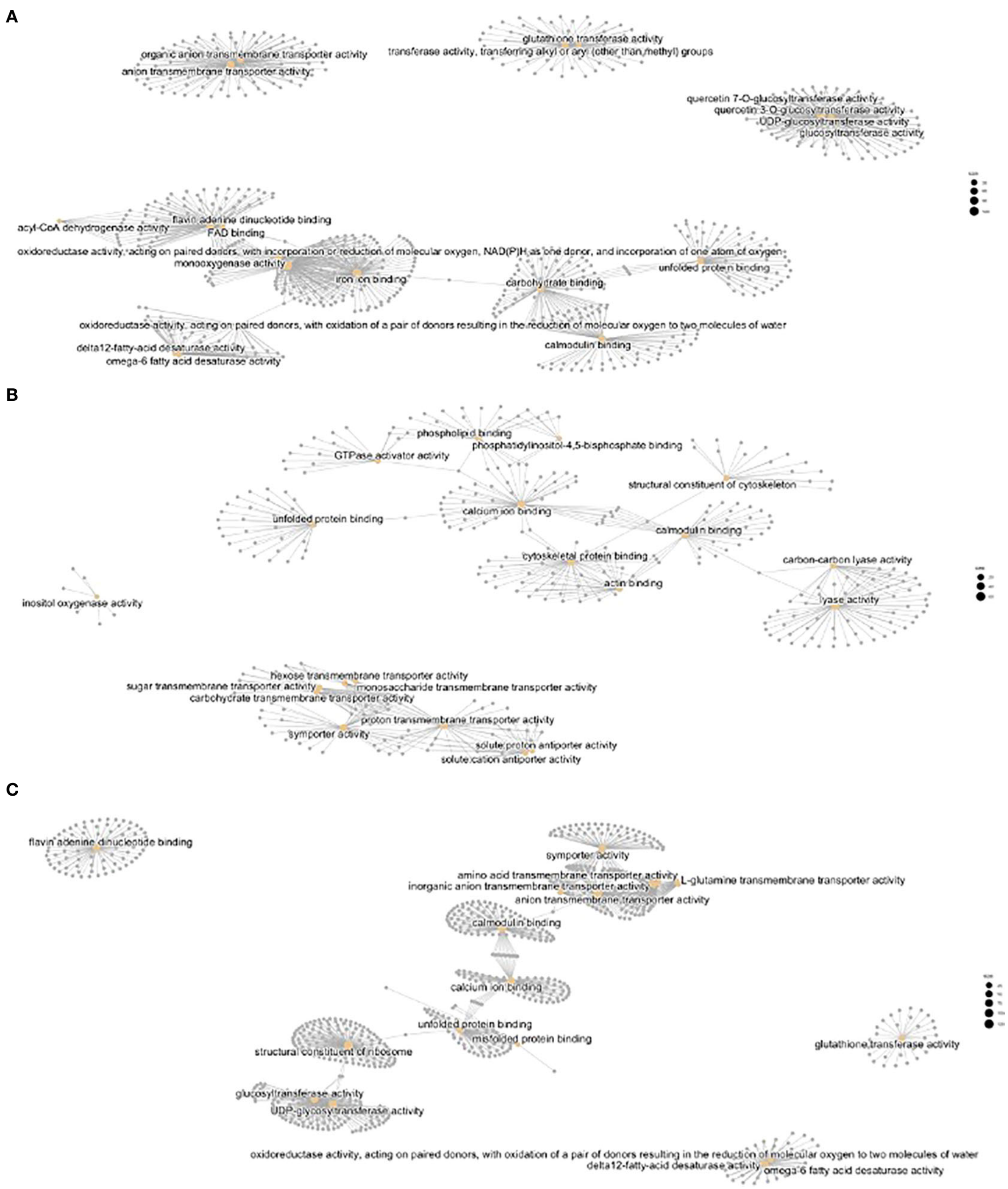


FIGURE 6
CNET plots of Molecular Functions with differential regulation. **(A)** Heat vs control, **(B)** cold vs control, **(C)** heat vs cold.

4 Discussion

In this work, we analyzed the long-term effect of non-optimal growing temperatures on flower development of *Antirrhinum majus*. We chose two temperatures ranges that may correspond to a cold spring with temperatures of 15°/5° C degrees day and night, and a mild summer with temperatures of 30°/23°C. While both temperature regimes are not extreme, they had a very strong impact on growth, as compared to standard (control) 22°/16°C day/night temperatures. This effect should be reflected in steady state transcriptomics after long periods of non-optimal temperatures.

We found that flower development was affected in almost every aspect, although some areas of robustness were identified. Floral organ development occurs from floral primordia, and just like leaves, it is comprised of an initial growth process driven by cell division, and a second part where cell expansion takes over (Reale et al., 2002; Delgado-Benarroch et al., 2009a; Gonzalez et al., 2012). Environmental changes, such as low or high temperatures, affect cell division and expansion (Granier et al., 2000). The most noticeable changes in floral organ size were observed in sepals and petals. Our transcriptomic analysis showed that the major genes involved in petal development i.e. *DEF* and *GLO*, were significantly affected by temperature. The only floral organ that remained unchanged was the petal tube. A number of studies have previously shown that the petal tube and the petal limb may be considered different in terms of regulatory networks related to growth or even pigment accumulation (Wheldale and Bateson, 1907; Almeida et al., 1989; Manchado-Rojo et al., 2014). Our work shows that the significant differences found in petal size under different temperature growth regimes were mainly due to changes in cell division. Similar results have been obtained under shorter growth periods in heat in Petunia (Sood et al., 2021). There are two mechanisms that render flowers with a lower or higher total cell number that are not mutually exclusive. The first is a change in the size of the flower meristem, and the second is a change in the period or speed of petal cell division. As our results showed that temperature only affected cell division, a differing floral size may be the result from changes in meristem size and petal growth (see below).

As other tissues in which meiosis occurs, pollen production is highly sensitive to temperatures. There is a large body of works that describe the effects of non-optimal temperatures on pollen development, including *Antirrhinum* species (Diers, 1971; Alaimo et al., 1997). Non-optimal temperatures affect pollen development in many plants. These include cold temperature in soja and tomato (Fernandez-Munoz et al., 1995; Domínguez et al., 2005; Ohnishi et al., 2010). High temperatures cause male sterility in rice (Endo et al., 2009), and again anther and pollen developmental defects in tomato (Müller et al., 2016). The defects in anther and pollen development in tomato were traced back to the B function genes that we found to be down-regulated (see below). Thus, it did not come as a surprise that high temperatures caused decreased pollen viability. Sets of enriched genes related to pollen and reproduction were identified despite using petals as a tissue. This indicates that the genes that are down regulated in petals, may be related, not only to pollen germination, growth, and viability, but may also play additional roles in other organs. Concerning seed germination, early studies have shown that temperatures play a key role in the process (Morinaga, 1926). Seed germination and viability is the result of developmental processes affected by environmental conditions. Abiotic stresses such as salt, cold and water stress negatively affect seed filling and impact seed germination in rice (Schmidt et al., 2013; Liu et al., 2019). As we found improved pollen germination at low temperatures. However, seed germination and quantity are optimal under control conditions. We conclude that fertilization and seed filling play key roles in *Antirrhinum* seed viability. Probably each of these processes are unequally affected by temperature but the optimal combination of pollen germination, fertilization and seed filling occurs at temperate temperatures.

The existence of both petal color and scent as cues for pollinators have been studied in many systems. Both color and scent are important for pollinator attraction in the genus *Anthirrinum* and other species (Hoballah et al., 2007; Suchet et al., 2010; Amrad et al., 2016; Ruiz-Hernández et al., 2021). Our results show an interesting coordination of both traits, as cold-grown plants showed a stronger and brighter color, while heat-grown plants were paler but had a richer scent profile, as compared to cold-grown flowers. Low temperatures activate anthocyanin biosynthesis while high temperatures have the opposite effect in apple fruits (Lin-Wang et al., 2011; Bai et al., 2014). Furthermore, the accumulation of anthocyanins in red oranges is controlled *via* a temperature sensitive retrotransposon (Butelli et al., 2012). Under cold conditions, having a bright color might be advantageous for pollinator attraction. Studies in Antirrhinum have shown the importance of anthocyanins for pollinator attraction (Dyer et al., 2007).

Volatile emission is affected by temperatures in many plants. Indeed, high temperatures modify scent profiles and emission in Petunia (Sagae et al., 2008; Cna'ani et al., 2015). Work in *Jasminun* has shown a maximum of emission at 25° with significantly lower levels at 20° or 30°C (Barman and Mitra, 2021).Similar to the results found for *Antirrhinum* flowers, we found in a previous work that the scent complexity of Narcissus cut flowers decreases when stored at cold temperatures and increased at higher temperatures (Terry et al., 2021). This indicates that the multicomponent functionality of flowers to attract pollinators may be advantageous given the climatic cycles observed in nature.

Several BP and MF categories appeared as a response to long-term temperature changes indicating basal adaptation. One was a major reorganization of ribosomal protein paralogs comprising over 200 genes. The ribosome acts as a cryosensor in plants, as reducing ribosomal activity induces cold signaling *via* CBF (Wigge et al., 2020). Changes in ribosome structure in response to short-period temperature changes have been recorded in *E.coli*, zebrafish and a variety of plants (VanBogelen and Neidhardt, 1990; Long et al., 2013; Martinez-Seidel et al., 2021). The number of paralogs coding for ribosomal proteins appears to be between two and over 30 in plants (Martinez-Seidel et al., 2020). Previous work describing modified ribosomal compositions coined the concept of ribosomal heterogeneity (Horiguchi et al., 2012). It appears that different ribosomal protein paralogs are not functionally redundant, as mutations in single genes cause severe developmental phenotypes such as altered leaf polarity or embryo lethality (Ma and Dooner, 2004; Fujikura et al., 2009). Recent work has shown that single amino acid changes in the ribosomal protein RPS23 have a strong impact on protein quality and aging in yeast, worms and flies, making them heat resistant (Martinez-Miguel et al., 2021). The *Antirrhinum* ortholog of RPS23 (Am01g28590) was differentially expressed in the dataset. This indicates that overall environmental impact may be channeled *via* changes in the protein synthesis machinery. These ribosomal types may be part of a basal adaptation to non-optimal temperatures.

Cellular membranes play a key role in every aspect of a cell's functions, including signaling and transport. The analysis shows that unsaturated fatty acid biosynthesis may be part of long-term adaptation to modified temperatures. The genes found indicate an adaptation of plasma and endoplasmic membranes. Adaptation to cold at the membrane level occurs in bacteria (Sakamoto and Murata, 2002), and functional studies have shown a positive effect of desaturation in tobacco (Kodama et al., 1994). This indicates that long-term cold stress may cause a reprograming of cell membranes as an adaptive step. Indeed, one group of enriched genes was identified as transporters of amino acids, cations etc. As these proteins are membrane bound, an emerging hypothesis is that there is a major reprogramming of both membrane structure and transporters. However, we do not have data to support changes at the paralog level versus decreased/increased activity of some transporters that may become limiting for growth and development.

Hypoxia has been extensively studied because of water logging in roots (Drew, 1997). The response to hypoxia in flooded roots occurs *via* a signal transduction pathway coordinated by type VII ETHYLENE RESPONSE FACTORS and the ABA signaling pathway (González-Guzmán et al., 2021). However, we sampled petals, a tissue with only three cell layers. We hypothesize that petals may be especially sensitive to temperature, as they are non-photosynthetic organs. This may compromise the equilibrium between sugar intake, photorespiration, and metabolism, making them vulnerable, and becoming hypoxic, despite their vicinity to the atmosphere. Hypoxia causes changes in protein degradation (Gibbs et al., 2011). Thus, the phenotypes observed may be the result of modified cellular processes that include protein synthesis and degradation. Due to the lengthy period required to flower in the cold, and the reduced number of flowers produced under heat conditions, we did not quantify floral number. However, considering the importance of the hypoxia niche in the shoot apical meristem (Weits et al., 2019), it could be that the observed changes in growth could be partly due to a modified state of hypoxia. This may cause changes in the speed of lateral organ formation, resulting in modified speeds of flower formation and changes in flower size due to differing size in primordia.

The hypoxia molecular footprint was different from that described for roots in other species such as tomato (Safavi-Rizi et al., 2020) or Arabidopsis (Gasch et al., 2016; Tang et al., 2021). Indeed, we found several AP2 transcription factors such as RAP2.2, DREB2 and DREB2A that respond to low oxygen levels. The ADH gene is a direct target of RAP2.2 in Arabidopsis in response to low oxygen levels (Papdi et al., 2015). As *AmADH1* was differentially expressed we infer that despite the sampling of petals, there is a hypoxic niche in this tissue occurring because of non-optimal temperatures. Although ABA is an inherent part of the hypoxia signaling response (González-Guzmán et al., 2021), only one paralog of PYL4 *Am07g22130*, showed differential expression indicating that the hypoxia signaling may vary between tissues. Indeed, hypoxia plays a role in root architecture *via* modification of the auxin signaling (Shukla et al., 2019). We did not find differential expression of auxin signaling genes. As petals do not undergo further growth, we conclude that the effects of hypoxia on auxin signaling could have occurred early in petal development but are absent at late stages of development.

While the GE analysis showed major pathways, small pathways with a few genes or unannotated components are difficult to find. We found two major players in flower development i.e. floral organ identity and circadian clock genes, and both showed differential expressions. Floral organ identity genes encoding the B -function involved in petal development have a major impact in floral organ size in *Antirrhinum* and petunia (Bey et al., 2004; Rijpkema et al., 2006). We indeed found differential expressions of DEF and GLO, involved in promoting flower size and scent emission (Manchado-Rojo et al., 2012). The plant's circadian clock coordinates cell division (Fung-Uceda et al., 2018), and *AmLHY* is a positive regulator of flower development (Terry et al., 2019). Furthermore, flower scent is misregulated in knockdown lines of *Antirrhinum* and petunia ZTL/CHL, GI1, or LHY (Fenske et al., 2015; Terry et al., 2019; Brandoli et al., 2020). While our sampling was at a single point, the deregulation of clock genes suggests that the identified changes in scent emission may be the result of a loss of both floral organ identity and clock gene coordination.

5 Conclusions

Exposure to short term heat or cold temperatures causes changes in gene expression, and our work showed that some of these processes, such as protein folding related to heat, ribosomal structure, or membrane fatty acid composition, are inherent to the process of adaptation. We propose that climate change has two levels of action in plants, one *via* the adaptation of basic cellular functions, and a second one related to tissue or development-specific processes. Differing genetic capacities in launching these processes may underlie the observed differences in resilience. Furthermore, not all biological processes may be equally affected by non-optimal temperatures. While *Antirrhinum majus* is an ornamental crop, the effects of non-optimal temperatures in flower development are profound. They affect all aspects including seed production. This indicates that obtaining resilient flowers is part of the requirements to provide food security. The effects on scent emission and flower color open another front related to pollination, with both ecological and economical implications for many crops.

Data availability statement

The data presented in the study are deposited in the European Nucleotide Archive (ENA) https://www.ebi.ac.uk/ena/browser/home (Study: PRJEB54068, Samples: ERS12336218-ERS12336232, Experiments: ERX9450224-ERX9450238, Runs: ERR9907396-ERR9907410). The description of this additional annotation process and the resulting files are available at https://github.com/jesualdotomasfernandezbreis/snapdragon-annotation.

Author contributions

RA: Conceptualization, methodology, investigation, data curation. JW: Conceptualization, methodology, investigation, validation, formal analysis, resources, data curation, writing, supervision, funding acquisition, project management. MT: Formal analysis, data Curation. NB: Formal analysis, data Curation. FV-N: Formal analysis, data curation. JF-B: Conceptualization, methodology, writing - review & editing, project management. ME-C: Conceptualization, methodology, investigation, validation, formal analysis, resources, data curation, writing, supervision, funding acquisition, project management. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1120183/full#supplementary-material

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Molecular mechanisms involved in fruit cracking: A review

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Several fleshy fruits are highly affected by cracking, a severe physiological disorder that compromises their quality and causes high economical losses to the producers. Cracking can occur due to physiological, genetic or environmental factors and may happen during fruit growth, development and ripening. Moreover, in fleshy fruits, exocarp plays an important role, acting as a mechanical protective barrier, defending against biotic or abiotic factors. Thus, when biochemical properties of the cuticle + epidermis + hypodermis are affected, cracks appear in the fruit skin. The identification of genes involved in development such as cell wall modifications, biosynthesis and transport of cuticular waxes, cuticular membrane deposition and associated transcription factors provides new insights to better understand how fruit cracking is affected by genetic factors. Amongst the major environmental stresses causing cracking are excessive water during fruit development, leading to imbalances in cations such as Ca. This review focus on expression of key genes in these pathways, in their influence in affected fruits and the potential for molecular breeding programs, aiming to develop cultivars more resistant to cracking under adverse environmental conditions.

KEYWORDS

environmental stress, exocarp-specific genes, fruit cracking, gene expression, molecular mechanisms

Introduction

Fruit cracking is a severe physiological disorder, common to many fruit crops. It affects fruit quality in numerous species of fleshy fruits (Brüggenwirth and Knoche, 2017; Butani et al., 2019; Lara et al., 2019; Schumann et al., 2019; Wang et al., 2021b).These include sweet cherry, plum, apricot, apple, litchi, pomegranate, citrus, banana, avocado, grape, persimmon, peach, tomato, and pistachio (Simon, 2006; Khadivi-Khub, 2015; Correia et al., 2018; Butani et al., 2019). However, sweet cherry, grape and tomato are the crops most affected by cracking due to their susceptibility to damage associated to the large scale of these industries (Brüggenwirth and Knoche, 2017; Schumann et al., 2019). Cracks on the fruit surface reduce the fruit marketability as they cause negative effects in fruit quality such as poor appearance, shelf life diminution and increased susceptibility to infections by fungi and other pathogens causing significant losses in the fresh market (Khadivi-Khub, 2015; Butani et al., 2019; Wang et al., 2021b). So, the cracked fruits can only be used in processing industries (especially for fruit juice) if they aren't infected by fungi (Simon, 2006; Khadivi-Khub, 2015).

In fruits, the skin, also designed as exocarp, supports the internal cell layers, being considered an essential element in fruits which provides a protective barrier against water loss and pathogen attack (Macnee et al., 2020). The fruit skin comprises three main layers, namely cuticle, epidermis and hypodermis (Knoche and Winkler, 2019). Among them, the hypodermis comprises one to several layers of hypodermal cells while the epidermis is located outside the hypodermis, consisting of in just one cell layer. Epidermis and hypodermis comprise the fruit skin (Knoche and Lang, 2017). The arrangement of hypodermal and epidermal cell layers as well as their thickness highly affect the fruit cracking (Wang et al., 2021b). Outside the epidermis, there is a cuticle or cuticular membrane consisting in lipid polymer that covers the fruit surface, acting as a primary barrier for transport of substances into and out of fruits (Weichert and Knoche, 2006). It also plays an important role in the mechanical properties of the skin (Knoche and Lang, 2017). Thus, the exocarp is a key target in many breeding programs related to cracking (Macnee et al., 2020).

Cracking index (CI) refers to the percentage of cracked fruits in the orchard (Correia et al., 2018). Determining the total number of cracked fruits according the orchards conditions is the most reliable method to access the CI (Christensen, 1972). However, this determination depends on climate conditions as well as the fruit stage development or cultivar, which compromises the method accuracy (Christensen, 1972). Considering the sweet cherry, in the lab, CI can be determined as CI= $\frac{(5a + 3b + c)*100}{250}$, where a, b and c indicate the number of cracked fruits after 2, 4 and 6h of fruits immersion in distilled water (Christensen, 1972; Correia et al., 2018). Concerning to the position of cracks, there are three main types of fruit cracking: (1) deep cracks in the side of fruits, also called as lateral cracking; (2) small/fine cracks at the fruit apical end, also called as pistillar end and (3) circular or semicircular cracks around the stem end in the cavity region (Simon, 2006; Khadivi-Khub, 2015; Rehman et al., 2015; Correia et al., 2018).

The combination of genetic and environmental factors makes fruit cracking difficult to study, even in controlled conditions. Thus, the basic mechanisms involved in cracking remain unclear. However, researchers have suggested that the high occurrence of fruit cracking can be influenced by several factors, namely physiological, biochemical, environmental, agronomical cultural, anatomical, genetic and postharvest storage factors (Simon, 2006; Khadivi-Khub, 2015; Rehman et al., 2015; Correia et al., 2018; Wang et al., 2021b). Simon (2006) suggested that the species and cultivars susceptibility to cracking is mainly genetic. The two main environmental factors involved in cracking are the quantity of rain and its distribution during the ripening period as well as the soil type (Simon, 2006). Cracking may occur during fruit growth, development and ripening (Khadivi-Khub, 2015). However, it mainly occurs during fruit ripening due to changes in the biochemical properties of the exocarp (Petit et al., 2017; Lara et al., 2019). When fruit tissues are subjected to pressures higher than the mechanical resistance of their cell walls and cuticle, the cracks appear in fruit skin (Brüggenwirth and Knoche, 2017). This mostly occurs when maturation and harvest time coincide with a period of high humidity, causing water movement from the branches and leaves to the fruits due to a large difference in their water potentials (Lara et al., 2014; Petit et al., 2017). Moreover, the combination of high temperatures and low humidity, which make the fruit's skin hard and inelastic, followed by heavy rains accelerates the growth and expansion of the internal tissues at a faster rate. Once the fruit's skin remains inelastic and their growth doesn't coup up with the internal tissues growth, cracks appears in the fruit skin (Butani et al., 2019). This leads to a constant stress supported by the fruit since, in most species, the fruit surface and volume increase during fruit development (Knoche and Lang, 2017). Thus, an uncoordinated internal growth associated to an external environment with high climatic variability results in the appearance of cracks in the fruit surface (Wang et al., 2021b).

Using sweet cherry as a model, cracking is commonly associated adverse environmental conditions. These include rain, wet weather and excessive osmotic water uptake through the fruit surface and skin, fruit peduncle cavity, and also fruit peduncle. Excessive water leads to an increase of flesh turgor, fruit volume and surface. Cracks develop when the limit of extensibility of its skin is exceeded (Weichert and Knoche, 2006; Winkler et al., 2016; Knoche and Lang, 2017). This possible explanation for cracking, known as a critical turgor hypothesis, suggests that fruit peduncle, presence of cracks and cuticle are potential pathways for water uptake in sweet cherry (Knoche and Peschel, 2002; Knoche and Winkler, 2019). Water uptake may occur during and after rainfall when water remains in sweet cherry surface as it is retained in the peduncle cavity and in the stylar end leading to a continuous water uptake after rain (Beyer et al., 2005; Knoche and Winkler, 2019). Another possible explanation for sweet cheery cracking, known as a zipper hypothesis, suggests that a localized skin rupture occurs like a zipper due to a local exposure of skin to water where a succession of events leads to cracks development (Winkler et al., 2016). Strain in the skin during the last stage of fruit growth occurs due to a down regulation of genes involved in wax and cutin biosynthesis, leading to a decrease in cuticle deposition (Alkio et al., 2012; Alkio et al., 2014). A thinner cuticle may not withstand increase of strain in the skin and, consequently, microcracks develop (Winkler et al., 2016).

Fruit cracking in sweet cheery can occur due to several additional factors, because of different cracking susceptibilities of cultivars. These include fruit size and firmness, fruit shape, skin and cuticular properties, osmotic concentration and stomata in the fruit skin, stage of fruit development, and water-retaining capacity of the fruit pulp (Simon, 2006; Balbontín et al., 2013; Khadivi-Khub, 2015; Rehman et al., 2015; Correia et al., 2018). Moreover, Li et al. (2021a) proposed that orchard management like irrigation, growth regulators or mineral applications as well as gene expression related to fruit traits may have a positive relationship with cracking. Simon et al. (2004) reported a positive correlation between cracking and soluble solids content.

Cuticle as an interface fruit-environment

The cuticle is very important in flesh fruits, as it acts as a mechanical protective barrier against external or internal stresses, either biotic or abiotic, and in defense against pathogens (Petit et al., 2017; Lara et al., 2019; Trivedi et al., 2019). The cuticle is composed of a lipophilic polymer of cutin, waxes, comprising a mixture of very-long-chain fatty acids and their derivatives, and polysaccharides (Knoche and Winkler, 2019; Trivedi et al., 2019). It is a primary barrier in water transport and fruit rot pathogens, responding to environmental conditions like water deficit, changes in relative humidity, temperature or light intensity (Knoche and Winkler, 2019; Lara et al., 2019). It also provides mechanical support for fruit integrity (Zarrouk et al., 2018). So, the cuticle weakening in ripe fruits can cause severe economic losses by developing several visual cuticle-associated traits which are dependent of the interaction among the cuticle and environment and/or the development stage of the fruit (Petit et al., 2017; Lara et al., 2019). Among the several visual cuticle-associated traits can be included fruit color in tomato (Gonzali and Perata, 2021), fruit cracking in sweet cherry (Lane et al., 2000; Simon, 2006; Rehman et al., 2015), tomato (Domínguez et al., 2012), pomegranate (Singh et al., 2020), grape (Sahadev et al., 2017) or litchi (Marboh et al., 2017), brightness in tomato (Petit et al., 2014), russeting in apple (Knoche et al., 2011; Straube et al., 2021) and pear (Zhang et al., 2020; Zhang et al., 2022), and browning in pear (Franck et al., 2007) and litchi (Jiang et al., 2004). The thickness and chemical composition of fruit cuticle is another factor in cuticle-associated traits, presenting a high variability according fruit tree species, cultivars, and fruit development (Knoche and Lang, 2017; Zarrouk et al., 2018). Although the cuticle-associated traits have a considerable phenotypic diversity, they can be linked to genotypic variation (Petit et al., 2017). However, to understand the cuticle-associated traits in crop species, it is essential to identify new cuticle-related genes and the alleles involved in the trait-of-interest to select beneficial cuticle-associated genetic variants for genetic improvement (Petit et al., 2017; Lara et al., 2019). Thus, the identification of genes that play a role in cuticle synthesis and deposition is important to obtain a better knowledge of its function and development (Lara et al., 2014; Lara et al., 2019; Wang et al., 2021b). Identifying genes involved in cuticle development may contribute to develop cracking resistant cultivars by maintaining the cuticle barrier function, and, thus prevent microcracks formation, keep low stomatal density and a thick cuticle (Peschel and Knoche, 2012).

Molecular mechanisms associated to cracking

Cracking susceptibility of cultivars is considerable among the different species affected by this disorder. Different fruit cultivars present different cracking phenotypes. It is interesting that a cultivar totally tolerant to the disorder has not been described (Balbontín et al., 2013; Butani et al., 2019), this maybe due to a quantitative gene effect based on multiple genes (Khadivi-Khub, 2015; Wang et al., 2021b). So, understanding the genetic factors involved in fruit cracking is essential to select and develop crack-resistant cultivars, which has been one of the major goals in most of the breeding programs (Balbontín et al., 2013; Khadivi-Khub, 2015).

One aim of sweet cherry breeding strategies is to develop more cracking resistant cultivars. Resistance may be associated with genotypes that present low cuticle strain and thick cuticle, maintaining an intact cuticle throughout fruit development. A second type may be genotypes that maintain cutin and wax deposition along fruit growth, especially in the last stage of fruit development (Peschel and Knoche, 2012) Indeed, the characterization of genes related to fruit cuticle development can provide more knowledge about the cuticle functions (Lara et al., 2014). Transcriptomic analyses, shows changes in the expression level of some genes, potentially involved in wax biosynthesis, consistent with wax concentrations (Lara et al., 2019). These include genes related to waxes and cutin biosynthesis and cuticular lipid transporters, whose downregulation leads to a cessation of cuticle deposition (Alkio et al., 2012; Alkio et al., 2014). The cuticular waxes composition varies among fruit species and cultivars (Trivedi et al., 2019).The major plant cuticular waxes components are derived from very-long-chain fatty acids (VLCFAs) and their derivatives like primary and secondary alcohols, alkanes, aldehydes, ketones, and esters (Samuels et al., 2008; Zarrouk et al., 2018; Trivedi et al., 2019). These biomolecules are generated by the *de novo* fatty acid biosynthesis in the plastid followed by fatty acid elongation in the endoplasmic reticulum of the epidermal cells (Zarrouk et al., 2018).

Genes involved in cell wall metabolism affect fruit cracking (Wang et al., 2021b). Cracking rate is influenced by cell wall protopectin and cellulose contents and cell wall thickness (Jiang et al., 2019a). Moreover, the mechanical characteristics of the pericarp, determined by cell wall disassembly, modification, and composition can also contribute to cracking susceptibility (Brüggenwirth and Knoche, 2017). Plant cell wall metabolism regulates the cell wall extensibility, determining cell size and shape (Le Gall et al., 2015). Cell wall degradation and modification has been linked to fruit ripening and softening (Teh et al., 2014). Cell wall-modifying enzymes designated as non-pectolytic enzymes are involved in cell enlargement and expansion by hemicellulose modifications. These include endo-1,4-β-glucanases (EGase), xyloglucan endotransglycosylase/hydrolases (XET/XTH) and expansins (Goulao and Oliveira, 2008; Le Gall et al., 2015). Other cell wall-modifying enzymes, including polygalacturonases (PG), pectin methylesterases (PME), pectin acetylesterases (PAE), pectin/pectate lyases (PL) and *β*-galactosidases (β-Gal), are involved in cell wall plasticity by cleavage or modification of the polysaccharide backbone. Thus, they act as pectolytic enzyme expansins (Goulao and Oliveira, 2008; Le Gall et al., 2015). The properties and structure of cell walls are affected by modifications on the cell wall polysaccharides during ripening (Brummell, 2006). These have been associated to the development of fruit cracking as a result of combined action of cell wall modifying enzymes during fruit ripening and softening (Brummell and Harpster, 2001). Xyloglucan endotransglycosylase is involved in cell wall expansion and re-modelling (Stratilová et al., 2020) by hydrolyzing and re-ligating xyloglucan to other polysaccharides, especially with cellulose. It may control the cell wall relaxation, as the interaction among xyloglucan and cellulose affects plant cells growth control and fruit softening (Kaur, 2019).

Plant growth, both in cell size and number drive fruit expansion and must overcome resistance from the protective cell wall (Marowa et al., 2016). The expansins are involved in cell wall extension acting as regulators of plant cell elongation. Expansins contribute to fruit ripening and softening (Li et al., 2003; Marowa et al., 2016). Expansins, act as zippers to break the hydrogen bonds and unlink cell wall polysaccharides (Marowa et al., 2016). Expansins have been associated with a decrease in cracking index, as they promote the fruit growth by cell walls extensibility and cell expansion (Marowa et al., 2016).

Plant cell wall-modifying enzymes play a key role in fruit ripening, being encoded by multigene families, highlighting their complexity (Brummell, 2006; Goulao and Oliveira, 2008). Moreover, there are increases in expression as well as *de novo* synthesis and activity of cell wall-modifying enzymes, promoting significant modifications in cell wall during ripening (Goulao and Oliveira, 2008).

Transcription factors (TFs) regulate gene expression acting as molecular switches of their target genes binding to different cis-regulatory elements (Franco-Zorrilla et al., 2014; Joshi et al., 2016; Javed et al., 2020). They control all developmental aspects in living cells (Javed et al., 2020). TFs present an important role in plant tolerance/resistance to both biotic and abiotic stresses (Shahzad et al., 2021), by suppressing or activating genes at the transcriptional level (Javed et al., 2020). Some TFs are crucial in biotic and abiotic stresses simultaneously, and also a single TF has the capacity to answer to several stresses (Shahzad et al., 2021). In plants, there are more than 50 TFs families, being WRKY, MYB, NAC (Javed et al., 2020; Shahzad et al., 2021), AP2/ERF (Javed et al., 2020), DREB, bZIP, Zinc-finger, HSF, Dof and NF-Y (Shahzad et al., 2021) the most important involved in biotic and abiotic stresses. So, a better knowledge about TF genes expressed under multiple stresses, may be useful in new crop breeding programs to develop climate-resilient cultivars as well as improve plants yield and health, since an upregulation of TFs is closely related to an increase of tolerance against biotic and abiotic stresses (Shahzad et al., 2021).

In this context, the review will focus on the main cuticle and cell wall related genes potentially involved in fruit cracking (Figure 1). The potentially cracking-related genes in the several fruits highly affected by cracking, such as sweet cherry (*Prunus avium*), apple (*Malus domestica*), watermelon (*Citrullus lanatus*), litchi (*Litchi chinensis*), tomato (*Solanum lycopersicum*), atemoya (*A. cherimola*× *A. squamosa*), grape (*Vitis vinifera*) and jujube (*Zizyphus jujuba*), are summarized in Tables 1–6 according their putatively role, namely cuticular membrane and cell wall metabolisms (Table 1), cutin biosynthesis and deposition (Table 2), cuticular waxes biosynthesis (Table 3), water transport, calcium transport and signaling, and starch and sucrose metabolism (Table 4), fruit hormone metabolisms (Table 5) and transcription factors (Table 6).

Cracking-related genes involved in cuticular membrane, cell wall, suberin and lignin biosynthesis

The first work about genes involved in the cuticle formation in sweet cherry was published by Alkio et al. (2012). Based on sequence similarity with *Arabidopsis*, they used the cultivar Regina to identify genes potentially relevant for cuticular membrane (CM) formation. Among the 18 CM target genes identified by Alkio et al. (2012), 15 of them were only detected in the exocarp, meaning that these genes are exocarp-specific. Moreover, 13 of the exocarp-specific genes present a positive correlation with CM deposition, that is, their transcription levels are high when the CM deposition rate is high and low when CM deposition is low. Generally, these genes have higher expression during the first stage of fruit development, when CM deposition is high (Alkio et al., 2012). In sweet cherry, the maximum β-galactosidase activity occurs in the early stages of active growth and then decrease abruptly during ripening (Kovács et al., 2008). Similarly, the results provided by Balbontín et al. (2014) also refer that transcript levels of *β-galactosidase* gene vary during fruit development, showing their highest transcript levels in the fruit set stage, declining as ripening advances (Table 1). Likewise, Correia et al. (2020b) found different expression levels during fruit development and under different applied compounds, like gibberellic acid, salicylic acid or calcium, in Sweetheart, a cultivar with moderate resistance to cracking. The expansin, *EXP1*, has higher expression in the ripening stage in Kordia, a cracking-resistant cultivar, while in Bing, a cracking-susceptible cultivar, has higher expression in the fruit setting and fruit color change stage (Balbontín et al., 2014). This data has been confirmed by Correia et al. (2020b). There is an increase in expression levels of *EXP1* during fruit development in a cracking-moderate resistant cultivar Sweetheart. Moreover, the expression of the most abundant expansins in sweet cherry (*A1.1*, *A8-like*, *A10.2*) are upregulated in a moderately resistant cultivar Regina compared to the cracking-susceptible cultivar Early Bigi (Table 1) (Michailidis et al., 2021). These findings are in agreement with Balbontín et al. (2014) when attested that the more cracking-resistant cultivar present higher gene expression in all stages as well as Correia et al. (2020b) who verified that cherries treated with biostimulant (*Ascophyllum nodosum*) and growth regulators (eg. abscisic acid, glycine betaine or salicylic acid) have lower cracking index, presenting higher transcripts levels of the studied genes, which increase their expression during fruit development. The gene related to pectin metabolism, *PEL.4*, has higher expression levels in the skin of a cracking-susceptible cultivar Early Bigi (Michailidis et al., 2021).

Expansins also play a role in apple fruit development. Wakasa et al. (2003) identified six expansin genes and studied their expression patterns during fruit growth, being *EXPA3* mainly expressed during the fruit enlargement phase. The same was later described by Joshi et al. (2018) for *EXPA4* gene. Moreover, *EXPA3* transcripts in the mesocarp are higher at the fruit color change stage. In the pericarp, *EXPA3* expression is higher at the begin of

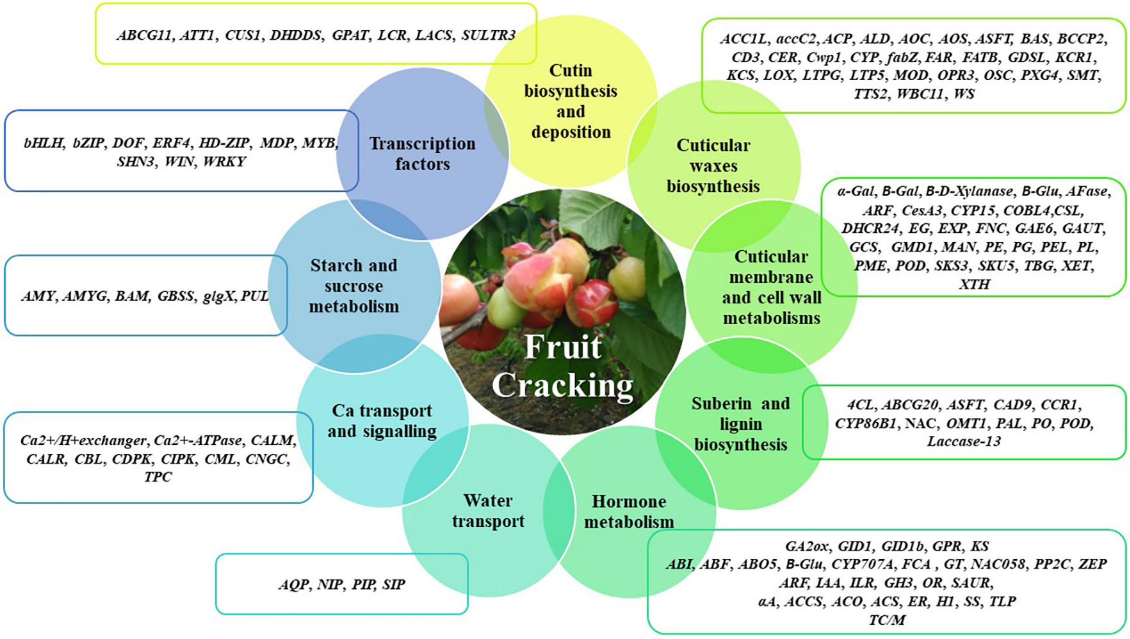

FIGURE 1
Main gene classes potentially involved in fruit cracking. The genes described belong to the molecular functions of cutin biosynthesis and deposition, cuticular waxes biosynthesis, cuticular membrane and cell wall metabolisms, suberin and lignin biosynthesis, hormone metabolism, water transport, Ca transport and signaling, starch and sucrose metabolisms and transcription factors. Cutin biosynthesis and deposition - *ABCG11 (ATP binding cassette transporter), ATT1 (Cytochrome P450 oxidase CYP86A2), CUS1 (cutin synthase 1), DHDDS (ditrans,polycis-polyprenyl diphosphate synthase), GPAT (Glycerol-3-phosphate acyltransferase), LCR (Cytochrome P450 oxidase CYP86A8), LACS (Long chain fatty acid–CoA synthetase), SULTR3 (sulfate transporter 3)*; Cuticular waxes biosynthesis - *ACC1L (acetyl-CoA carboxylase 1-like), accC2 (biotin carboxylase 2, chloroplastic), ACP (Acyl carrier protein), ALD (aminotransferase ALD, chloroplastic-like), AOC (oxide cyclase), AOS (allene oxide synthase), ASFT (Aliphatic suberin feruloyl-transferase), BAS (Beta-amyrin synthase), BCCP2 (biotin carboxyl carrier protein of acetyl-CoA carboxylase 2, chloroplastic), CD3 (cutin deficient 3), CER (Eceriferum family), Cwp1 (cuticular water permeability), CYP (cytochrome-P450 family), fabZ (3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ-like), FAR (fatty acyl reductase), FATB (Fatty acyl–ACP-thioesterase B), GDSL (GDSL lipase), KCR1 (b-Ketoacyl-CoA reductase 1), KCS (β-ketoacyl-CoA synthase), LOX (lipoxygenase), LTPG (glycosylphosphatidylinositol-anchored lipid protein), LTP5 (lipid transfer protein 5), MOD (Microsomal oleate desnaturase), OPR3 (12-oxophytodienoate reductase 3), OSC (oxidosqualene cyclase), PXG4 (Putative peroxygenase 4), SMT (24-methylenesterol C-methyltransferase), TTS2 (triterpene synthase 2), WBC11 (ABC-transporter WBC11), WS (wax synthase)*; Cuticular membrane and cell wall metabolisms - *α-Gal (α- galactosidase), B-Gal (β-galactosidase), B-Glu (β-glucosidase), AFase (alpha-L-arabinofuranosidase), ARF (arabinofuranosidase), CesA3 (cellulose synthase), CYP15 (cytochrome P450 monooxygenase/hydrolase), COBL4 (COBRA-like gene 4), CSL (Cellulose synthase-like), DHCR24 (Delta24-sterol reductase), EG (endoglucanase), EXP (Expansin), FNC (Fruit netted-cracking gene), GAE6 (UDP-glucuronate 4-epimerase), GAUT (alpha-1,4-galacturonosyltransferase), GCS (Gamma-glutamylcysteine synthetase, gamma-GCS), GMD1 (GDP-mannose 4,6-dehydratase 1), MAN (Beta-mannanendohydrolase), PE (pectinesterase), PG (polygalacturonase), PEL/PL (pectate lyase), PME (pectin methylesterase), POD (Peroxidase), SKS3 (Pectinesterase-like), SKU5 (Pectinesterase-like), TBG (tomato β-galactosidase), XET (xyloglucan: xyloglucosyl transferase), XTH (Xyloglucan endotransglycosylase/hydrolase)*; Suberin and lignin biosynthesis - *4CL (4-coumaric acid, CoA ligase), ABCG20 (ATP-Binding cassette G20), ASFT (aliphatic suberin feruloyl-transferase), CAD9 (cinnamyl alcohol dehydrogenase9), CCR1 (cinnamoyl CoA reductase), CYP86B1 (Cytochrome P450), NAC (NAC domain containing protein), OMT1 (O-methyltransferase1), PAL (phenylalanine ammonia lyase), PO/POD (Peroxidase)*; Hormone metabolism - *GA2ox (Gibberellin 2-oxidase), GID1 (GA insensitive DWARF1), GPR (Gibberellin-regulated protein), KS (ent-kaurene synthase), ABI (ABA insensitive), ABF (ABRE-binding factors), ABO5 (ABA overly-sensitive 5), B-Glu (β-glucosidase), CYP707A (ABA 8′-hydroxylase), FCA (Flowering time control protein A), GT (ABA glycosyltransferase), NAC058 (ABA signaling gene - NAC Domain containing protein58), PP2C (Protein phosphatase 2C), ZEP (Zeaxanthin epoxidase), ARF (Auxin response Factor), IAA (indole-3-acetic acid), ILR (IAA-amino acid hydrolase), GH3 (IAA-amido synthetase), OR (Oxidoreductase) (NAD+ oxidoreductase), SAUR (Small Auxin Up-Regulated genes), αA (alpha-amylase), ACCS (Aminocyclopropanecarboxylate synthase), ACO (1-aminocyclopropane-1-carboxylic acid oxidase), ACS (1-aminocyclopropane-1-carboxylic acid synthase), ER (Ethylene receptor), H1 (Hydrolase), SS (sucrose synthase), TLP (Thaumatin-like protein), TC/M (terpene cyclase/mutase)*; Water transport - *AQP (Aquaporin), NIP (Aquaporin), PIP (Plasma membrane intrinsic protein), SIP (Aquaporin)*; Ca transport and signalling - *CALM (Calmodulin), CALR (Calreticulin), CBL (calcineurin B-like protein), CDPK (Ca^{2+}-dependent protein kinases), CIPK (CBL-interacting serine/threonine-protein kinase), CML (Calmodulin-like protein), CNGC (Cyclic nucleotide-gated ion Channel), TPC (putative voltage-gated Ca^{2+})*; Starch and sucrose metabolism - *AMY (alpha-amylase), AMYG (glucoamylase), BAM (beta-amylase), GBSS (granule-bound starch synthase), glgX (glycogen debranching enzyme), PUL (pullulanase)*; Transcription factors - *bHLH (bHLH transcription factor), bZYP (bZIP transcription factor), DOF (DOF transcription factor), ERF4 (ethylene-responsive transcription factor 4), HD-ZIP (homeobox-leucine zipper protein), MDP (MADS box transcription factor), MYB (MYB domain protein), SHN3* (ethylene response subfamily member), *WIN (AP2/EREBP-type transcription factor), WRKY (WRKY family transcription factor).*

fruit development and in the ripening stage (Wakasa et al., 2003). This indicates that an accumulation of *EXPA3* mRNA in pericarp reduces the susceptibility of fruit cracking. Early symptoms of fruit cracking coincide with situations in which *EXPA3* gene expression in the mesocarp exceeds the expression in the pericarp (Table 1) (Kasai et al., 2008). Expression levels of *β-Gal* genes increase during apple fruit growth and are higher in the mature fruits of cultivar Fuji, a softer and crisper apple, than in fruits of cultivar Qinguan, a firmer and tougher apple (Yang et al., 2018). Among them, *β-Gal1*, *β-Gal2*, and *β-Gal5* genes are highly expressed in fruits, presenting a

TABLE 1 Potentially cracking-related genes involved in metabolisms of cuticular membrane, cell wall, suberin and lignin biosynthesis.

Genes	Species	Reference
ABCG20, CAD9, CCR1, CYP86B1, NAC038, NAC058, OMT1	Apple	Joshi et al. (2018); Straube et al. (2021)
AFase, CesA3	Grape	Martins et al. (2020); Yu et al. (2020)
ARF, COBL4, CSLA9, CSL12, GAE6, *GMD1,* SKS3, *SKU5*	Jujube	Hou et al. (2018)
ASFT, 4CL, Laccase-13, PAL	Tomato	Zhang et al. (2021b)
α-GAL, AGAL2, AGAL1	Atemoya, Jujube	Hou et al. (2018); Li et al. (2019)
β-D-Xylanase	Litchi	Wang et al. (2021a)
β-Gal (1, 2, 5, 8)	Apple, Atemoya, Grape, Jujube, Litchi, Sweet Cherry	Kovács et al. (2008); Balbontín et al. (2014); Li et al. (2014); Hou et al. (2018); Joshi et al. (2018); Yang et al. (2018); Li et al. (2019); Correia et al. (2020b); Yu et al. (2020)
β-Glu, BGLU17	Apple, Atemoya, Grape	Joshi et al. (2018); Chen et al. (2019a); Yu et al. (2020)
CYP15	Grape	Martins et al. (2020)
EG	Atemoya, Grape, Litchi	Li et al. (2014); Li et al. (2019); Yu et al. (2020); Zhu et al. (2020)
EXP, EXP1, EXP2, EXP6, EXPA3, EXPA4, EXPA11, EXPA15, A1.1, A8-like, A10.2	Apple, Atemoya, Grape, Jujube, Litchi, Sweet Cherry, Tomato	Brummell et al. (1999); Moctezuma et al. (2003); Yong et al. (2006); Kasai et al. (2008); Balbontín et al. (2014); Li et al. (2014); Hou et al. (2018); Joshi et al. (2018); Jiang et al. (2019a); Li et al. (2019); Correia et al. (2020b); Martins et al. (2020); Xue et al. (2020); Michailidis et al. (2021); Wang et al. (2021a); Zhang et al. (2021b)
FNC, GCS, TBG4, TBG6	Tomato	Smith et al. (2002); Moctezuma et al. (2003); Xue et al. (2020); Zhang et al. (2021b)
DHCR24	Watermelon	Jiang et al. (2019b)
GAUT	Atemoya	Chen et al. (2019a)
MAN, MAN5	Jujube, Tomato	Hou et al. (2018); Xue et al. (2020)
PE, PEL, PEL.4	Atemoya, Grape, Litchi, Sweet Cherry, Tomato	Li et al. (2014); Chen et al. (2019a); Li et al. (2019); Xue et al. (2020); Zhu et al. (2020); Michailidis et al. (2021)
PG, PG1, PG2	Atemoya, Grape, Jujube, Litchi, Tomato	Li et al. (2014); Hou et al. (2018); Chen et al. (2019a); Li et al. (2019); Jiang et al. (2019a); Martins et al. (2020); Xue et al. (2020); Yu et al. (2020); Zhu et al. (2020); Zhang et al. (2021b)
PL, PL1, PL2	Grape, Jujube	Hou et al. (2018); Yu et al. (2020)
PME, PME1, PMEI, PME3	Atemoya, Grape, Jujube	Li et al. (2019); Martins et al. (2020); Yu et al. (2020)
PO1, PO2	Litchi	Wang et al. (2019a)
POD, POD1, POD2	Grape, Tomato, Watermelon	Jiang et al. (2019b); Xue et al. (2020); Zhu et al. (2020)
XET, XET1, XET2, XET3	Atemoya, Grape, Litchi, Watermelon	Lu et al. (2006); Li et al. (2014); Jiang et al. (2019b); Li et al. (2019); Zhu et al. (2020)
XTH, XTH7, XTH9	Jujube, Tomato	Hou et al. (2018); Xue et al. (2020)

TABLE 2 Potentially cracking-related genes involved in cutin biosynthesis and deposition.

Genes	Species	Reference
ABCG11	Apple	Straube et al. (2021)
ATT1	Sweet Cherry	Alkio et al. (2012); Declercq et al. (2014)
CUS1	Tomato	Zhang et al. (2021b)
DHDDS, SULTR3	Watermelon	Jiang et al. (2019b)
GPAT	Watermelon	Jiang et al. (2019b)
GPAT4	Tomato	Romero and Rose (2019)
GPAT5		Zhang et al. (2021b)
GPAT6	Apple	Straube et al. (2021)
GPAT4/8, LCR	Sweet Cherry	Alkio et al. (2012)
LACS1	Sweet Cherry	Alkio et al. (2012)
	Tomato	Romero and Rose (2019)
LACS2	Sweet Cherry	Alkio et al. (2012); Declercq et al. (2014)

significant increase of expression patterns until fruit ripening, which suggest that these genes can affect the fruit texture in both types of apple cultivars (Yang et al., 2018). Similarly, there is an upregulation during apples development for *BGAL8* and *BGLU17* cell wall related genes (Table 1) (Joshi et al., 2018). Furthermore, an upregulation of genes involved in suberin and lignin synthesis, namely *ABCG20*, *CYP86B1*, *NAC038* and *NAC058*, leads to an increase in suberin content and periderm formation, and thus, to the microcracks development and russet apples (Table 1) (Straube et al., 2021). In contrast, an upregulation of the lignin-biosynthesis genes, *CAD9*, *CCR1* and *OMT1* can prevent crack initiation (Table 1) (Joshi et al., 2018).

Regarding to suberin and lignin related genes in watermelon, *POD1* gene is upregulated in the cracking-resistant watermelon, while *POD2* is downregulated in cracking-susceptible watermelon. Similarly, the genes involved in cell wall mechanisms, *XET1*, *XET2*

TABLE 3 Potentially cracking-related genes involved in cuticular waxes biosynthesis.

Genes	Species	Reference
ACC1L, accC2, BCCP2, fabZ, PXG4	Jujube	Li et al. (2020); Li et al. (2021c)
ACP, MOD	Litchi	Wang et al. (2019a); Wang et al. (2021a)
ALD1, ALD4, ALDH3F1, AOC, FAR2, OPR3	Jujube	Li et al. (2020); Liu et al. (2020)
AOS	Jujube	Liu et al. (2020)
	Litchi	Wang et al. (2019a); Wang et al. (2019b)
ASFT, BAS, OSC (1, 3, 4, 5)	Apple	Joshi et al. (2018); Falginella et al. (2021)
CD3, Cwp1, FAR, TTS2	Tomato	Hovav et al. (2007); Romero and Rose (2019); Zhang et al. (2021b)
CER1	Jujube	Li et al. (2020); Li et al. (2021b)
	Sweet Cherry	Alkio et al. (2012)
CER3	Apple	Joshi et al. (2018)
	Jujube	Li et al. (2021b)
	Sweet Cherry	Alkio et al. (2012)
CER5	Jujube	Li et al. (2021b)
	Sweet Cherry	Alkio et al. (2012)
CER6	Apple	Straube et al. (2021)
	Tomato	Vogg et al. (2004); Romero and Rose (2019)

(Continued)

TABLE 3 Continued

Genes	Species	Reference
CER9	Grape	Martins et al. (2020)
CER (4, 7, 8, 14, 15, 6, 18, 26, 29)	Jujube	Li et al. (2021b)
CER1L1	Jujube	Li et al. (2021c)
CYP716A1	Apple	Joshi et al. (2018)
CYP (86A, 86A22, 86B1,94A2)	Jujube	Li et al. (2020); Li et al. (2021c)
FATB, KCR1, WBC11, WS	Sweet Cherry	Alkio et al. (2012); Balbontín et al. (2014); Correia et al. (2020b)
KCS	Jujube	Liu et al. (2020)
	Tomato	Zhang et al. (2021b)
	Watermelon	Jiang et al. (2019b)
KCS1	Jujube	Li et al. (2020)
	Sweet Cherry	Alkio et al. (2012)
KCS6	Sweet Cherry	Alkio et al. (2012); Balbontín et al. (2014)
KCS10	Apple	Straube et al. (2021)
KCS12	Jujube	Li et al. (2020)
LOX	Litchi	Wang et al. (2019a)
LOX2	Jujube	Liu et al. (2020)
LTPG1	Sweet Cherry	Alkio et al. (2012); Balbontín et al. (2014)
LTPG (2, 3, 5, 6, 7, 8, 11, 15)	Apple	Joshi et al. (2018); Gao et al. (2021)
LTP5, GDSL, NLTP9	Tomato	Zhang et al. (2021b)
SMT	Watermelon	Jiang et al. (2019b)
WSD1	Apple	Straube et al. (2021)

and *DHCR24* (Table 1) are downregulated in cracking-susceptible watermelon (Jiang et al., 2019b).

Concerning to cell wall related genes in litchi, the analysis of *XET1*, *XET2* and *XET3* genes has different expression patterns among a cracking-resistant cultivar Huaizhi and a cracking-susceptible cultivar Nuomici, but only *XET1* is fruit-specific, once *XET1* transcripts accumulation appeared in pericarp while *XET2* and *XET3* transcripts accumulation enhanced in aril tissues, suggesting that they may play different roles in litchi aril and pericarp growth, and thus, *XET1* is more likely to play a role in reducing litchi fruit cracking than *XET2* and

TABLE 4 Potentially cracking-related genes involved in water transport, calcium transport and signaling, and starch and sucrose metabolisms.

	Genes	Species	Reference
Water Transport	*AQP, NIP, SIP*	Litchi	Li et al. (2014)
	PIP	Jujube	Ren et al. (2017)
		Litchi	Li et al. (2014); Wang et al. (2021a)
	PIP1;4	Sweet Cherry	Breia et al. (2020)
	PIP2;1		Michailidis et al. (2021)
	PIP2A	Apple	Joshi et al. (2018)
Calcium transport and signaling	*Ca^{2+}/H^+exchanger, Ca^{2+}-ATPase, CBL, CDPK, TPC*	Litchi	Li et al. (2014)
	CALM, CALR	Jujube	Ren et al. (2017)
	CIPK, CML, CNGC	Litchi	Wang et al. (2021a)
Starch and sucrose metabolisms	*AMY, AMYG, BAM, GBSS, PUL*	Atemoya	Chen et al. (2019a)
	glgA, glgB, glgC, glgP, glgX		

TABLE 5 Potentially cracking-related genes involved in fruit hormone metabolisms.

	Genes	Species	Reference
Gibberellins metabolic pathway	*GID1b*	Apple	Joshi et al. (2018)
	GPR	Litchi	Wang et al. (2021a)
	GA2ox, GID1, KS		Li et al. (2014)
ABA metabolic pathway	*ABI, ABF2, ABF3, ABO5, FCA*	Sweet Cherry	Michailidis et al. (2021)
	ABI1, ABI5, β-Glu, GT	Litchi	Li et al. (2014)
	CYP707A		Li et al. (2014); Wang et al. (2019a); Wang et al. (2019b)
	NAC058	Apple	Joshi et al. (2018)
	PP2C	Litchi	Li et al. (2014); Wang et al. (2021a)
	ZEP		Wang et al. (2021a)
Auxin metabolic pathway	*ARF, IAA, ILR, SAUR*	Litchi	Wang et al. (2021a)
	GH3		Wang et al. (2019a); Wang et al. (2019b); Wang et al. (2021a)
	OR1, OR3		Wang et al. (2019a); Wang et al. (2019b)
Ethylene metabolic pathway	*aA, SS, TLP*	Litchi	Wang et al. (2019b)
	ACCS, H1		Wang et al. (2019a); Wang et al. (2019b)
	ACO, ACS	Litchi	Wang et al. (2021a)
		Sweet Cherry	Michailidis et al. (2021)
	ER	Tomato	Xue et al. (2020)
Brassinosteroid metabolic pathway	*TC/M*	Litchi	Wang et al. (2019a); Wang et al. (2019b)

XET3 (Table 1) (Lu et al., 2006). Additionally, the expression of a *XET* gene is upregulated in fruits without cracks compared to cracked fruits (Li et al., 2014). The expression of two genes encoding expansins in litchi pericarp, *Exp1* and *Exp2*, appear to have a closely association with fruit growth and cracking, since the expression of both genes is detected from the early stage of fruit rapid growth and then increase and reach to the highest level at the end of the growth phase in pericarp of the cracking-resistant cultivar Huaizhi, while *Exp1* gene is detected at the stage of rapid fruit growth, and then increase slightly and finally kept almost constant in pericarp of the cracking-susceptible cultivar Nuomici, not being detected expression of *Exp2* in this cultivar (Yong et al., 2006). Similar results were obtained by Wang et al. (2021a), whose an upregulation of cell wall related genes (*EXP* and *β-D-Xylanase*) leads to a low mechanical strength and by Li et al. (2014), who found an upregulation of five *EXP* in fruits without cracks compared to cracked fruits. The same is observed on nine *β-Gal* genes, which are upregulated in fruits without cracks compared to cracked fruits, while five *PG*, one *EG* and three *PE* genes are upregulated in cracked fruits compared to fruits without cracks (Table 1) (Li et al., 2014). Regarding to suberin and lignin related genes, an upregulation of *PO1* and *PO2* genes leads to an increase of lignin biosynthesis, resulting in differences in cuticle structure of litchi fruits pericarps with different susceptibilities to cracking compared to cracked pericarps (Wang et al., 2019a).

Bargel and Neinhuis (2005) studied the biomechanics of tomato fruit skin and isolated cuticles, from three cultivars differing in

TABLE 6 Transcription factors genes potentially involved in fruit cracking.

Genes	Species	Reference
bHLH, bZIP, DOF, WRKY	Litchi	Wang et al. (2021a)
ERF4	Tomato	Xue et al. (2020)
	Watermelon	Liao et al. (2020)
HD-ZIP, MDP	Watermelon	Jiang et al. (2019b)
MYB	Litchi	Wang et al. (2021a)
MYB93, MYB42	Apple	Falginella et al. (2021); Straube et al. (2021)
SHN3	Apple	Falginella et al. (2021); Straube et al. (2021)
WINA, WINB	Sweet Cherry	Alkio et al. (2012); Balbontín et al. (2014)

cracking susceptibility and fruit shape, concluding that the cuticle is a mechanically important component of the tomato fruit. Other important contribution to understand the multiple metabolic and genetic phenomena that occur in the fruit skin during ripening was made in tomato by. Mintz-Oron et al. (2008), which describes the differential gene expression at different fruit developmental stages and in different tissues of the fruit and allowed to identify genes expressed specifically in the skin at ripening, such as genes involved in cell wall modification. Also in tomato, a higher expression of *FCN* gene also increase the expression of other genes involved in different metabolic pathways such as suberin metabolism genes (*ASFT* and *GPAT5*), lignin metabolism genes (*4CL*, *PAL* and *Laccase-13*), and cell wall metabolism genes (*EXPA11* and *PG*), which affects the cell wall extensibility, fruit softening, pericarp firmness leading to the appearance of cracks in all fruit surface (Table 1) (Zhang et al., 2021b). The simultaneous suppression of *PG* and *EXP1* in ripening fruits reduces cell wall disassembly since *pg/exp* fruits are more firm, present more protopectin and thicker cell walls, concluding that a ripe fruit with more intact pectins in its primary walls is more resistant to cracking (Jiang et al., 2019a). Likewise, antisense inhibition of PE and PG activity affects the level of fruit cracking, while suppression of the ripening related expansin gene (*Exp1*) (Brummell et al., 1999) and tomato β-galactosidase 4 (*TBG4*) (Smith et al., 2002) increases fruit firmness. On the other hand, the relationship between activity of cell wall enzymes and cuticular layer was demonstrated in tomato by Moctezuma et al. (2003) as a result of antisense suppression of a β-galactosidase gene (*TBG6*), observing a positive correlation between cracked fruit number with low levels of β-galactosidase transcripts. The results suggest that the TBG6 product may have an important function in cell wall galactosyl residue metabolism during cell elongation, so the various altered phenotypes observed as a result of *TBG6* gene down-regulation in tomato fruit are further evidence that β-galactosidases have important functions in the overall growth and development of tomato fruit (Table 1) (Moctezuma et al., 2003). Additionally, the main genes involved in tomato cracking are associated with different metabolic pathways such as cell wall organization, oxidoreductase activity and catalytic activity, which included genes as *MAN*, *PE*, *POD*, *EXP*, *XTH7*, *XTH9*, *PG2*, *ER*, *ERF4* and *gamma-GCS* (Xue et al., 2020). The genes involved in cell wall loosening and expansion, namely *XTH7*, *XTH9*, *PE* and *POD*, are downregulated in a cracking-resistant cultivar and upregulated in a cracking-susceptible cultivar. Likewise, cell-wall degrading enzyme-associated genes are also upregulated, namely *GCS*, *MAN* and *PG* genes as well as ethylene and auxin responsive genes such as *PG*, *PE*, *EXP* and *XTH7* which can be related to cell wall regulation once ethylene influences fruit development and ripening (Xue et al., 2020).

In atemoya, several genes related to cell wall mechanisms were identified, namely 34 *PGs*, 21 *PEs*, 19 *EXPs*, 17 β-*GALs*, 13 *EGs*, 6 α-*GALs*, 6 *PME*, 4 *PELs*, 4 *XETs* and 3 cellulases, which, in general, present higher expression levels in cracked fruits than fruits without cracks, that leads to a reduction in skin elasticity and, consequently, fruits cracking (Li et al., 2019). In addition, *GAUT* gene, involved in pectin synthesis, is upregulated as well as the genes involved in pectin degradation, namely *PE*, *PG* and *PEL*. Likewise, β-glucosidase gene, responsible for cellulose degradation, also is upregulated (Table 1) (Chen et al., 2019a).

The effect of calcium in genes involved in cell modifications was studied by Yu et al. (2020) using a cracking-susceptible cultivar Xiangfei, whose expression was analyzed in grape berry skins after 1, 2 and 3 weeks of the calcium applications comparing to fruits without calcium application (as a control). Among then, five polygalacturonases (*PG*) and three endoglucanases (*EG*) were studied, showing a downregulation of almost all *PG* genes by calcium at all weeks as well as a downregulation of two *EG* genes in the first and second weeks, and then an upregulation of these genes in the third week (Table 1) (Yu et al., 2020). Similar results were obtained by Zhu et al. (2020) using grapes of the same cultivar, Xiangfei, during the ripening stage, finding an upregulation of two *EGs* and one *PG* along the fruit maturation. Other genes involved in cell modifications namely one pectin methylesterase (*PME1*), two polygalacturonases (*PG1* and *PG2*), one expansin (*EXP6*) and one cellulose synthase (*CesA3*) as well as a cuticle biosynthesis gene, cytochrome P450 monooxygenase/hydrolase (*CYP15*), were studied at pulp and skin of grape berry, also with and without calcium treatment in the cultivar Vinhão, showing a downregulation of the genes *PME1*, *PG1*, *PG2*, *EXP6* and *CYP15* promoted by calcium in skin and pulp, while *CesA3* gene is not significantly affected by calcium in the skin and pulp (Table 1) (Martins et al., 2020). This suggest a regulation by calcium at transcriptional level and also that calcium can inhibit additional enzymatic pathways involved in cell wall mechanisms (Martins et al., 2020). The work performed by Yu et al. (2020) also analyzed four pectate lyases (*PL*), ten pectin methylesterase (*PME*), two PME inhibitors (*PMEI*), one alpha-L-arabinofuranosidase (*AFase*), four β-galactosidase (β-*Gal*), and one β-glucosidase (β-*Glu*), all involved in cell wall modifications, in grape berry skins after 1, 2 and 3 weeks of the calcium applications. Among the analyzed genes, the *AFase*, the β-*Glu*, almost all β-*Gal* genes and one *PME* are downregulated by calcium at all weeks, while two *PME* genes are downregulated in the first week after calcium application, four *PME* genes are downregulated in the second week and three *PME* genes are downregulated in the third week (Yu et al., 2020). Regarding *PL* genes, two are downregulated by calcium at first and second weeks and upregulated at third week, while the other two *PL* genes are upregulated during the three weeks. Moreover, there is also one *PMEI* gene continuously and significantly upregulated by calcium and another *PMEI* gene downregulated in the first week (Table 1) (Yu et al., 2020). Based in the results, calcium applications in grape berry appear to induce specific modifications both in skin and in pulp, inhibiting pectin degradation and cell wall loosening, and changing the cuticle structure (Martins et al., 2020) as well as leading to cell wall disassembly inhibition, and promoting cell wall strengthening (Yu et al., 2020), playing an important role in preventing cracking. In order to analyze the transcriptome and identify important metabolisms related to grape berry cracking, Zhu et al. (2020) made a RNA-Seq analysis to assess the expression of pericarp genes during the ripening stage, namely at 1 (W1), 2 (W2) and 3 (W3) weeks after veraison in cultivar Xiangfei. The three groups (W1, W2 and W3) presented a similar number of expressed genes, however with different expression in each group (some genes express in all groups, but some genes only express in one unique group) (Zhang et al., 2021a). Comparing W1, W2 and W3, the authors detected an increase of cracking during repining highlighting great changes in gene expression during this period, which can be correlated with 303 DEGs up-regulated and 354 DEGs down-regulated in W2 and W3 relatively to W1 (Zhu et al., 2020). During the fruit development, the cell wall mechanical properties have an important role (Brüggenwirth and Knoche, 2017), so Zhu et al. (2020) validated the RNA-Seq results with a qRT-PCR analysis of genes involved in cell mechanisms, obtaining highly consistent results for both analysis. Among the DEGs involved in cell wall mechanisms, one peroxidase (*POD*), one pectinesterase (*PE*) and eleven xyloglucan endotransglycosylase (*XET*), beyond two *EGs* and one *PG*, were identified (Table 1). These genes have differential expression when W2 and W3 groups are compared to W1, showing an up-regulated expression along fruit maturation, which suggested that cell wall related genes play important roles in grape berry cracking regulation (Zhu et al., 2020).

By a transcriptomic analysis of jujube using young and mature fruit, Hou et al. (2018) found several differentially expressed genes, 19 of them related to cell wall mechanisms and fruit ripening. The analysis of gene expression revealed that *AGAL2*, *BGAL*, *EXP2*, *EXPA15*, *PL2*, *SKU5*, *GAE6* and *XTH* genes are upregulated in young fruit stage while *ARF*, *CSL12*, *CSLA9*, *COBL4*, *GMD1*, *PL1* and *PG1* genes are upregulated in ripe fruit stage. However, *AGAL1*, *MAN5*, *PME3* and *SKS3* genes, don't present significant expression differences among the two ripening fruit stages (Table 1) (Hou et al., 2018). Thus, these differences in gene expression during fruit development leads to important changes in cell wall metabolisms playing important roles in preventing or enhancing jujube cracking (Hou et al., 2018).

Cracking-related genes involved in cutin biosynthesis and deposition, and in cuticular waxes biosynthesis

In sweet cherry, the expression of genes involved in cutin biosynthesis and deposition, namely *LCR* and *LACS1* genes, is detected in the beginning of fruit development, while expression of *ATT1* and *LACS2* (Table 2) have higher expression at later fruit stages (Alkio et al., 2012). The previous data was confirmed by Declercq et al. (2014) for *LACS2* and *ATT1* genes, concluding that the expression of these genes increase cutin deposition and decrease cuticle permeability. They may be involved in the molecular mechanisms of sweet cherry cracking. Additionally, expression of *GPAT4/8* is detected in the beginning of fruit development and increase again in the final phase of fruit development (Alkio et al., 2012). Concerning cuticular waxes related genes, more specifically involved in biosynthesis of VLCFAS, *KCS6* and *KCS1* present higher expression in the beginning of fruit development and increased again in the final phase of fruit development as well as the expression of *Lipase* (Table 3) (Alkio et al., 2012). Likewise, Balbontín et al. (2014) found higher expression of *KCS6* at fruit setting (begin of fruit development) and fruit color change stage in a cracking-susceptible cultivar Bing, and higher expression in a cracking-resistant cultivar Kordia, coinciding with the ripening of fruits. Additionally, the wax synthase gene, *WS*, presents similar expression levels to *KCS6* (Balbontín et al., 2014). These results have been again corroborated by Correia et al. (2020b) analyzing the expression patterns of *WS* gene in a cracking-moderate resistant cultivar Sweetheart. *WS* increases during fruit development, leading to higher wax content (Correia et al., 2020b). The genes involved in lipid transport, *LTPG1*, *WBC11* and *CER1* show higher expression in the begin of fruit development and, again, in the fruit ripening stage (Table 3) (Alkio et al., 2012). The expression of *KCR1* and *FATB* genes is higher in the exocarp in the beginning of fruit development and again in the final fruit development phase. The expression of *CER5* and *CER3* genes is also exocarp-specific at all developmental stages but do not correlate with the CM deposition rate (Alkio et al., 2012).

In apples, the presence of microcracks can lead to russeting (rough and brownish patches on the fruit skin) which can occur due to water uptake on the fruit's surface (Khadivi-Khub, 2015; Straube et al., 2021). Straube et al. (2021) studied the effect of moisture exposure in apples verifying a decrease in cutin and wax contents due to a down-regulation of genes involved in cutin and wax synthesis (*ABCG11*, *GPAT6*, *KCS10*, *WSD1* and *CER6* genes) (Table 2 and Table 3). This leads to a decrease in cuticle formation and, consequently, to microcracks formation. The *LTPG* genes codify proteins involved in lipid transport. Studies performed by Joshi et al. (2018) show a significant upregulation of *LTPG5* gene as well as of *CER3* and *ASFT* during apple fruit development. The expression of *LTPG* genes is responsive to abiotic stresses and stress hormones such as drought, cold, salt, salicylic acid and jasmonate (Gao et al., 2021). Among 26 potential *LTPG* genes, 9 of them are highly expressed in fruits, including *LTPG2*, *LTPG3*, *LTPG5*, *LTPG6*, *LTPG7*, *LTPG8*, *LTPG11*, *LTPG15*, and *LTPG19*, highlighting the function of this gene family in wax biosynthesis and cracking prevention (Table 3) (Gao et al., 2021). Triterpenes are components of surface waxes (Thimmappa et al., 2014). There is a close relationship between the expression of oxidosqualene cyclase (*OSC*) genes and russeting level in apples. *OSC1* and *OSC3* genes present low expression in cultivar Rugiada that generally shows fully russeted skin. In contrast, *OSC1* and *OSC3* are highly expressed in cultivars Smoothee and Golden Delicious with low and moderate russeting, respectively (Falginella et al., 2021). *OSC4* and *OSC5* genes are downregulated in cultivars Smoothee and Golden Delicious and upregulated in Rugiada, correlating with high russeting level in this cultivar (Table 3) (Falginella et al., 2021). Likewise, the β-amyrin biosynthesis-related genes, *BAS* and *CYP716A1*, are upregulated during fruit development (Joshi et al., 2018).

The watermelon rind has an important role in fruit cracking, being the rind hardness positively correlated with cracking resistance of this fruit (Liao et al., 2020). The expression of genes related to cracking was studied in watermelon (*Citrullus lanatus*), namely cutin related genes, *GPAT*, *DHDDS* and *SULTR3* (Table 2) and genes involved in cuticular waxes biosynthesis, *SMT* and *KCS* (Table 3), using a cracking-resistant and a cracking-susceptible watermelon cultivars (Jiang et al., 2019b). The results show that the *DHDDS* and *SULTR3* genes are upregulated in the cracking-resistant watermelon, while *GPAT*, *SMT* and *KCS* genes are downregulated in cracking-susceptible watermelon (Jiang et al., 2019b).

In litchi (*Litchi chinensis*), the pericarps of fruits without cracks from cultivars with different cracking susceptibilities and cracked fruits present differences in cuticle structure as a result of an upregulation of genes related to fatty acids such as *LOX*, *MOD* and *AOS* (Table 3) (Wang et al., 2019a) as well as a downregulation of lipid synthesis genes, like *ACP*, which is responsible by a low pericarp mechanical strength (Wang et al., 2021a). Moreover, the upregulation of *AOS* gene is found in cracked fruits (Wang et al., 2019b).

The expression of genes related to cutin in tomato, like *LACS1* (long-chain acyl-CoA synthase 1), *CUS1* (cutin synthase) and *GPAT4* (glycerol-3-phosphate acyltransferase 4), decreases during the fruit development and is higher in fruits under water stress, being consistent with the developmental regulation of cuticle (Table 2) (Romero and Rose, 2019). Further evidence of the relationship between fruit cracking and properties and composition of the cuticle in tomato is provided by Vogg et al. (2004), where the mutation of the *CER6* gene (β-ketoacyl-CoA synthase) leads to an alteration of the cuticular wax composition and increases water permeability (Hovav et al., 2007). Additionally, the genes *TTS2* (triterpene synthase 2) and *CD3* (cutin deficient 3) beyond *CER6* (eceriferum 6), all involved in wax biosynthesis, transport, deposition and regulation, decrease their expression during the fruit development and are higher in fruits under water stress, suggesting that water availability affects the cuticle properties (Table 3) (Romero and Rose, 2019). Moreover, the *Cwp1* gene (cuticular water permeability) when expressed leads to fruit dehydration and consequently causes microcracks in tomato cuticle (Hovav et al., 2007). The higher expression of *FNC* gene is responsible by cracking all over the tomato pericarp, whose expression affects other cracking related genes, namely by increasing the expression of genes involved in lipid metabolism such as *GDSL*, *KCS* and *FAR*, and lipid transport like *NLTP9* and *LTP5*, affecting the cuticle elasticity and wax content and thud, cracks appear in fruit surface (Table 3) (Zhang et al., 2021b).

Concerning to genes related to wax formation in grape, Martins et al. (2020) verified a downregulation of one E3 ubiquitin ligase (*CER9*) when calcium was applied in the cultivar Vinhão (Table 3).

In last years, several studies related to biosynthesis of cuticular waxes and related genes in jujube have been developed (Li et al., 2020; Liu et al., 2020; Li et al., 2021b; Li et al., 2021c). A RNA-Seq analysis, using fruits with and without cracking, to analyze the genes differentially expressed in cracked and non-cracked jujube fruits, allowed to find 785 up-regulated and 251 down-regulated genes in cracked fruits, which are involved in several metabolic processes, namely surface wax production in cracked fruits (Liu et al., 2020). The expression of genes involved in fatty acid biosynthesis (*FAR2*) and fatty acid elongation (*KCS1* and *KCS12*) was studied in jujube fruits at different maturation stages, namely white-ripe, coloring, and full-red development stages, collected from cultivars with different cracking susceptibilities (a highly cracking-resistant cultivar Popozao, a cracking-resistant cultivar Banzao, and a cracking-susceptible cultivar Hupingzao), showing higher expression for *FAR2* and *KCS12* genes in coloring stage, being higher in the more resistant cultivar, and lower in the more susceptible cultivar while *KCS1* gene presents similar expression during fruit development and for all cultivars (Li et al., 2020). Moreover, the 3-ketoacyl-CoA synthase (*KCS*) gene, related to the synthetic pathway of cutin, is highly upregulated in cracked fruits, which leads to alterations in biosynthesis in cuticle wax and, consequently, jujube cracking (Table 3) (Liu et al., 2020). Furthermore, Li et al. (2020) also analyzed the genes involved in fatty acid degradation, namely *ALD1*, *ALD4* and *ALDH3F1* genes, whose expression of *ALD1* and *ALDH3F1* genes is upregulated in coloring stage in cracking-resistant cultivar while the expression of *ALD4* gene is upregulated in cracking-susceptible cultivar at the same stage (Table 3). Likewise, a transcriptomic analysis carried out by Li et al. (2021c) to access the wax metabolism pathways in jujube using RNA from fruit pericarp at different maturation stages of a cracking-resistant cultivar and a cracking-susceptible cultivar allowed to identify different metabolic pathways related to wax metabolism, namely fatty acid biosynthesis, fatty acid metabolism and cutin, suberin and wax biosynthesis. All identified genes, in general, increase their expression during fruit development; however, genes *CER1L1* (eceriferum 1-like), *BCCP2* (biotin carboxyl carrier protein of acetyl-CoA carboxylase 2, chloroplastic), *ACC1L* (acetyl-CoA carboxylase 1-like) and *CYP86A22* (cytochrome-P450 86A22) have more expression in cracking-resistant cultivar, while genes *accC2* (biotin carboxylase 2, chloroplastic) and *fabZ* (3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ-like) present higher expression in cracking-susceptible cultivar (Table 3) (Li et al., 2021c). Concerning to genes involved in jujube wax biosynthesis (*CYP94A2*, *PXG4*, *CYP86A*, *CER1* and *CYP86B1*), in general, are higher in white-ripe period, however *PXG4*, *CYP86A*, *CER1* and *CYP86B1* genes maintain higher expression in the cracking-resistant cultivar than in the other cultivars in coloring stage (Table 3) (Li et al., 2020). *CER* genes represent a family of genes with an important role in waxes biosynthesis in jujube, being identified 29 candidate genes (named *CER1* to *CER29*), twelve of them present differences in expression among two cultivars with different susceptibilities to cracking (Li et al., 2021b). Among them, *CER7*, *CER14*, *CER15* and *CER16* genes have higher expression in cracking-susceptible cultivar, while *CER29* has more expression in cracking-resistant cultivar. In addition, *CER26* only has expression in cracking-resistant cultivar (Table 3) (Li et al., 2021b). In the biosynthesis of cuticular waxes, in general, the highly cracking-resistant cultivar present higher gene expression in coloring period while cracking-susceptible cultivars have higher expression in white-ripe, decreasing during fruit development and, thus, during the wax formation, the cracking-resistant cultivar synthesize more very-long-chain alkanes and aldehydes, accompanying the fruit surface enlargement, which reduces the jujube cracking (Li et al., 2020). The biosynthesis of jasmonic acid (JA) associated with α-linolenic metabolism (as the precursor of JA) is the main associated to cracking in which the allene oxide cyclase (*AOC*), allene oxide synthase (*AOS*) and 12-oxophytodienoate reductase 3 (*OPR3*) genes are upregulated, while lipoxygenase 2 (*LOX2*) gene is downregulated in cracked jujube fruits when compared with non-cracked jujube fruits (Table 3) (Liu et al., 2020).

Cracking-related genes involved in water transport, calcium transport and signaling, and starch and sucrose metabolisms

Aquaporins (AQPs) transport water, and water uptake is associated to rain-induced cracking in sweet cherries. A better knowledge about these proteins in exocarp and the involvement of AQPs in water penetration through microcracks as well as the transcriptional profile of the genes that codify AQPs can provide new finds about sweet cherry cracking (Chen et al., 2019b). Chen et al. (2019b) identified 25 putative aquaporins genes in sweet cherry, 16 of them express in fruit. An example of the aquaporin role in sweet cherry cracking was provided by Michailidis et al. (2021), who verified that the expression of aquaporin gene, *PIP2;1*, is upregulated in the skin of Early Bigi cultivar (Table 4). Moreover, Breia et al. (2020) found an upregulation of *PIP1;4* gene under pre-harvest application of $CaCl_2$ in cultivar Skeena, suggesting that this AQP is involved in water transport and, possibly, in crack prevention. Likewise, the aquaporin gene *PIP2A* (Table 4) is upregulated during apple development, which may prevent crack initiation (Joshi et al., 2018).

By a high-throughput RNA sequencing (RNA-Seq), the transcriptome of litchi pericarp revealed four genes (*AQP*, 1; *PIP*, 1; *NIP*, 1; *SIP*, 1) involved in water transport and 13 genes (*TPC*, 1; *Ca^{2+}/H^+ exchanger*, 3; *Ca^{2+}-ATPase*, 4; *CDPK*, 2; *CBL*, 3) involved in Ca transport (Table 4), whose expression present significant differences among cracked fruits and fruits without cracks (Li et al., 2014). Furthermore, a downregulation of calcium transport and signaling genes, like *CIPK*, *CML* and *CNGC* provoke a decrease of the mechanical strength of pericarp of a cracking-susceptible cultivar Nuomici (Wang et al., 2021a).

The genes involved in starch and sucrose metabolism pathways also appear to have an important role in atemoya cracking, since genes involved in starch synthesis such as *glgA*, *glgB*, *glgC* and *GBSS* are mainly downregulated in cracked fruits, while genes involved in starch degradation like *AMYG*, *PUL*, *AMY*, *BAM*, *glgX* and *glgP* are mainly upregulated in cracked fruits (Table 4) (Chen et al., 2019a).

By a transcriptomic analysis, a set of 12 cracking-resistant fruits and 12 cracking-susceptible fruits were analyzed to study the gene expression in both types of fruits, finding 218 upregulated genes and 173 downregulated genes, being the aquaporin *PIP* (involved in water absorption), *CALM* and *CALR* (both involved in calcium transport and regulation) genes (Table 4), the most related to jujube cracking (Ren et al., 2017). All these genes have higher expression in cracking-resistant fruits than in cracking-susceptible fruits, playing an important role in preventing cracking (Ren et al., 2017).

Cracking-related genes involved in fruit hormone metabolism

In sweet cherry, the *ABF2*, *ABF3*, *ABO5*, *ABI* and *FCA* genes, involved in abscisic acid metabolism, are downregulated in the cracking-susceptible-cultivar Early Bigi, while *ACS* and *ACO* genes, involved in ethylene biosynthesis, are upregulated in a cracking-moderate resistant cultivar Regina (Table 5) (Michailidis et al., 2021). As these plant growth regulators are directly involved in abiotic stress signaling they may pose a mechanistic mode of action to understand the environmental effects on fruit cracking in cherry.

During apple fruit development, the ABA signaling gene, *NAC058* (Table 5), is upregulated, and may prevent the cracking initiation (Joshi et al., 2018).

In watermelon, *CHDH* and *GST* genes (Table 5) are possibly related to hormone metabolism, whose expression of *GST* gene is upregulated in the cracking-resistant watermelon, while *CHDH* gene is downregulated in cracking-susceptible watermelon (Jiang et al., 2019b).

A balance among pericarp strength and aril expanding pressure can be responsible by litchi cracking, which can occur due an unbalance of plant hormone metabolisms (Wang et al., 2019b; Wang et al., 2021a). Different expression levels of genes related to hormone metabolism was described by Li et al. (2014), namely in five genes (*KS*, 2; *GA2ox*, 2; *GID1*, 1) involved in GA metabolism and 21 genes (*CYP707A*, 2; *GT*, 9; *β-Glu*, 6; *PP2C*, 2; *ABI1*, 1; *ABI5*, 1) involved in ABA metabolism in fruits with and without cracks (Table 5). Moreover, genes related to auxins (*GH3*, *IAA* and *ARF*), gibberellins (*GPRs*), and ethylene (*ACO* and *ACS*) are downregulated in litchi pericarp, while genes involved in auxin metabolism (*ILR*, *SAUR* and *ARF*) and ABA metabolism (*ZEP* and *PP2C*) are upregulated in litchi aril, leading to differences in fruit development and pericarp mechanical strength (Table 5) (Wang et al., 2021a). Using the transcriptomic and metabolomics analysis, Wang et al. (2019a); Wang et al. (2019b) and Wang et al. (2021a) suggested that the susceptibility to litchi cracking may be associated with the difference in hormone balance of the two analyzed cultivars, that is, differences in metabolism of IAA (indoleacetic acid, natural auxin), ABA (abscisic acid), ethylene, BR (brassinosteroid) and JA (jasmonic acid) once the different metabolites generated by different hormone metabolism are cultivar specific and have distinct expression patterns in the three types of litchi pericarps. Thus, changes in gene expression and metabolites can explain the cracking susceptibility, namely a downregulation of *OR1* and *OR3* genes, and *ACCS* and *H1* genes, involved in IAA and ethylene metabolisms, respectively, and an upregulation of *CYP707A* and *TC/M* genes involved in ABA and BR metabolisms, respectively, in cracking-resistant cultivar Feizixiao; in contrast, *AOS* (BR metabolism) gene present highest expression in cracked fruits from cracking-susceptible cultivar Baitangying (Table 5) (Wang et al., 2019a; Wang et al., 2019b). Also, in the ethylene metabolism associated to sucrose synthesis and sweetness increase, *aA* and *SS* genes are downregulated in a cracking-resistant cultivar while *TLP* gene is upregulated in cracked fruits from cracking-susceptible cultivar (Wang et al., 2019a; Wang et al., 2019b). Additionally, *GH3* gene, involved in IAA, is upregulated in cracking-resistant cultivar (Wang et al., 2019a; Wang et al., 2019b; Wang et al., 2021a).

Cracking in atemoya seems to have a close relation with phytohormones, so, several hormones related genes has been identified. The majority is related to auxin and ABA pathways, with 18 and 12 genes, respectively, while cytokinin pathway presented 6 genes, salicylic acid pathway comprised 5 genes, gibberellin and jasmonic acid pathways included 3 genes and ethylene pathway only presented one gene. Comparing with fruits without cracks, the auxin and jasmonic acid related genes were downregulated in cracked fruits, while ABA, cytokinin, gibberellin, ethylene and salicylic acid related genes were upregulated, showing that these genes can have an important role in cracking (Li et al., 2019).

Transcription factors genes involved in fruit cracking

It's known that different final products can be generated due to differential regulation by transcription factors according to environmental or development stimuli (Falginella et al., 2021).

In sweet cherry, *WINA* and *WINB* genes encode AP2/EREBP-type transcription factors, regulating several genes involved in cutin and wax biosynthesis (Table 6) (Alkio et al., 2012; Balbontín et al., 2014). According to Alkio et al. (2012), these genes present higher expression in the initial stage of fruit development. Balbontín et al. (2014) obtained similar results for *WINB*, finding higher expression levels in the beginning of fruit development in a cracking-resistant cultivar Kordia. This indicates that *WINB* can influence the expression of other cuticular waxes related genes during the fruit growth.

Likewise, a down-regulation of *SHN3* leads to low cutin and wax contents and, consequently, to microcracks development in apple (Table 6) (Straube et al., 2021). *SHN3* expression compromises cuticle formation, being considered an essential regulator of apple cuticle biosynthesis (Lashbrooke et al., 2015). Similar results were obtained by Falginella et al. (2021) in which high levels of *SHN3*, are related to high cutin and wax contents, and low russet development, while low levels of *SHN3* transcripts leads to a decrease in cutin and wax contents as well as microcracks development and consequently, high russet development. In contrast, a lower expression of transcription factor *MYB93*, related to suberin and lignin synthesis, leads to low suberin content, resulting in low russet development, while a higher gene expression promotes an increase in suberin, allowing the microcracks development and, consequently, high russet development (Falginella et al., 2021) as also described by Straube et al. (2021) for the *MYB93* and *MYB42* genes (Table 6).

The ethylene-responsive transcription factor 4, *ERF4*, is considered the major gene underlying watermelon rind hardness regulation and thus an important factor in cracking resistance. However, beyond the function of *ERF4* in rind hardness variability and consequently in cracking resistance, their expression can be affected by the regulation of other genes such as genes involved in cell wall modification and/or degradation as well as genes related to lignin biosynthesis (Liao et al., 2020). Moreover, *MDP* gene is upregulated in the cracking-resistant watermelon, while the expression level of *HD-ZIP* in cracking-resistant watermelon is lower than in cracking-susceptible watermelon (Jiang et al., 2019b).

In litchi, a decrease of the pericarp mechanical strength can occur due a downregulation of transcription factor genes, such as *WRKY*, *bZIP*, *bHLH* and *MYB* causing a development retardation in fruit and (Table 6) (Wang et al., 2021a). In contrast, an upregulation of transcription factor genes, like *WRKY*, *bHLH*, *DOF* and *MYB* cause an upregulation of starch/sucrose metabolism related genes and sugar/water transport and, thus, differences in mechanical strength of pericarp (Wang et al., 2021a).

Other potential genes/pathways involved in fruit cracking

During apple development, Joshi et al. (2018) found a significant upregulation in the majority of the genes during fruit development, suggesting that a high expression of cuticle-related genes can prevent crack initiation and possibly enhancing cuticular cracking repair.

Atemoya cracking can occur due to an unbalance among genes involved in hormone metabolisms (Li et al., 2019) as well as genes involved in starch metabolism and genes related to cell wall, whereby the transformation of starch into soluble sugars leads to an increase in turgor pressure and, consequently, in cells and tissues rupture. At the same time, the pectin and cellulose degradation decreases cell wall toughness, which together with starch metabolism leads to cracking development in the fruit pericarp (Chen et al., 2019a).

Furthermore, Zhang et al. (2021a) carried out an enrichment analysis during veraison and maturity stages using grapes that were treated with calcium as cracking mitigation strategy, to analyze the transcriptome and secondary metabolites in grape berry cracking. The enrichment analysis under the application of calcium sprays revealed that the main DEGs are related to flavone and flavonol biosynthesis pathway and also to flavonoid metabolism pathway, meaning that the balance of up and down regulation of genes involved in both pathways determine the grape berry cracking rate, and thus, allowing to identify new pathways and genes involved in grape berry cracking and also explain the role of calcium sprays in modulating these pathways and their effect in reducing cracking rate (Zhang et al., 2021a). However, Zhang et al. (2021a) suggest that other metabolic pathways can be involved in grape berry cracking.

In jujube, Hou et al. (2022) used cracked and non-cracked fruits of two cracking-susceptible cultivars, and non-cracked fruits of a cracking-resistant cultivar to elucidate cracking-related molecular mechanisms. Comparing samples from the cracking-resistant cultivar to samples with and without cracking of each cracking-susceptible cultivar, the authors found several cracking related genes which are involved in different metabolic pathways, namely in water transport, cell wall metabolism, starch and sucrose metabolism, cuticle structure, calcium transport, ABA metabolism, indoleacetic acid metabolism, jasmonic acid metabolism, gibberellic acid metabolism and transcription factors (Hou et al., 2022). In general, all cracking related genes involved in these pathways are upregulated in cracked fruits, compared to the non-cracked fruits and, thus, the authors propose that the high expression levels in cracked fruits leads to an increase in the turgor pressure and a decrease in the exocarp mechanical strength, which can lead to the fruit cracking development (Hou et al., 2022).

Importance for new molecular breeding

Genomics with the development of complete reference genomes, allows to find interesting molecular opportunities for the identification of candidate genes linked to agronomic traits (Bianchi et al., 2015; Soundararajan et al., 2019). Moreover, by the development of omics, such as metabolomics and transcriptomics, associated with whole-genome sequences provides great information about the molecular mechanisms in fruits (Zhang and Hao, 2020; Wang et al., 2021b). Thus, the combination of genomic, transcriptomic and metabolomic analyses can reveal important knowledge and genetic basis for crop's molecular breeding (Zhang and Hao, 2020). Although the gene expression can be affected by internal and external factors, by the combined information provided by different omics, it is possible to identify transcription factors and key genes of different biosynthesis pathways as well as to understand how environmental conditions affects the traits of interest at molecular level, and consequently improve fruit quality and molecular breeding programs (García-Gómez et al., 2020).

Fruit trees are economically important, but the lengthy life cycles of several years slow the study at the genetic level. However, molecular tools represent a good strategy to understand adaptation to abiotic stresses and environmental conditions (Licciardello et al., 2021). So, it's important to understand how thousands of genes can interact with each other as well as how the related metabolic pathways contribute to plant development and adaptation to the environment (Licciardello et al., 2021). Thus, the identification and characterization of genes controlling agricultural traits and tagging molecular markers constitutes advances for development of new breeding techniques (Soundararajan et al., 2019). In this follow up, the functional genomics in fruit trees has deployed several methodologies to improve the molecular breeding techniques in fruit crops, such as gene expression-based biomarkers, transcriptomic and metabolomics, whole-genome variations and sequence, among others (Licciardello et al., 2021). With the available information, several fruit quality traits can be improved, namely the development of new cultivars with small/larger size, good-flavored fruits, attractive color, sugar and acid levels, reduced juvenile phase, massive and constant yields, reduced susceptibility to fruit cracking, self-compatibility, and improved resistance or tolerance to disease as well as resistance to abiotic stresses like adverse environmental conditions (Soundararajan et al., 2019).

Regarding cracking, it's known that there are several factors that affect fruit cracking, such as physiological, genetics, environmental and postharvest storage (Khadivi-Khub, 2015; Wang et al., 2021b). Among the several mitigation strategies to prevent cracking, calcium is a key mineral in plant physiology (Winkler and Knoche, 2021). It plays an important role in the pre- and postharvest physiology of most plant and particularly of fruit, being considered a critical nutrient in determining fruit quality (Winkler and Knoche, 2019). For example, calcium application in grape berry appears to induce specific modifications both in skin and in pulp, changing the cuticle structure, playing an important role in preventing cracking (Martins et al., 2020) as well as the inhibition of cell wall disassembly promoting cell wall strengthening and, thus, calcium can prevent grape cracking (Yu et al., 2020). Likewise, the use of calcium to mitigate the risk of pre-harvest rain-cracking of sweet cherry increases the fruit quality, firmness and shelf-life, reducing the cracking susceptibly (Correia et al., 2019; Winkler and Knoche, 2019; Breia et al., 2020; Winkler et al., 2020; Correia et al., 2020a; Matteo et al., 2022). Furthermore, in sweet cherry, the combination of calcium with growth regulators highly reduce the cracking incidence, promoting differential gene expression of some exocarp related genes (Correia et al., 2020b). Thus, a better knowledge about the cuticle related genes can provide new insights about molecular mechanisms involved in cracking of flesh fruits. For example, the understanding of exocarp development in sweet cherry as well as the expression of exocarp-specific genes during fruit growth, maturation, softening, cuticle deposition and sugar transport, can provide great information about their role in conferring cracking resistance (Alkio et al., 2014). Likewise, in watermelon, the identification of genes related to rind hardness and the associated molecular markers can help to understand rind hardness and fruit cracking resistance and, thus, used in future breeding programs, by CRISPR-Cas9 or marker-assisted selection, to create more resistant cultivars (Liao et al., 2020). Moreover, in jujube, the knowledge about the differences in the cuticular wax and the expression of related genes, using cultivars with different susceptibilities to cracking, may provide new insights about prevent cracking (Li et al., 2014), which coupled by possible enhancing or silencing of related genes by gene modification technology, can change the cell wall structure and arrangement, and, thus, help in fruit cracking prevention (Hou et al., 2018).

Actually, the effects of climate changes, like excessive rains, leads to different physiological responses of the crops, affecting fruit growth, development and quality (Hirpo and Gebeyehu, 2019). The fruit ripening, that is, from a green fruit to a ripe fruit, represents a synchronized process with changes in physiological structure and biochemical composition according to interactions among fruits and their environment (García-Gómez et al., 2020). Fruit cracking emerges as a physiological disorder during fruit development as response to genetic or environmental factors (Wang et al., 2021b). The fruit development is controlled by expression of several genes (polygenic expression regulated by hundreds to thousands of genes), with different associated molecular mechanisms. These include biochemical, transcriptional, hormonal or metabolites levels. Thus, when physiological disorders appear, like cracking, the different underlying mechanisms make it challenging to study (García-Gómez et al., 2020). However, in general, an increase of expression of genes related to cell wall mechanisms arises in cracking-resistant cultivars of sweet cherry (Balbontín et al., 2014; Michailidis et al., 2021), apple (Kasai et al., 2008; Joshi et al., 2018) or watermelon (Jiang et al., 2019b). This indicates a common mechanism causing cracking. Likewise, an upregulation of cuticular waxes related genes in apple (Gao et al., 2021; Straube et al., 2021), watermelon (Jiang et al., 2019b) or jujube (Li et al., 2021c) is also correlated with a decrease of cracking index. Moreover, an unbalance of genes related to hormone metabolisms can increase the cracking susceptibility in litchi (Wang et al., 2019a; Wang et al., 2019b; Wang et al., 2021a) and in atemoya (Li et al., 2019). Hou et al. (2022) reported several metabolic pathways involved in cracking jujube fruits, highlighting the complexity of this disorder.

Thus, the combination of genomic, transcriptomic and metabolomic analyses can reveal important knowledge and genetic basis for crops molecular breeding (Zhang and Hao, 2020). Moreover, the understanding about the differences in gene expression during fruit development elucidates the molecular mechanisms of cuticle related genes, playing important roles in preventing or enhancing fruit cracking (Hou et al., 2018).

Conclusion

Although fruit cracking remains a great challenge to the producers, it is known that the genetic factors play a crucial role in its development. The genetic component makes cracking an attractive field for researchers who work with molecular breeding. The development of different omics technologies, open news perspectives to understand how this disorder occurs at molecular level. The molecular mechanisms involved in cracking are based on correlations as direct proof of concept based on mutations or reverse genetics are still missing. It is known that exocarp-specific transcripts play a crucial role in cracking development, namely genes involved in cuticular membrane cuticular, cell wall mechanisms or cuticular wax biosynthesis. Additionally, by the analysis of the metabolome, which is closely related to phenotype, the regulation of several metabolic pathways can affect the expression of exocarp-specific genes and, consequently, affects the development of fruit cracking. This physiological disorder is enhanced by environmental conditions, such as high temperatures and heavy rain. The scenario of climate change, foreseen for the future, makes it even more urgent to understand the responses of plants to stress (simple or combined), at various levels, especially at the transcriptomic level. Many genes are repressed or induced in stress response, involving a precise regulation of complex stress-gene networks. It is therefore crucial to understand the function of the genes involved, to determine the functional relationships between genes and how they are affected by biotic or abiotic factors. Obtaining a set of candidate genes for molecular breeding programs by genome editing technologies should bring forward crop performance under changing environmental conditions.

Author contributions

MS, ME-C, BG and MM contributed to the conceptualization of the review. MS wrote the first draft of the manuscript. ME-C, BG and MM supervised, reviewed and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The roles of plant proteases and protease inhibitors in drought response: a review

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Upon exposure to drought, plants undergo complex signal transduction events with concomitant changes in the expression of genes, proteins and metabolites. For example, proteomics studies continue to identify multitudes of drought-responsive proteins with diverse roles in drought adaptation. Among these are protein degradation processes that activate enzymes and signalling peptides, recycle nitrogen sources, and maintain protein turnover and homeostasis under stressful environments. Here, we review the differential expression and functional activities of plant protease and protease inhibitor proteins under drought stress, mainly focusing on comparative studies involving genotypes of contrasting drought phenotypes. We further explore studies of transgenic plants either overexpressing or repressing proteases or their inhibitors under drought conditions and discuss the potential roles of these transgenes in drought response. Overall, the review highlights the integral role of protein degradation during plant survival under water deficits, irrespective of the genotypes' level of drought resilience. However, drought-sensitive genotypes exhibit higher proteolytic activities, while drought-tolerant genotypes tend to protect proteins from degradation by expressing more protease inhibitors. In addition, transgenic plant biology studies implicate proteases and protease inhibitors in various other physiological functions under drought stress. These include the regulation of stomatal closure, maintenance of relative water content, phytohormonal signalling systems including abscisic acid (ABA) signalling, and the induction of ABA-related stress genes, all of which are essential for maintaining cellular homeostasis under water deficits. Therefore, more validation studies are required to explore the various functions of proteases and their inhibitors under water limitation and their contributions towards drought adaptation.

KEYWORDS

plant proteases, protease inhibitors, proteolysis, protein degradation, drought stress, drought response, comparative proteomics, protein homeostasis

1 Introduction

Plants require an optimum supply of light, water, temperature, and mineral nutrients for normal growth and development (Taiz and Zeiger, 2012). In nature, however, plants are often exposed to diverse abiotic stresses, including drought, high salinity, extreme temperatures, chemical toxicity and nutrient deficiency, which negatively affect their survival (Wang et al., 2003; Mahajan and Tuteja, 2005). In the case of crops, these stress factors may reduce yield, causing negative impacts on food supply chains (Mittler, 2006). Among the environmental stresses, drought is the major limiting factor of crop production worldwide (Farooq et al., 2009; da Silva et al., 2013), and its effects are further exacerbated by climate change and global warming. As climate models continue to predict the occurrence of frequent and severe drought episodes, more famines are likely to be experienced in drought-prone areas (IPCC, 2007; Nelson et al., 2009).

Plants are exposed to water deficit stress when rainfall declines during the growing season and when the rate of transpiration exceeds that of water absorption by roots due to hot and dry conditions (Turner and Begg, 1981; Bray, 1997). Consequently, osmotic stress develops, causing adverse effects on plant physiology, metabolism, and growth patterns (Taiz and Zeiger, 2012). For example, water deficit disrupts cell structure and function, including membrane integrity, photosynthesis, respiration, and growth processes (Anjum et al., 2011; Kumar et al., 2018; Kapoor et al., 2020). The extent of such effects, however, depends on the duration and severity of the water limitation, the plant species, genotype and/or developmental stage (Mullet and Whitsitt, 1996; Bray, 1997; Anjum et al., 2011), and whether water scarcity occurs in combination with other biotic and/or abiotic stresses (Rizhsky et al., 2004; Suzuki et al., 2014; Zandalinas et al., 2018). Nonetheless, plants have developed various mechanisms to cope with the prevailing environmental stresses to maintain cell structure, function and growth (Xiong and Zhu, 2002; Farooq et al., 2009; Shao et al., 2009; Osakabe et al., 2014).

One of the earliest responses of plants to dehydration stress is stomatal closure which is induced by the phytohormone abscisic acid (ABA) (Zhang et al., 2006; Lim et al., 2015; Kuromori et al., 2018). ABA is synthesised in roots and leaves in response to reduced water content in drying soil (Davies and Zhang, 1991; Taiz and Zeiger, 2012). As stomata close, transpiration water loss is reduced, and water is conserved. However, stomatal closure also restricts the absorption of CO_2 required for photosynthesis and the uptake of nutrients for plant growth (Basu et al., 2016). The reduction in CO_2 absorption and assimilation leads to an electron-rich environment in cells that is conducive for increased accumulation of reactive oxygen species (ROS) (Salehi-Lisar and Bakhshayeshan-Agdam, 2016), causing oxidative stress (Gill and Tuteja, 2010; Sharma et al., 2012), a secondary effect of many environmental stresses, including drought (Levitt, 1980a; Levitt, 1980b). If ROS molecules are not maintained at relatively low levels and/or effectively detoxified, they may damage lipids, nucleic acids and proteins, causing catastrophic disruptions to cell structure and metabolism (Wang et al., 2003).

To mitigate the adverse effects of both osmotic and oxidative stresses on cells, plants accumulate or activate a variety of stress-related genes, proteins, and metabolites through changes in cellular metabolism (Xiong and Zhu, 2002; Shao et al., 2009). Some of these molecular changes are mediated by ABA *via* the ABA-dependent pathway of stress response, while others form part of the ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2007; Yoshida et al., 2014), and have various signalling, gene regulatory and protective functions (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2007). Ultimately, cellular homeostasis is maintained to promote plant survival under stressful conditions (Xiong and Zhu, 2002; Vinocur and Altman, 2005).

Apart from ABA, other phytohormones such as jasmonic acid, salicylic acid, ethylene, auxins, gibberellins, cytokinins, brassinosteroids, and small molecular peptides also mediate plant responses to drought through complex interactions of their signalling pathways (Clarke and Durley, 1981; Hale and Orcutt, 1987; Ullah et al., 2018; Jogawat et al., 2021; Salvi et al., 2021; Iqbal et al., 2022). The crosstalk between phytohormones may have positive or negative effects on the interacting hormones and their ultimate effect in alleviating drought stress (Ullah et al., 2018; Jogawat et al., 2021). Furthermore, the biosynthesis, catabolism, and transport of phytohormones, and their conversion between bioactive, inactive, and storage forms also influence how plants reprogram growth and developmental processes to survive drought stress (Clarke and Durley, 1981; Hale and Orcutt, 1987). For example, the levels of ABA, auxins, salicylic acid, jasmonic acid, brassinosteroids, and ethylene may increase in response to water deficits to facilitate stomata regulation of transpiration, osmotic adjustment, ROS scavenging, and increased root growth.

Conversely, the contents of gibberellins and cytokinins tend to decline under drought, and these hormones have opposite effects on stomata conductance and shoot and root meristem activity compared to ABA (Clarke and Durley, 1981; Hale and Orcutt, 1987; Ullah et al., 2018; Salvi et al., 2021; Iqbal et al., 2022). Furthermore, a decline in gibberellins content in plants under drought results in the accumulation of growth-repressor proteins such as DELLA and the subsequent development of growth-retarded plant phenotypes that are more tolerant to drought (Achard and Genschik, 2009; Salvi et al., 2021). Likewise, drought-induced reduction in cytokinins is associated with shoot growth inhibition and enhanced root growth facilitating water absorption from drying soils (Salvi et al., 2021). Collectively, phytohormone interactions in plants under drought stress modulate plant responses through complex morpho-physiological and molecular mechanisms, including transcriptional regulation of drought stress-related genes to maximise survival.

Research on plant responses to drought has increased exponentially in the last two decades, as evidenced by the growing number of related publications available on the PubMed database https://pubmed.ncbi.nlm.nih.gov/. In addition, genomes of over 788 different plant species (Sun et al., 2022), including those of the model plant Arabidopsis (*Arabidopsis thaliana*) (The Arabidopsis Genome Initiative, 2000), and major crops (Bolger et al., 2014) have been sequenced. Several reviews have discussed the applications of genome sequences (Edwards and Batley, 2010; Bolger et al., 2014) and genome editing tools (Arora and Narula, 2017; Bao et al., 2019; Zhang et al., 2020) for crop improvement, including drought resilience. Furthermore, genome sequence data are invaluable reference tools for high-throughput "omics" studies involving many aspects of plant biology. Consequently, innumerable transcriptomics, proteomics, and metabolomics studies on plant responses to drought have been published and reviewed (Cramer et al., 2011; Ngara and Ndimba, 2014; Shanker et al., 2014; Barkla, 2016; Ngara et al., 2021; Singh et al., 2022; Thanmalagan et al., 2022). In addition, the generalised functional catalogue of plant genes outlined by Bevan et al. (1998) has become an invaluable tool for assigning putative roles to drought-responsive proteins identified in proteomics studies (Nouri and Komatsu, 2010; Aranjuelo et al., 2011; Zhao et al., 2011; Mohammadi et al., 2012a; Wang et al., 2014; Tamburino et al., 2017; Goche et al., 2020).

Undoubtedly, the drought models employed in such studies are quite diverse, and so are the results generated (Osmolovskaya et al., 2018). Nonetheless, "omics" studies continue to broaden our understanding of how plants reprogram cellular metabolism to maximise survival under unfavourable environmental conditions (Kido et al., 2016; Ngara et al., 2021; Baldoni, 2022; Rakkammal et al., 2022; Singh et al., 2022). Furthermore, comparative proteomics studies of plant genotypes with contrasting drought phenotypes provide new insights into drought response mechanisms (Barkla, 2016). For example, common plant responses to water deprivation between drought-tolerant and sensitive cultivars include the down-regulation of metabolism-related proteins, possibly as an energy-saving mechanism, and the up-regulation of defence-related proteins for protective functions (Ford et al., 2011; Jedmowski et al., 2014; Faghani et al., 2015; Cheng et al., 2016; Wu et al., 2016; Zeng et al., 2019; Goche et al., 2020; Moosavi et al., 2020). Conversely, increased alternative splicing events (Fracasso et al., 2016) and higher constitutive expression of secondary metabolism, redox homeostasis, and translation-related genes (Fracasso et al., 2016; Azzouz-Olden et al., 2020) possibly contribute towards the drought-superior traits of some varieties. These tolerant varieties also exhibit greater drought-induced accumulation of proteins involved in signal transduction, osmolyte biosynthesis, transcription, translation, and several protective roles such as antioxidant enzymes, dehydrins, late embryogenesis abundant proteins, chaperons, and regulators of proteolysis (Wang et al., 2015; Cheng et al., 2016; Chmielewska et al., 2016; Goche et al., 2020). Overall, such genes and proteins are pivotal during drought adaptation (Ingram and Bartels, 1996; Ramanjulu and Bartels, 2002) and could serve as potential biomarkers for crop improvement strategies (Barkla, 2016).

Here, we review the differential expression and functional activities of plant protease and protease inhibitor proteins under drought stress, mainly focusing on comparative studies of cereal crops involving genotypes of contrasting drought phenotypes. We further explore studies of transgenic plants that either overexpress or repress proteases or their inhibitors under drought conditions and discuss the potential roles of these transgenes in drought response.

2 Plant proteases and protease inhibitors

Plant proteases are proteolytic enzymes that hydrolyse peptide bonds in proteins and are found in various plant tissues and organs (Palma et al., 2002; Schaller, 2004; van der Hoorn, 2008; Sharma and Gayen, 2021). Their activities are tightly regulated through the transcriptional control of protease transcripts, post-translational modifications of their proenzymes, actions of endogenous protease inhibitors, and/or compartmentalization into organelles and cellular compartments to avoid random acts of protein degradation (Brzin and Kidrič, 1996; Vierstra, 1996; Diaz-Mendoza et al., 2016). For instance, plant proteases are located in the cytosol, chloroplasts, vacuoles, nuclei, endoplasmic reticulum, proteasome, mitochondria, and cell walls (Vierstra, 1996; Kidric et al., 2014; Diaz-Mendoza et al., 2016), and also secreted into the extracellular matrix (Ngara et al., 2018; Ngcala et al., 2020; Godson and van der Hoorn, 2021). Each of these cellular compartments may possess specialised proteolytic pathways. For example, in the cytosol, protein degradation is mainly carried out by the highly selective ubiquitin-proteasome system (UPS), which consists of ubiquitin, the proteasome and associated components (Hopkins and Huner, 2009; Xu and Xue, 2019). Other proteolytic machinery found in plants include the caseinolytic protease (Clp) system in plastids and mitochondria and the vacuolar processing enzymes in vacuoles (Kato and Sakamoto, 2010; Vaseva et al., 2012; Nishimura and van Wijk, 2015; van Wijk, 2015; Ali and Baek, 2020).

Proteases are structurally and functionally diverse and are classified based on their catalytic activity, such as aspartic, cysteine, serine and threonine peptidases (Callis, 1995; Schaller, 2004). Alternatively, proteolytic enzymes are grouped into endo- and exo-peptidases depending on the site of cleavage on the peptide chain (Palma et al., 2002; Schaller, 2004). Examples of endopeptidases include serine, cysteine, aspartic, threonine and metalloendopeptidases, while exopeptidases include aminopeptidases, dipeptidases, carboxypeptidase, didpeptidyl-peptidases, omega peptidases and peptidyl-dipeptidases (Beers et al., 2000; Palma et al., 2002; Vaseva et al., 2012; Kidric et al., 2014). Likewise, protease inhibitors are diverse small molecules of either protein or non-protein nature (Polya, 2003); and are located in various plant organs and cellular compartments (Mosolov and Valueva, 2011; Kidric et al., 2014). They are differentiated based on their reaction mechanism or the type of proteases they inhibit (Mosolov and Valueva, 2011; Kidric et al., 2014). Examples of endogenous plant protease inhibitors include phytocystatins, serpins, Kunitz protease inhibitors, Bowman-Birk inhibitors, a-amylase inhibitors, bifunctional trypsin inhibitors, metallocarboxypeptidase inhibitors, mustard trypsin inhibitors, and potato-type inhibitors (Mosolov and Valueva, 2011; Vaseva et al., 2012; Kidric et al., 2014; Hellinger and Gruber, 2019). Additional information and online resource tools for plant proteases and their endogenous inhibitors can be found in the listed databases (Table 1).

2.1 Putative protein substrates and functions of plant proteases

The mechanism of action of proteases, their inhibitors, and different proteolytic pathways in plants have been extensively reviewed elsewhere (Vierstra, 1996; Vaseva et al., 2012; Kidric et al., 2014) and can be accessed for in-depth reading. Other

TABLE 1 Online databases and resource tools for plant proteases and protease inhibitors.

Database/resource tool	Website	Supported information	References
MEROPS	http://merops.sanger.ac.uk	Proteolytic enzymes, their substrates and inhibitors	Rawlings et al. (2018)
PLANT-PIs	http://plantpis.ba.itb.cnr.it/	Proteases inhibitors and their genes in higher plants	De Leo et al. (2002)
PINIR	http://pinir.ncl.res.in	Potato type inhibitor-II proteins	Yadav et al. (2021)
ProtIdent	http://www.csbio.sjtu.edu.cn/bioinf/Protease/	Proteolytic enzymes	Chou and Shen (2008)

reviews have discussed proteolytic machinery in plastids, mitochondria and peroxisomes, as well as their roles in maintaining protein homeostasis, also called proteostasis in these organelles (Kato and Sakamoto, 2010; Nishimura and van Wijk, 2015; van Wijk, 2015; Nishimura et al., 2016; Nishimura et al., 2017; Sun et al., 2021). The extensive review by van Wijk (2015) also lists substrates of plastidal, mitochondrial and peroxisomal proteases and is recommended for further reading, together with other references therein. Readers are also encouraged to access the comprehensive review chapter by Kato and Sakamoto (2010) on major chloroplast proteases, their substrates and functions. Nishimura et al. (2016) also provide a comprehensive pictorial depiction of the different types of metallo, serine, and aspartic proteases in plastids of land plants, their suborganellar location, and apply case studies to illustrate how the major chloroplastic proteases maintain organellar proteostasis. These earlier reviews highlight the different types of proteolytic machinery in plant cells, organelles and compartments, as well as the integral role of proteolysis during protein processing, maturation and quantity and quality control.

A common notion from these reviews is the extensive diversity of plant proteases, their multiple protein substrates, subcellular locations, and specialised functions. These proteolytic systems also work in a coordinated manner to maintain protein homeostasis (Kato and Sakamoto, 2010). However, the identities of specific protein substrates and mechanisms of action for many of these proteases are only partially known (Kato and Sakamoto, 2010; van Wijk, 2015). Nevertheless, enormous progress has been made in profiling the physiological substrates of various plant proteases. For example, several studies have used *in vitro* recombinant proteins, mutant systems, substrate-trapping assays, and N-terminal degradome techniques coupled with quantitative proteomics and mass spectrometry technologies for substrate identification (Moberg et al., 2003; Tsiatsiani et al., 2013; Sako et al., 2014; Carrie et al., 2015; Galiullina et al., 2015; Opalinska et al., 2017; Moreno et al., 2018; Welsch et al., 2018; Rowland et al., 2022).

In a recent study, Rowland et al. (2022) used several mutant types, the terminal amine stable isotopic labelling of substrates (TAILS), and label-free proteomic methods to investigate the coordination between Clp and presequence protease (PreP) proteases in maintaining chloroplast proteostasis. The results showed synergistic interactions between the two protease systems, their effects on embryo lethality, and changes in N-terminal protein processing activities. Furthermore, the loss-of-function of the Clp systems resulted in the over-accumulation of chloroplast stromal chaperons such as heat shock protein 90 (HSP90), chaperon protein CLPB3, chloroplastic (CLPB3) and chaperonins (CPN60/10/20), which possibly re-fold and stabilise abnormally folded and aggregated proteins. However, none of these three chaperons was affected in the *prep1prep2* mutant plants relative to the wild-type (Rowland et al., 2022) further highlighting the different effects of proteases in plants. Using several *clp* mutants, co-immunoprecipitation, and proteomic analyses, Welsch et al. (2018) experimentally showed that phytoene synthase, an enzyme in carotenoid biosynthesis, is a substrate for the Clp protease system. Therefore, the authors argued that proteolysis is critical in ensuring quantity control of phytoene synthase for adequate carotenoid biosynthesis (Welsch et al., 2018).

We acknowledge that a complete listing of known substrates of various plant proteases is beyond the scope of the current review. However, below is a brief account of how plants maintain protein homeostasis using diverse proteolytic machinery and their substrates. In many cases, they operate in tandem to complete target protein degradation (Kato and Sakamoto, 2010; van Wijk, 2015). A summary list of protein substrates of selected plant proteolytic machinery is also presented in Table 2. For example, once imported into their target organelles, nucleus-encoded chloroplast and mitochondrial proteins are further processed through the proteolytic cleavage of chloroplast transit peptides (cTP) and mitochondrial targeting peptides (mTP) before maturation. These activities are carried out by the stromal processing peptidase (SPP) (Richter and Lamppa, 1999; Richter et al., 2005) and mitochondrial processing peptidase (MPP) (Ghifari et al., 2019) metalloproteases in chloroplasts and mitochondria, respectively or other yet to be identified peptidases (Rowland et al., 2022). In peroxisomes, the cleavable N-terminal peroxisomal targeting signal (PTS2) of nucleus-encoded proteins is cleaved off by the degradation of the periplasmic protein (Deg)15 proteases, while PTS1 in non-cleavable (Schuhmann et al., 2008). Deg proteases are ATP-independent serine endopeptidases with essential roles in protein quality control in nearly all organisms, including plants (Schuhmann et al., 2008; Schuhmann et al., 2012).

Schuhmann et al. (2008) experimentally verified that PTS2-containing presequences of glyoxysomal malate dehydrogenase, 3-keto-acyl-CoA thiolase, and a long-chain acyl-CoA synthetase 6 are substrates of Deg15. The cleaved cTP, mTP and PTS2 peptides are subsequently degraded by PreP (Moberg et al., 2003; Kmiec and Glaser, 2012) or organellar oligopeptidase (OPP) (Ghifari et al., 2019) to prevent the accumulation of potentially toxic non-functional peptides and to facilitate amino acid recycling (van Wijk, 2015; Rowland et al., 2022). Other *in vitro* studies using recombinant proteins have shown that various Deg family proteins may be involved in maintaining chloroplast proteostasis by degrading thylakoid lumen proteins such as plastocyanin and the oxygen-evolving complex (OEC) protein 33 of the photosystem II (PSII), and heat stressed or photo-damaged D1 reaction centre protein (Kato and Sakamoto, 2010; van Wijk, 2015). Chloroplasts and mitochondria also contain other specialised endopeptidases, such as the type-I signal peptidase family (SPase I) and the plastidal type-I signal peptidase (Plsp1), that process luminal and intermembrane precursor proteins by cleaving off signal peptides for full maturation and activation (Tuteja, 2005; Kato and Sakamoto, 2010). For example, Plsp1 is involved in the maturation of the translocon at the outer envelope membrane of chloroplasts, 75kDA (Toc75), a member of the protein translocation complex at the outer envelope membrane of plastids (Baldwin et al., 2005). Other chloroplast and mitochondrion-encoded proteins are further post-translationally modified by the proteolytic removal of an N-terminal methionine using methionine aminopeptidases (MAPs) to achieve normal protein function and leaf development (Ghifari et al., 2019).

Due to their wide structural diversity, substrate specificities, selectivity, and/or subcellular locations (Vierstra, 1996; Kato and Sakamoto, 2010; Vaseva et al., 2012; Kidric et al., 2014; van Wijk, 2015), plant proteases are involved in numerous functions during

TABLE 2 Examples of protein substrates for selected proteases and peptidases during plant growth and development.

Name	Class	Examples of substrates[1]	Physiological functions of proteolytic activity	References[2]
Clp	ATP-dependent serine-type protease	Phytoene synthase; molecular chaperons, CAO, various chloroplast proteins involved in protein homeostasis, photosynthesis, redox homeostasis, plastid biosynthesis pathway, and plastid gene expression	Chloroplast biogenesis, chloroplast housekeeping, protein homeostasis, regulation of chlorophyll b synthesis, protein quality control, folding, and activity, quantity control for adequate carotenoid biosynthesis	Kato and Sakamoto (2010); van Wijk, 2015; Moreno et al. (2018); Welsch et al. (2018); Liao and van Wijk (2019); Rodriguez-concepcion et al. (2019); Bouchnak and van Wijk (2021); Rowland et al. (2022)
Deg	ATP-independent serine-type protease	Thylakoid lumen proteins, plastocyanin, OEC33 of PSII; photo-damaged, heat and high light intensity pretreated D1 protein of the PSII	PSII repair cycle, response to photo-oxidative stress, maintenance of chloroplast homeostasis	Kato and Sakamoto (2010)
FtsH	ATP-dependent metalloprotease	D1 protein of PSII; oxidatively damaged membrane proteins, MPC4, Pam18-2, Tim17-2, oxidatively damaged mitochondrial proteins, Slp1& 2, GCD, several metabolism-related proteins	PSII repair cycle, plastid development and thylakoid membrane formation, Response to photo-oxidative stress, high light acclimation, senescence, thermotolerance	Kato and Sakamoto (2010); Opalinska et al. (2017)
Metacaspase9	Cysteine aspartate protease	Several substrates including PEPCK, LEAs, chaperons, proteases, protease inhibitors, HSPs, Toc159, protein synthesis-related proteins, metacaspase	PCD, maintenance of plant growth and development, regulation of cell metabolism, possible involvement in drought stress response	Tsiatsiani et al. (2013)
Icp55	N-terminal aminopeptidase	Several substrates including HSPs; antioxidant enzymes, proteases, PRR proteins, ATP synthases, metabolism and protein synthesis-related proteins	Secondary processing of mitochondrial presequences; maintenance of protein stability in mitochondria	Carrie et al. (2015)
Oct1 (or MIP)	N-terminal aminopeptidase	PRR proteins, valyl-tRNA synthetase, PRORP1, PMH1, PMH2, B13 NADH complex, glycine-tRNA synthetase	Secondary processing of mitochondrial presequences; maintenance of protein stability in mitochondria	Carrie et al. (2015)
Phytaspase	Subtilisin-like serine-dependent protease	Various peptide-based caspase substrates	PCD triggered by biotic and abiotic factors such oxidative and salt stresses	Galiullina et al. (2015)
Plsp1	Serine endopeptidase	Toc75, OEC33	Biogenesis of chloroplast internal membranes, protein maturation	Kato and Sakamoto (2010)
PreP	Metalloendopeptidase	Cleaved cTP, mTP and PTS2 peptides	Prevention of over-accumulation of potentially toxic non-functional peptides; amino acid recycling	Moberg et al. (2003); Kato and Sakamoto (2010); Kmiec and Glaser (2012); van Wijk (2015); Rowland et al. (2022)
26S proteasome	ATP-dependent protease complex	DELLA, JAZ, LTA2, PDH E1α; various unimported or misfolded	Regulation of phytohormone activity and signaling. Degradation of unimported and	Achard and Genschik (2009); Sako et al. (2014)

(Continued)

TABLE 2 Continued

Name	Class	Examples of substrates[1]	Physiological functions of proteolytic activity	References[2]
		plastid precursor proteins in the cytosol	misfolded plastid precursors to avoid cytotoxicity, maintenance of cellular homeostasis	
VPE	Cysteine-type endopeptidase	Proprotein precursors of various vacuolar proteins such as seed storage proteins, hydrolytic enzymes, protease inhibitors, stress proteins e.g. chitinases	Maturation and activation of various vacuolar proteins, mediation of PCD, production of cyclic peptides due to peptide ligating activity, supports plant development and responses to environmental stresses, processing of seed storage proteins, and hydrolytic enzymes, protease inhibitors	Yamada et al. (2020)

[1]To manage long lists of protein substrates from the reviewed research paper, major functional groups of substrates are given; [2]The list of protein substrates was retrieved from both research papers and reviews.
Caspases-cysteine-dependent death and inflammation-related proteases of animal origin; CAO, chlorophyll a oxidase; Clp, caseinolytic protease; cTP, chloroplast transit peptide; Deg, degradation of periplasmic proteins; FtsH, filamentous temperature-sensitive H; HSPs, heat shock proteins; Icp55, intermediate cleaving peptidase of 55 kDa; JAZ, jasmonate ZIM-domain; LEA, late embryogenesis abundant; LTA2, nuclear encoded dihydrolipoamide s-acetyltransferase encoding the pyruvate decarboxylase E2 subunit; MIP, mitochondrial intermediate peptidase; MPC4, mitochondrial pyruvate carrier 4; mTP, mitochondrial targeting peptides; Oct1, octapeptidyl aminopeptidase 1; OEC33, oxygen-evolving complex protein 33; PAM18-2, presequence translocase-associated motor 18-2; PCD, programmed cell death; PDH E1α, pyruvate dehydrogenase E1 alpha subunit; PlsP1, plastidal type-I signal peptidase; PMH1, mitochondrial RNA helicase 1; PMH2, mitochondrial RNA helicase 2; PreP, presequence protease; PRORP1, proteinaceous RNase P1; PRR, pentatricopeptide repeat; PSII, photosystem II; PTS2, N-terminus peroxisomal targeting signal; Slp1, stomatin-like protein 1; Slp2, stomatin-like protein 2; TIM-17-2, translocase inner membrane subunit 17-2; Toc75, translocon at the outer envelope membrane of chloroplast 75; Toc159, translocon at the outer envelope membrane of chloroplast 159; VPE, vacuolar processing enzyme.

normal plant growth and development and in response to biotic and abiotic stresses (Figure 1). For instance, proteolytic activities are essential during nitrogen recycling and remobilisation, leaf senescence and programmed cell death, controlled degradation of damaged or abnormal proteins, activation and maturation of proteins and peptide hormones, seed germination, seedling growth, cellular housekeeping, regulating protein turnover, and in response to biotic and abiotic stresses (Brzin and Kidrič, 1996; Vierstra, 1996; Schaller, 2004; van der Hoorn, 2008; Hopkins and Huner, 2009; Kato and Sakamoto, 2010; Kidric et al., 2014; van Wijk, 2015; Diaz-Mendoza et al., 2016). On the other hand, protease inhibitors regulate the function of proteases by inhibiting their catalytic activity and may participate in plant defence and protective roles in response to biotic and abiotic stresses (Brzin and Kidrič, 1996; Mosolov and Valueva, 2005; Mosolov and Valueva, 2011).

2.2 Plant protease and protease inhibitor proteins under drought stress

2.2.1 Comparative proteomics studies

The involvement of plant proteases and protease inhibitors in drought response has been reported in various plant species using transcriptomics, proteomics and/or enzyme activity assays. Earlier reviews have also discussed the roles of plant proteases and their inhibitors under normal growth and in response to various biotic and abiotic stresses (Brzin and Kidrič, 1996; Mosolov and Valueva, 2011; Kidric et al., 2014; Godson and van der Hoorn, 2021), including senescence (Diaz-Mendoza et al., 2016). Xu and Xue (2019) reviewed the components of the UPS and their regulation in response to biotic and abiotic stresses. Others have outlined the potential function of plant proteases in signal transduction systems under environmental stresses (Sharma and Gayen, 2021), including

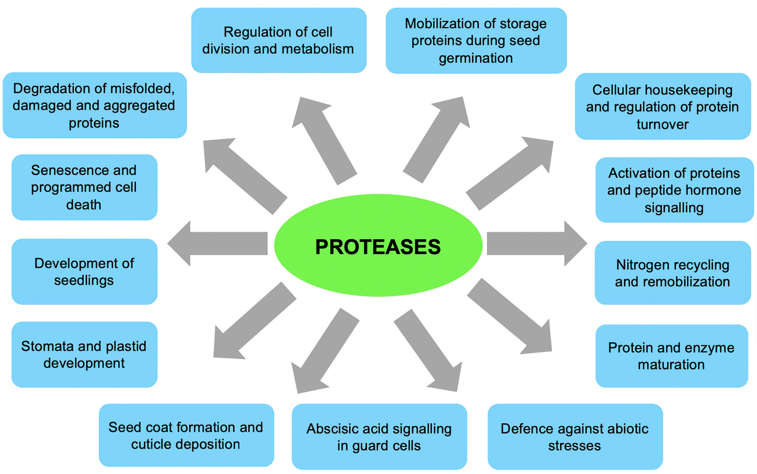

FIGURE 1
General functions of plant proteases in plant growth and development. (Brzin and Kidrič, 1996; Vierstra, 1996; Schaller, 2004; van der Hoorn, 2008; Hopkins and Huner, 2009; Kato and Sakamoto, 2010; Kidric et al., 2014; van Wijk, 2015; Diaz-Mendoza et al., 2016; Sharma and Gayen, 2021).

drought (D'Ippólito et al., 2021). Due to the extensive collection of "omics" studies available in the public domain, we opted to focus this review on the drought-induced expressional changes of protease and protease inhibitor proteins as depicted in comparative proteomic studies of cereal crops (Table 3). However, the general literature on proteomics approaches (Monteoliva and Albar, 2004; Wu et al., 2006; Abdallah et al., 2012; Zargar et al., 2016) and plant proteome analyses under drought stress and applications in crop improvement (Mohammadi et al., 2012b; Barkla, 2016; Wang et al., 2016; Ghatak et al., 2017) has been extensively reviewed elsewhere and will not be discussed in the current review.

Wu et al. (2016) used label-free and tandem mass tag multiplexing methods to analyse leaf proteome changes in two rice (*Oryza sativa*) varieties subjected to water deficits and re-watering. Amongst the observed unique proteome changes was the

TABLE 3 Studies showing differential accumulation and functional activities of protease and protease inhibitor proteins in plants under drought stress.

Plant species	Tissue	Genotypes	Proteomic methods	Differential expression of proteases and inhibitor proteins under drought stress	References
Sorghum					
	Roots	SA1441-tolerant ICSB338-suseptible	iTRAQ, LC-MS/MS, qRT-PCR	Up-regulation of peptidase C1A family in both varieties, aspartic peptidase A1 family peptidase in the susceptible variety, and proteinase inhibitor, potato inhibitor I in the tolerant variety.	Goche et al. (2020)
	Leaves	11434-tolerant 11431-sensitive	2D-DIGE, MALDI-TOF-MS	Up-regulation of aspartate protease, mitochondrial processing peptidase in the susceptible variety.	Jedmowski et al. (2014)
Rice	Leaves	IAC1131-tolerant Nipponbare-sensitive	SDS PAGE, Label-free, TMT and western blotting analyses	Up-regulation of ClpD1 protease in the drought tolerant variety.	Wu et al. (2016)
Wheat					
	Leaves	Xihan No.2-tolerant Longchun 23-sensitive	2D gel electrophoresis, MALDI-TOF/TOF MS	Up-regulation of proteasome subunit alpha type-7-A, proteasome subunit alpha type-1 and ATP-dependent Clp protease proteolytic subunit in the drought sensitive variety. The drought tolerant variety increased the abundance of proteasome subunit alpha type-2, 20S proteasome beta 7 subunit, aspartic proteinase nepenthesin-1 precursor and triticain alpha subunit.	Cheng et al. (2016)
	Leaves Roots	SERI M 82-tolerant SW89.5193-sensitive	2D gel electrophoresis, MALDI-TOF/TOF MS, qRT-PCR	Up regulation of proteasome alpha type protein in leaves of the drought sensitive variety.	Faghani et al. (2015)
	Leaves	Excalibur-tolerant RAC875-tolerant Kukri-sensitive	iTRAQ and LC-MS/MS	Up-regulation of type II metacaspase and leucine aminopeptidase in drought-tolerant cultivars.	Ford et al. (2011)
Barley					
	Leaves	15141-tolerant 15163-sensitive	2D gel electrophoresis, MALDI-TOF/TOF MS	Up-regulation of ATP-dependent Clp proteases and leucine aminopeptidase in both varieties, while zinc metalloprotease increased in abundance in the drought tolerant variety.	Ashoub et al. (2013)
	Leaves	XZ5-tolerant XZ54-sensitive	2D gel electrophoresis, MALDI-TOF-TOF, qRT-PCR	Up-regulation of FtsH protease 1 metallopeptidase in the drought tolerant genotype.	Wang et al. (2015)
	Leaves Roots	Cam/B1/CI-tolerant Maresi-sensitive	2D gel electrophoresis, MALDI-TOF-TOF	Up-regulation of ClpP protease in the leaves of the drought tolerant variety.	Chmielewska et al. (2016)

SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TMT, Tandem Mass Tags; iTRAQ, isobaric tags for relative and absolute quantification; LC-MS/MS, Liquid chromatography mass spectrometry; 2D-DIGE, two- dimensional difference gel electrophoresis qRT-PCR-quantitative real time polymerase chain reaction; MALDI-TOF-MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; Clp, caseiolytic protease; FtsH, filamentous temperature sensitive H; ATP, adenosine triphosphate.

increased accumulation of a ClpD1 protease in the drought-tolerant IAC1131 rice variety under severe drought conditions and its decreased accumulation upon re-watering. ClpD1 belongs to the caseinolytic protease (Clps) family of proteins (Kato and Sakamoto, 2010; Roberts et al., 2012; Vaseva et al., 2012; Nishimura and van Wijk, 2015) and is encoded by the drought-inducible gene *OsClpD1* in rice (Wu et al., 2016). Clp proteases are involved in degrading damaged proteins in the plastids (Kato and Sakamoto, 2010; Ali and Baek, 2020). Wu et al. (2016) suggested that the drought-induced accumulation of ClpD1 in the drought-tolerant variety increased the genotype's chances of coping with extreme drought conditions through the regulation of protein quality.

In a comparative gel-based proteomic study, an array of proteolysis-related proteins were up-regulated in leaves of wheat (*Triticum aestivum*) plants subjected to drought stress for 18, 24 and 48 hours (Cheng et al., 2016). For example, proteasome subunit alpha type-2, 20S proteasome beta 7 subunit, an aspartic proteinase precursor protein and a triticain alpha subunit were up-regulated in Xihan No. 2, a drought-tolerant wheat variety. Likewise, increased abundances of proteasome subunit alpha type-7-A, proteasome subunit alpha type-1 and an ATP-dependent Clp protease proteolytic subunit were observed in the drought-sensitive variety following water deprivation. The authors suggested that proteolysis is an essential mechanism for maintaining protein quality under stress, irrespective of a cultivar's degree of drought resilience. Furthermore, when coupled with adequate protein refolding by chaperons, proteolytic activity helps plants to maintain protein homeostasis under dehydration stress (Cheng et al., 2016). In another study, a proteasome alpha-type protein accumulated in leaves of a drought-sensitive wheat variety but not the drought-tolerant one upon exposure to water limitation (Faghani et al., 2015). Overall, the observations highlight the importance of selective protein degradation by the ubiquitin-proteasome system in plants under water deficit stress.

In another comparative gel-based proteomic study of sorghum (*Sorghum bicolor*) leaves, an aspartate protease and a mitochondrial processing peptidase were up-regulated in the drought-susceptible variety in response to drought stress (Jedmowski et al., 2014). Aspartic proteases are widely distributed within the plant kingdom and are involved in protein degradation during normal plant development, nitrogen recycling, programmed cell death, and stress response (Mutlu and Gal, 1999; Simoes and Faro, 2004). As discussed earlier, mitochondrial processing peptidases are involved in the removal of N-terminal signal peptides also called the presequence, from nuclear-encoded mitochondrial proteins during or after their import into the mitochondrion (Glaser et al., 1998; Kwasniak et al., 2012; Ghifari et al., 2019). As such, the observed drought-increased accumulation of aspartic protease could indicate high levels of degraded proteins in the susceptible variety, while the mitochondrial processing peptidase could be responsible for presequence removal (Jedmowski et al., 2014), and thus maturation of various newly synthesised mitochondrial precursors (Glaser et al., 1998; Gakh et al., 2002; van Wijk, 2015).

In one of our studies, Goche et al. (2020) conducted a comparative root proteomic analysis of two contrasting sorghum varieties subjected to limited watering. Among the observed differences in protein expression patterns were the up-regulation of proteolytic enzymes in ICSB338, the drought-sensitive variety, while the more drought-tolerant SA1441 accumulated proteins involved in transcription, protein synthesis, and the inhibition of protease activities (Goche, 2018; Goche et al., 2020). For example, dipeptidyl peptidase, serine-type, cysteine-type, and aspartic-type endopeptidases were up-regulated in the drought-susceptible sorghum variety upon exposure to drought stress. On the other hand, the drought-tolerant variety predominantly up-regulated protease inhibitors such as the proteinase inhibitor, potato inhibitor I (Goche, 2018; Goche et al., 2020). Although the significance of such contrasting proteome expression patterns warrants further functional validation studies, it is evident that the drought-resilient SA1441 reprograms gene expression, possibly to facilitate the accumulation of diverse stress proteins and protect them from proteolytic degradation. It is also plausible that the drought-sensitive sorghum variety requires extensive protein degradation to remove damaged proteins in many cellular locations and to recycle amino acids for targeted protein synthesis (Hopkins and Huner, 2009).

Ford et al. (2011) also conducted an extensive comparative leaf proteomic analysis of one drought-sensitive Kukri and two drought-tolerant RAC875 and Excalibur wheat cultivars in response to cyclic drought treatment. Leaf samples were subsequently taken during the initial period of water deficit, two wilting time-points, and following re-watering for relative water content (RWC) estimations and proteome analysis. As observed from the RWC results, this experimental system provided a platform for investigating complex physiological changes of wheat under repetitive cycles of water deficit and recovery, similar to those experienced under natural field conditions. Comparative analyses of the drought-responsive proteins were conducted at the four sampling time points using isobaric tags for relative and absolute quantification (iTRAQ) and tandem mass spectrometry. Overall, the results revealed complex proteome responses of wheat to the cyclic drought treatment, genotypic differences between cultivars irrespective of their levels of drought resistance, and some common trends in metabolic changes under conditions of water deprivation (Ford et al., 2011). For example, proteins related to photosynthesis and the Calvin cycle were down-regulated across the three cultivars, while ROS-scavenging enzymes and dehydrins were up-regulated.

The study also revealed increased accumulation of type II metacaspase and leucine aminopeptidase proteins in drought-tolerant cultivars, especially towards the later stages of water deprivation. However, after re-watering, the type II metacaspase protein levels reverted to control levels in both drought-tolerant cultivars but increased in the drought-susceptible cultivar (Ford et al., 2011). Conversely, the accumulation pattern of the leucine aminopeptidase protein was not consistent between the tolerant cultivars during the water deficit but increased upon re-watering in both cultivars. Generally, the study highlights the complexity of plant proteome responses during drought stress and recovery, the diversity of proteases and their selective functions during drought stress, and the differential responses of proteases in drought-stressed wheat plants with different drought tolerance levels. For example, since metacaspases play a role in programmed cell death

(Tsiatsiani et al., 2011), their early expression in drought-tolerant cultivars may implicate them in protein degradation processes that rapidly sacrifice some cells to ensure plant survival under stress (Ford et al., 2011). In addition, leucine aminopeptidases activate proteins and regulate protein turnover in plants (Walling and Gu, 1996; Matsui et al., 2006). As such, their increased accumulation upon re-watering in the drought-tolerant cultivars may indicate the importance of protein degradation, amino acid recycling and activation processes as cells reset their protein component during recovery from stress.

In another comparative proteomic study, proteases were differentially expressed in barley (*Hordeum vulgare*) plants subjected to drought stress (Ashoub et al., 2013). The study used gel-based proteomic methods to analyse the leaf proteome changes between accessions #15141 and #15163, which are tolerant and sensitive to drought, respectively. For example, two ATP-dependent Clp proteases were up-regulated in both varieties in response to drought stress. In contrast, a third ATP-dependent Clp protease and two leucine aminopeptidases were up-regulated only in the drought-susceptible accession, while a zinc metalloprotease had increased accumulation only in the tolerant accession. The authors also reported that the drought-susceptible variety showed an increased accumulation of proteases in drought response compared to the drought-tolerant variety, which accumulated more proteins involved in protective mechanisms against drought stress (Ashoub et al., 2013). Overall, the studies mentioned above emphasize the diversity of proteolytic enzymes, their selective specificities, and pivotal roles in various protein degradation processes during drought response in plants.

Although the current review aimed at surveying the drought-induced expression changes of protease and protease inhibitor proteins as depicted in comparative proteomic studies, we also scanned through a few similar studies on comparative transcriptomics to establish trends in transcript levels of these two groups of proteins. Indeed, the correlation between mRNA and proteins of various biological systems, including the human liver (Anderson and Seilhame, 1997), yeast (Gygi et al., 1999), and plants (Carpentier et al., 2008; Ponnala et al., 2014) has been debated for years, with poor correlation trends being attributed to various factors including the differential turnover rates of mRNA and proteins and posttranslational modifications of proteins (Abbott, 1999; Salekdeh et al., 2002; Vogel and Marcotte, 2012). Nevertheless, different "omics" technologies and the data thereof should be regarded as complementary, as each analytical technique may over and/or under-represent particular groups of biological molecules depending on their inherent characteristics (Carpentier et al., 2008). However, we found recent comparative transcriptomics studies that reported the drought-induced differential gene expression patterns of proteases and/or their inhibitors between plant genotypes with contrasting levels of drought tolerance (Zenda et al., 2019; Abdel-Ghany et al., 2020; Bhogireddy et al., 2020; Shivhare et al., 2020).

For example, Abdel-Ghany et al. (2020) conducted an extensive comparative transcriptome analysis between two drought-resistant and two drought-sensitive sorghum varieties in response to 20% polyethylene glycol (PEG)-induced osmotic stress. The results revealed differential expression of protein degradation-related transcripts between seedlings of the two groups of genotypes in response to 1 and 6 hours of osmotic stress treatment. For example, a cysteine protease gene and a senescence-associated gene were up-regulated in all four sorghum genotypes in response to the 1 hour and 6 hours of stress, respectively, highlighting the critical role of proteolysis and senescence during drought response in plants. In addition, following 1 hour of PEG treatment, a cysteine protease inhibitor and three cysteine protease genes were among the top 20 up-regulated genes in both drought-resistant sorghum varieties. However, at 6 hours, four seed storage protein-related protease inhibitors are down-regulated in the same drought-resistant varieties. Surprisingly, no protease or protease inhibitor genes were responsive to the PEG treatments at either time point in the drought-susceptible sorghum varieties. The authors suggested that these proteases and inhibitor genes possibly functioned in early-stress responses in the drought-resistant varieties (Abdel-Ghany et al., 2020).

Bhogireddy et al. (2020) also conducted a comparative leaf transcriptome analysis of two peanut (*Arachis hypogaea* L.) genotypes subjected to drought stress. The study reported a higher constitutive expression of several drought-responsive genes including protease inhibitors and senescence-related proteases in the drought-tolerant genotype than the sensitive one. Following water deprivation, both genotypes had increased expression of several proteolysis-related genes, but the change was generally much greater in the drought-susceptible variety. The authors suggested that high constitutive expression of drought-stress genes contributes towards the increased drought resilience of the drought-tolerant peanut genotype (Bhogireddy et al., 2020), and these findings are consistent with those reported in other drought transcriptomics studies of sorghum (reviewed in Ngara et al., 2021).

Protein degradation-related genes were also differentially expressed in maize (*Zea mays*) plants subjected to limited watering (Zenda et al., 2019). For example, several UPS-related genes were up-regulated in leaves of the drought-tolerant maize genotype following stress. Furthermore, a comparative analysis of transcripts between the drought-stressed samples of the susceptible genotype versus the tolerant genotype revealed an up-regulation of several ubiquitin-related and the really interesting new gene (RING) zinc-finger protein related genes. The authors suggested that protein ubiquitination and proteolytic processes are critical in facilitating protein turnover and homeostasis in plants under drought stress (Zenda et al., 2019). While the above account describes the trends in a few studies, future systematic reviews with meta-analyses of various studies must evaluate and synthesise the trends in constitutive and drought-induced expression of these proteolysis-related genes and proteins between multiple plants and genotypes.

2.2.2 Functional validation studies

Indeed, proteomics technologies continue to broaden our understanding of plant molecular changes under drought. Such alterations affect many functional classes of plant genes (Bevan et al., 1998), including protein degradation. However, expression proteomics data still needs to be validated to ascertain the biological functions of the identified stress-responsive proteins (Rabilloud and Lescuyer, 2014; Handler et al., 2018). Undoubtedly, such proteomic to biological inferences are laden with hurdles that have been critically reviewed by Rabilloud and Lescuyer (2014). Nevertheless, different system biology approaches (Kitano, 2002), such as western blotting, quantitative polymerase chain reaction (qPCR), and enzyme activity assays, are often used to validate quantitative proteomics data (Rabilloud and Lescuyer, 2014; Handler et al., 2018). Other functional validation approaches include transgenic plant systems that either overexpress or repress specific genes under altered environmental conditions (Oh et al., 2005; Kondou et al., 2010; Bolle et al., 2011; Rhee and Mutwil, 2014; Watanabe and Hoefgen, 2019). Therefore, here we discuss a few studies that have utilised protease activity assays in cereals and legumes (Table 4), and transgenic overexpression (Table 5) and knockout or knockdown mutant (Table 6) plant systems to elucidate the involvement and functions of proteases and/or protease inhibitors in plants subjected to drought stress. The reviewed transgenic studies are mainly on the model plant Arabidopsis. The study of plant gene function using gain-of-function or loss-of-function mutants, such as overexpression or knockout/down, has been reviewed (Kondou et al., 2010; Bolle et al., 2011). However, the pros and cons of the above-mentioned experimental validation systems are outside the scope of the current review and, thus, will not be discussed. Readers are directed to excellent reviews where such issues are critically discussed (Bajaj et al., 1999; Sharma et al., 2002; Bhatnagar-Mathur et al., 2008; Kondou et al., 2010; Bolle et al., 2011; Rabilloud and Lescuyer, 2014; Rhee and Mutwil, 2014; Khan et al., 2016; Handler et al., 2018).

2.2.2.1 Enzyme activity assays

Using a wide range of substrates and protease inhibitors, Hieng et al. (2004) evaluated the activities of different proteolytic enzymes in leaf extracts of common bean (*Phaseolus vulgaris*) cultivars subjected to water limitation. The cultivars used, Tiber, Zorin and Starozagorski exhibited various degrees of sensitivity to drought stress, with Tiber being more drought-tolerant. The results showed a general decrease in leaf protein content in all three cultivars, possibly due to increased proteolytic activity. However, the drought-tolerant cultivar exhibited the least protein content decrease and the least amount of proteolytic activity under severe drought stress. Activity assays for a 65 kDa serine protease with a pH optimum of 8.5 showed the greatest increase in the drought-susceptible varieties Starozagorski and Zorin, and a decrease in Tiber, the tolerant cultivar under severe stress. In contrast, the activities of two other serine proteases increased in all three cultivars, possibly illustrating the existence of a large group of proteases with different roles under drought stress in common beans. The study also reported increased activity of an aminopeptidase in the most drought-sensitive Starozagorski and a decrease in the drought-tolerant cultivar. Overall, the findings of the study support the notion of the presence of a wide range of proteolytic enzymes with different substrate specificities, subcellular locations and specific inhibitors in plants. The authors also suggested that regulation of serine proteases in the tolerant variety could guard against premature drought-included leaf senescence in common bean (Hieng et al., 2004).

In another study, Simova-Stoilova et al. (2010) investigated the activities of acidic proteases and aminopeptidases in winter wheat genotypes under drought and recovery at biochemical and transcriptional levels. The cultivars used were Katya (drought-resistant), Pobeda (cold-resistant) and Sadovo (disease-resistant), with Pobeda and Sadovo being more drought-sensitive than Katya. The results showed significant variations in leaf total protein content of control plants of all three cultivars. Furthermore, upon exposure to water limitation, the protein content of the drought-susceptible Pobeda and Sadovo varieties decreased by almost 50% relative to the respective controls but was restored after re-watering. On the other hand, minimal protein loss was observed in the

TABLE 4 Studies showing differential functional activities of proteases in plants under drought stress.

Plant species	Tissue	Genotypes	Functional assay	Differential functional activities of proteases under drought stress	References
Wheat	Leaves	Katya-resistant Pobeda-susceptible Sadovo-susceptible	Endopeptidase and aminopeptidase activity assays	Increased endopeptidase activity in all three wheat cultivars. The susceptible varieties exhibited the highest activity.	Simova-Stoilova et al. (2010)
Common bean	Leaves	Tiber-tolerant Starozagorski-susceptible Zorin-susceptible	Endoproteolytic and aminopeptidase activity assays	Serine protease activity highest in drought-susceptible varieties. Increased activity of aminopeptidase in Starozagorski.	Hieng et al. (2004)
Cowpea & Common bean	Leaves	EPACE-1-tolerant TI83D-tolerant Carioca-sensitive IPA-sensitive	Endoproteolytic activity assays	Increased endoproteolytic activity higher in drought-susceptible bean cultivars compared to drought tolerant cowpea cultivars. The highest activity of aspartic protease was observed in Carioca, the most drought-susceptible bean cultivars.	Cruz de Carvalho et al. (2001)

TABLE 5 List of transgenic overexpression studies illustrating the roles of protease and protease inhibitors in plants under drought stress.

Gene ID	Name	Transgenic plant	Drought-induced performance of transgenic plants	References
Proteases				
ASPG1	*Aspartic protease in guard cell 1*	Arabidopsis	Overexpression of *ASPG1* gene in drought-stressed Arabidopsis plants enhanced ABA sensitivity in guard cells and triggered stomatal closure to reduced water loss. The transgenic plants also showed increased activities of superoxide dismutase and catalase compared to wild-type plants. The *ASPG1* gene played an essential role in drought avoidance through ABA signalling in guard cells and displayed more tolerance.	Yao et al. (2012)
APA1	*Aspartic Protease*	Arabidopsis	*APA1* expression was induced in response to drought stress and ABA treatment in the transgenic plants. The overexpression of the *APA1* gene in the transgenic plants enhanced drought tolerance to mild water deficit through ABA-dependent signalling.	Sebastián et al. (2020)
SPCP2	*Sweet potato papain-like cysteine protease*	Arabidopsis	Transgenic Arabidopsis plants overexpressing *SPCP2* exhibited early flowering, higher percentage of incompletely developed siliques, reduced average fresh weight and lower seed germination rates compared to the wild-type. *SPCP2* gene expression was induced during natural leaf senescence in salt and drought stress tolerance compared to wild-type plants.	Chen et al. (2010)
Protease Inhibitors				
BBI	*Bowman-Birk inhibitor*	Arabidopsis	The transgenic plants expressing *BBI* maintained normal growth and higher relative water content when compared to the wild-type plants. The transgenic plant overexpressing *BBI* showed increased activities of glutathione-s-transferase and ascorbate peroxidase antioxidants and reduced malondialdehyde content when compared to the wild-type plants. The expression of *BBI* in the transgenic plants enhanced drought tolerance.	Malefo et al. (2020)
OCPI1	*Oryza sativa chymotrypsin inhibitor-like 1*	Rice	*OCPI1* overexpressing transgenic plants had higher total protein content than wild-type under drought stress. *OCPI1* overexpressing plants also had higher grain yield compared to the wild-type. Chymotrypsin activity was also inhibited in the *OCPII*-overexpressing transgenic plant.	Huang et al. (2007)
OCP12	*Oryza sativa chymotrypsin inhibitor-like 2*	Arabidopsis	*OCPI2* overexpressing plants maintained higher biomass, relative water and proline content compared to the wild-type. Chymotrypsin protease activities were lower in the transgenic compared to the wild-type plants. The transgenic plants overexpressing *OCP12* showed enhanced tolerance to salt and osmotic stresses when compared to wild-type plants.	Tiwari et al. (2015)
MpCYS4	*Malus prunifolia* cystanin	Arabidopsis and Apple	Transgenic plants overexpressing *MpCYS4* exhibited higher relative water content, chlorophyll content and stomatal closure, and reduced electrolyte leakage compared to the wild-type. Overexpression of MpCYS4 in transgenic plants led to up-regulation of ABA-and drought-related genes and enhanced drought tolerance.	Tan et al. (2017)

ABA-abscisic acid.

TABLE 6 List of transgenic knockout/down studies illustrating the roles of protease and protease inhibitors in plants under drought stress.

Gene ID	Name	Transgenic plant	Drought-induced performance of knockout plants	References
ZmSAG3	*Zea mays Senescence-associated gene*	Maize	Increased levels of MDA, ion leakage, ROS content, wilting and senescence in *ZmSAG3-OE* compared to wild-type. Increased seed germination rate and levels of antioxidant enzymes, high levels of chlorophyll synthesis and stress-related genes in knockout plants with reduced levels of senescence and chlorophyll degradation-related genes compared to wild-type.	Wang et al. (2022)
HvPap1 & *HvPap19*	*Cathepsin F-like & Cathepsin B-like genes*	Barley	*HvPap1* knockdown plants exhibited delayed leaf senescence under control conditions, thicker cuticle under control and drought conditions. Jasmonic acid and JA-isoleucine levels increased in *HvPap1* knockdown and wild-type plants following drought. ABA content high in wild-type, and the two knockdown lines, was significantly higher in knockdown plants after drought.	Gomez-sanchez et al. (2019)
Tr-KPI	*Kunitz proteinase inhibitor*	White clover	High proline levels before and after drought stress in *tr-kdi1* and *tr-kdi5* knockdown plants than wild-type. Increased drought-induced expression of genes involved in ethylene biosynthesis in knockdown plants.	Islam et al. (2017)
AtFtsHi3	*Arabidopsis thaliana Pseudo-protease AtFtsHi3*	Arabidopsis	*ftshi3-I(kd)* knockdown lines had a pale green phenotype, reduced growth, and distorted chloroplast ultrastructure following drought stress but high endogenous ABA content. Early onset of drought sensitivity in wild-type and *ftshi3-1(Comp)* lines that *ftshi3-I(kd)*. Increased levels of ABA-responsive genes in the *AtFTsHi3* knockdown mutant than the wild-type plants.	Mishra et al. (2021)

ABA, abscisic acid; MDA, malondialdehdye; ROS, reactive oxygen species; kd, knockdown.

drought-resistant cultivar under drought stress. The results also showed increased endopeptidase activity for all three cultivars under severe drought stress, with both susceptible varieties exhibiting the highest activity. The high basal level of azocaseinolytic activity observed in the drought-resistant variety, but the least drought-induced effect is noteworthy. As suggested in sorghum transcriptomics studies under water limitation (Fracasso et al., 2016; Azzouz-Olden et al., 2020) high constitutive expression of genes in drought-tolerant genotypes could contribute towards superior traits to dehydration stress. Overall, the authors suggested that low levels of proteolytic activity observed in the drought-resistant Katya cultivar could be regarded as a marker for drought tolerance (Simova-Stoilova et al., 2010).

Likewise, Cruz de Carvalho et al. (2001) investigated the leaf endoprotease activity of common bean and cowpea (*Vigna unguiculata)* cultivars in response to drought stress. The cowpea cultivars EPACE-1 and TI83D were reportedly more drought-tolerant than the common bean cultivars Carioca and IPA, with an overall tolerance trend of EPACE-1 > TI83D > IPA > Carioca. The results showed differences in endoproteolytic activity across all four cultivars, being higher in the most drought-susceptible common bean cultivars than in cowpea. For example, under mild-drought conditions, the total endoproteolytic activity increased by 235%, 119%, 95% and 58% for cultivar Carioca, IPA, TI83D and EPACE-1, respectively. The study also investigated the involvement of cysteine, aspartic, serine, and metalloproteases in the drought response of the most drought-sensitive Carioca cultivar using class-specific protease inhibitors. The results suggested limited involvement of metalloproteases in the common bean cultivar under mild stress, but some enzyme activity of cysteine and serine proteases. Furthermore, pepstatin A inhibited about 25% of the total proteolytic activity under mild-drought conditions, thus indicating the presence of drought-responsive aspartic proteases in the bean leaf extract. Subsequent analysis of aspartic protease activity in all four cultivars using a specific peptide substrate revealed that water deficit induced aspartic protease activity in cowpea and bean leaf tissues. However, this enzyme activity increased with increasing levels of sensitivity to drought. The authors suggested that this increase in aspartic protease enzyme activity in the drought-sensitive bean cultivars could be a drought-adaptive response for remobilizing nitrogen to other plant parts under stressful environments (Cruz de Carvalho et al., 2001).

2.2.2.2 Transgenic plant biology

2.2.2.2.1 Using overexpression mutant lines

Drought studies using transgenic plants overexpressing protease and protease inhibitors genes (Table 5) are unravelling the roles of protein degradation under conditions of water deprivation (Huang et al., 2007; Zhang et al., 2008; Chen et al., 2010; Yao et al., 2012; Tiwari et al., 2015; Tan et al., 2017; Roux et al., 2019; Malefo et al., 2020; Sebastián et al., 2020). For example, Yao et al. (2012) conducted an extensive molecular study of an Arabidopsis gene *ASPG1* (*aspartic protease in guard cell* 1) and its biological functions in drought response. The study used wild-type and *ASPG1*-overexpressing *(ASPG1*-OE) Arabidopsis plants to investigate tissue-specific gene expression patterns and the roles of *ASPG1* in ABA-signalling systems associated with drought. After exposure to a 14-day drought stress treatment followed by re-watering, *ASPG1*-OE plants exhibited a greater recovery from wilting symptoms and higher survival rates. Gene expression analysis revealed that *ASPG1* is drought-inducible in both wild-type and *ASPG1*-OE plants and is expressed in young seedlings, leaves, stems, flowers, and siliques but not in roots. The gene is also preferentially localized in guard cells where it participates in the ABA-signalling processes, including ROS accumulation triggering stomatal closure. The increased hydrogen peroxide contents are further detoxified by high antioxidant activities of superoxide dismutase and catalase, thus alleviating oxidative stress effects. In addition, increased ABA sensitivity was observed in *ASPG1*-OE plants under drought stress which could account for increased stomatal closure and a significant reduction in transpiration water loss. The authors suggested that *ASPG1* participates in drought response *via* the ABA-dependent pathway (Yao et al., 2012).

Sebastián et al. (2020) also investigated the participation of an aspartic protease gene (*APA1*) in drought response using transgenic Arabidopsis plants overexpressing *APA1* (OE-*APA1*), wild-type and *apa1* insertional lines under well-watered and mild-water deficit conditions. The study revealed that OE-*APA1* lines tolerated drought stress better than the wild-type and *apa1* lines. In addition, drought stress did not affect the overall plant phenotype, total chlorophyll content, and principal root length of the OE-*APA1* lines relative to the well-watered plants. However, *APA1* overexpressing plants exhibited reduced stomatal pore aperture and water loss under mild-drought stress compared to the wild-type and *apa1* lines, yet exogenous ABA did not intensify stomatal closure. *APA1* was shown to be ABA-responsive, exhibiting a 4-fold increase in expression in well-watered wild-type plants supplemented with exogenous ABA. Furthermore, under well-watered conditions, OE-*APA1* plants up-regulated the expression of ABA biosynthetic and signalling genes relative to the wild-type, while the same genes were down-regulation in *apa1* insertional lines. As such, the authors suggested that *APA1* participates in drought response *via* the regulation of the ABA-signalling pathway and its location in leaves, vascular tissues, epidermal, and guard cells implicate it in stomatal closure as a water-saving mechanism under conditions of water scarcity (Sebastián et al., 2020).

Likewise, Chen et al. (2010) also investigated the role of sweet potato (*Ipomoea batatas*) papain-like cysteine protease (*SPCP2)* in transgenic Arabidopsis plants. Qualitative phenotypic analyses showed that transgenic Arabidopsis plants overexpressing *SPCP2* contained a higher number of incompletely developed siliques, and exhibited earlier flowering, reduced fresh weight per seed, and lower germination rates compared to the wild-type controls. It is plausible that the SPCP2 protein degraded silique storage proteins resulting in incomplete development. Furthermore, *SPCP2* gene expression was induced during natural leaf senescence, thus suggesting a gene function in senescence. *SPCP2* transgenic Arabidopsis plants also showed high tolerance to salt and drought stresses compared to the wild-type, further implicating the gene in osmotic-stress response (Chen et al., 2010).

Bowman-Birk inhibitors (BBI) are compound inhibitors that inhibit both trypsin and chymotrypsin (Habib and Fazili, 2007;

Vaseva et al., 2012). Malefo et al. (2020) generated transgenic *A. thaliana* plants overexpressing a *BBI* gene from maize and studied its role in drought tolerance. Drought stress was imposed on 4-week-old plants by withholding water for 9 days, and comparisons were made between the wild-type and transgenic plants under well-watered and drought-stress conditions. The results showed that the transgenic plants exhibited less wilting symptoms, greater survival rate, higher RWC, and higher fresh leaf biomass than the wild-type under drought conditions. Furthermore, lipid peroxidation was reduced, while glutathione-S-transferase activity was enhanced in transgenic lines compared to the wild-type exposed to water limitation. The authors suggested that the improved performance of the *BBI*-overexpressing transgenic plants under drought could be attributed partly to the reduction in drought-induced oxidative stress (Malefo et al., 2020).

In another study, an *Oryza sativa chymotrypsin inhibitor-like 1* (*OCPI1*) gene was transformed into rice plants and characterized at the reproductive stage under drought-stressed field conditions (Huang et al., 2007). The results showed that the positive transgenic plants inhibited endogenous chymotrypsin activity resulting in higher total protein content of leaves and panicles compared to the wild-type under drought conditions. Furthermore, the *OCPI1*-overexpressing plants had higher grain yield due to higher seed setting rates than the wild-type under similar drought conditions, further supporting the potential usefulness of *OCPI1* in crop improvement strategies (Huang et al., 2007). Likewise, the over-expression of an *OCPI2* gene in Arabidopsis resulted in enhanced tolerance to salt and PEG- and mannitol-induced osmotic stresses in transgenic plants relative to the wild-type (Tiwari et al., 2015). Transgenic Arabidopsis *OCPI2* plants also exhibited greater vegetative and reproductive potential, higher biomass, seed yield, RWC, membrane stability and proline content. The authors suggested that these enhanced characteristics could have contributed towards the better performance of transgenic plants under salt and osmotic stresses (Tiwari et al., 2015).

Apart from serine protease inhibitors discussed above, plants also contain cystatins which inhibit cysteine proteases of papain and legumain families (Grudkowska and Zagdańska, 2004; Martínez et al., 2012). Some phytocystatins are drought-inducible as evidenced by increased gene expression of a triticale cystatin *TrcC-8* in triticate leaf and root tissue under dehydration (Chojnacka et al., 2015). Western blotting analysis also showed increased drought-induced accumulation of the protein but its decline upon re-watering in the same tissues. In addition, the recombinant TrcC-8 protein exhibited inhibitory effects on wheat and triticale leaf cysteine proteases under water deficit stress (Chojnacka et al., 2015). Therefore, cystatins may function as regulatory elements of cysteine protease activity under drought stress (Diop et al., 2004; Zhang et al., 2008).

Tan et al. (2017) studied the biological roles of cystatins under drought stress by overexpressing a *Malus prunifolia* cystatin gene (*MpCYS4*) in transgenic Arabidopsis and apple (*Malus domestica*). The study showed that the *MpCYS4* protein is localized in the nucleus, plasma membrane and cytoplasm, consistent with its known cellular locations (Kidric et al., 2014; Diaz-Mendoza et al., 2016). A range of drought assays showed that transgenic Arabidopsis plants overexpressing *MpCYS4* had improved drought tolerance as exhibited by the plants' decreased water loss, increased survival rate, increased RWC and greater stomatal closure under drought, relative to the wild-type. Physiological assessments of the transgenic apple plants overexpressing *MpCYS4* also exhibited higher RWC, chlorophyll content and stomatal closure, and reduced electrolyte leakage under water deficits compared to the wild-type. Generally, the *MpCYS4* gene resulted in higher transcriptional expression of known ABA- and stress-responsive genes in both transgenic Arabidopsis and apple plants, further highlighting the involvement of *MpCYS4* in ABA-signalling processes and thus drought tolerance (Tan et al., 2017).

2.2.2.2.2 Using knockout or knockdown mutant lines

Plant gene function can also be studied using knockout or knockdown mutants with total or partial loss of gene function (Bolle et al., 2011). Several studies have utilized such mutants of proteases or protease inhibitors to study their role during plant growth and drought response (Islam et al., 2017; Gomez-sanchez et al., 2019; Mishra et al., 2021; Wang et al., 2022). A summary of these studies is also given in Table 6. For example, Wang et al. (2022) investigated the role of the maize (*Z. mays*) senescence-associated gene, *ZmSAG39* encoding a cysteine protease in maize plants subjected to either darkness or drought stress. The genotypes used were wild-type, transgenic *ZmSAG39* overexpression (OE) lines and *ZmSAG39* knockout lines. The results indicated that *ZmSAG39* gene expression was induced by darkness and drought stress in the leaves of wild-type plants. The study also investigated seed germination rates in wild-type, *ZmSAG39* knockout and *ZmSAG39*-OE plants under 8% and 12% polyethylene glycol (PEG)-6000 treatment. The results showed that the knockout lines had higher germination rates when compared to the wild and *ZmSAG39*-OE. Furthermore, the *ZmSAG39*-OE lines exhibited severe leaf senescence under complete darkness for 5 days, with lower chlorophyll content but higher membrane ion leakage rate and malondialdehyde (MDA) content than wild-type and knockout lines.

Similarly, following a 14-day drought-stress treatment imposed by withholding water, the *ZmSAG39*-OE lines showed greater levels of leaf wilting and senescence than the wild-type. In contrast, the *ZmSAG39* knockout lines had an upright and greener phenotype. In addition, the *ZmSAG39*-OE lines exhibited reduced survival rates and chlorophyll content, higher membrane leakage levels, MDA, ROS, and oxidative stress damage compared to wild-type and knockout lines. Interestingly, the activities of a range of antioxidant enzymes were much greater in the *ZmSAG39* knockout lines than in the wild-type and *ZmSAG39*-OE lines, thus, indicating the enhanced antioxidant capacity of knockout mutants. The expression levels of various stress-responsive genes were also analyzed between the wild-type and transgenic plants. The results showed expression levels of stress-related genes (lipid-transfer protein, caleosin-related family protein, and salt-overly sensitive 1) and chlorophyll synthesis-related genes (chlorophyll *a* oxygenase) were higher in the drought-stressed knockout lines compared to the wild-type.

On the other hand, senescence-related genes (microRNA 3, cysteine protease, and senescence-associated gene 12) and a chlorophyll degradation-related gene (non-yellow colouring 1) were higher in the drought-stressed *ZmSAG39*-OE lines compared to the wild-type. These results indicate the negative regulatory effect of *ZmSAG39* on the analysed stress-related and chlorophyll-synthesis-related genes under drought stress. However, under the same conditions of water deficits, *ZmSAG39* exhibited a positive regulatory effect on genes associated with senescence and chlorophyll degradation. The authors concluded that *ZmSAG39* is associated with leaf senescence, and its increased expression under darkness and drought enhances the sensitivity of maize plants to stress. As such, the knocking out of *ZmSAG3* from maize enhanced the resistance of maize plants to darkness and drought tolerance and delayed leaf senescence (Wang et al., 2022).

Gomez-sanchez et al. (2019) also investigated the effect of individually knocking down drought-induced cysteine protease genes, *HvPap1 and HvPap19*, in leaves of barley (*Hordeum vulgare*) under drought stress. Seven-day-old plants of wild-type, knockdown *HvPap1* (*KDPap1*), and knockdown *HvPap19* (*KDPap19*) were subjected to drought stress by withholding water for 14 days. The wild-type plants were more susceptible to water deprivation, as evidenced by reduced leaf turgor, while both knockdown genotypes remained upright. Furthermore, *KDPap1* plants exhibited delayed leaf senescence during their normal growth cycle under well-watered conditions compared to the wild-type. *KDPap1* also had a thicker upper epidermis cuticle under normal and drought conditions compared to the wild-type and *KDPap19*. However, the drought-induced reduction in chlorophyll and carotenoid contents was not significantly different between the three genotypes. Nevertheless, drought-stressed *KDPap1* and *KDPap19* plants exhibited higher total protein content than wild-type plants, possibly indicating reduced proteolysis in the knockdown mutant lines.

The study also analysed the levels of selected phytohormones in the three genotypes under well-watered and drought-stress conditions (Gomez-sanchez et al., 2019). The results showed differences in the responses of the phytohormones to water deprivation. For example, ABA levels increased upon exposure to drought in wild-type, *KDPap1* and *KDPap19* lines, possibly underscoring the critical role of this hormone during drought response. However, ABA levels were much higher in both knockdown lines compared to the wild-type. Conversely, 12-oxo-phytodienoic acid (OPDA), a precursor for jasmonic acid (Taiz and Zeiger, 2012), decreased in all three genotypes following water deprivation. This decline could be attributed to the observed increase in jasmonic acid and its bioactive form, jasmonic-isoleucine, in drought-stressed wild-type and *KDPap1* plants. However, independent lines of *KDPap19* gave inconsistent trends in jasmonic acid and jasmonic-isoleucine levels. On the other hand, salicylic acid remained unchanged under drought stress conditions in wild-type and transgenic plants. Although increases in ABA and jasmonic acid levels are known to regulate stomatal closure and water loss under drought stress conditions (Salvi et al., 2021), the observed stomatal behaviour in the knockdown plants was unusual. The authors, however, concluded that *HvPap1 and HvPap19* are drought-inducible genes and may cause differential effects on barley plants subjected to drought (Gomez-sanchez et al., 2019).

Islam et al. (2017) investigated the functions of Kunitz proteinase inhibitors (KPIs) in white clover (*Trifolium repens* L.) plants subjected to water deficits using several knockdown lines of different *Tr-KPIs*. The study utilised two distinct water withholding regimes, a Non-PreStress treatment with direct exposure of plants to water deficits and a PreStress treatment in which plants were initially exposed to drought stress and rehydration, followed by a final drought stress treatment. Both *Tr-KPI1* and *Tr-KPI5* genes were drought-inducible in three untransformed white clover genotypes with varying degrees of drought tolerance. However, significantly higher gene expression levels were observed in the PreStress treatment, and the genes exhibited different induction levels in leaf and root tissues. The study also showed that proline accumulation and gene expression of *9-cis-epoxycarotenoid dioxygenase* (*NCED1*), a key enzyme in the ABA biosynthesis pathway (Taiz and Zeiger, 2012) are drought-inducible in Non-PreStress treated plants but not PreStress treatment. The authors suggested that PreStress water deprivation treatment primes plants for future exposure to water limitation, resulting in reduced proline accumulation and no significant changes in *NCED* gene expression.

To further study the role of *Tr-KPI* genes in drought response, four knockdown mutant lines were generated, *35S::tr-kpi1*, *35S::tr-kpi2*, *35S::tr-kpi4* and *35S::tr-kpi5*; however, for the sake of brevity in this review, the knockdown lines will be referred to as *kpi1*, *kpi2*, *kpi4* and *kpi5*, respectively. Leaf proline levels were much higher in well-watered plants of *kpi1* and *kpi5* lines and even greater during PreStress treatment than the wild-type plants. However, *kpi2* and *kpi4* lines exhibited lower proline levels under well-watered conditions compared to the wild-type and were not used further in the study. These results possibly highlight the additive effects of high endogenous proline levels in *kpi1* and *kpi5* lines in response to PreStress treatment. Both *kpi1* and *kpi5* plants also exhibited increased drought-induced transcript abundance of ethylene biosynthesis genes, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase 1 (ACS1) and ACC oxidase 1 (ACO1) compared to the wild-type plants. The authors suggested that knockdown lines may experience some level of constitutive stress under well-watered conditions; hence the increased proline accumulation possibly acts as a ROS scavenger. Furthermore, *Tr-KPIs* may have different tissue-specific expression patterns and target various proteases, resulting in a functionally diverse group of active proteinase inhibitors during plant growth and drought response (Islam et al., 2017).

In an extensive study, Mishra et al. (2021) investigated the effect of an *A. thaliana filamentous temperature-sensitive H* (*FtsH*) pseudo-protease (*AtFtsHi3*) on the growth and drought tolerance of Arabidopsis plants using knockdown mutants. Pseudo-proteases are catalytically inactive proteolytic enzymes with cell regulatory functions (Reynolds and Fischer, 2015). Gene expression analysis of *AtFtsHi3* indicated that the transcript was expressed in seedlings, young flowers, roots, leaves, siliques, and stems during the growth of wild-type Arabidopsis plants. The generated knockdown mutant, *ftshi3-1 (kd)*, exhibited a 99% reduction in the transcript expression of the target gene *AtFtsHi3* relative to the wild-type. In addition, the mutant seedlings and plants had a pale-green phenotype, were much smaller in rosette size, and had reduced root growth, with lower numbers of lateral roots than the wild-type. The study also generated complementation lines by expressing the *FtsHi3* gene in the *ftshi3-I (kd)* background and designated them *ftshi3-I (Comp)*. Further analysis of chloroplasts ultrastructure between the wild-type, *ftshi3-I (Comp)*, and *ftshi3-I (kd)* showed that the knockdown mutant lines had distorted chloroplasts with thinner membranes, fewer and distorted thylakoid membranes, and fewer starch granules relative to the other wild-type and *Comp* lines.

Severe drought stress was also imposed on 4-week-old Arabidopsis plants of wild-type, *ftshi3-I (Comp)*, and *ftshi3-I (kd)* lines by withholding watering for up to 20 days and observing the drought-related phenotypes. The results showed that *ftshi3-I (kd)* plants were more tolerant to 12-days of drought treatment, while the wild-type and *ftshi3-I (Comp)* plants were more drought sensitive with wilting and chlorotic phenotypes. Furthermore, 80% of the *ftshi3-I(kd)* plants recovered after 20 days of drought stress, followed by 14 days of re-watering, while only 20% of the wild-type and *ftshi3-I (Comp)* recovered under the same conditions. While all three genotypes had relatively similar leaf ABA levels under control conditions, upon exposure to water limitation, the wild-type and *ftshi3-I (Comp)* plants had higher ABA content in leaves than the knockdown line. The authors suggested that high endogenous levels of ABA in cotyledons and roots under well-watered conditions could better prime the *ftshi3-I(kd)* plants for drought than the wild-type. Furthermore, the *ftshi3-I(kd)* plants had reduced stomatal density but larger stomatal size than the other two genotypes. Overall, this study showed that *ftshi3-I(kd)* plants had improved drought tolerance compared to wild-type and *ftshi3-I (Comp)* plants (Mishra et al., 2021).

2.3 Overall functions: more than just acts of protein degradation and its regulation

In summary, plants encounter drought-induced osmotic and oxidative stresses, which disrupt cell structure and function (Levitt, 1980b; Ingram and Bartels, 1996; Bray, 1997). In turn, drought signalling events alter the expression patterns of various genes and proteins with diverse functions in drought adaptation, such as proteases and protease inhibitors (Figure 2A). Consequently, damaged, misfolded, or aggregated proteins are either removed through protein degradation activities of proteases (Vierstra, 1996; Vaseva et al., 2012) or stabilized by chaperons and osmoprotectants (Ingram and Bartels, 1996; Bray, 1997) to restore cellular homeostasis. Such proteolytic activities are essential for preventing the accumulation of potentially toxic non-functional proteins and peptides, recycling nitrogen sources and providing free amino acid pools for renewed protein synthesis well-suited for stressful conditions (Vierstra, 1996; van Wijk, 2015; Rowland et al., 2022). The accumulated drought-responsive protease inhibitor proteins primarily regulate the catalytic activities of proteases (Mosolov and Valueva, 2011). Nonetheless, the roles of plant proteases and their inhibitors under drought stress are more than just acts of protein degradation and regulation (Figure 2).

Results from comparative proteomics and enzyme activity assays (Figure 2B) and transgenic biology studies (Figure 2C) provide experimental evidence for the diverse roles of plant proteases and their inhibitors under drought stress (Tables 3-6). For example, while most plants undergo protein damage due to osmotic and/or oxidative stresses, the drought-sensitive genotypes suffer the most, as evidenced by their increased endoproteolytic activities and reduced protein content under drought stress (Cruz de Carvalho et al., 2001; Hieng et al., 2004; Simova-Stoilova et al., 2010). On the contrary, drought-tolerant genotypes protect their proteins from drought-induced damage by increasing the efficiency of their protective machinery, such as antioxidant capacity and chaperon activities, and inhibiting proteolysis (Cheng et al., 2016; Goche et al., 2020). Furthermore, the reviewed transgenic plant biology studies (Tables 5, 6) add new insights into other roles of proteases and protease inhibitors, including maintaining high RWC and water conservation through enhanced stomatal control (Yao et al., 2012; Tiwari et al., 2015; Tan et al., 2017; Malefo et al., 2020; Sebastián et al., 2020). In addition, proteases and their inhibitors participate in ABA-signalling events and the up-regulation of ABA-responsive genes essential for drought adaptation (Yao et al., 2012; Tan et al., 2017; Sebastián et al., 2020). As a result, transgenic plants overexpressing either protease or protease inhibitor genes exhibit enhanced performance, plant growth and yield, survival rate, and resilience to water deficits (Huang et al., 2007; Yao et al., 2012; Tiwari et al., 2015; Tan et al., 2017; Malefo et al., 2020).

Furthermore, gene function studies using loss-of-function technologies such as knockout/down mutants also provide additional experimental evidence on the broader roles of plant proteases and their inhibitors in drought response (Tables 5, 6; Figure 2C). Nevertheless, the experimental challenges related to the unintended effects of gene manipulation technologies on the overall plant phenotype are real. For example, Gomez-sanchez et al. (2019) suggested that the observed unexpected increase in cuticle thickness of *KDPap1*, an *HvPap1* knockdown mutant line, could have been due to pleiotropic effects associated with the knocked-down cysteine protease gene. In addition, knockdown mutants exhibit some residual mRNA expression or protein accumulation of the knocked-down gene to varying extents (Islam et al., 2017; Gomez-sanchez et al., 2019; Mishra et al., 2021), hence the knockdown character (Gomez-sanchez et al., 2019). For example, while Mishra et al. (2021) reported a 99% reduction in the transcript expression of the target *AtFtsHi3* gene in the generated knockdown mutant, *ftshi3-I (kd)*, relative to the wild-type, other studies reported much lower reduction rates of the target genes (Islam et al., 2017).

Furthermore, although the knockdown procedure would have specifically intended to reduce the expression level of a target gene, in some cases, untargeted genes are also affected (Islam et al., 2017). For example, Islam et al. (2017) produced different knockdown mutants of specific *Tr-KPIs* in their study. However, the RNA interference procedure also affected the expression of other untargeted members of the *Tr-KPI* gene family. Consequently, evaluating resultant plant mutant phenotypes after exposure to drought stress becomes challenging. Therefore, despite the steady progress in gene function investigations using transgenic mutant plants, more studies with multiple transgenic lines are required to ascertain the *bona fide* physiological effects and roles of target protease and protease inhibitor genes.

As discussed earlier, phytohormones may interact during plant growth and development, as well as in response to drought stress, ultimately contributing towards the overall plant phenotype (Clarke and Durley, 1981; Hale and Orcutt, 1987; Ullah et al., 2018; Jogawat et al., 2021; Salvi et al., 2021; Iqbal et al., 2022). In some cases, the proteolytic machinery regulates phytohormone activity and signalling (Ullah et al., 2018; Salvi et al., 2021; Iqbal et al., 2022). This is evidenced by the results of the reviewed transgenic studies (Tables 5, 6) involving genes of aspartic proteases (Yao et al., 2012; Sebastián et al., 2020), a cystatin (Tan et al., 2017), senescence-associated gene (Wang et al., 2022), cathepsin-like proteases (Gomez-sanchez et al., 2019), Kunitz proteinase inhibitors (Islam et al., 2017) and a pseudo-protease FtsHi3 (Mishra et al., 2021).

For example, gibberellins regulate gene expression through DELLA proteins, which are repressors of plant growth and development (Achard and Genschik, 2009). Under normal growth conditions, gibberellins facilitate optimal plant growth and development by mediating the 26S proteasome degradation of DELLA proteins. Conversely, when exposed to drought conditions, gibberellins levels decrease, and DELLA proteins accumulate, resulting in plant growth inhibition and the development of dwarf plant phenotypes (Achard and Genschik, 2009; Taiz and Zeiger, 2012; Colebrook et al., 2014; Salvi et al., 2021). Similarly, the 26S proteasome degradation pathway is involved in the jasmonic acid signalling pathway (Taiz and Zeiger, 2012). During drought stress, the biosynthesis and accumulation of jasmonic acid increases and jasmonate ZIM-domain (JAZ) proteins are degraded by the 26S proteasome, thus allowing transcription of downstream stress-related

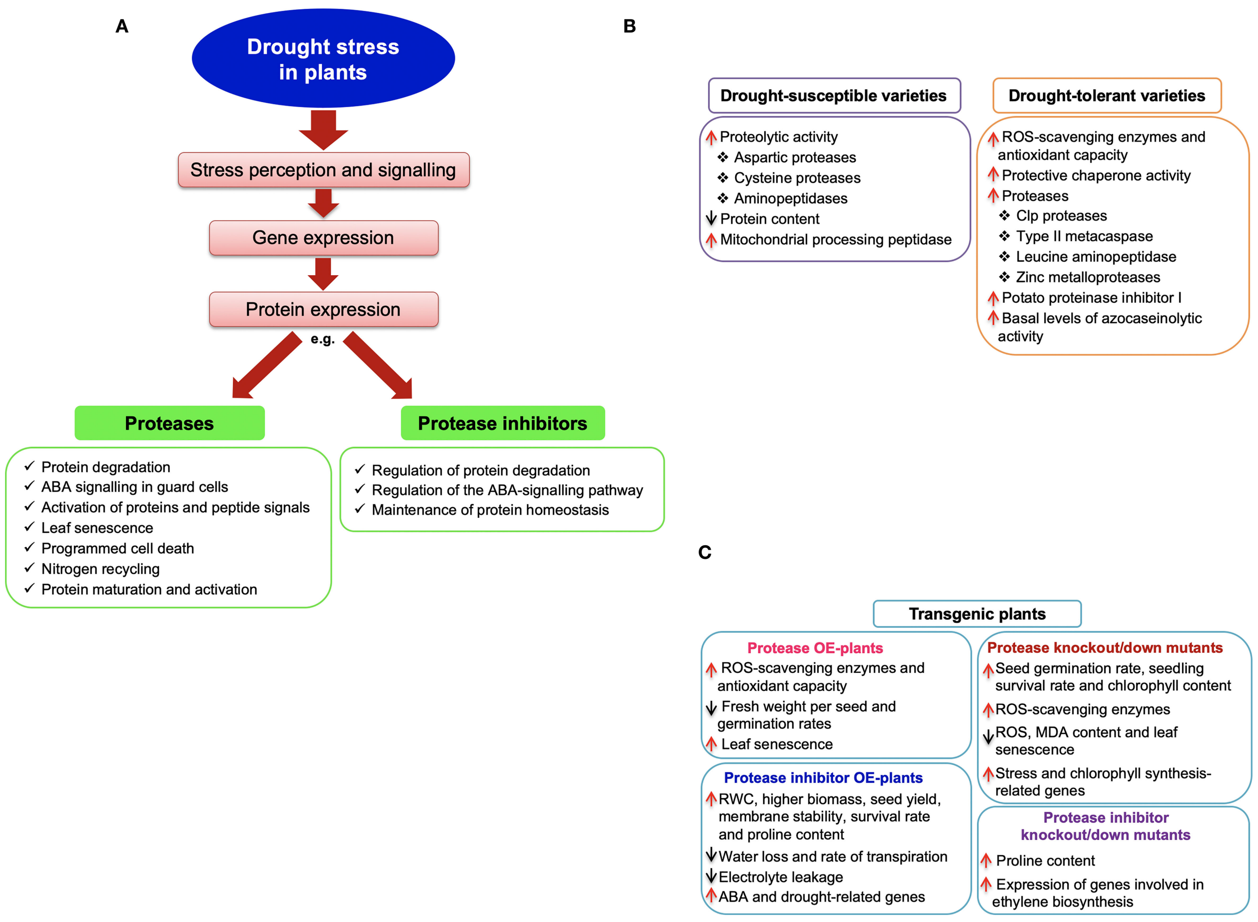

FIGURE 2
Regulation and functions of plant proteases and protease inhibitors extrapolated from various drought-stress studies. **(A)** A simplified schematic diagram showing that drought stress signalling in plants results in changes in gene and protein expression patterns such as those of proteases and protease inhibitors. **(B)** Differential regulation of plant proteases and protease inhibitors in drought-susceptible and tolerant plant varieties under drought stress. **(C)** Physiological effects of transgenic overexpression and repression of proteases and protease inhibitors in plants under drought stress. Red arrows indicate up-regulation or increase, while black arrows indicate down-regulation or decrease. ABA, abscisic acid; Clp, caseinolytic protease; MDA, malondialdehyde; ROS, reactive oxygen species; RWC, relative water content; OE-plants, overexpression-plants. (Cruz de Carvalho et al., 2001; Hieng et al., 2004; Huang et al., 2007; Chen et al., 2010; Simova-Stoilova et al., 2010; Ford et al., 2011; Yao et al., 2012; Ashoub et al., 2013; Jedmowski et al., 2014; Faghani et al., 2015; Tiwari et al., 2015; Wang et al., 2015; Cheng et al., 2016; Chmielewska et al., 2016; Wu et al., 2016; Islam et al., 2017; Tan et al., 2017; Gomez-sanchez et al., 2019; Goche et al., 2020; Malefo et al., 2020; Sebastián et al., 2020; Mishra et al., 2021; Wang et al., 2022).

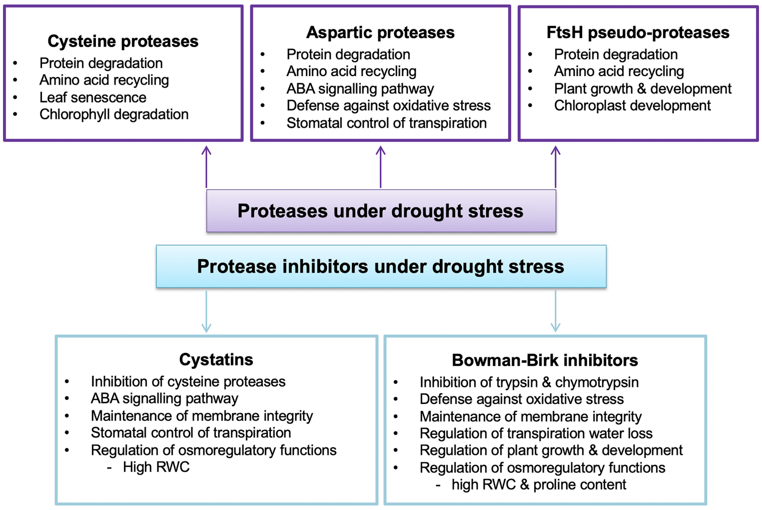

FIGURE 3
Physiological functions of selected proteases and protease inhibitors in drought-stressed plants inferred from transgenic studies. ABA, Abscisic acid; RWC, relative water content. (Chen et al., 2010; Yao et al., 2012; Tan et al., 2017; Malefo et al., 2020; Sebastián et al., 2020; Mishra et al., 2021).

genes. JAZ proteins are repressors of DNA-binding transcriptional factors of the jasmonate hormonal signalling system (Taiz and Zeiger, 2012; Wager and Browse, 2012). Therefore, their presence under normal growth conditions regulates the transcriptional control of jasmonic acid-related gene expression (Wager and Browse, 2012). Thus, the 26S proteasome machinery regulates phytohormone signalling systems in plants.

Other studies have reported that exogenous application of ABA increases chymotrypsin inhibitory activity in barley leaves, while jasmonic acid treatment enhances trypsin inhibitor activity (Casaretto et al., 2004). These observations highlight the role of phytohormones in regulating proteolytic activities and vice versa during normal plant growth and drought response. Indeed, the current review of comparative proteomics, enzyme activity assays and transgenic studies expands our understanding of the broader functions of proteases and protease inhibitors in drought response. Some of the inferred physiological roles of protein degradation activities and their regulatory processes discussed above are summarised in Figure 3.

3 Concluding remarks and perspectives

Protein degradation and its regulatory processes in plants are crucial for maintaining cellular homeostasis under water deficits; otherwise, damaged proteins would accumulate and disrupt cell structure and metabolism. However, the drought-induced differential expression of proteases and protease inhibitor proteins in contrasting genotypes are also unravelling the complex networking events during drought responses in plants. For example, increased proteolysis and reduced total protein in drought-sensitive genotypes could indicate massive protein damage events and less robust protective machinery against drought and its secondary effects, which result in sub-optimal metabolic and growth processes. In contrast, the more drought-tolerant genotypes exhibit reduced levels of proteolysis due to fewer proteases and more protease inhibitors. Together with enhanced protective machinery and improved targeted stress-induced gene transcriptional and translational activities, these factors collectively contribute towards greater performance and survival rates under water-limitation conditions.

Nevertheless, the types and functions of proteases and protease inhibitors are quite diverse, and these proteins could be involved in many more physiological roles, such as stomatal control and ABA, jasmonic acid, and ethylene signalling. Furthermore, with this wide structural and functional diversity, their differential expression patterns between drought-tolerant and susceptible genotypes possibly make proteases and protease inhibitors important groups of proteins with potential use in crop improvement strategies. For instance, could the reduced level of protein degradation in drought-tolerant genotypes be used as a marker for drought tolerance and vice versa? Nevertheless, more functional validation studies are required to unravel additional roles of protein degradation events in plants under drought stress as we continue to find ways of generating crops with enhanced resilience to hot and drier climates. Furthermore, meta-analyses of protease and protease inhibitor gene and/or protein expression datasets between contrasting plant species and genotypes are required to unravel the expansive role of protein degradation in plant drought response.

Author contributions

Conceptualisation, RN. Writing-manuscript preparation, SJM, RN. Writing-review and editing, RN. Funding acquisition, supervision, RN. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Morphological, physiological, and molecular scion traits are determinant for salt-stress tolerance of grafted citrus plants

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Introduction: Citrus productivity has been decreasing in the last decade in the Mediterranean basin as a consequence of climate change and the high levels of salinity found in the aquifers. Citrus varieties are cultivated grafted onto a rootstock, which has been reported as responsible for plant tolerance to adverse situations. However, other important factors for stress tolerance relying in the scion have been less studied. The aim of this study was to evaluate the effect of the grafted scion on citrus tolerance to salt stress.

Methods: Four different citrus rootstock/scion combinations were subjected to salt stress for 30 days, using Carrizo citrange (CC) or *Citrus macrophylla* (CM) as rootstocks, and Navelina orange (NA) or Oronules mandarin (OR) as scions. CM-OR was the most tolerant combination, whereas CC-NA was the most sensitive one.

Results and discussion: Our results support the idea that the rootstock plays an important role in salt stress tolerance, but scion is also crucial. Thus, photosynthesis and transpiration, processes regulated by abscisic acid and jasmonic acid, are determinant of plant performance. These photosynthetic parameters were not affected in plants of the salt-tolerant combination CM-OR, probably due to the lower intoxication with Cl^- ions, allowing a better performance of the photosynthetic machinery under stress conditions. The different stomatal density of the two citrus scions used in this work (higher in the sensitive NA in comparison to the tolerant OR) also contributes to the different tolerance of the grafted plants to this adverse condition. Additionally, *CsDTX35.1* and *CsDTX35.2*, genes codifying for Cl^- tonoplast transporters, were exclusively overexpressed in plants of the salt-tolerant combination CM-OR, suggesting that these transporters involved in Cl^- compartmentalization could be crucial for salt stress tolerance. It is concluded that to improve citrus tolerance to high salinity, it is important that scions have a versatile photosynthetic system, an adequate stomatal density, and a proper modulation of genes coding for Cl^- transporters in the tonoplast.

KEYWORDS

abiotic stress, grafting, photosynthesis, phytohormones, salinity, vacuole

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1 Introduction

Salt stress is one of the major environmental constrains affecting agriculture, whose incidence has increased in the last decades due to aquifer overexploitations and seawater intrusion, threatening agriculture sustainability (İbrahimova et al., 2021). Recent studies have concluded that approximately 1 billion hectares of soil are salinized worldwide (Ivushkin et al., 2019), which comprises 20% of the total cultivated and 33% of irrigated soils for agriculture (Shrivastava and Kumar, 2015). Furthermore, it is expected that this percentage dramatically increased during the 21st century as a consequence of the increase in world population and the climate change (Hassani et al., 2021).

High salinity is a combination of osmotic and ion toxic components. At initial stages of salt stress, there is an osmotic effect caused by the accumulation of sodium (Na^+) and chloride (Cl^-) ions in the soil, which reduces soil water potential and subsequently challenges water and nutrient uptake (Acosta-Motos et al., 2017). This adverse effect can be countered by the plant following different strategies, including accumulation of compatible osmolytes, such as proline, trehalose, or glycine-betaine (Baggett et al., 2021). The toxic component is linked to the uptake of water with excess of Na^+ or Cl^- ions. The excess of Na^+ and Cl^- contributes to enzyme inactivation and macromolecule disruption (Kamran et al., 2020). To mitigate this part of salt stress, plants have developed different strategies that lead to plant tolerance, such as (i) the maintenance of high K^+/Na^+ ratios through the exclusion of Na^+ uptake; (ii) the dilution of Na^+ and/or Cl^- ions through their translocation to the canopy; (iii) the control of transpiration, limiting the entrance of salinized water; and/or (iv) the compartmentalization of the Na^+ and Cl^- ion excess in the vacuoles through tonoplast Na^+/H^+ antiporters (Kamran et al., 2020).

The main mechanisms to cope with salinity in citrus include (i) stomatal closure (López-Climent et al., 2008); (ii) proline accumulation (Vives-Peris et al., 2018a); (iii) alterations in the phytohormone concentrations, which play an essential role in the signaling response to stress (Vives-Peris et al., 2018b); and (iv) changes in expression of genes involved in salt stress tolerance, such as *Na^+/H^+ eXchanger* (*NHX*) and *High Affinity K^+ Transporter* (*HKT*) that encode for tonoplast Na^+ transporters responsible for their sequestration in the vacuoles (Martínez-Alcántara et al., 2015). However, with Cl^- excess being the most toxic component of salt stress in citrus (López-Climent et al., 2008), it seems necessary to study the behavior of its tonoplast transporters. Among them, the *ChLoride Channel* (*CLC*), *ATP-Binding Cassette* (*ABC*), and *Multidrug And Toxin Extrusion* (*MATE)* families contain members (*CLCa*, *CLCc*, *Aluminum-activated Malate Transporter 9* [*ALMT9*], or *DeToXifyng efflux carriers 33* and *35* [*DTX33*, and *DTX35*]) that have been reported as Cl^- transporters in the vacuole membrane, responsible for the compartmentalization of this element in the vacuole (Baetz et al., 2016; Zhang et al., 2017).

Among other regions, citrus are widely cultivated in the Mediterranean basin with scarce precipitations that force to use additional water supplies, usually with underground water that often contains high levels of ions (Ziogas et al., 2021). This fact, along with the low tolerance of citrus plants to salinity (Prior et al., 2007), leads to important reductions of their productivity (Ziogas et al., 2021). To mitigate the deleterious effects of salinity on citrus, several cultural practices have been traditionally proposed, including (i) the use of tolerant rootstocks as Cleopatra mandarin or *Citrus macrophylla* (CM) instead of the sensitive Carrizo citrange (CC) (López-Climent et al., 2008; Vives-Peris et al., 2017); (ii) the irrigation management, watering the plants with additional volume to hinder the formation of a salt bulk around roots (Bello et al., 2021); and/or (iii) the use of beneficial microorganisms (Vives-Peris et al., 2020). However, selecting appropriate tolerant scions that could cope with the adverse effect of salt stress and maintain crop performance and yield has not been usually considered. This is in part due to the lack of information on the physiological and molecular processes beyond their different scion tolerance (Ben Yahmed et al., 2016; Brito et al., 2021).

The main objective of this work was to elucidate the role of the grafted scion on citrus tolerance to salt stress. For this purpose, an experiment with four rootstock/scion combinations was conducted subjecting plants to salt stress conditions. Results have revealed the importance of different morphological, physiological, and molecular traits from the scion in the tolerance of the rootstock/scion combination to adverse soil-dependent environmental conditions, such as high salinity or drought. Thus, leaf stomatal density and the regulation of the stomatal closure seem to play an essential role since these parameters highly influence transpiration and, consequently, water and ion uptake. Additionally, Cl^- tonoplast channels' *CsDTX35.1* and *CsDTX35.2* overexpression has been detected exclusively in tolerant scions, suggesting that tolerant plants could improve the compartmentalization of Na^+ or Cl^- ions.

2 Materials and methods

2.1 Plant material and treatments

Two-year-old certified Navelina orange (NA; *Citrus sinensis* L. Osbeck) or Oronules mandarin (OR; *Citrus clementina* Hort. Ex Tan.) grafted onto the rootstocks Carrizo citrange (CC; *Citrus sinensis* L. Osbeck x *Poncirus trifoliata* L. Raf.), or *Citrus macrophylla* Wester (CM), having similar size and development, was used as plant material. The four different rootstock/scion combinations obtained were (1) Carrizo citrange–Navelina (CC-NA), (2) Carrizo citrange–Oronules (CC-OR), (3) *Citrus macrophylla*–Navelina (CM-NA), and (4) *Citrus macrophylla*–Oronules (CM-OR). Certified plants were provided by an authorized plant nursery (Beniplant, Peniscola, Castelló, Spain). Plant material was grown in 2.5-L black PVC pots containing peat moss as substrate under greenhouse conditions (22/30 ± 2°C day/night, 50%–70% Hr, and natural photoperiod), and watered with half-strength Hoagland solution (Manzi et al., 2015). Plants were acclimated for 2 months previously to stress imposition. The experiments were performed in the greenhouses of the Universitat Jaume I (Castelló de la Plana, Castelló, Spain; 39.991774N, 0.071120W) during the months of May to July of 2021. Following the Spanish legislation, certified plants were used in the experiments. No additional legislation applies to these plant species and the used methodologies.

Salt stress was applied by increasing the sodium chloride (NaCl) concentration in the irrigation solution to 90 mM. Plants were watered three times per week at field capacity. Plants watered without NaCl were added as controls. Leaf and root tissue samples were collected at 30 days after stress imposition. Both tissues were immediately frozen with liquid N_2 and maintained at −80°C for further analyses (Supplementary Figure 1). The experiment was replicated three times with five plants per group and replicate. The analytical determinations described below were performed with three replicates from each biological sample.

2.2 Malondialdehyde determination

Malondialdehyde (MDA) was spectrophotometrically quantified with the methodology described by Hodges et al. (1999) with some modifications. Briefly, 200 mg of frozen material, ground to fine powder, was extracted in 2 ml of 80% ethanol by 30 min of sonication (Elma S30, Elmasonic, Elma Schmidbauer GmbH, Singen, Germany). After this, samples were centrifuged for 20 min at 4,500 rpm, and 800 µl of the supernatant was mixed with 20% trichloroacetic acid (Merck, Darmstadt, Germany) or a mix of 20% trichloroacetic acid with 0.5 thiobarbituric acid (Merck, Darmstadt, Germany). Samples were incubated in a bath at 90°C for 1 h, and cooled down in ice for 10 min. Finally, samples were centrifuged and measured with a spectrophotometer (Spectronic Genesys 10 UV, Thermo, Waltham, MA, USA) at 440, 532, and 600 nm. The concentration of MDA content was achieved following the calculations described in Arbona et al. (2008).

2.3 Chloride and sodium analysis

The analysis of total Cl^- ion content was performed in leaf and root tissues by automatic titration with a chloride analyzer (M926, Sherwood Scientific Ltd., Cambridge, UK) as described in López-Climent et al. (2008) with some modifications. This methodology consisted in the extraction of 50 mg of fresh material by incubation in 5 ml of a buffer with 0.1 N HNO_3 (Panreac, Barcelona, Spain) and 10% glacial acetic acid (Labbox Labware S.L., Barcelona, Spain) for 12 h at 25°C in darkness. After this period, 0.5 ml was taken for determinations in the chloride meter, using a commercial standard solution of 200 mg L^{-1} of Cl^- for the instrument calibration (Sherwood Scientific Ltd., Cambridge, UK).

2.4 Gas exchange and quantum yield parameters

Gas exchange parameters, including net photosynthesis rate (A), transpiration rate (E), and stomatal conductance (g_s), were measured at 30 days of stress with a portable photosynthesis system (LI-6800, LI-COR Environmental, Lincoln, NE, USA) between 9 and 11 a.m. Light lamp was established at 1000 µmol m^{-2} s^{-1}, air flow at 150 µmol mol^{-1}, and CO_2 of reference at 400 ppm. Three undamaged mature leaves from three randomly chosen plants were measured in each group, obtaining four measures per leaf after instrument stabilization at 1–2 min (Zandalinas et al., 2016).

In parallel, at each sampling point (10, 20, and 30 days of stress), photosystem II quantum yield (Φ_{PSII}) was measured with a portable fluorometer (FluorPen FP-MAX 100, Photon Systems Instruments, Drasov Czech Republic) between 9 and 11 a.m. in nine light-adapted undamaged leaves from three different plants (Zandalinas et al., 2016).

2.5 Stomatal density

Stomata preparation was achieved by the obtaining of imprints with the help of dental adhesive resin (Aquasil Ultra+ Smart Wetting Impression Material, Dentsply Sirona, York, PA, USA) from the abaxial epidermis from mature full expanded leaves, taking the middle part between the midrib and the leaf edge, which has been demonstrated as a representative part of the whole leaf (Geisler et al., 2000; Beaulieu et al., 2008). After this, transparent nail polish was distributed over the leaf mold, and carefully removed after 5 min, placing it in microscopy slides (Beaulieu et al., 2008). Stomata preparations were viewed in the optical microscope (Nikon Eclipse 80i microscope equipped with Nikon DXM1200F digital camera, Nikon, Tokyo, Japan), and the obtained images were used for counting the stomatal density.

2.6 Phytohormone analysis

Analysis of leaf and root content of the phytohormones ABA, SA, JA, and IAA was conducted as described in Durgbanshi et al. (2005), involving the extraction of 200 mg of freshly ground material in 2 ml of deionized water with a mill ball equipment (MillMix 20, Domel Železniki, Slovenija), adding 25 ng of $[^2H_6]$-ABA, $[^{13}C_6]$-SA, dehydro-jasmonic acid (DHJA), and 2.5 ng $[^2H_5]$-IAA as internal standards. After this, samples were centrifuged, the supernatant was collected, and the pH was adjusted between 2.8 and 3.2. After this, a double liquid:liquid partition with diethyl ether (Fisher Scientific, Hampton, NH, USA) was performed, recovering the organic phase, which was dried in a vacuum centrifuge evaporator (Speed Vac, Jouan, Saint Herblain Cedex, France). Finally, the dried pellet was resuspended in 0.5 ml of 90:10 (v:v) water:methanol through sonication (Elma S30) for 10 min, and filtered through 0.22-µm PTFE syringe filters. The filtrate was diluted 1:3 (v:v) with 90:10 (v:v) water:methanol and transferred to glass liquid chromatography vials.

Processed samples were injected to the UPLC-MS system, consisting of a UPLC equipment connected to a triple quadrupole through an orthogonal Z-spray interface (Xevo TQ-S, Waters Corp., Milford, MA, USA). A sample volume of 15 µl was injected in the equipment and separated at 40°C through a reversed-phase C_{18} column (50 × 2.1 mm, 1.6 µm particle size, Luna Omega, Phenomenex, Torrance, CA, USA), using a gradient of ultrapure water and acetonitrile, both supplemented with 0.1% formic acid, with a constant flow rate of 300 µl min^{-1} (Supplementary Table 1). The mass spectrometer gas flow was fixed at 250 L h^{-1}, with a desolvation gas flow of 1,200 L h^{-1} at 650°C in multiple reaction monitoring (MRM) mode. The transitions and retention times used for phytohormone quantification are provided in Supplementary Table 2. Quantification was achieved through the injection of a standard curve prepared with commercial standards, using the internal standards mentioned above, and processed with the software MassLynx V4.2 (Waters Corp., Milford, MA, USA).

2.7 Gene expression analysis

RNA was extracted from fresh frozen tissue ground to fine powder with the RNeasy extraction kit from Qiagen according to the manufacturer's instructions (Qiagen, Hilden, Germany), and the quality of the extracted RNA was measured with a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA) to determine the RNA concentration and the absorbance ratios 260/280 and 260/230 nm to check for contaminations or impurities. A total amount of 5 µg from the extracted RNA was treated with DNase to remove the possibly extracted DNA (DNase I, Fermentas, Waltham, MA, USA), again measuring the quality with the Nanodrop spectrophotometer. Finally, a total amount of 1 µg of the extracted RNA was retrotranscribed to cDNA using the Primescript RT Reagent Kit (Takara, Shiga, Japan). Target gene accessions were obtained by searching the Arabidopsis TAIR database (Berardini et al., 2015) to obtain the protein sequence, which was used to perform a TBLASTN in the Citrus sinensis genome v1.1 from Phytozome (Goodstein et al., 2012), finally obtaining the CDS sequence used for the primer design. The primers used for the analysis of gene expression are provided in Supplementary Table 3. Actin (ACT) and tubulin (TUB) were used as housekeeping genes to normalize gene expression levels (Vives-Peris et al., 2018b).

The RT-qPCR analysis was performed to analyze gene expression in an ABI Step One detection system (Applied Biosystems, Foster City, CA, USA). Briefly, the amplification was conducted in reactions containing 1 µl of cDNA solution, 5 µl of Maxima SYBR Green/ROX qPCR mix (Thermo Scientific, Wilmington, DE, USA), 1 µl of a 10 µM mix of forward and reverse primers (Supplementary Table 3), and 3 µl of sterile deionized water to achieve a final volume of 10 µl per reaction. The amplification curve of temperatures consisted in 10 min at 95°C for preincubation and 40 cycles of amplification (each one with 10 s at 95°C for denaturation followed by 10 s at 60°C for annealing and an extension of 20 s at 72°C). The obtained results were processed with StepOne Software v2.3 and Relative Expression Software Tool v2 (REST; Pfaffl, 2001; Pfaffl et al., 2002).

2.8 Statistical analysis

Statistical analysis was performed with Infostat 2020 software (Universidad Nacional de Córdoba, Córdoba, Argentina). Data were subjected to one- or two-way analysis of variance (ANOVA) to compare the data between the four rootstock/scion combination and a Tukey *post-hoc* test ($p \le 0.05$) to compare the data obtained from stressed plants with their respective control. Microsoft Excel 365 (Microsoft, Albuquerque, NM, USA) was used for the representation of column graphs, whereas Sigmaplot v14.0 (Systat Software, Chicago, IL, USA) was used for representing radar plots and the principal component analysis (PCA). This statistical method consists in the description of the variation from *p* random variables in new uncorrelated ones (principal components) and ordering them by the percentage of the total variation explained by each one (Pearson, 1901; Hotelling, 1933).

3 Results

3.1 Plant phenotype

After 30 days of stress, mild phenotypic damage was observed in salt-stressed plants, but only in those plants grafted onto CC. In plants of both rootstock/scion combinations, CC-NA and CC-OR, some leaves (<5% of total leaves) were slightly curved under salt stress conditions (Supplementary Figure 2). In addition to the leaf phenotype, total fresh biomass was also evaluated, but no significant differences were appreciated among the different groups (Supplementary Table 4).

3.2 Malondialdehyde and proline content

MDA concentration was evaluated in leaves and roots after 30 days of salt stress (Figure 1). Only plants with the rootstock/scion combination CC-NA exhibited a significant increase of MDA leaf content as a consequence of salt stress, with values 2.0-fold higher than those determined in leaves from CC-NA non-stressed plants (Figure 1A). On the other hand, salt treatment did not affect root MDA content, but higher values were observed in roots of plants grafted onto CM in both control and salt stress conditions, yielding a 2.8-fold mean increase in roots of CM-grafted plants in comparison to CC-grafted plants, regardless of the salt stress treatment (Figure 1B).

Meanwhile, proline content did not exhibit statistically significant differences neither in leaves nor in roots after 30 days, depending on neither the rootstock/scion combination nor the salt stress treatment (Supplementary Figure 3).

3.3 Chloride and sodium accumulation

The accumulation of Cl^- varied among groups of plants, considering both salt stress treatment and rootstock/scion combination, and in both organs, leaves and roots (Figure 2). In leaves (Figure 2A), CC-NA plants were the fastest in the accumulation of Cl^- ions, being the only one that exhibited differences related to their control at 20 days (1.8-fold increase). This difference increased at 30 days, reaching values 2.8-fold higher

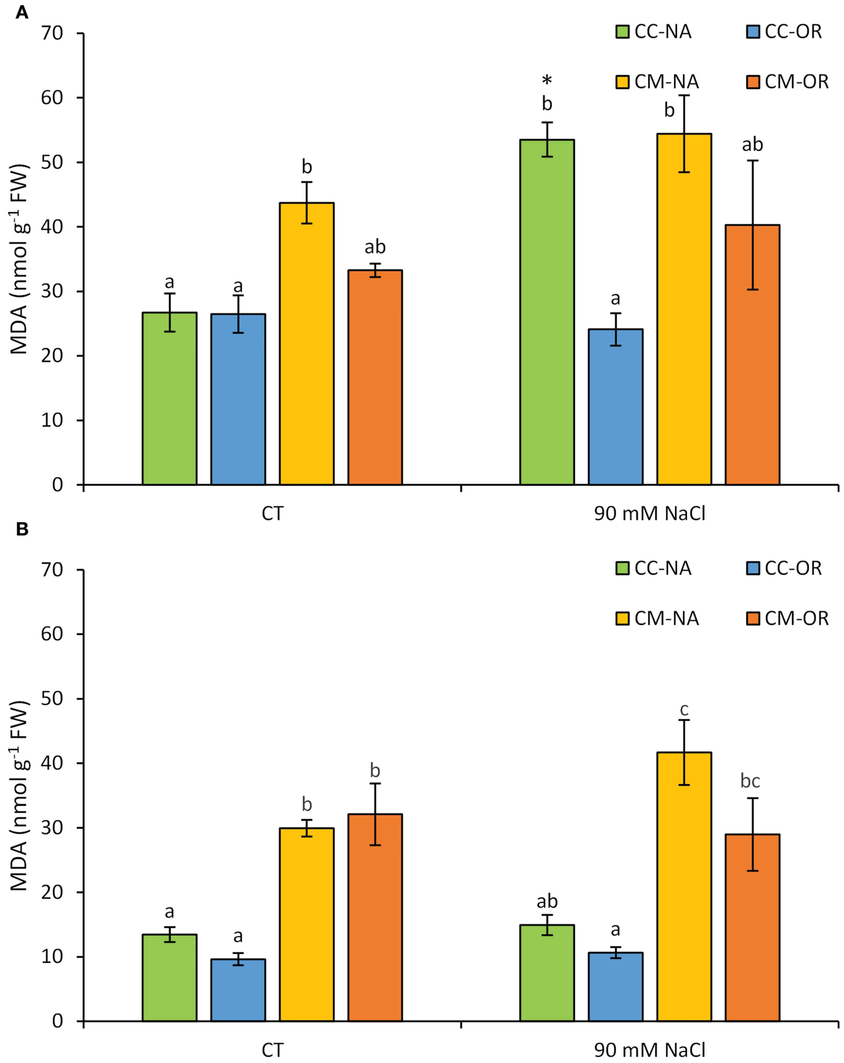

FIGURE 1
Leaf **(A)** and root **(B)** malondialdehyde content. Malondialdehyde content in leaves and roots of control and salt-stressed plants of CC-NA (green bars), CC-OR (blue bars), CM-NA (yellow bars), and CM-OR (orange bars) after 30 days. Values are the mean of three replicates ± standard error. Asterisks denote statistically significant differences in the stressed plants related to control at $p \leq 0.05$. Different letters denote statistically significant differences among the rootstock/scion combinations for each treatment at $p \leq 0.05$.

in salt-stressed plants. However, at 30 days, CC-OR and CM-NA salt-stressed plants accumulated higher amounts of Cl^- ions as well (89.1% and 70.1% higher than controls, respectively), whereas leaves of CM-OR stressed plants did not show an increase of Cl^- content in comparison to control. Meanwhile, after 30 days of stress, root Cl^- content (Figure 2B) increased in salt-stressed plants in all rootstock/scion combinations in comparison to their respective controls, with the highest accumulation observed in CC-NA salt-stressed plants (4.4-fold increase).

In parallel to chloride, sodium leaf content exclusively increased in plants grafted onto CC after 30 days, with levels 4.35 and 4.47 times higher than those in the non-stressed CC-NA and CC-OR plants, respectively (Supplementary Table 5). After 30 days from the stress onset, Na^+ root content also increased independently of the rootstock/scion combination, with values in salt-stressed plants approximately four times higher than their respective controls (Supplementary Table 5).

3.4 Gas exchange, chlorophyll fluorescence parameters, and stomatal density

Plants from CM-OR combination exhibited the lowest values of A, E, and g_s under control conditions, but this situation was reverted under 30 days of salt stress, when CC-NA plants exhibited high decreases of these three parameters (Figure 3). As a result of salt stress treatment, A decreased in plants from all rootstock/scion combinations except for CM-OR (49.7%, 22.0%, and 28.6% in CC-NA, CC-OR, and CM-NA, respectively, Figure 3A). Similarly, E and g_s levels were reduced after 30 days of salt stress treatment in all plant combinations except CM-OR, with this decrease being more evident in CC-NA plants (reductions of approximately 50% in both parameters, Figures 3B, C).

As shown in Figure 3D, only plants with the combination CC-NA had significant reductions in quantum yield of photosystem II (Φ_{PSII})

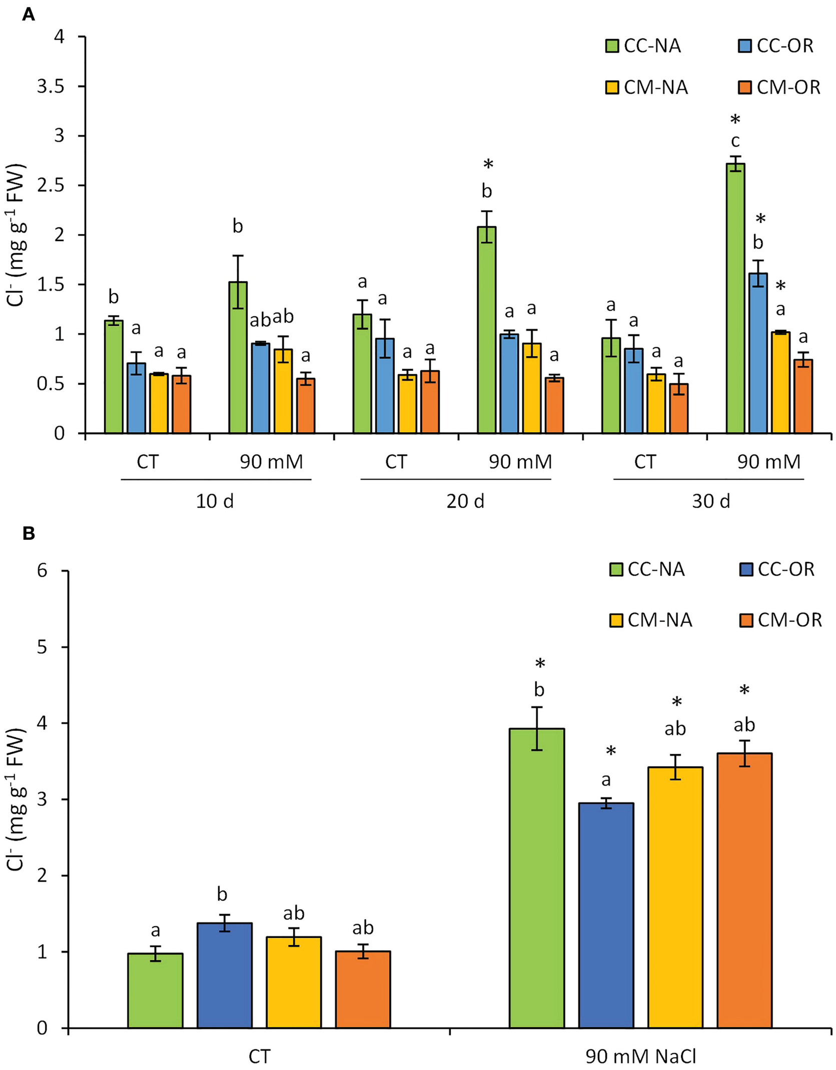

FIGURE 2
Leaf **(A)** and root **(B)** chloride content. Chloride ion content in leaves and roots of control and salt-stressed plants of CC-NA (green bars), CC-OR (blue bars), CM-NA (yellow bars), and CM-OR (orange bars). Values are the mean of three replicates ± standard error. Asterisks denote statistically significant differences in the stressed plants related to control at $p \leq 0.05$. Different letters denote statistically significant differences among the rootstock/scion combinations for each treatment at $p \leq 0.05$.

under stress (1.0% and 1.2% with respect to control at 20 and 30 days, respectively), with this parameter reflecting the efficiency of photosystem II, a key element for the light energy absorption, which is usually damaged under stressful conditions. Salt-stressed plants from the other rootstock/scion combinations (CC-OR, CM-NA, and CM-OR) did not exhibit variations in this parameter during all the experiments in comparison to their respective controls.

A scion-dependent effect was detected in the stomatal density, since combinations with OR as the aerial tissues exhibited a mean diminution of stomatal density of 19.9% in comparison to those plants grafted with NA, independently of the salt stress treatment (Figure 4).

3.5 Chlorophyll and carotenoid content

Slight differences were observed in chlorophyll and carotenoid leaf content between control and salt-stressed plants after 30 days (Supplementary Figure 4). Under stress, Chl_b and total chlorophyll content increased exclusively in leaves of CC-NA plants. Additionally, contrasting concentrations of Chl_a were observed in stressed plants grafted onto CC, with values 20.7% higher in CC-NA leaves in comparison to CC-OR. No differences in the total carotenoid content were caused by salt stress treatment.

3.6 Phytohormone content

After 30 days of salt stress treatment, leaf and root phytohormone content was differentially affected depending on the rootstock/scion combination (Figures 5, 6; Supplementary Table 6). Only plants with CM-OR combination increased leaf ABA content after this period (1.41-fold higher than its respective control), whereas no differences were appreciated in the root

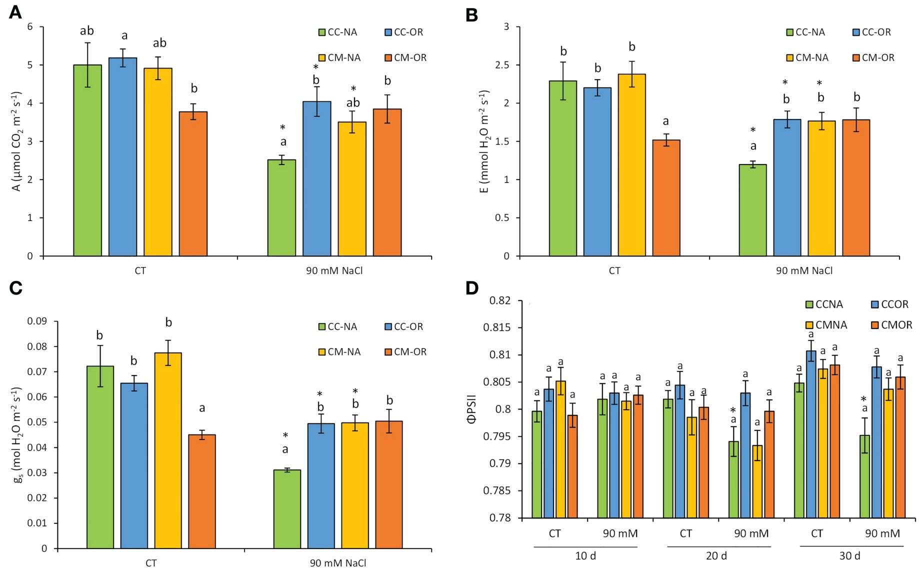

FIGURE 3
Photosynthesis-related parameters. Gas exchange parameters of leaves from control and salt-stressed plants of CC-NA (green bars), CC-OR (blue bars), CM-NA (yellow bars), and CM-OR (orange bars). **(A)** Net photosynthetic rate at 30 days. **(B)** Transpiration at 30 days. **(C)** Stomatal conductance at 30 days. **(D)** Quantum yield at 10, 20, and 30 days. Values are the mean of the data obtained from three leaves with four measurements per leaf ± standard error. Asterisks denote statistically significant differences in the stressed plants related to control at $p \le 0.05$. Different letters denote statistically significant differences among the rootstock/scion combinations for each treatment at $p \le 0.05$.

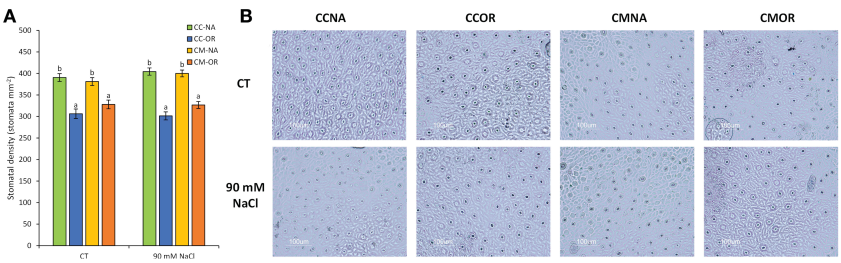

FIGURE 4
Stomata-related parameters. **(A)** Stomatal density in leaves of CC-NA (green bars), CC-OR (blue bars), CM-NA (yellow bars), and CM-OR (orange bars) control and salt-stressed plants. **(B)** Microscope photographs from stomata preparations from leaves of control and salt-stressed plants after 30 days. Values are the mean of 20 leaves ± standard error. Different letters denote statistically significant differences among the rootstock/scion combinations for each treatment at $p \le 0.05$.

content of this plant hormone between control and salt-stressed plants of any rootstock/scion combination (Figures 5, 6). Figures 5, 6 show that JA accumulation pattern was contrasting between leaves and roots, with increased concentrations in CC-NA leaves (values 2.4 times higher than control), but decreased concentrations in roots (47.4% and 71.3%, in CC-NA and CM-OR plants, respectively). Meanwhile, salt stress only caused a decline in SA content in CC-NA plants (24.5% and 45.3% of reduction in leaves and roots with respect to non-stressed plants). Finally, the leaf content of the auxin IAA only varied in CM-NA plants watered with NaCl supplemented solution, which showed concentrations 36.7% higher than control (Figure 5), while in roots, its content decreased in the combinations that had OR as aerial tissues, with reductions of 28.5% and 39.2% in the root IAA content of CC-OR and CM-OR plants (Figure 6).

3.7 Principal component analysis

Additionally, a PCA was performed in order to check the most important variables involved in the different tolerance of each rootstock/scion combination to salt stress after 30 days of 90 mM NaCl treatment (Figure 7). The first principal component (PC1) comprised 40.0% of the total variation and the second component (PC2) comprised 23.9%, as shown in the PCA graph with 63.9% of the variation. Additionally, the first component was the most important one separating the data obtained from control and salt-stressed groups of plants. According to the positioning in the principal components graph (Figure 7B), the rootstock/scion combination CC-NA (the most sensitive) showed the highest difference between control and stressed plants, whereas in the most tolerant one (CM-OR), this difference was the lowest.

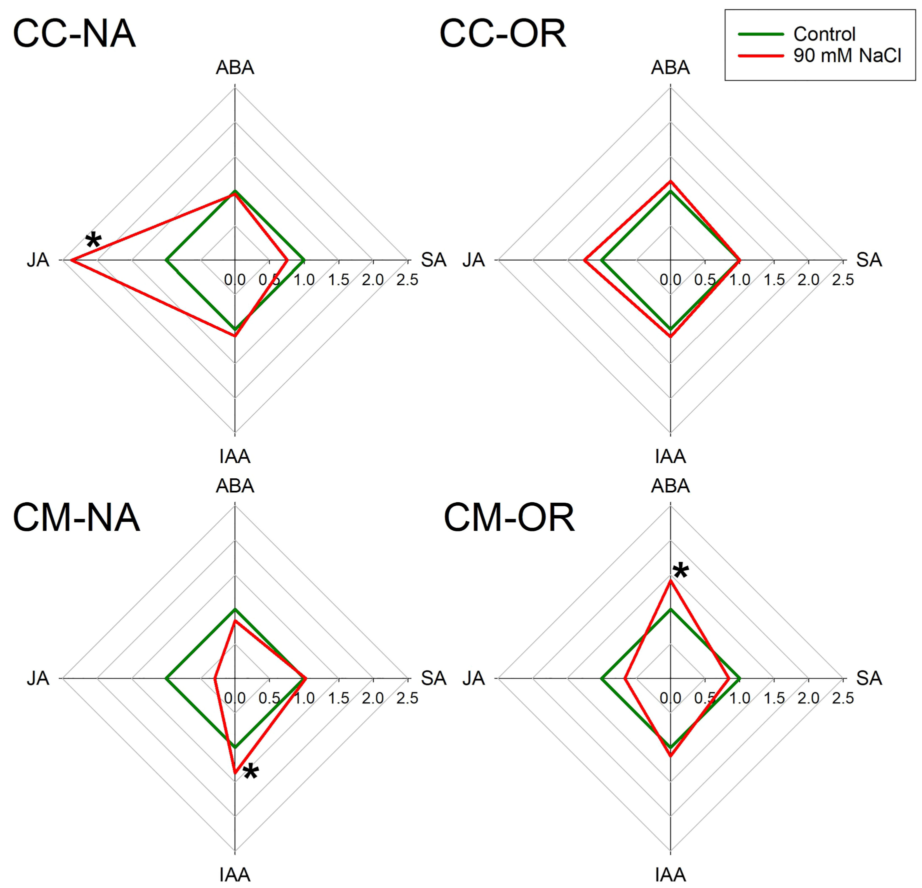

FIGURE 5
Leaf phytohormone content. Radar plots with leaf relative content of abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and indole acetic acid (IAA) in control (green lines), and 90 mM NaCl stressed plants (red lines) of CC-NA, CC-OR, CM-NA, and CM-OR after 30 days. Data are mean of three replicates. Asterisks denote statistically significant differences between control and salt-stressed plants of each scion/rootstock combination at $p \leq 0.05$.

3.8 Gene expression

The expression of genes codifying for tonoplast Cl^- and Na^+ transporters was analyzed in leaf samples obtained from control and salt-stressed plants after 30 days of treatment (Figure 8). Thus, some of the genes related to Cl^- uptake-related transporters, including *CsCLCa*, *CsCLCc*, and *CsDTX33*, were not affected by salt stress (Figure 8). However, under salt stress conditions, *CsDTX35.1* was downregulated in CC-NA and CM-NA plants (reduction of 83.3% and 77.2% of their relative expression, respectively), but in CM-OR plants, its expression suffered an overregulation 2.0 times higher than control (Figure 8). Similarly, leaves of CM-OR plants also exhibited a 2.5-fold induction of the gene *CsDTX35.2* due to salt stress, but a downregulation was recorded in CC-OR plants (50.0% related to control; Figure 8). This downregulation of *CsDTX35.2* in CC-OR plants subjected to salt stress was correlated with an induction of *CsALMT9* (2.1 times higher than controls, Figure 8).

The expression of the gene *CsHKT1.2*, codifying for a Na^+ tonoplast transporter, was repressed in leaves from all the combinations except in CC-OR plants, with downregulation between 50.0% and 61.3% in salt-stressed plants in comparison to their respective controls (Figure 8). No differences were observed in the expression of the gene related to other studied Na^+ transporters in vacuole membrane, *CsNHX1* (Figure 8).

4 Discussion

Citrus is one of the most relevant fruit crops worldwide, whose productivity is being limited by the effects of climate change. Among abiotic stresses affecting this crop, high salinity is one of the most important, since citrus are usually grown in coastal areas where salinization of irrigation water is a main concern. Traditionally, approaches such as the use of tolerant rootstocks or the application of additional water to dilute the salt bulk have been used for mitigating the deleterious effect of this harmful situation, but strategies focused on the use of tolerant scions are practically unexplored (Ziogas et al., 2021). Some authors have intended to develop a platform to screen the rapid tolerance of citrus varieties to salt stress by using excised young twigs without roots (Ben Yahmed et al., 2016), which does not seem to be a realistic approach since citrus are commercially cultured grafted onto different rootstocks.

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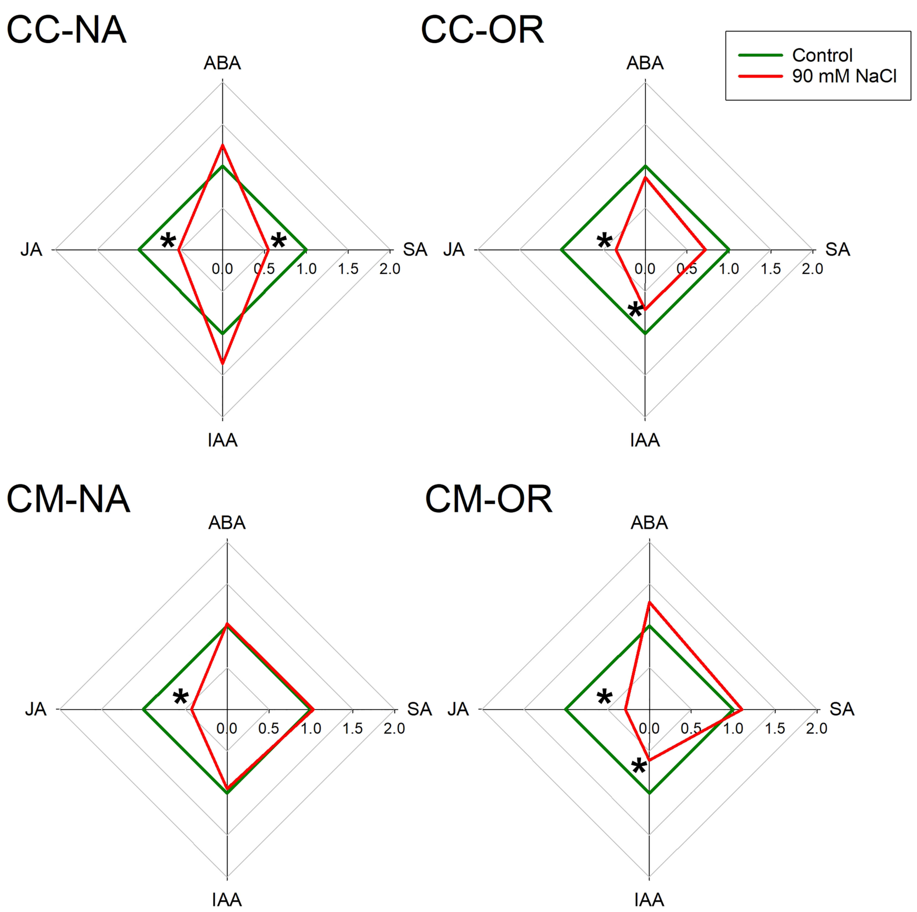

FIGURE 6
Root phytohormone content. Radar plots with root relative content of abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and indole acetic acid (IAA) in control (green lines) and 90 mM NaCl stressed plants (red lines) of CC-NA, CC-OR, CM-NA, and CM-OR after 30 days. Data are mean of three replicates. Asterisks denote statistically significant differences between control and salt-stressed plants of each scion/rootstock combination at $p \leq 0.05$.

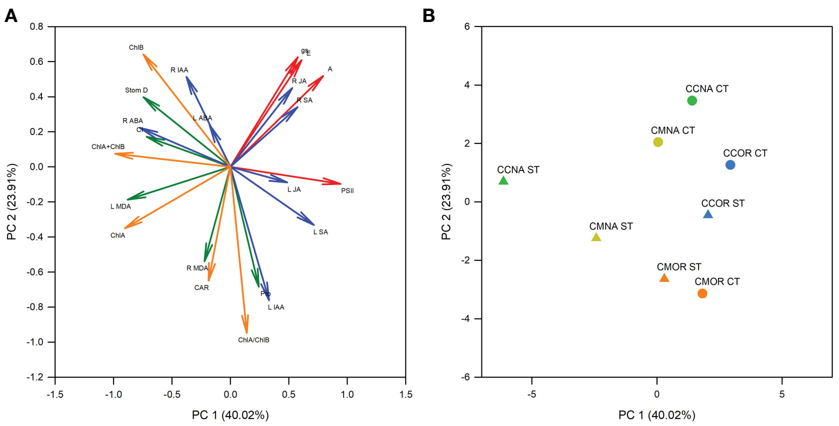

FIGURE 7
Principal component analysis. PCA with the physiological and biochemical data presented in this work. **(A)** Loading plot, representing photosynthesis related parameters (red arrows), phytohormone content (blue arrows), chlorophyll and carotenoid content (orange arrows), and other biochemical and physiological parameters (green arrows). **(B)** Scores plot, including the different groups of rootstock/scion combinations, CC-NA (green symbols), CC-OR (blue symbols), CM-NA (yellow symbols), and CM-OR (orange symbols) under control (circles) and salt stress (triangles) conditions.

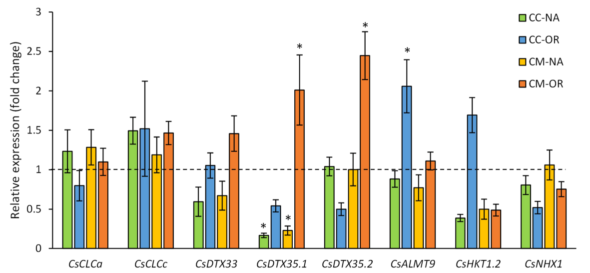

FIGURE 8
Gene expression. Relative expression of genes involved in Cl^- and Na^+ uptake in the vacuoles in CC-NA (green bars), CC-OR (blue bars), CM-NA (yellow bars), and CM-OR (orange bars) in control and 90 mM NaCl stressed plants. Data are mean of three replicates ± standard error. Asterisks denote statistically significant differences between control and salt-stressed plants of each scion/rootstock combination at $p \leq 0.05$.

In this work, a comparative analysis of the response of the four different citrus rootstock/scion combinations to salt stress was performed. To ensure that the observed responses were due to tolerance mechanisms and not to an excessive damage, a mild salt stress was applied, selecting a 90 mM NaCl concentration as used in previous works with citrus (Vives-Peris et al., 2018a), instead of higher concentrations used in other works, which could lead to an excessive damage not representing field situations. The low severity of the applied stress was supported by the small differences observed in the proline accumulation pattern, an amino acid that acts as a compatible osmolyte to reduce the osmotic stress induced by high salinity (Supplementary Figure 3; Vives-Peris et al., 2018a). Apart from its osmoprotectant role, other beneficial properties have been previously described for proline, including a high metal chelating capacity (Dar et al., 2016) and a role in the maintenance of the redox potential through reactive oxygen species (ROS) inactivation, since it is considered a non-enzymatic antioxidant (Kavi Kishor et al., 2022).

The maintenance of chlorophyll and carotenoid levels (Supplementary Figure 4) revealed a slight increase in Chl_b content in leaves of CC-NA plants, which could indicate a higher sensitivity of this genotype combination to the stress and a response to ameliorate the decrease in photosynthesis-related parameters. In fact, a similar increase in Chl_b concentration under mild salt stress conditions compared to control has been reported in lettuce plants subjected to 50 mM NaCl for 8 days, with its endogenous content being reduced in the presence of higher concentrations of NaCl (Shin et al., 2020). Chlorophyll and carotenoid contents have been described as markers of salt stress tolerance, with their levels usually decreased in sensitive plants subjected to this adverse condition (Ashraf and Harris, 2013). The few variations of their concentration observed in our work support the low severity of the applied stress conditions in both terms, NaCl concentration and duration.

The results obtained in this work provide evidence on some of the main scion-related morphological, physiological, and molecular mechanisms that are involved in citrus tolerance to high salinity in the irrigation solution. One of the most important factors contributing to this tolerance is the regulation of the photosynthetic apparatus. Thus, in a multitude of plant species, including citrus, the decrease in photosynthetic related parameters (including A, E, g_s, and Φ_{PSII}) under salt stress conditions has been widely studied, which are maintained when plants tolerate this adverse situation (Vives-Peris et al., 2018a), and their regulation may contribute to salt stress tolerance through the maintenance of the intrinsic water use efficiency (iWUE) levels, ensuring an optimal use of water and carbon (Fernández-García et al., 2014). This agrees with our results, since under salt stress, photosynthesis-related parameters decreased the most in CC-NA plants but were not affected in CM-OR plants (Figure 3). The decrease in stomatal conductance and transpiration is mainly, but not exclusively, mediated by ABA, and minimizes water loss and reduces the uptake of Na^+ and Cl^- ions (Acosta-Motos et al., 2015). Moreover, Φ_{PSII} only decreased in CC-NA plants under stress (Figure 3D), confirming the importance of the maintenance and reparation of the photosynthetic system in citrus plants when they are subjected to abiotic stress conditions, as it has been recently described for other stresses such as drought, high light, high temperatures, and their combinations (Balfagón et al., 2022).

All these parameters are highly involved in stress tolerance, since the maintenance of the photosynthetic system is crucial in the defense against hazardous environmental conditions, and allow to point out that the tolerance of OR to salt stress is higher than that of NA. This, along with the higher oxidative damage in CC-NA plants subjected to salt stress, marked an increase in MDA content, a subproduct of membrane lipid peroxidation (Figure 1), which is in concordance with the higher sensitivity of CC to salt stress in comparison to CM (which is moderately tolerant), although MDA basal levels were higher in CM-grafted plants, maybe due to the enhanced antioxidant system of CC, as it has been previously reported even under *in vitro* conditions (Vives-Peris et al., 2017). This higher MDA content in roots and leaves of plants grafted onto CM could be due to other parameters differentially affecting the rootstocks, such as soil texture or temperature, since previous works have reported that they have different tolerance to other abiotic stress conditions, such as low temperatures (Primo-Capella et al., 2022). Additionally, the higher MDA content could also be considered as beneficial, since it has been proposed as a signaling molecule whose transient accumulation under abiotic conditions can improve plant antioxidant responses to abiotic stress conditions through the induction of the expression of ALDHs (reviewed in Morales and Munné-Bosch, 2019).

There are several factors that can contribute to the enhanced tolerance of OR to salt stress, including the lower stomatal density of this genotype (Figure 4), which would contribute to better control the transpiration and, consequently, the uptake of water and Na^+ or Cl^- excess, as it is shown by the correlation among leaf Cl^- and Na^+ levels found in the different genotypes (the lowest in the tolerant CM-OR and the highest and fastest in the sensitive CC-NA, Figure 2; Supplementary Table 5). Although Cl^- is an essential element for plants, involved in key processes such as photosynthesis, stomatal regulation, nitrogen metabolism, or osmoregulation (Colmenero-Flores et al., 2019), excessive levels of this ion as a consequence of soil salinity are the main factor determining leaf damage in citrus under salt stress in comparison with Na^+ toxicity or the osmotic component (López-Climent et al., 2008). The improved photosynthetic regulation could be partially mediated by phytohormones such as ABA, whose content was only increased in leaves of CM-OR plants after 30 days of stress (Figure 5; Supplementary Table 6), this molecule being one of the main signals inducing the stomatal closure in plants cultured under several adverse conditions (Bharath et al., 2021). Although this phytohormone is often accumulated under salt stress conditions, in this work, no increase was observed in plants of remaining rootstock/scion combinations, which could confirm that the stress intensity was moderated. Thus, the increase in ABA content in CM-OR leaves was relatively low and could not be enough for an efficient control of stomatal closure, and consequently, this difference could be due to an ABA-independent stomatal control as described by previous works (Hsu et al., 2018), which could be regulated by other hormones such as SA or oxylipins (Montillet et al., 2013) or even calcium (Wang et al., 2012).

The accumulation of JA under abiotic stress conditions has been previously studied and is often accompanied by an SA content decrease as in this case; thus, both phytohormones have been usually reported as antagonists (Wang et al., 2021). In our work, a JA accumulation was observed in leaves of the sensitive combination CC-NA, while a decrease in leaf SA content was observed only in this combination. However, a general decrease in JA root levels was observed, which is more evident in those plants grafted onto the salt-tolerant rootstock CM. This would also support the idea of the incipient grade of salt stress at this point, since existing literature describes lower JA contents in plants tolerant to salt stress (Shahzad et al., 2015). It could also verify the higher tolerance of CM to high salinity with respect to CC. Interestingly, root IAA levels decreased in salinized plants having OR as scion. Therefore, the grafted scion would be essential for IAA synthesis and transport to the roots. Hence, low IAA translocation to roots could improve salt stress tolerance by the reduction of root branching, thus limiting the surface of Na^+ or Cl^- absorption. In fact, the main biosynthesis route of this auxin derives from tryptophan, which is produced in the chloroplasts via the shikimate pathway, stressing the importance of the aerial tissues in the production of this phytohormone (Casanova-Sáez et al., 2021).

The accumulation of Cl^- and Na^+ ions in the vacuole could be another strategy to reduce the negative impact of salt stress, as it has been previously described in other plant species such as Arabidopsis or soybean (reviewed in Wu and Li, 2019). Thus, several tonoplast transporters have been identified, including Cl^- transporters such as CLCa, CLCc, DTX33, DTX35, and ALMT9 (De Angeli et al., 2009; Baetz et al., 2016; Zhang et al., 2017) or Na^+ transporters HKT1 or NHX1 (Martínez-Alcántara et al., 2015). The analyses of the expression of genes codifying for Cl^- or Na^+ transporters resulted in an overexpression of *CsDTX35.1* and *CsDTX35.2* genes in the tolerant combination CM-OR (Figure 8). Consequently, these results suggest that *CsDTX35.1* and *CsDTX35.2* (two isoforms from *AtDTX35*) are crucial for Cl^- transport through the tonoplast in citrus, as it has been previously described for Arabidopsis (Zhang et al., 2017), whereas the role of CLC transporters would be less relevant since their gene expressions are not affected by salt stress. This information could confirm the enhanced capability of CM-OR plants to synthesize these transporters, responsible for the vacuole compartmentalization of Cl^- ions from the cytosol under salt stress conditions (considering that Cl^- is the most damaging component of salt stress in citrus) whereas Na^+ transport through tonoplast could be less relevant (López-Climent et al., 2008). Thus, since Cl^- content was lower in CM-OR plants, a double strategy could be used in these plants, excluding ion root uptake and enhancing its compartmentalization in the vacuoles. In the sensitive plant combinations, higher levels of Cl^- ions would be dispersed in the cytosol or moved through xylem to other tissues following the transpiration stream (Geilfus, 2018). The identification of these candidate genes, *CsDTX35.1* and *CsDTX35.2*, can be useful for future breeding citrus programs, either by selecting new varieties with higher expression of these genes in response to high salinity, or by increasing their expression through genetic engineering techniques. In this sense, Arabidopsis plants overexpressing the gene codifying for the tonoplast Na^+ transporter *At*NHX1 improve their tolerance to salt stress (Pabuayon et al., 2021).

5 Conclusion

In addition to the rootstock, scion plays an important role in salt stress tolerance in citrus. This work shows some of the main scion-related morphological, physiological, and molecular mechanisms that are involved in citrus tolerance to high salinity and can be the bases for future breeding programs and could also provide mechanisms for selecting the best rootstock–scion combination under each particular condition, contributing to a more productive and sustainable citrus industry.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

VV-P and ML-C performed all experiments, VV-P, AG-C and RP-C conceived the study and the experimental design and wrote the manuscript. VV-P and MM-S performed the sample and data analysis. AG-C and RP-C supervised the work and provided financial resources. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1145625/full#supplementary-material

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Short-term water stress responses of grafted pepper plants are associated with changes in the hormonal balance

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Phytohormones play an important role in regulating the plant behavior to drought. In previous studies, NIBER® pepper rootstock showed tolerance to drought in terms of production and fruit quality compared to ungrafted plants. In this study, our hypothesis was that short-term exposure to water stress in young, grafted pepper plants would shed light on tolerance to drought in terms of modulation of the hormonal balance. To validate this hypothesis, fresh weight, water use efficiency (WUE) and the main hormone classes were analyzed in self-grafted pepper plants (variety onto variety, V/V) and variety grafted onto NIBER® (V/N) at 4, 24, and 48h after severe water stress was induced by PEG addition. After 48h, WUE in V/N was higher than in V/V, due to major stomata closure to maintain water retention in the leaves. This can be explained by the higher abscisic acid (ABA) levels observed in the leaves of V/N plants. Despite the interaction between ABA and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in relation to stomata closure is controversial, we observed an important increase of ACC at the end of the experiment in V/N plants coinciding with an important rise of the WUE and ABA. The maximum concentration of jasmonic acid and salicylic acid after 48h was found in the leaves of V/N, associated with their role in abiotic stress signaling and tolerance. Respect to auxins and cytokinins, the highest concentrations were linked to water stress and NIBER®, but this effect did not occur for gibberellins. These results show that hormone balance was affected by water stress and rootstock genotype, where NIBER® rootstock displayed a better ability to overcome short-term water stress.

KEYWORDS

Capsicum annuum, drought stress, leaves, phytohormones, root, water use efficiency

1 Introduction

Drought stress is one of the most important environmental factors negatively affecting agriculture production and it has been aggravated in the last decade by climatic change worldwide (Gray and Brady, 2016). Most crops are highly vulnerable to drought stress, resulting in growth and production impairment with relevant economic consequences (Vicente-Serrano, 2007).

Plants have developed several adaptive strategies to mitigate the negative effects of water scarcity, evolving morpho-physiological, phenological, biochemical, and genetic mechanisms (Basu et al., 2016; Ullah et al., 2018). Plant roots are the first organs sensing soil water deficit and this perception induces a complex signaling network from root to shoot (and shoot to root), in which hormones, reactive oxygen species (ROS), sugars, other metabolites, and small nucleotides are mainly involved (Albacete et al., 2014). Among them, phytohormones are the key mediators of plant responses to drought stress, they are involved in the tolerance strategies (Pérez-Alfocea et al., 2011; Ullah et al., 2018) by producing chemical messengers which activate various physiological processes to overcome drought stress (Fahad et al., 2015).

Drought provokes osmotic stress that induces abscisic acid (ABA) synthesis, which is implicated in the synthesis of compatible osmolytes, the regulation of drought-responsive genes expression, and the regulation of stomatal closure. Generally, ABA synthesis occurs in the roots from where it is translocated to the leaves *via* the xylem sap, inducing stomatal closure to decrease water loss. However, several experiments have demonstrated that ABA biosynthesis also takes place in leaves (Holbrook et al., 2002; Manzi et al., 2017; López-Serrano et al., 2020), but also stomatal closure can occur without the assistance of ABA root synthesis (Holbrook et al., 2002).

Ethylene or its direct precursor 1-aminocyclopropane-1-carboxyl acid (ACC) is highly mobile within the cell and can be translocated basipetally *via* the phloem or acropetally through the xylem (Druege, 2006). Both have been considered important regulators of water stress responses by inducing leaf senescence, epinasty, organs abscission, and leaf growth inhibition (Acosta-Motos et al., 2020; Fatma et al., 2022).

Other hormones, such as auxins (IAA), cytokinins (CKs), and gibberellins (GAs) are directly involved in the control of plant growth and their concentrations can be environmentally modulated (Werner et al., 2001; Sachs, 2005), playing critical roles during water stress. However, they can have an opposite effect since high auxin levels have been associated with drought tolerance, while GA accumulation decreased tolerance (Ullah et al., 2018). CKs have shown a dual role under water stress since positive but also negative effects on drought tolerance have been reported (Zwack and Rashotte, 2015; Li et al., 2016). A decrease in CK transport from the root to the shoot could inhibit leaf growth while a low CK content would promote root growth and modify the root/shoot ratio (Rahayu, 2005).

Furthermore, jasmonic acid (JA) and salicylic acid (SA) are hormones classically involved in biotic stress tolerance signaling (Li et al., 2003), and it is only recently that their importance in abiotic stress responses has been revealed (Muñoz-Espinoza et al., 2015). Water deficit increased JA levels in several species (Brossa et al., 2011; Chen et al., 2016; de Ollas et al., 2018). Moreover, JA and SA regulate stomatal conductance, increase root hydraulic conductivity, enhance the scavenging of ROS by antioxidant activity stimulation, and promote root development, thus contributing to drought tolerance (Munné-Bosch and Peñuelas, 2003; Saruhan et al., 2012; Aslam et al., 2021). Their function is directly related to their relative and absolute concentrations, when SA and JA were equally applied externally at low concentrations they acted synergistically, whereas applying high concentrations of one hormone antagonized the other one (Mur et al., 2006).

It is important to note that the role of each phytohormone has been frequently described considering individual signaling pathways and not the hormonal interaction network, the spatial organ distribution, the long-distance hormonal signaling, and the type of crosstalk between hormones (positive or negative), which can be dependent of the magnitude (time and intensity) of the water stress. Indeed, different studies have demonstrated the hormonal interactions taking place under drought stress, highlighting the complexity of hormonal signaling cascades (Davies et al., 2005; Muñoz-Espinoza et al., 2015; de Ollas et al., 2018; Ullah et al., 2018; Devireddy et al., 2021; Huntenburg et al., 2022).

An important approach for discovering how long-distance hormonal communication and how roots can alter the shoot perception (or vice versa) under stress is the use of grafted plants. Vegetable grafting has become an effective strategy to increase tolerance under water stress (Rouphael et al., 2008; Penella et al., 2014; Sánchez-Rodríguez et al., 2016; López-Serrano et al., 2019; Gisbert-Mullor et al., 2020; Padilla et al., 2021) by the use of tolerant rootstocks that improve the physiological performance of plants under drought conditions. Some studies have demonstrated that the efficiency of tolerant rootstocks in overcoming water stress is related to their higher capacity to absorb water and nutrients, maintain root growth, achieve an active osmotic adjustment, and activate the antioxidant defense systems (Rouphael et al., 2008; Yao et al., 2016; Zhang et al., 2019a). This higher root efficiency under water stress contributes to maintain the metabolic processes taking place in the scion, sustaining plant growth and productivity. In addition, hormonal communication plays an important role in achieving water stress adaptation of grafted plants. Different combinations of rootstocks and scions have different modes of phytohormone synthesis transport (Lacombe and Achard, 2016; Lu et al., 2020) and affect plant adaptability to stress. ABA is the main hormone studied in grafted plants under water stress, because of its function in controlling stomata closure. Most studies have been done in tomato (Holbrook et al., 2002; Dodd et al., 2009; Ghanem et al., 2011; Cantero-Navarro et al., 2016; Gaion et al., 2018; Zhang et al., 2019b) and cucumber (Liu et al., 2016). However, there are no reports on hormonal balance regulation in grafted pepper plants exposed to water stress, being sweet pepper an important vegetable crop, thoroughly cultivated in the Mediterranean area, where water shortage is a major problem limiting productivity (Penella et al., 2014). Even more, the availability of rootstocks tolerant to water stress is lacking in pepper plants (Lee et al., 2010; Penella et al., 2014; Kyriacou et al., 2017). Nevertheless, to fill this gap we have obtained by a classic breeding program a water stress tolerant rootstock, NIBER®, an F1 hybrid that has been tested under field conditions achieving greater yields than the ungrafted variety (Gisbert-Mullor et al., 2020). Mechanisms by which NIBER® rootstock modulates plant performance under water stress, particularly hormonal balance, neither have not been fully unraveled.

Therefore, the present work aimed to determine whether the water stress tolerance observed in pepper plants grafted onto NIBER® in terms of productivity is associated with changes in the hormonal balance in early stage of grafted plant development and identify the hormones role responsible for the drought tolerance in rootstock and scion. Understanding the interactive hormonal mechanism can be effective for the development to tolerant rootstocks.

To fulfill this, we compared the hormonal profiles (ACC, CKs, GAs, ABA, IAA, JA, and SA) in roots and leaves of two pepper graft combinations (variety grafted onto NIBER® and self-grafted variety) under optimal and short-term water stress conditions.

2 Materials and methods

2.1 Plant material

Based on our previous studies (Gisbert-Mullor et al., 2020), a hybrid pepper rootstock tolerant to water stress i.e., NIBER® (*Capsicum annuum* x *C. annuum*, abbreviated as N) was employed in this study. Two plant combinations were herein used: the commercial pepper variety "Maestral F1" (sweet pepper, California-type, Semillas Fitó, Spain, abbreviated as V) was grafted onto NIBER® (abbreviated as V/N) and the self-grafted plants (abbreviated as V/V), thus considering the grafting effect. Early in March, the seeds of V and N were sown in 104 seedling trays filled with a peat-based substrate for germination. The grafts were performed after 2 months using the tube-grafting method (Penella et al., 2015). The grafted plants were maintained in a chamber with relative humidity above 95% and air temperature around 28-29°C for a 4-6 day period to successfully join rootstock and scion (Penella et al., 2014).

2.2 Hydroponic greenhouse conditions

Three weeks after grafting (by the beginning of June), seedlings were removed from the substrate and their roots were cleaned before being placed in 2L polyethylene pots in a Venlo-type greenhouse situated in Valencia (Spain, 39° 29' 1.135" N 0° 20' 27.315" W) under natural light conditions (610-870 µmol m^{-2} s^{-1}) and temperature and relative humidity ranges of 21-25°C and 52-72%, respectively. Pots were filled with a nutrient solution containing (in mmol L^{-1}): 13.0 NO_3^-, 1.0 $H_2PO_4^-$, 2.45 SO_4^{2-}, 1.6 Cl^-, 1.0 NH_4^+, 6.0 K^+, 4.0 Ca^{2+}, 2.5 Mg^{2+}, 0.5 Na^+ and micronutrients (15.8 µM Fe^{2+}, 10.3 µM Mn^{2+}, 4.2 µM Zn^{2+}, 43.5 µM B^+, 2.14 µM Cu^{2+}), that were artificially aerated with an air pump. The electrical conductivity and pH of the nutrient solution were 2.1 dS m^{-1} and 6.7, respectively. After 7 days of seedling acclimation to the pots, the water stress treatment was initiated by adding 5% PEG 8000 (Sigma Co.) to the nutrient solution. The osmotic potential of the nutrient solutions, measured by a vapor osmometer (Digital osmometer, Wescor, Logan, USA), were -0.55 MPa for 5% PEG and -0.05 MPa for the control solution (0% PEG). The layout was a completely randomized design with 20 plants per combination (V/V and V/N) and treatment (5% PEG and control).

2.3 Fresh weight determination

Fresh weight determinations were performed at the end of the experiment (48h) using the plants that were not used for the hormonal analysis. Four plants per graft combination and treatment were analyzed by measuring the fresh weight of leaves and roots. The data are shown as a percentage of water stress over control conditions for self-grated (V/V) and variety grafted onto NIBER® (V/N).

2.4 Photosynthesis analysis

Gas exchange measurements were performed at the beginning (T0) and the end of the experiment (T48). The gas exchange measurements were taken in the morning (9.30 am to 10.30 am GMT) with four plants per graft combination and treatment. The net CO_2 assimilation rate (A_N, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), and transpiration rate (E, mmol H_2O m^{-2} s^{-1}) were determined on fully expanded leaves (3rd - 4th leaf from the apex) in the steady-state under saturating light conditions (1000 µmol m^{-2} s^{-1}) and with 400 ppm CO_2 by a LI-6400 infrared gas analyzer (LI-COR, Nebraska, USA) at 24 ± 2°C and 65 ± 10% relative humidity. Parameters A_N/g_s and A_N/E_{leaf} were calculated as intrinsic water use efficiency and instantaneous water use efficiency, respectively.

2.5 Sampling for hormonal analysis

The samples (leaves and roots) for hormonal analysis were taken before PEG addition (T0), and 4h (T4), 24h (T24), and 48h (T48) after water stress treatment began. Measurements were taken in fully expanded mature leaves (3rd – 4th leaf from the shoot apex) and 2 cm from distal roots. The layout was randomized with 4 samples of independent plants. The samples were frozen in liquid nitrogen immediately after harvest, conserved at −80°C, and afterwards freeze-dried.

2.6 Hormone extraction and analysis

Cytokinins (*trans*-zeatin, tZ, zeatin riboside, ZR, and isopentenyl adenine, iP), gibberellins (GA1, GA3, and GA4), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and the ethylene precursor 1-

aminocyclopropane-1-carboxylic acid (ACC) were analyzed according to Albacete et al. (2008) and Großkinsky et al. (2014) with some modifications. Briefly, 40 mg of freeze-dried plant material were homogenized and dropped in 1 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20000 g, 15 min) and re-extracted for 30 min at 4°C in additional 1 mL of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and evaporated at 40°C under vacuum either to near dryness or until the organic solvent was removed. The residue was dissolved in 0.5 mL methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA).

Ten µL of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each analyzed component (1, 10, 50, and 100 µg L^{-1}) and corrected for 10 µg L^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%.

2.7 Statistical analysis

Data for each measure time (T0, T4, T24 and T48) and parameter were subject to an analysis of variance using Statgraphics Centurion 18 (Statgraphics Technologies, Inc., The Plains, Virgina, USA). The mean comparisons were performed using Fisher's least significance difference (LSD) test at $P \leq 0.05$.

3 Results

3.1 Fresh weight

The fresh weight of leaves (Figure 1A) was affected by water stress at the end of the experiment with significant differences for both plant combinations, with a 28% and 83% reduction in V/N and V/V respectively, compared with control conditions. The fresh root weight (Figure 1B) was less affected by water stress without significant differences, the reduction was 12% and 8% for V/V and V/N, respectively and respect to their controls.

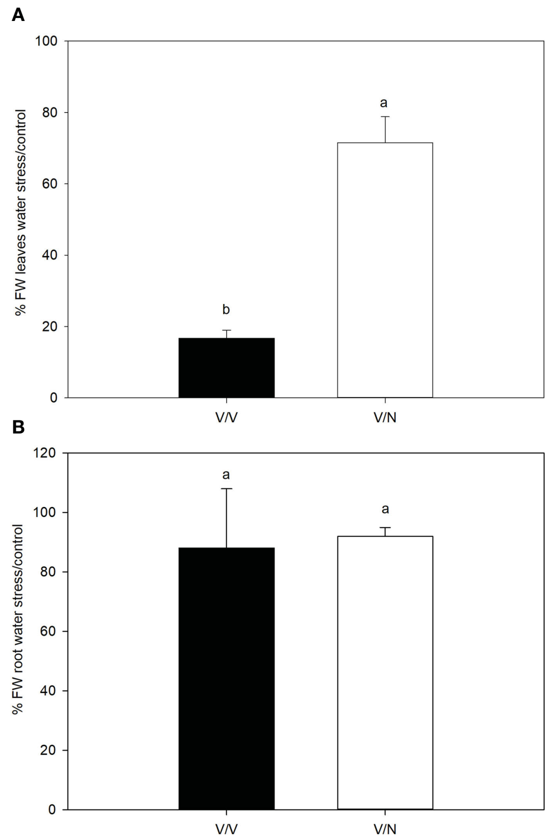

FIGURE 1
Percentage fresh weight in leaves **(A)** and roots **(B)** under water stress conditions compared to control conditions in the selfgrafted plants (V/V) and V grafted onto NIBER® (V/N). Values are mean for n = 4. Different letters are statistically different according with LSD test ($P \leq 0.05$).

3.2 Photosynthetic parameters

Instantaneous water use efficiency (A_N/E) (Figure 2A) did not show significant differences at T0 for V/V and V/N with values between 1.8-2.2. After 48h, all plants with PEG treatment increased significantly the A_N/E values. The increase with respect to its control plants was 47% for V/V and 44% for V/N, being the highest values for V/N-WS Regarding intrinsic water use efficiency (A_N/g_s) (Figure 2B), differences between genotypes were already observed at T0, V/V showed lower values compared with V/N. At the end of the experiment, plants under PEG treatment exhibited higher values with significant differences respect to control plants, plus the highest rise was observed in V/N plants.

3.3 Hormonal profiling

3.3.1 ACC

In general terms, ACC levels were higher in roots than in leaves, reaching up to 4.5-fold as a mean value for all times and all plant combinations. At T0, in the control treatment, V/V and V/N did not show significant differences either in roots or in leaves (Figures 3A, B). From T4 to T48, ACC concentration remained constant for each plant combination and treatment except at T24 for V/V in roots and at T48 for V/N in leaves, when the highest ACC levels were observed in response to water stress.

3.3.2 ABA

In contrast to ACC, the ABA concentrations were higher in leaves than in roots. Similar to ACC at T0, ABA levels in roots and leaves (Figures 3C, D) did not display significant differences between V/V and V/N. In roots, at T4 and T24 the highest values were found in V/N-WS, while at the end of the experiment (T48) the ABA levels for this plant combination decreased by 54%. In leaves,V/N-WS ABA concentrations reached the highest values at T48 with significant differences. At T48 in roots and leaves, the lowest ABA values were found in V/V control plants, with significant differences to the rest of the plant combinations and treatments.

3.3.3 IAA

In roots, IAA concentration (Figure 4A) remained constant in V/N control and in V/V-WS decreased along the experiment. Nevertheless, for the rest of the plant combinations and treatments there was an erratic behavior, highlighting the IAA decrease from T24 to T48 for V/V control and the increase for V/N-WS. In leaves, IAA levels (Figure 4B) increased along the experiment (except for V/V control), reaching the maximum

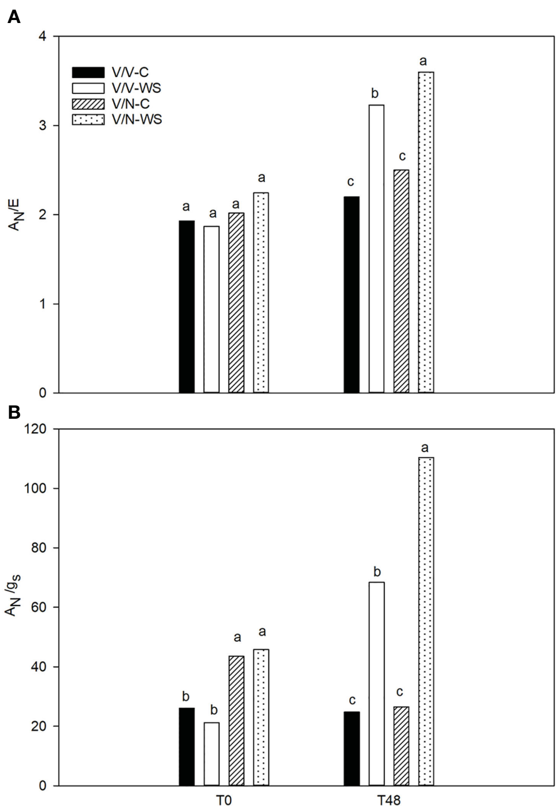

FIGURE 2
Instantaneous water use efficiency (A_N/E) **(A)** and intrinsic water use efficiency (A_N/g_s) **(B)** in the self-grafted pepper plants (V/V) and the plants grafted onto NIBER® (V/N) at 0% PEG (control, C) or 5% PEG (water stress, WS). Measurements were taken on T0 and T48 (hours after treatment with PEG began). Data are the mean values for n = 4. For each studied time, different letters indicate significant differences at $P \leq 0.05$ (LSD test).

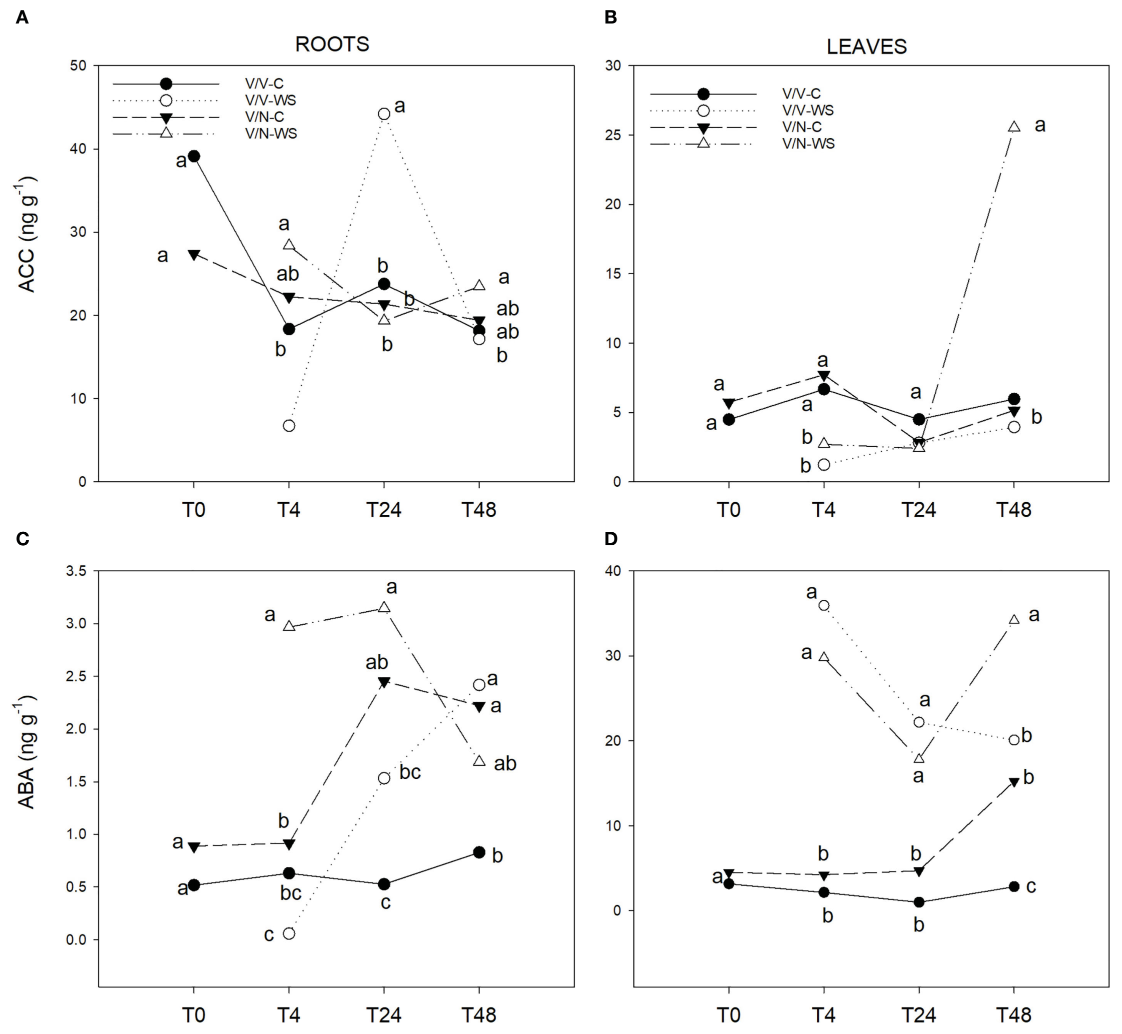

FIGURE 3
ACC **(A, B)** and ABA **(C, D)** levels in roots and leaves of self-grafted pepper plants (V/V) and the plants grafted onto NIBER® (V/N) at 0% PEG (control, C) or 5% PEG (water stress, WS). Measurements were taken on T0, T4, T24 and T48 (hours after treatment with PEG began). Data are the mean values for n = 4. For each studied time, different letters indicate significant differences at P ≤ 0.05 (LSD test).

values at the end of the experiment in V/V-WS and V/N control, without significant differences with V/N-WS.

3.3.4 CKs

CKs levels were 9-fold lower in leaves than roots, showing different dynamics in both organs. In roots, CKs levels (Figure 4C) remained constant after a decrease from T0 to T4, except for a sustained increase in V/N-WS at T24, following a decrease to the lowest CKs concentrations at the end of the experiment. In leaves (Figure 4D), CKs behavior resembled IAA role, with an increase from T4 to T48 for all plant combinations and treatments without significant differences between them at the end of experiment.

3.3.5 GAs

The concentrations of GAs were similar in leaves and roots (Figures 4E, F). In roots, an increase was observed in V/N control and V/V-WS from T4 to T48. In leaves, in response to water stress, a peak of GAs was detected at T24 in V/N-WS, decreasing later to reach similar values to the control plants. The lowest GA values were found in V/V plants under control and water stress conditions.

3.3.6 JA

In roots, the levels of JA (Figure 5A) were 9-fold (as average) higher than in leaves. In roots, both treatments showed a differential trend. The highest values were observed in control conditions with a peak in both plant combinations at T24. Under water stress, V/V and V/N displayed the lowest values, without significant differences between them. However, in leaves (Figure 5B), a peak at T24 in the JA levels was observed for all plant combinations and treatments, following a decrease until T48, being the highest values for V/N-WS and the lowest values for V/V-WS, with significant differences.

3.3.7 SA

In roots, increased SA concentrations in response to water stress were detected at T24 for V/V and V/N (Figure 5C). From T24 to T48 SA levels decreased to similar values for all plant combinations

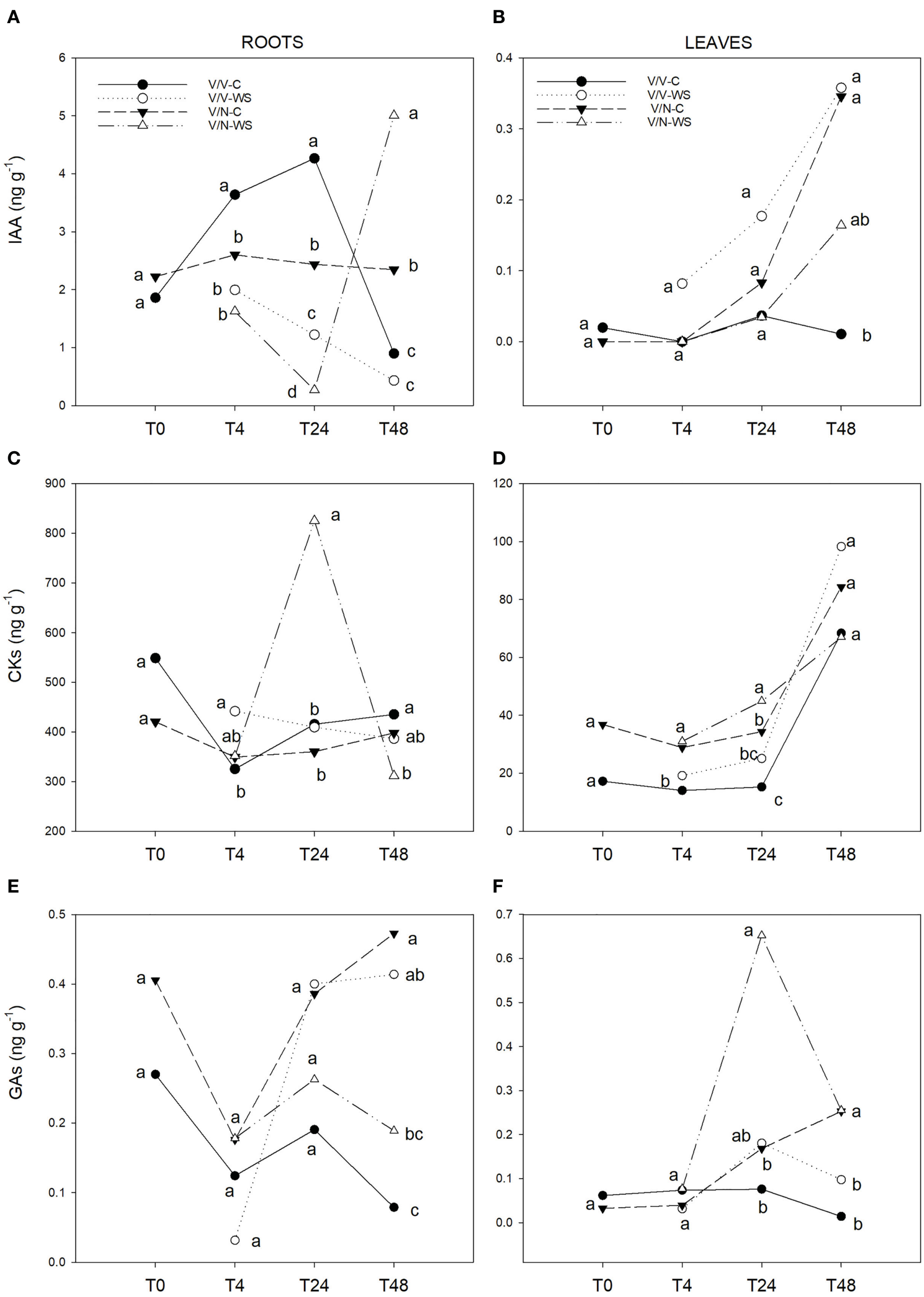

FIGURE 4
IAA **(A, B)**, CKs **(C, D)** and GAs **(E, F)** concentration in roots and leaves of self-grafted pepper plants (V/V) and the plants grafted onto NIBER® (V/N) at 0% PEG (control, C) or 5% PEG (water stress, WS). Measurements were taken on T0, T4, T24 and T48 (hours after treatment with PEG began). Data are the mean values for n = 4. For each studied time, different letters indicate significant differences at P ≤ 0.05 (LSD test).

and treatments, without significant differences between them. A different evolution was observed in leaves (Figure 5D) with respect to roots, with a constant drop along the experiment in all plants and treatments, standing up V/N-WS with the highest concentration at T4. At the end of the experiment, two groups were separated, with highest SA levels belonging to water-stressed plants and lowest values to control plants.

4 Discussion

The study of hormone signaling fine-tuning during the early state of water stress exposure could help to distinguish and understand the tolerance responses in grafted plants mediated by efficient rootstocks. Indeed, plant hormones play a key role in controlling the adaptive processes to water stress, involving long-

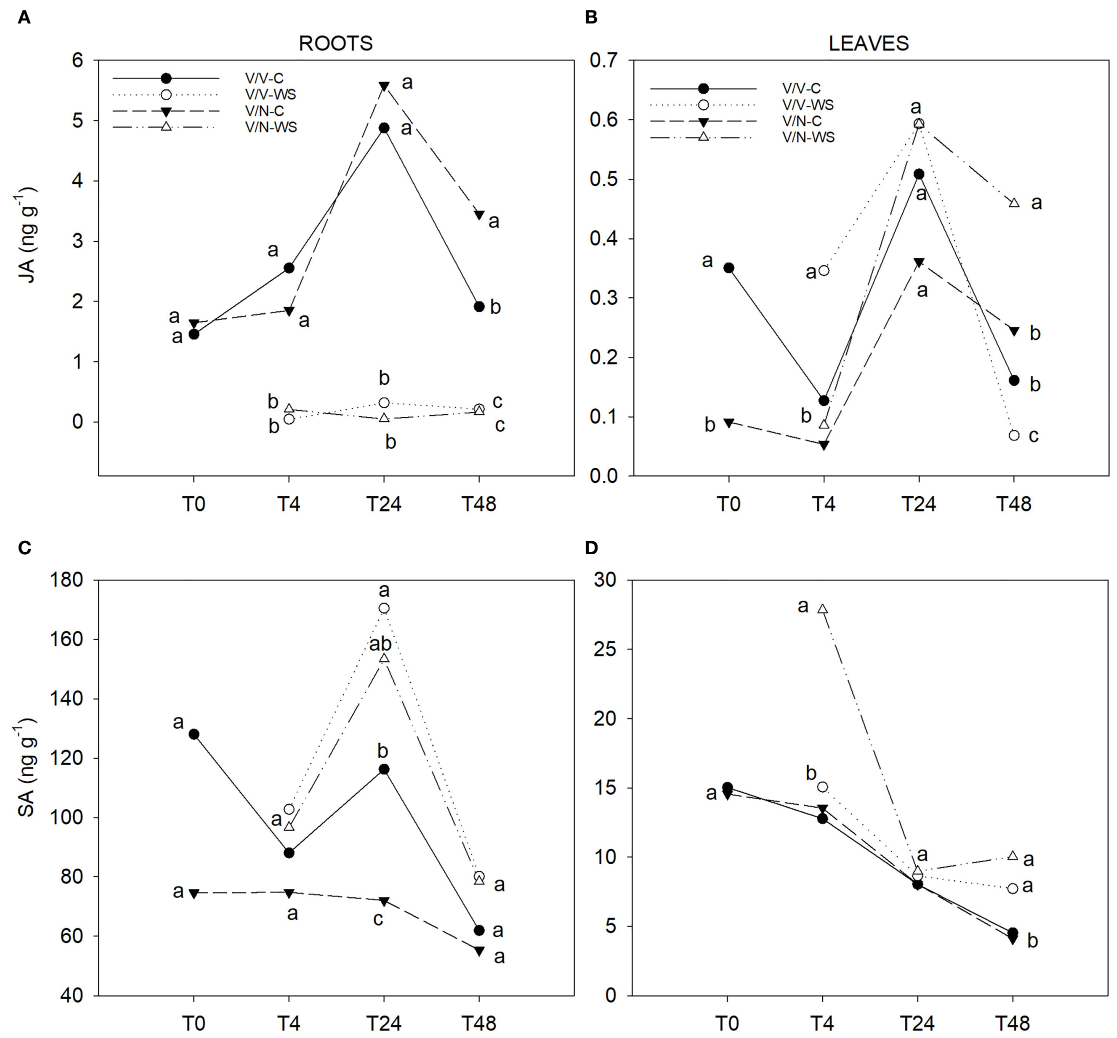

FIGURE 5
JA **(A, B)** and SA **(C, D)** levels in roots and leaves of self-grafted pepper plants (V/V) and the plants grafted onto NIBER® (V/N) at 0% PEG (control, C) or 5% PEG (water stress, WS). Measurements were taken on T0, T4, T24 and T48 (hours after treatment with PEG began). Data are the mean values for n = 4. For each studied time, different letters indicate significant differences at P $\leq$ 0.05 (LSD test).

distance communication between different organs of plants together with *in situ* phytohormone synthesis (Albacete et al., 2008; Acosta-Motos et al., 2020; Lu et al., 2020).

Under water stress, roots are the first organs to perceive the osmotic stress, causing a rapid loss of shoot turgor and stomata closure, within minutes and hours (Munns, 2002). In this study, we observed at 48h that osmotic stress provoked a higher increase in A_N/g_s in V/N than in V/V. This indicates that the regulation of stomatal closure is more efficient in the V/N combination, thus allowing major water accumulation in the leaves. In this sense, the fresh weight loss on leaves caused by osmotic stress was reduced in V/N plants compared to V/V, since N was considered as a tolerant rootstock. However, no effect was detected in the fresh weight of the roots in both graft combinations, indicating that the leaves were more sensitive to osmotic stress than the roots. Several authors (Hsiao and Xu, 2000; Sharp, 2002; López-Serrano et al., 2019) have also observed this differential response between roots and leaves. This is interesting because V/N water requirements and use should be lower, implicating positive economic and environmental effects. Indeed, graft technology has been used as an effective tool to increase WUE under water stress in vegetable crops like tomato (Cantero-Navarro et al., 2016; Gaion et al., 2018; Fullana-Pericàs et al., 2020), pepper (Gisbert-Mullor et al., 2020; Padilla et al., 2021) or cucumber (Liu et al., 2016).

Water relations traits are controlled by hormonal signals from root to shoot and shoot to root. Currently, ABA is the primary hormone that modulates stomatal performance contributing to the regulation of water-mediated stomatal closure and plays a key role in drought resistance (Holbrook et al., 2002; Gaion et al., 2018; Yang et al., 2022). Under short-term water stress, it has been described that ABA is synthesized mainly in the roots and afterward transported into guard cells to trigger stomata closure in leaves (Wilkinson and Davies, 2002; Allario et al., 2013; Sarwat and Tuteja, 2017). We found a fast induction (T4) of ABA concentration under water stress in V/N for roots and leaves, but only for the leaves in V/V. This suggests that the roots of a tolerant water stress rootstock such as NIBER® were more sensitive to changes in the osmotic potential of the nutrient solution. As a consequence, V/N speeded up ABA synthesis in roots and transport to the leaves (Liu et al., 2016), as it can be observed in ABA rise at T48 in leaves coinciding with ABA decrease in roots. Hence, a differential rootstock behavior was observed under water stress, with an ABA hypersensitivity or biosynthesis to ABA in V/N plants that can increase WUE in the scion thus enhancing stress tolerance. Similar responses were observed in different vegetable-grafted plants using tolerant rootstocks under water stress, such as cucumber grafted onto luffa (Liu et al., 2016) and tomato grafted onto different recombinant inbred lines from *Solanum pimpinellifolium* (Cantero-Navarro et al., 2016). Considering that the ABA levels in leaves were higher than in the roots in all plant combinations and treatments, the synthesis of ABA in the scion cannot be ruled out (Manzi et al., 2017; López-Serrano et al., 2020).

ABA and ethylene (or its precursor ACC) regulate stress responses in coordinated ways, in senescence, flooding, drought, and wounding stresses, and have been considered important WUE regulators under stress conditions (Wilkinson, 2004; Cantero-Navarro et al., 2016). However, the interaction between ethylene and ABA in relation to stomata closure is controversial and still not fully understood (Wilkinson et al., 2012; Chen et al., 2013). Generally, ABA and JA are positive regulators of stomata closure, while IAA and CKs have been described as negative regulators. However, the regulatory role of ethylene on stomata behavior is ambiguous, acting as a positive or negative regulator depending on the tissue and environmental conditions (Nemhauser et al., 2006; Huang et al., 2008; Daszkowska-Golec and Szarejko, 2013). In this sense, under water stress, elevated ABA levels usually limited ethylene production in maize plants (Sharp et al., 1994). In *Arabidopsis thaliana*, ethylene physiologically inhibited ABA-dependent stomata closure through the ethylene signaling pathway (Tanaka et al., 2005). Despite the apparent antagonist relation between ABA and ethylene under water stress (Spollen et al., 2000), in *A. thaliana* ethylene signaling was promoted during short-term ABA treatment (*ERF1*, *EDF1* and *EDF4* up-regulated) (Yang et al., 2014). In citrus (Tudela and Primo-Millo, 1992) and pea (Belimov et al., 2009), water stress induced an increase in ACC concentrations. Additionally, in grafted tomato plants, ACC in the roots could increase agronomic WUE (Cantero-Navarro et al., 2016). These results show that ethylene also plays an important role in stomatal control (Desikan et al., 2006; Vysotskaya et al., 2011). We did not find dramatic changes in ACC levels in leaves, except for an important increase at the end of the experiment (after 48h of water stress) in plants grafted onto NIBER®, coinciding with a significant rise of intrinsic WUE and ABA. These results could indicate that ACC is promoted at the initial stage of ABA-dependent control of water stress, using ACC as a rapid response to accelerate tolerance in V/N (Yang et al., 2014). Importantly, this effect was not observed in V/V plants, and only a maximum ACC concentration was measured at T24 in roots following an important decrease at T48h. This drop was not associated with an increase in leaves, which could indicate ACC degradation in the roots.

In addition to ABA and ethylene, JA and SA are also involved in the stomata response under water stress (Nazareno and Hernandez, 2017; Munemasa et al., 2019; Müller and Munné-Bosch, 2021). JA and 12-OPDA (JA precursor) are positive regulators of stomata closure, leading to increased drought stress tolerance (Savchenko and Dehesh, 2014). However, we did not find any change in JA concentrations in the root system under water stress, indicating that JA is not a primary hormonal factor controlling drought stress and/or there was an early transient increase. Similarly, other studies did not find changes in JA under water stress, possibly due to JA accumulation being characterized as early transient (within 3h), therefore being dependent on the measure time (Luo et al., 2019; Wang et al., 2020; Huntenburg et al., 2022). In the leaves, the highest JA levels at the end of the experiment were found in V/N under water stress, which coincides with increased levels of ABA and ACC and stomata closure. Regarding SA, its role has been associated with biotic stress defense responses (Vlot et al., 2009). Recently, different research works have suggested that SA can have an important contribution to abiotic stress-induced signaling and tolerance (Miura and Tada, 2014; Zandalinas et al., 2016). However, the effect of SA on water stress tolerance is still unclear (Borsani et al., 2001). In our experimental conditions, SA increased at T24h in the roots mainly under water stress in both plant combinations, thus indicating that SA may be involved in drought responses. SA content augmented approximately 2-fold with water stress in barley roots associated to ABA increase (Bandurska and Stroinski, 2005), corresponding to our observations at T24h. In leaves, SA has been described to be implicated in stomata closure (Mori et al., 2001; Liu et al., 2013; Prodhan et al., 2018), and in the enhancement of antioxidants and antioxidant enzymes mainly to protect the photosynthetic apparatus (Miura and Tada, 2014; Khan et al., 2015; Zandalinas et al., 2016). The endogenous SA accumulation in leaves has been detected in several crops like citrus (Zandalinas et al., 2016), mustard (Alam et al., 2013), and *Phyllyrea angustifolia*, where SA levels were correlated with the water stress degree, increasing up to 5-fold under severe stress, thus suggesting a role for SA in drought tolerance (Munné-Bosch and Peñuelas, 2003). In pepper leaves, a drastic SA increase occurred immediately after water stress was applied only in V/N plants. Afterwards, SA concentrations decreased to reach values similar to V/V values, but higher than V/N control plants. This could indicate that SA accumulation is related to water stress, but it is also dependent on the rootstock genotype.

IAA, CKs, and GAs are hormones related to plant growth and development, and they are also involved in regulating drought responses (Devireddy et al., 2021; Raza et al., 2022). However, the variations of these hormones content under water stress are contradictory in our experiment. In roots under water stress, IAA content showed a gradual decline in V/V from T4 until the end of the experiment, but in V/N plants the IAA decrease occurred at T24 and, thereafter, IAA concentration increased up to a maximum. Regarding the concentrations of CKs under water stress, a transient increase at T24 in V/N was observed in roots and then CK levels declined to reach values similar to the optimal watering conditions and to the rest of the plant combinations and treatments. In both hormones, the highest concentrations were linked to water stress and to water stress tolerant rootstock (NIBER®), but this effect was not observed for GAs. IAA and CKs promote root branching and root growth, having a potential role in drought-tolerance mechanisms (Verma et al., 2016; Ullah et al., 2018). By using NIBER® as rootstock under salinity conditions for 10 days, a significant increase in root length was stated (López-Serrano et al., 2020), which could explain the increase in IAA and CKs when NIBER® is used under the osmotic treatment. In addition, increasing endogenous IAA levels in roots under osmotic stress have been associated with enhanced tolerance in Arabidopsis (Kim et al., 2013) and *Prosopis strombulifera* (halophyte) (Llanes et al., 2014) due to an increase in lateral root formation and enlarged root system (Llanes et al., 2016).

However, the GA trend in roots did not seem to be dependent on either water stress or rootstock genotype, considering that there were no significant differences between V/V and V/N at the end of the experiment.

In the leaves, IAA and CK levels increased along the experiment, but no significant differences were observed between both rootstock and treatments, which could indicate a poor relation with water stress. Increasing IAA in maize leaves was observed on the first day under water stress (provoked by PEG addition, -0.4MPa) (Wang et al., 2008) with an osmotic potential similar to the one used in this experiment. The increase of CKs has been related to an amelioration of the effect of water stress by stimulating osmotic adjustment and allowing water absorption. However, the increase in IAA and CKs in the majority of studies is associated with stimulated stomata opening and they are considered as ABA antagonists (Pospíšilová, 2003; Gaion et al., 2018). The stomata closure observed in our study could be the consequence of crosstalk between concentration and action place (Ullah et al., 2018; Iqbal et al., 2022).

Regarding GAs in leaves, they can modulate drought responses through stomata development and responses (Nir et al., 2014; Gaion et al., 2018). In our results, an important transient increase in leaves at T24 was recorded in V/N under water stress. Subsequently, GA levels declined to reach control values, and no differences in GA content associated with water stress were observed at the end of the experiment, although there were significant differences between rootstocks. Several studies have demonstrated that the reduction of GA levels contributes to plant growth restriction under drought (Llanes et al., 2016). Besides, in halophyte and some no-halophyte tolerant plants, GA concentrations in leaves increased in response to an osmotic potential decrease to maintain the growth (Li et al., 2012; Colebrook et al., 2014; Llanes et al., 2016). The transient increase observed in V/N under water stress could be associated with GA modulation and signaling for growth preservation.

The knowledge about endogenous phytohormone modulation in response to water stress remains scarce given that most plants' hormonal studies are based on exogenous applications. Overall, this work reflects the fast modulation of the balance of major phytohormones during short-term water stress in young pepper plants, self-grafted or grafted onto a water stress tolerant rootstock such as NIBER®. Phytohormone levels during early water stress exposure (up to 48h) revealed natural variability present in V/V and V/N and how V/N integrates various hormonal signals to tolerate drought imposition. It is essential to determine the water stress tolerance mechanisms and to find the key factors responsible for short-term tolerance, such as hormones. Therefore, this study will allow to understand the early differential responses to water stress in grafted pepper plants and the contribution of NIBER® rootstock hormonal balance to scion water stress improvement. This study will be crucial to extend knowledge and open the door to future biotechnological strategies to improve drought tolerance. However, due to the high level of complexity of the phytohormones network, further studies are required.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author. The authors declare that all data generated during this work are included in the manuscript.

Author contributions

YP: conducted experiments, methodology, investigation, data curation, review. RG-M: conducted experiments, investigation, review. SL-G: Conceptualization, investigation, review. PM-M: methodology, investigation, analytical tools, ÁC and AA: Conceptualization, methodology, investigation, writing – review & editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Exogenous spermine alleviates the negative effects of combined salinity and paraquat in tomato plants by decreasing stress-induced oxidative damage

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Plants are frequently exposed to different combinations of soil constraints including salinity and different herbicides. These abiotic conditions negatively affect photosynthesis, growth and plant development resulting in limitations in agriculture production. To respond to these conditions, plants accumulate different metabolites that restore cellular homeostasis and are key for stress acclimation processes. In this work, we analyzed the role of exogenous spermine (Spm), a polyamine involved in plant tolerance to abiotic stress, in tomato responses to the combination of salinity (S) and the herbicide paraquat (PQ). Our findings showed that application of Spm reduced leaf damage and enhanced survival, growth, photosystem II function and photosynthetic rate of tomato plants subjected to the combination of S and PQ. In addition, we revealed that exogenous Spm reduced H_2O_2 and malondialdehyde (MDA) accumulation in plants subjected to S+PQ, suggesting that the role of exogenous Spm in alleviating the negative effects of this stress combination could be attributed to a decrease in stress-induced oxidative damage in tomato plants. Taken together, our results identify a key role for Spm in improving plant tolerance to combined stress.

KEYWORDS

spermine, stress combination, ROS, tomato, polyamine, climate change, salinity, herbicide

1 Introduction

Plants growing in nature normally experience different combinations of climate threats, including heat, drought, sudden flooding, or cold snaps, among others, that negatively affect their productivity, growth and development (Yadav et al., 2020; Masson-Delmotte et al., 2021; Pascual et al., 2022). In addition, the devastating effect of human activities on soils results in poor soil quality characterized by increased amounts of salinity, herbicides, microplastics, heavy metals, nutrition deficiencies or changes in pH and microbial diversity that pose a serious challenge for agricultural production (Rillig et al., 2019; Zandalinas et al., 2021b; Pascual et al., 2022; Sinha et al., 2022; Speißer et al., 2022). Therefore, plants are constantly subjected to different combinations of two or more of these conditions (Zandalinas et al., 2021a; Zandalinas and Mittler, 2022). The excessive use of herbicides along with increased salt toxicity are among the major soil-associated abiotic stresses that diminish agricultural production all over the world (Pitman and Läuchli, 2002). Paraquat (PQ; also known as methyl viologen; 1,1′-dimethyl-4,4′-bipyridinium dichloride) is a reactive oxygen species (ROS)-producing, rapidly acting and non-selective herbicide (Hawkes, 2014). In turn, excess salinity in soils disturb ion balances and result in ROS production, oxidative stress and decreases in photosynthetic plant capability (Apel and Hirt, 2004; Wang et al., 2008). To prevent salt-induced damages in plants, these changes result in raises in the root/canopy ratio, and modifications in the leaf anatomy, xanthophyll cycle, photorespiration pathway, and water-water cycle (Acosta-Motos et al., 2017). Therefore, both stresses can impede plant growth and development by hindering nutrient absorption, inhibiting cell division and elongation, and disturbing the metabolic and photosynthetic system, that result in losses in crop yield (Li et al., 2013; Ullah et al., 2021).

Polyamines (PAs) are small aliphatic amines detected in all living organisms. In plants, the three major PAs include spermine (Spm), putrescine (Put) and spermidine (Spd), and are implicated in root elongation, leaf senescence, floral development, fruit ripening, programmed cell death, transcript expression and protein translation, and chromatin organization (Drolet et al., 1986). In addition, different genetic studies have revealed a key role for PAs in plant tolerance to different abiotic stresses. For example, a mutational approach to increase polyamine biosynthesis in *Oryza sativa* resulted in an enhanced oxidative stress tolerance by preventing the accumulation of ROS (Jang et al., 2012). Spd has been involved in modulating cell rescue and defense as well as antioxidant pathways in tomato seedlings subjected to heat stress (Sang et al., 2017), has been shown to increase the expression of transcripts encoding heat shock proteins to protect Arabidopsis plants from high temperatures (Sagor et al., 2012), and has been suggested to improve salinity tolerance of tomato plants (Zhang et al., 2015) and sorghum seedlings (Yin et al., 2016). In addition, Arabidopsis mutants deficient in Spm (*acl5/spms*) were hypersensitive to salinity and drought stress (Jang et al., 2012). Applications of exogenous PAs have been also reported to improve the tolerance of different plant species to several abiotic stresses, including salinity, high temperatures or drought stress (Mitsuya et al., 2009; Hu et al., 2012; Kamiab et al., 2014; Ahanger et al., 2019; Marco et al., 2019; Seifi et al., 2019; Acosta-Motos et al., 2020; Upadhyay et al., 2020; Sharma and Garg, 2021; ElSayed et al., 2022; Li et al., 2022). For example, Spm and Spd treatments have been correlated with an increased ROS scavenging, enhanced photosynthesis, improved plant growth, and decreased damaging impacts of salinity stress compared to non-treated plants (ElSayed et al., 2018; ElSayed et al., 2022). Moreover, applications of Put or Spm, or a mixture of them on wheat seedlings resulted in a positive modulation of drought responses by enhancing osmolyte accumulation, increasing free PA levels and regulating PA biosynthetic genes (Ebeed et al., 2017). Therefore, the role of the different PAs in promoting tolerance of several plant species to different abiotic stresses has been widely demonstrated. However, the effects of exogenous treatments of Spm on tomato responses to the combination of two important soil constraints, *i.e.*, salinity and the herbicide PQ have not been reported yet. In this work, the impact of the combination of salinity and PQ in the survival, growth, physiology, oxidative stress and the activity of different antioxidant enzymes in tomato Spm-treated and non-treated plants was evaluated. Our results show that plants treated with Spm improved their survival, growth, leaf damage, photosynthesis and photosystem (PSII) function when subjected to the combination of salinity and PQ, compared to stressed plants not treated with Spm. We further reveal that Spm function could be associated to reductions in oxidative stress induced by the impact of combined salinity and PQ, suggesting that Spm could alleviate the negative effects of this stress combination in tomato plants.

2 Materials and methods

2.1 Plant material and growth conditions

Montecarlo hybrid tomato seeds purchased from a commercial nursery (Seminis, Barásoain, Navarra, Spain) were used as plant material for exogenous Spm experiments. Tomato seeds were cultivated in seedling trays filled with peat moss, perlite and vermiculite (80:10:10). After germination, seedlings were transplanted to 10-cm diameter pots filled with peat moss and maintained under greenhouse conditions (70% relative humidity, 200 μmol photons m^{-2} s^{-1} light intensity, natural photoperiod day/night cycle with temperatures averaging 25.0°C and 18.0°C, respectively) and watered three times a week with half-strength Hoagland solution. Temperature and relative humidity were recorded regularly with a portable USB datalogger (OM-EL-WIN-USB, Omega, NJ, United States).

2.2 Stress treatments and experimental design

To study the effect of exogenous Spm in the tolerance of plants to different stresses and their combination, we treated 4-week-old tomato plants with Spm by watering with 0.5 L of distilled water containing 0.5 mM Spm (Sigma-Aldrich, St. Louis, MO, USA; Xu et al., 2020) once a day during a week, and control (CT) plants were watered in parallel with 0.5 L of distilled water (Supplementary Figure 1). After finishing Spm treatments, Spm-treated and not treated plants were subjected to four different conditions: CT, salinity (S, 150 mM NaCl), herbicide paraquat (PQ, 1.5 μM PQ), and the combination of salinity and paraquat (S+PQ, 150 mM NaCl + 1.5 μM PQ). Plants were watered three times a week for 15 days with each stressor described above (S, PQ and S+PQ) in a half-strength Hoagland solution. Therefore, eight experimental groups were designed (CT, CT+Spm, S, S+Spm, PQ, PQ+Spm, S+PQ, S +PQ+Spm), subjecting 5 plants to each stress treatment, and all experiments were repeated at least three times (Supplementary Figure 1). Once all stress treatments were completed, the number of healthy leaves (leaves with no symptoms of damage; Balfagón et al., 2019) and plant height were scored for all control and stressed plants, followed by sampling leaves in an intermediate position in the canopy in liquid N_2. Samples were stored at -80°C until further use. For each analysis described below, at least three independent technical repeats per biological repeat and experimental group were performed.

2.3 Photosynthetic parameters and photosystem II efficiency

Photosynthetic rate, stomatal conductance, transpiration rate and PSII efficiency were measured simultaneously on plants subjected to the different stress treatments between 9:30 and 11:30 A.M. Leaf gas exchange parameters (photosynthetic rate, stomatal conductance and transpiration rate) were measured by using a LCpro$^+$ portable infrared gas analyzer (LI-6800, LICOR, Lincoln NE, USA) under ambient CO_2 and moisture. After instrument stabilization, six measurements were taken on three different mature fully expanded leaves in three replicate plants from each treatment. PSII efficiency was analyzed on the same leaves and plants using a portable fluorometer (FluorPen FP-MAX 100, Photon Systems Instruments, Czech Republic).

2.4 Malondialdehyde analysis

Approximately 200 mg of ground frozen leaf tissue was homogenized in 2 mL of 80% ethanol (Panreac) by sonication for 30 min. Homogenates were then centrifuged at 12000 g for 10 min and different aliquots of the supernatant were mixed either with 20% trichloroacetic acid or with a mixture of 20% trichloroacetic acid and 0.5% thiobarbituric acid. Both mixtures were incubated in a water bath at 90°C for 1 h and, after cooling down, samples were centrifuged at 5000 g for 5 min at 4°C. The absorbance of the supernatant was read at 440, 534 and 600 nm against a blank, and malondialdehyde (MDA) concentration was calculated as described in Zandalinas et al. (2017).

2.5 H_2O_2 accumulation

H_2O_2 accumulation in leaves was measured by using a commercial kit (Amplex Red hydrogen peroxide-peroxidase assay, Molecular Probes/Invitrogen, Eugene, OR, USA) with few modifications. Briefly, 500 µL of 50 mM sodium phosphate buffer at pH 7.4, containing 50 µM of Amplex Red reagent and 0.05 U mL^{-1} of horseradish peroxidase, was added to approximately 40 mg of frozen leaf tissue and incubated for 30 min at room temperature in darkness. Then, samples were centrifuged at 12000 g for 12 min at 4°C and 50 µL of supernatants were transferred into new opaque tubes. Absorbance at 560 nm was measured by using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The concentration of H_2O_2 in each sample was determined from a standard curve consisting of 0, 0.5, 1, 3, 6, and 9 µM H_2O_2. After absorbance measurements, H_2O_2 accumulation per mg of fresh weight was calculated.

2.6 RNA isolation, primer design and RT-qPCR

RNA was extracted from frozen leaf tissue using an RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Total RNA concentration and purity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) from the ratio of absorbance readings at 260 and 280 nm. Reverse transcription was performed from 1 µg of total RNA using Primer script RT reagent with oligo(dT) primer (Takara Bio Inc., Kusatsu, Japan). The specific primers used for the amplification of each gene are included in Supplementary Table 1. Primer pairs used for the tomato amplification were designed using free surface Primer3Plus (https://www.primer3plus.com) and NCBI database. Relative expression analysis by RT-qPCR were performed in a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, United States). The reaction mixture contained 1 µL of cDNA, 5 µL of SYBRGreen (Applied Biosystems) and 1 µM of each gene-specific primer pair in a final volume of 10 µL. The thermal profile used to analyze the relative gene expression consisted of 10 min at 95°C for pre-incubation, followed by 40 cycles of 10 s at 95°C for denaturation, 10 s at 60°C for annealing and 20 s at 72°C for the extension. Amplicon specificity of the PCR reaction was evaluated by the presence of a single peak in the dissociation curve after the amplification steps. The expression levels of all genes were normalized against the expression of two endogenous control genes (actin [Solyc03g078400] and GAPDH [Solyc05g014470]) based on previous housekeeping selection for tomato (Mascia et al., 2010) and the relative expression was calculated by using REST (Pfaffl et al., 2002). For all genes studied, the reference for S, PQ and S+PQ samples was the expression value obtained for the CT conditions, whereas the reference for S+Spm, PQ+Spm and S+PQ+Spm was the expression value obtained for the CT+Spm conditions. Reference conditions were set as 1. Three technical replicates were analyzed on each biological replicate.

2.7 Antioxidant enzyme activities

To determine the superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) activities, about 100 mg of frozen leaf tissue was extracted in 2 mL of phosphate buffer at pH 7 in a ball mill (MillMix20, Domel, Železniki, Slovenija). After centrifugation at 14000 g at 4°C for 10 min, supernatants were recovered. Different buffers were used for each enzyme extraction: for SOD, 50 mM phosphate buffer (pH 6.8) with 1.33 mM diethyl-diamino-pentaacetic acid; for CAT and GR, 50 mM phosphate buffer (pH 6.8 and pH 7.5, respectively). The SOD activity was determined following the O_2^--induced reduction of nitroblue tetrazolium using the xanthine–xanthine oxidase system. CAT activity was determined using the H_2O_2-dependent reduction of titanium chloride. The GR activity was studied following the increase in the absorbance at 412 nm during 2 min as result of the production of the adduct DTNB-GSH after GSSG reduction. The reaction was initiated by adding 10 µL of enzyme extract and the increment in absorbance was recorded during 2 min at 265 nm. Enzyme activity was expressed as enzyme unit (U) per mg of protein. Total protein amount of each extract was determined following the method of Bradford (1976) using Bovine Serum Albumin (BSA) as a protein standard. Further details on enzyme assays are provided in Hossain et al. (2009).

2.8 Total antioxidant enzyme activity inhibition

To determine the total antioxidant enzyme activity inhibition (Lado et al., 2016), 50 mg of frozen leaf tissue was extracted in 1 mL of MeOH 80% in a ball mill (MillMix20, Domel, Železniki, Slovenija). After centrifugation at 14000 g at 4°C for 10 min, supernatants were recovered. A buffer containing 738 µM ABTS (Sigma-Aldrich, St. Louis, MO, USA) and 245 µM $K_2S_2O_8$ (Sigma-Aldrich, St. Louis, MO, USA) was prepared and incubated at room temperature in darkness. Then, a blank with the previous solution buffer was adjusted to an absorbance of 700 nm, and 10 µL of each extract was added to 1 mL of buffer mix. Measurements at 734 nm were recorded at different times (0, 0.5, 1 and 2 mins). Antioxidant activities were determined using ABTS reduction in potassium persulfate and were calculated as U per mg of protein contained in each extract.

2.9 Statistical analysis

Results are presented as the mean ± SD. Statistical analyses were performed by two-tailed Student's *t*-test. * denotes statistical significance at $P < 0.05$ with respect to CT and $\underline{*}$ means statistical significance at $P < 0.05$ between Spm-treated and non-treated plants exposed to the same stress condition.

3 Results

3.1 Survival, healthy leaves and growth of Spm-treated and non-treated tomato plants subjected to combined salinity and paraquat

To study the survival and growth of Spm-treated and non-treated tomato plants in response to S, PQ and their combination (S+PQ), plants were subjected to 150 mM NaCl, 1.5 µM PQ or 150 mM NaCl and 1.5 µM PQ, respectively, for 15 days (Supplementary Figure 1). As shown in Figure 1A, survival of tomato plants not treated with Spm significantly decreased in

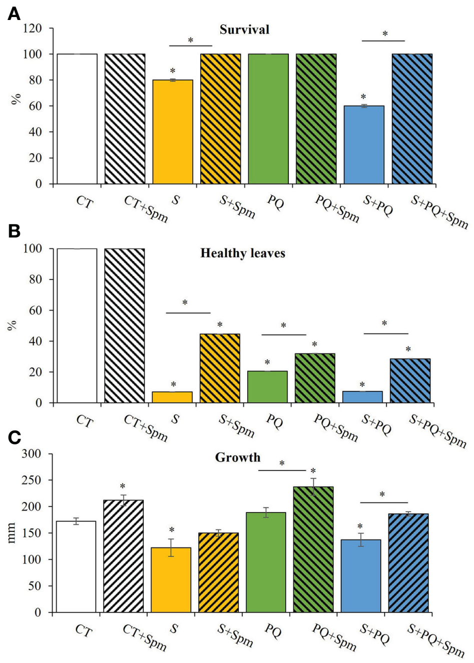

FIGURE 1
Involvement of Spm treatment in the survival, percentage of healthy leaves and growth of tomato plants subjected to salinity, paraquat and the combination of salinity and paraquat. **(A)** Survival, **(B)** percentage of healthy leaves, and **(C)** growth of non-treated and Spm-treated tomato plants subjected to the different stresses. All experiments were repeated at least three times with similar results. Values indicate the mean ± standard deviation. * refers to statistical significance at $P < 0.05$ with respect to CT and $\underline{*}$ means statistical significance at $P < 0.05$ between Spm-treated and non-treated plants exposed to the same stress condition. CT, control; S, salinity; Spm, spermine; PQ, paraquat.

response to S (around 80%) and more markedly in plants subjected to S+PQ (around 60%), whereas all plants survived when subjected to PQ. Interestingly, all Spm-treated plants subjected to the different stresses survived at the end of the different stress treatments (Figure 1A). Similarly, plants treated with Spm and subjected to S, PQ and S+PQ showed more healthy leaves and less visible toxicity symptoms such as chlorosis and necrosis (Balfagón et al., 2019), compared to plants not treated with Spm and subjected to the different stresses (Figure 1B). In addition, Spm treatment significantly increased tomato growth under control or stress conditions of PQ and S+PQ (Figure 1C).

3.2 Photosynthetic and gas exchange parameters of Spm-treated and non-treated tomato plants subjected to combined salinity and paraquat

To determine whether Spm could affect the photosynthesis and different gas exchange parameters of control plants, and plants subjected to S, PQ and S+PQ, photosynthetic rate, PSII efficiency, transpiration rate and stomatal conductance were monitored in plants treated and non-treated with Spm (Figure 2). Photosynthetic rate levels significantly decreased in response to all individual and combined stresses in non-treated plants compared to CT, reaching the lowest value when plants were subjected to S+PQ (Figure 2A). Interestingly, exogenous Spm application enhanced the photosynthetic rate under each individual and combined stress. In this sense, Spm-treated plants subjected to S, PQ and S+PQ increased by 6.78%, 7.67% and 33.3%, respectively, their photosynthetic rate. PSII efficiency was negatively affected by S and S+PQ, and Spm treatment increased these values by 13.21% and 8.29%, respectively (Figure 2B). Not surprisingly, stomatal conductance of plants treated and non-treated with Spm from the different stresses similarly corresponded to the transpiration rates measured in these plants (Figures 2C, D). Therefore, whereas plants subjected to S and S+PQ, and non-treated with Spm reduced transpiration rate and stomatal conductance levels, PQ did not change or slightly increased these values. Interestingly, Spm treatment increased both gas exchange parameters in CT plants as well as in plants subjected to PQ and S+PQ with respect to the corresponding non-treated stressed plants (Figures 2C, D). Taking together, Spm could be involved in

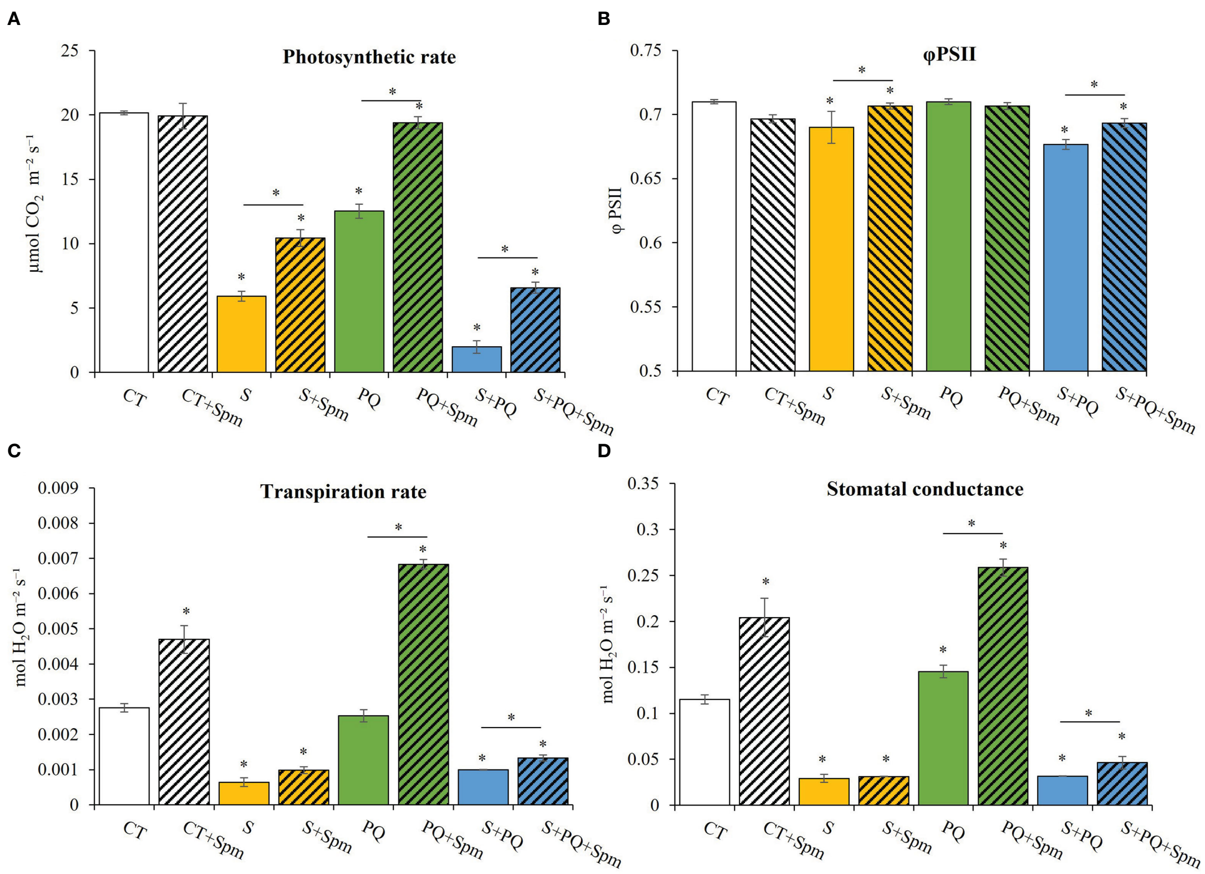

FIGURE 2
Involvement of Spm treatment in the photosynthetic rate, PSII function, and gas exchange parameters of tomato plants subjected to salinity, paraquat and the combination of salinity and paraquat. **(A)** photosynthetic rate, **(B)** PSII efficiency, **(C)** transpiration rate, and **(D)** stomatal conductance of non-treated and Spm-treated tomato plants subjected to the different stresses. All experiments were repeated at least three times with similar results. Values indicate the mean ± standard deviation. * refers to statistical significance at P< 0.05 with respect to CT and * means statistical significance at P< 0.05 between Spm-treated and non-treated plants exposed to the same stress condition. CT, control; S, salinity; Spm, spermine; PQ, paraquat; PSII, photosystem II.

promoting stomata aperture in response to PQ and S+PQ, as well as under control conditions.

3.3 Oxidative stress and antioxidant response of Spm-treated and non-treated tomato plants subjected to combined salinity and paraquat

To gain a better understanding of the impact of each stress condition and the Spm-associated changes on the oxidative stress, H_2O_2 levels, MDA content, RT-qPCR analyses of transcripts involved in detoxification pathways, as well as different antioxidant enzyme (SOD, GR and CAT) activities were studied (Figures 3, 4; Supplementary Figure 2). Plants not treated with Spm and subjected to the different stresses significantly increased their leaf H_2O_2 levels compared to CT, whereas Spm treatment reduced the amount of H_2O_2 accumulated in plants subjected to S, PQ and S+PQ with respect to the corresponding stressed plants not treated with Spm (Figure 3A). In addition, the degree of lipid peroxidation in tomato plants was studied by monitoring changes in MDA levels (Taulavuori et al., 2001). As shown in Figure 3B, the level of lipid peroxidation decreased by about 56% and 12% in plants subjected to PQ+Spm and S+PQ+Spm, with respect to PQ and S+PQ, respectively.

The activation of detoxification pathways in Spm-treated and non-treated tomato plants subjected to the different stress conditions was evaluated by assessing the expression of FeSOD, Cu/ZnSOD1, Cu/ZnSOD2, MnSOD, CAT1, GPX, cAPX and GR transcripts as well as enzymatic activities of SOD, GR and CAT (Figure 4; Supplementary Figure 2). In general, Spm treatment modified the expression of all the transcripts tested involved in detoxification processes compared to that observed in stressed plants not treated with Spm (Figure 4A; Supplementary Figure 2). The expression of Cu/ZnSOD1, Cu/ZnSOD2, CAT1 and GPX was, in general, attenuated due to the effect of Spm treatment in plants subjected to the different stresses, whereas the expression of cAPX and GR1 was enhanced as a result of exogenous Spm application compared to that shown in stressed plants not treated by Spm (Figure 4A; Supplementary Figure 2). To further analyze the role of Spm in ROS scavenging processes, the activities of SOD, GR and CAT were evaluated (Figures 4B–D). S or S+Spm did not alter any enzymatic activity, and PQ increased GR activity. Under S+PQ conditions, a significant decrease of SOD and GR enzymatic activities in Spm-treated plants by 21.21% and 66.67%, respectively, was observed compared to non-treated plants subjected to S+PQ. On the contrary, CAT activities did not show any alteration in response to stress in both Spm-treated and non-treated plants (Figure 4D).

Additionally, to determine the total antioxidant capacity of plants, analysis of the inhibition of total antioxidant activities was performed (Table 1). Spm treatment enhanced the total antioxidant capacity of plants under CT conditions. Moreover, PQ and S+PQ resulted in increased levels of total antioxidant activity compared to CT values. By contrast, values of total inhibition of antioxidant activities decreased in plants subjected to S+PQ+Spm, compared to S+PQ (Table 1).

4 Discussion

The critical influence of PAs in protecting plant cells from several abiotic stresses has been extensively demonstrated in different plant species (reviewed in Zandalinas et al., 2022). However, the potential role of the different plant PAs in

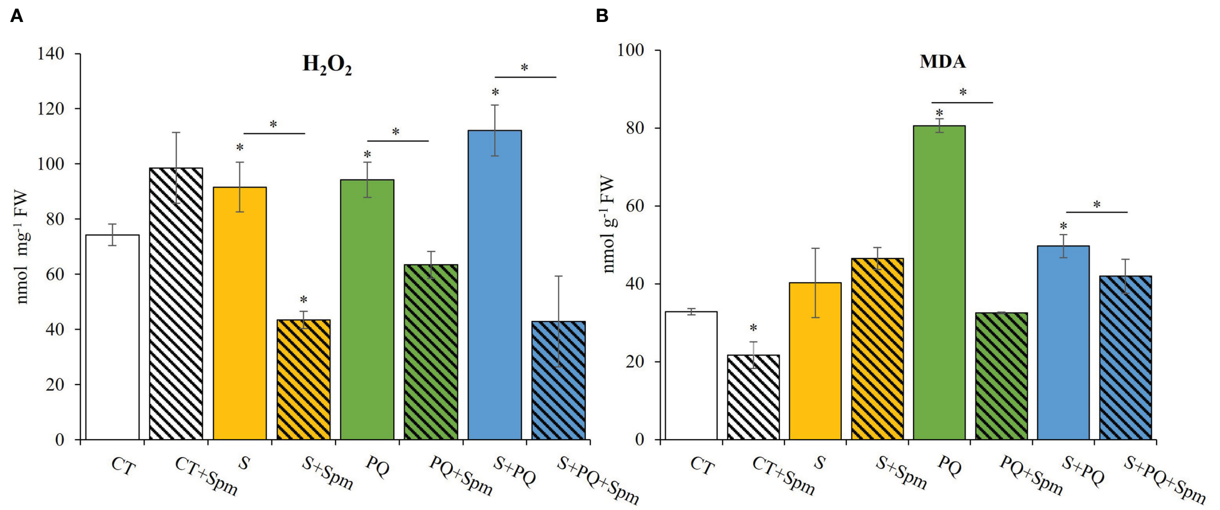

FIGURE 3
Involvement of Spm treatment in H_2O_2 and MDA accumulation in tomato plants subjected to salinity, paraquat and the combination of salinity and paraquat. **(A)** H_2O_2 accumulation, and **(B)** MDA levels of non-treated and Spm-treated tomato plants subjected to the different stresses. All experiments were repeated at least three times with similar results. Values indicate the mean ± standard deviation. * refers to statistical significance at P< 0.05 with respect to CT and $\underline{*}$ means statistical significance at P< 0.05 between Spm-treated and non-treated plants exposed to the same stress condition. CT, control; FW, fresh weight; MDA, malondialdehyde; S, salinity; Spm, spermine; PQ, paraquat.

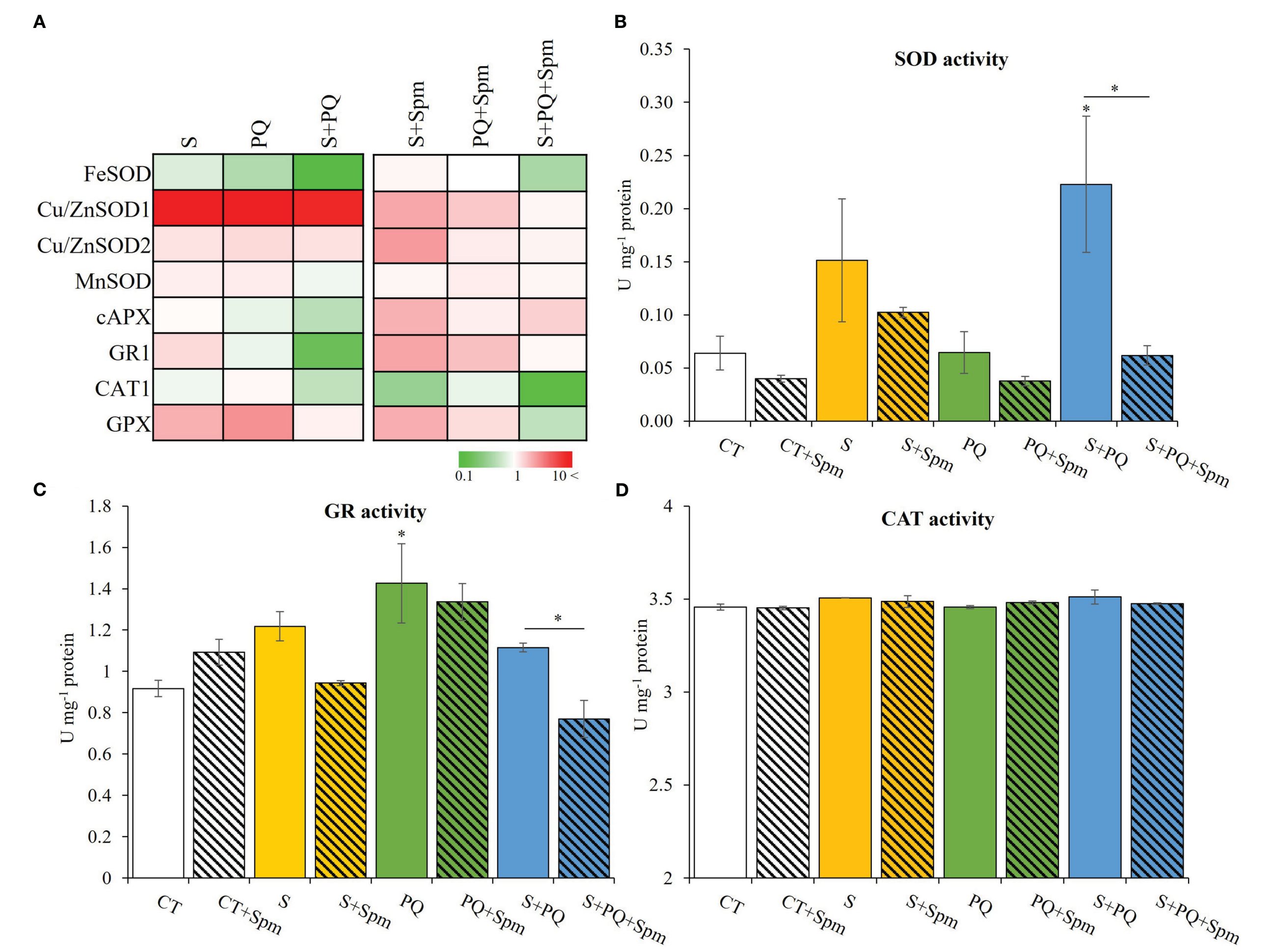

FIGURE 4
Involvement of Spm treatment in the expression of transcripts encoding antioxidant enzymes and their activities in tomato plants subjected to salinity, paraquat and the combination of salinity and paraquat. **(A)** Relative expression of transcripts encoding the antioxidant enzymes FeSOD, Cu/ZnSOD1, Cu/ZnSOD2, MnSOD, cAPX, GR1, CAT1 and GPX. **(B–D)** Activities of the antioxidant enzymes SOD **(B)**, GR **(C)** and CAT **(D)** in non-treated and Spm-treated tomato plants subjected to the different stresses. All experiments were repeated at least three times with similar results. Values indicate the mean ± standard deviation. * refers to statistical significance at P< 0.05 with respect to CT and * means statistical significance at P< 0.05 between Spm-treated and non-treated plants exposed to the same stress condition. cAPX, cytosolic ascorbate peroxidase; CAT, catalase; CT, control; GPX, glutathione peroxidase; GR, glutathione reductase; S, salinity; SOD, superoxide dismutase; Spm, spermine; PQ, paraquat.

TABLE 1 Involvement of Spm treatment in the total antioxidant enzymatic inhibition of tomato plants subjected to salinity, paraquat and the combination of salinity and paraquat.

	Average inhibition antioxidant enzymatic activity (% compared to time 0)		
Treatments	0.5 min	1 min	2 min
CT	68.37 ± 10.70	71.59 ± 9.72	74.22 ± 8.77
CT+Spm	81.97 ± 2.48*	86.22 ± 0.72*	89.46 ± 2.37*
S	56.99 ± 11.73	62.96 ± 10.48	68.79 ± 10.93
S+Spm	64.58 ± 4.34	74.89 ± 1.53	83.28 ± 6.54*
PQ	127.93 ± 3.65*	135.86 ± 5.52*	142.21 ± 5.01*
PQ+Spm	126.11 ± 9.54*	130.30 ± 10.94*	137.69 ± 10.46*
S+PQ	122.46 ± 4.61*	130.75 ± 3.02*	137.55 ± 4.40*
S+PQ+Spm	105.14 ± 3.04*†	112.30 ± 0.62*†	121.02 ± 2.33*†

Values indicate the mean ± standard error. * refers to statistical significance at P< 0.05 with respect to CT and † means statistical significance at P< 0.05 between Spm-treated and non-treated plants exposed to the same stress condition. CT, control; PQ, paraquat; S, salinity; Spm, spermine.

regulating plant responses to different abiotic stress combinations is not fully understood (Zandalinas et al., 2022). For example, previous reports suggested a key role for Spm in conferring tolerance of trifoliate orange seedlings exposed to the combination of heat and drought (Fu et al., 2014). In addition, modulation of PA biosynthesis was correlated with a better protection of tobacco plants against the combination of heat and drought (Cvikrová et al., 2013). On the contrary, other stress combinations including high light and heat stress, induced the repression of PAs such as Put, suggesting that Put may have a marginal effect on plant acclimation to this stress combination (Balfagón et al., 2022). In this work, we expanded the knowledge about the action of Spm in tomato plant tolerance to the combination of another important abiotic stress combination (*i.e.*, salinity and the herbicide PQ). Our results revealed that application of exogenous Spm enhanced survival, growth, photosynthetic rate and PSII function of tomato plants subjected to the combination of S and PQ (S+PQ) compared with plants subjected to S+PQ and not treated with Spm, while decreasing the leaf damage associated with the different stresses (Figures 1, 2A, B). In agreement with previous reports proposing Spm as a stomatal regulator (*e.g.*, Hassan et al., 2018; Berahim et al., 2021), our results indicate that Spm treatments induced stomatal aperture and enhanced transpiration in tomato plants under PQ and S+PQ (Figures 2C, D). Therefore, the Spm-induced improvement in photosynthetic rate of tomato plants subjected to PQ and S+PQ (Figure 2A) could be partially attributed to the increased stomatal aperture associated with Spm treatment (Figure 2D), resulting in a potential increment of internal CO_2 concentration in plant cells. These results suggest that Spm could be involved in modulating stomata aperture resulting in contrasting outcomes depending on the plant species and/or the stress or stress combination involved. Moreover, the improvement in the photosynthetic rate observed in response to stresses involving S (S and S+PQ; Figure 2A) in plants treated with Spm could be attributed to the Spm-induced increase in PSII function under these stress conditions (Figure 2B). Further studies are needed to determine the specific molecular mechanisms of Spm in controlling stomata changes as well improving PSII activity under these stress situations.

It has been previously reported that S and PQ stresses applied individually result in oxidative damage to plants (Wang et al., 2008; Hawkes, 2014), and that an increase in antioxidant capacities of cells is a key strategy against stress-induced oxidative damage (Yadav et al., 2011). Our results indicate that Spm treatments significantly reduced the amount of H_2O_2 accumulated in response to S, PQ and S+PQ (Figure 3A), as well as stressed-induced MDA levels in response to PQ and S+PQ (Figure 3B). Therefore, Spm played a key role in alleviating a potential stress-induced oxidative damage as well as reduced lipid peroxidation processes that could occur in plants subjected to PQ or S+PQ. In agreement with our data, Li et al. (2022) reported a positive correlation between Spm treatment and improvements in photosynthetic performance, antioxidant capacity and redox homeostasis in creeping bentgrass subjected to salinity.

The exact mechanism by which PAs confer protection to different stresses remains unclear (Marco et al., 2011). Their role on plant tolerance to stress has been associated with their capacity to regulate transcription and translation, maintain membrane stability, and modulate antioxidant processes (reviewed in Liu et al., 2015). The lower oxidative stress of Spm-treated tomato plants subjected to S+PQ (assessed by reduced content in H_2O_2 and MDA; Figure 3), could result in alterations in the expression of transcripts encoding antioxidant enzymes, in a reduction of their activities (Figure 4), as well as in a lower total antioxidant capacity of plant cells under stress (Table 1). In addition, the Spm-induced decline in the oxidative damage associated to S+PQ could be also correlated to less PSII damage (Figure 2B) and, in turn, in enhanced photosynthetic rates (Figure 2A). Possible mechanisms by which Spm could trigger ROS reduction include its role in inhibiting the auto-oxidation of metals that leads to impairment of the electron supply for ROS generation (Shi et al., 2010), or its function as direct antioxidants and ROS scavengers as proposed previously (Liu et al., 2015) and suggested in our data by the increment of the total antioxidant capacity of CT plants treated with Spm (Table 1). Further studies are needed to decipher the specific mechanism utilized by Spm to reduce the oxidative pressure of plant cells subjected to S+PQ.

Taking together, our findings show that tomato plants treated with Spm improved their survival, growth, leaf damage, photosynthesis and PSII function in response to S+PQ. In addition, our results indicate that Spm is involved in ameliorating the negative effects of S+PQ by reducing the stress-associated oxidative pressure and suggest that Spm could play a key role in tomato plant responses to combined stress. Further studies are of course required in order to determine the exact molecular mechanism by which Spm and the other main polyamines (putrescine and spermidine) may function under this stress combination.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

LP and CS-M performed the research; LP and SZ designed the research, prepared figures, and wrote the manuscript with contributions of AG-C and ML-C. SZ and AG-C supervised the research, provided laboratory infrastructure and funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

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Potential role of the regulatory *miR1119-MYC2* module in wheat (*Triticum aestivum* L.) drought tolerance

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MicroRNA (miRNA)-target gene modules are essential components of plants' abiotic stress signalling pathways Little is known about the drought-responsive miRNA-target modules in wheat, but systems biology approaches have enabled the prediction of these regulatory modules and systematic study of their roles in responses to abiotic stresses. Using such an approach, we sought miRNA-target module(s) that may be differentially expressed under drought and non-stressed conditions by mining Expressed Sequence Tag (EST) libraries of wheat roots and identified a strong candidate (*miR1119-MYC2*). We then assessed molecular and physiochemical differences between two wheat genotypes with contrasting drought tolerance in a controlled drought experiment and assessed possible relationships between their tolerance and evaluated traits. We found that the *miR1119-MYC2* module significantly responds to drought stress in wheat roots. It is differentially expressed between the contrasting wheat genotypes and under drought versus non-stressed conditions. We also found significant associations between the module's expression profiles and ABA hormone content, water relations, photosynthetic activities, H_2O_2 levels, plasma membrane damage, and antioxidant enzyme activities in wheat. Collectively, our results suggest that a regulatory module consisting of *miR1119* and *MYC2* may play an important role in wheat's drought tolerance.

KEYWORDS
noncoding RNAs, miRNA, transcription factor, systems biology, expressed sequence tag, abiotic stress, drought

1 Introduction

Abiotic stresses like drought compromise plant survival and significantly reduce their growth and yield (Pereira, 2016). Drought stress can result in various physiological and biochemical reactions in plants (Ilyas et al., 2021). It leads to a substantial change in plant water relations, which drives osmotic and oxidative stresses that cause the partial closure of stomata and reductions in photosynthesis, generation of reactive oxygen species (ROS), activation of plant antioxidant system (AOS), and altered cell homeostasis (Rane et al., 2022). To adjust these reactions and to minimize the adverse effects of drought stress, plants recruit multiple and sometimes overlapping signalling pathways. These pathways provide highly variable genotype-dependent tolerance of drought stress, and associated variation in survival and productivity (Shamloo-Dashtpagerdi et al., 2019; Shamloo-Dashtpagerdi et al., 2022b; Zhang et al., 2022b). The regulatory components located in the heart of signalling pathways are the leading players in controlling responses to drought (Shinozaki and Yamaguchi-Shinozaki, 2007; Zhang et al., 2022b). Thus, identifying and characterizing the key regulatory components is crucial for efforts to improve crops' drought tolerance.

Plant miRNAs (typically 20-24 nucleotides long) play key roles in post-transcriptional gene regulation (Nogoy et al., 2018). Genes encoding them are transcribed by RNA polymerase II. Processing of the transcripts by 5′ capping, splicing, and polyadenylation at the 3′ end yields pri-miRNA. Further processing results in precursor RNA (pre-miRNA) with a stem-loop structure, which is cleaved by DCL1, giving rise to mature miRNA-miRNA* duplexes with a two nucleotide overhang at the 3′ end (Tiwari et al., 2021a; Tiwari et al., 2021b). Mature miRNA binds to target messenger RNA (mRNA) and negatively regulate target genes (Hou et al., 2019). miRNAs and their target genes form regulatory modules that are key components of abiotic stress signalling pathways that fine-tune various adaptive stress responses in plants (Millar, 2020; Pagano et al., 2021; Zhang et al., 2022a). Clearly, identifying miRNA-target modules involved in responses to abiotic stresses is highly desirable because it can provide important new insights into plants' stress tolerance mechanisms. Thus, both experimental and computational methods have been used in efforts to identify and characterize modules involved in the responses of diverse plant species to various environmental stresses. For instance, Liu et al. (2019) showed that several miRNA-target modules (including *miR164-MYB*, *miR164-NAC*, *miR159-MYB*, *miR156-SPL* and *miR160- ARF*) are differentially regulated in maize inbred lines with contrasting drought sensitivities. Water deficits also reportedly reduce expression of the miRNA-target modules *miR159-MYB*, *miR396-GRF*, *miR535-SPL*, *miR166b-HD-ZIP* III and *miR167-ARF* in cardamom (*Elettaria cardamomum* Maton) (Anjali et al., 2019). In addition, the *miR168a-OMT1* module contributes to the salinity tolerance of *Brassica rapa* L., mainly through the regulation of melatonin biosynthesis (Shamloo-Dashtpagerdi et al., 2022). There is also some evidence that the *miR1118-PIP1;5* module participates in wheat plants' salinity tolerance through adjustment of their water status and ion homeostasis, and mitigation of membrane damage (Shamloo-Dashtpagerdi et al., 2022c).

Bread wheat is one of the world's most important cereal crops, that drought stress significantly reduces its productivity, thus threatening global food security (Juliana et al., 2019; Sallam et al., 2019). This is because drought reduces plants' Relative Water Content (RWC) and induces stomatal closure, thereby reducing stomatal conductance and photosynthetic rates, often accompanied by chloroplast damage, chlorophyll photo-oxidation, and metabolic distortions that further limit photosynthesis rates and reduce grain yields (Sallam et al., 2019). Moreover, peroxidation of cell membranes through ROS production, changes in morphological characteristics, and nutrient uptake reduction have been frequently reported in wheat exposed to drought stress (Ahmad et al., 2018; Sallam et al., 2019). Therefore, identifying the genes controlling such physiological and biochemical traits, and elucidating the regulation of wheat genes under drought stress, is crucial to accelerate the genetic improvement of wheat's drought tolerance. Little is known about the drought-modulated miRNAs, their target genes, and related physiochemical responses in wheat. However, advances in systems biology approaches involving combinations of computational biology, statistical and experimental methods, have enabled detailed study of miRNA-target modules and related physiochemical changes in responses to abiotic stresses (Baltoumas et al., 2021; Pagano et al., 2021). Therefore, we designed the study presented here to identify possible miRNA-target module(s) involved in wheat responses to drought stress. For this purpose, we first mined EST libraries of wheat exposed to drought stress using various computational and bioinformatics methods to identify possible drought-responsive miRNA-target modules and identified a strong candidate (*miR1119-MYC2*). Next, we characterized molecular and physiochemical differences between two genotypes with different degrees of stress tolerance in a controlled drought experiment and explored the relationships between drought tolerance and evaluated characteristics. The results indicate that the *miR1119-MYC2* module might contribute to wheat drought tolerance by regulating multiple physiochemical responses.

2 Materials and methods

2.1 Computational analysis

2.1.1 Data sources

EST data were retrieved from resources available *via* the website of the US wheat genome project (http://wheat.pw.usda.gov/NSF/data.html) (Chao et al., 2006). These included 5′EST libraries obtained from roots of hexaploid wheat (*Triticum aestivum* L. cv Chinese Spring) under both unstressed conditions (TA058E1X, containing 1025 sequences, named NS), and the drought conditions (TA055E1X, containing 1310 sequences, named DS) (Zhang et al., 2004). Briefly, the experimental procedure was as follows:

Plants of genotype Chinese Spring were subjected to drought conditions so that RWC values in their leaves reached between 60 and 80%. Then, total RNA was extracted from roots of control and treated plants at the full tillering stage. Extracted total RNA samples were mixed and used for cDNA synthesis and sequencing (https://harvest.ucr.edu/).

2.1.2 EST processing

EST sequences of the NS and DS libraries were subjected to stepwise processing using the EGassembler online bioinformatics service (http://egassembler.hgc.jp) (Masoudi-Nejad et al., 2006).

Vector contaminants were removed, and sequences similar to plastids and repetitive sequences were also excluded. If the trimmed sequences were less than 100 bp or had more than 4% ambiguous bases they were excluded from further analysis (Masoudi-Nejad et al., 2006; Bouck and Vision, 2007). The remaining high-quality ESTs were used for downstream analysis.

2.1.3 *In silico* gene expression analysis

All high-quality ESTs of the NS and DS libraries identified in the previous steps were clustered and assembled into unigenes (contigs and singletons), using EGassembler with an overlap percent identity cutoff >80%. A Python script was developed to quantify expression levels for each contig (gene) based on the number of ESTs of each library contributing to it. To identify differentially expressed genes (DEGs) under the drought and control treatments, Audic and Claverie (AC) statistical test (Audic and Claverie, 1997) implemented in ACDtool (http://www.igs.cnrs-mrs.fr/acdtool/) (Claverie and Ta, 2019) was used. A gene was considered to be differentially expressed if the p-value obtained with Benjamini–Hochberg False Discovery Rate (FDR) (Benjamini and Hochberg, 1995) was ≤ 0.05.

2.1.4 Functional annotation of DEGs

BLASTN search against National Center for Biotechnology Information (NCBI) Non-redundant nucleotide sequences (nt) database and IWGCS RefSeq v2.1 (https://www.wheatgenome.org/) were done for identified DEGs using CLC Genomics Workbench 9.5 software and an E-value ≤ 10^{-5}. In addition, BLASTX search vs NCBI Non-redundant protein sequences (nr), UniProt, and The Arabidopsis Information Resource (TAIR) protein databases were performed using the same software and the same E-value. The DEGs were then subjected to gene set enrichment analysis using GeneCodis4 (https://genecodis.genyo.es/) (Garcia-Moreno et al., 2022). In GeneCodis4, the hypergeometric statistical test, the minimum number of genes in each category (n = 3) and p-value ≤ 0.05 were selected. In addition, all informative ESTs from the two libraries (2335 sequences that yielded a significant hit in the BLASTX searches) were used as the background set. Statistically significant functional categories were illustrated by bubble plot using the SRplot online tool (http://www.bioinformatics.com.cn/srplot).

2.1.5 Computational identification of differentially expressed miRNA-target modules

Potential genes encoding miRNAs in the identified DEGs were predicted using the miRkwood online tool (https://bioinfo.cristal.univlille.fr/mirkwood/mirkwood.php) (Guigon et al., 2019). Therefore, putative coding sequences, tRNAs, and rRNAs among the DEGs were excluded using BLAST searches implemented in miRkwood (E-value=1E-5). The Minimal Folding Free Energy Index (MFEI) of pre-miRNAs was adjusted to less than or equal to -0.6 (Zhang et al., 2006).

A set of the revised criteria of plant miRNAs provided by Axtell and Meyers (2018) was also applied to strengthen our computational predictions, by:

- Excluding miRNA/miRNA* duplexes containing secondary stems or large loops
- Allowing up to five mismatched positions in miRNA/miRNA* duplexes, with at most three in asymmetric bulges
- Including only mature miRNAs that were 20 to 24 nucleotides long

The psRNATarget server (https://www.zhaolab.org/psRNATarget/) (Dai et al., 2018) was used to find the putative target gene(s) of identified miRNAs among the DEGs, with the following default parameters:

- Maximum expectation for complementarity, 5
- Penalty for G:U pair, 0.5
- Penalty for other mismatches, 1
- Extra weight in seed region, 1.5
- Seed region, 2-13 Nt
- Number of mismatches allowed in seed region, 2
- Length of scoring region for complementary analysis (HSP size), 19
- Bulge (gap), allowed

Therefore, the predicted miRNA(s) identified in the previous step were searched against all sequences of the identified DEGs. Genes with a lower expectation value and target accessibility-maximum energy to unpair the target site (UPE) were selected as target genes (Dai and Zhao, 2011; Dai et al., 2018). Accordingly, based on the applied parameters, the most reliable miRNA-target module was selected for downstream analysis.

2.1.6 Pathway analysis

All identified DEGs with significant BLAST scores along with the predicted miRNA were categorized into significant pathways using the Pathway Studio software (https://www.pathwaystudio.com/) (Nikitin et al., 2003) with the following parameters:

- Neighbour groups to assess: small molecules, functional class, complex, protein
- Connection type: expression, regulation, molecular transport, promoter binding, molecular synthesis, and chemical reaction
- Enrichment p-value cut-off: 0.05

Then significantly associated pathways were networked using the same software with a hierarchical layout.

2.2 Drought stress experiment

2.2.1 Plant material and drought treatment

Seeds of the drought stress-tolerant wheat genotype Sirvan and susceptible genotype Moghan3 (Riasat et al., 2018; Mehrabad Pour-Benab et al., 2019; Shamloo-Dashtpagerdi et al., 2022a) were surface-sterilized and stratified in Petri dishes on soaked filter paper at 4 °C for three days to synchronize germination. The resulting seedlings were transferred to pots, each containing 2 kg of sandy-clay soil. The pots were then kept in a controlled greenhouse with 24 °C/18 °C day/night temperatures, 10–12 h natural photoperiods and irrigation based on the soil field capacity (FC), from late December to early February 2021. The pots were divided into non-stressed (control) and stressed groups at the 5-leaf growth stage. The former were fully irrigated with tap water and the latter were subjected to drought stress by withholding irrigation and keeping them at 60% FC until the end of the experiment. Roots were sampled at three time points (3, 24, and 48 h after the drought stress treatment was initiated) from five replicates (pots) with a single plant per treatment and time point.

2.2.2 qPCR analyses of the identified miRNA-target module

Total RNA was extracted from root samples of both wheat genotypes under drought conditions using a Column RNA isolation kit (DENAzist Asia, Iran) following the manufacturer's recommended protocols. The quality and quantity of RNA were checked by separating the major ribosomal RNA bands in 2% agarose gels and spectrophotometry at two wavelength ratios of $A_{260/230}$ and $A_{260/280}$ nm (NanoDrop, Technologies Inc). Possible genomic DNA contamination was eliminated with RNase-free DNase I (Jena Bioscience, Germany) treatment following the manufacturer's recommendations.

Specific stem-loop RT primers for predicted miRNA expression profiling were designed according to Varkonyi-Gasic et al. (2007) (Table S1) and applied to reverse transcribe the total RNA to cDNA using an Easy cDNA Synthesis Kit (Pars, Iran), following the manufacturer's instructions. They were then used in quantitative polymerase chain reaction (qPCR) assays with a Bioer thermal cycler system (Bioer Technology Co., Ltd., China), 2X SYBR Green Real-Time PCR kit (Pars, Iran) and five biological and two technical replicates per sample. The *T. aestivum* rRNA26 homolog was used as the internal standard of qPCR to normalize the miRNA expression (Table S1). Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

To evaluate the expression profile of the predicted target gene, 2 mg samples of DNase-treated RNA were used to synthesize the first strand cDNA using an Easy cDNA Synthesis Kit (Pars, Iran) following the manufacturer's recommendations. Gene-specific primers for predicted target gene were designed according to the corresponding contig sequence using AlleleID software 7.7. Its expression levels were then assessed by qPCR, again using the Bioer thermal cycler system, a 2X SYBR Green Real-Time PCR kit (Pars, Iran), five biological and two technical replicates per sample, and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). However, the *Actin* gene (GenBank: AB181991.1) was used as an internal standard (Table S1).

2.2.3 Determination of ABA content

Roots of untreated and drought-treated plants were thoroughly washed with tap water to remove soil, then samples were extracted with 80% ethanol in a 1:10 *w/v* ratio at room temperature. The resulting extract was separated by filtration, evaporated to an aqueous residue, and diluted with distilled water. Then the residues were acidified to pH 2.5 with dilute HCl (ratio of organic to aqueous phases being 3:1). The resulting solutions were shaken with three volumes of saturated $NaHCO_3$ solution (pH 8-9) to neutralize acidic compounds, then ABA was extracted from the aqueous phase with diethyl ether (Vysotskaya et al., 2004), and quantified by enzyme-linked immunoassay (ELISA) using specific antibodies as described by Arkhipova et al. (2020).

2.2.4 Evaluation of photosynthetic parameters

Photosynthetic parameters were evaluated using the method described by Figueroa-Bustos et al. (2020) with a few modifications. Briefly, fully expanded leaves of each genotype were used to evaluate their photosynthesis rate (A_N; net CO_2 assimilation), transpiration rate (E), and stomatal conductance (g_s), with a Li-6400 portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA). All measurements were carried out between 10:00 am and 1:00 pm, with a leaf temperature of 26 °C and photon flux density of 500 mmol m^{-2} s^{-1}. The flow rate through the chamber was 500 mmol s^{-1}. The leaves' chlorophyll contents were determined using a Portable Minolta SPAD 502 Plus chlorophyll meter (Delta T, UK) in terms of SPAD values. In addition, the Intrinsic Water Use Efficiency (WUEi) was calculated as the ratio between A_N and g_s (A_N/g_s; µmol CO_2 mol^{-1} H_2O) (Franco-Navarro et al., 2016).

2.2.5 Evaluation of antioxidant enzyme activities

Portions of sampled roots were ground and homogenized in liquid nitrogen, then extracted with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM EDTA, 5 mM β-mercaptoethanol and 4% (*w/v*) PVP. The resulting extracts were centrifuged at 30,000 g for 30 min at 4 °C and the supernatants were immediately used to determine activities of antioxidant enzymes as follows. Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined from the inhibition of nitro blue tetrazolium (NBT) reduction under light (Stewart and Bewley, 1980). Peroxidase (POD; EC 1.11.1.7) activity was measured by determining guaiacol oxidation from changes in absorbance at 470 nm after adding H_2O_2 in a spectrophotometer (Shah and Nahakpam, 2012). Catalase (CAT; EC 1.11.1.6) activity was measured by monitoring absorbance at 240 nm to follow the decomposition of H_2O_2 to H_2O (Patra et al., 1978).

2.2.6 Physiochemical assays

The RWC of leaves of both wheat genotypes was measured using the protocol described by Sade et al. (2015). The proline content of the root samples was evaluated according to Bates et al. (1973). H_2O_2 contents were measured by detecting titanium-peroxide complex formation at 415 nm spectrophotometrically (Yu et al., 2019). Cell membrane damage due to stress was measured by monitoring electrolyte leakage (EL) from roots of the wheat genotypes, following Sairam and Srivastava (2002).

2.2.7 Statistical analysis

The acquired expression and physiochemical data were subjected to analysis of variance (ANOVA) with comparisons of means by Duncan's multiple range test (p-value ≤ 0.01) using STATISTICA v. 12 software (https://www.statistica.com/en/). To investigate the relationships among the measured characteristics, including expression profiles of the identified miRNA-target module, ABA content, photosynthetic properties, antioxidant activities and physiochemical parameters, Principal Component Analysis (PCA) was performed based on Pearson correlations with varimax rotation (Corner, 2009) implemented in XLSTAT software 2019.2.2 (https://www.xlstat.com).

3 Results

3.1 EST sequences analysis and identification of DEGs

Of the 2335 raw EST sequences of wheat roots in the non-stress (NS) and drought stress (DS) libraries, 238 were trimmed during sequence processing by EGassembler, and no sequence was removed. These high-quality sequences, with an average length of 637 bp, were clustered and assembled into 1462 unigenes (475 contigs and 987 singletons). The minimum, maximum, and average lengths of the contigs were 120, 2243, and 675 bp, respectively (Table S2). The number of ESTs in contigs ranged from 2 (279 contigs) to 67 (one contig), indicating different expression levels of genes.

The AC test revealed that a total of 93 contigs (genes) were differentially expressed in response to drought (FDR ≤ 0.05). Of those, 85 genes were upregulated, and 8 genes were downregulated under the drought treatment. Contig 455 (encoding MTO3 S-adenosylmethionine synthetase), contig 399 (encoding extensin-like), and contig 392 (encoding ricin B-like lectin) were upregulated most strongly (4.85-fold), and contig 330 encoding ribulose bisphosphate carboxylase small subunit, chloroplastic 3 was most strongly downregulated (-5.64 folds) (Figure 1 and Supplementary File 1).

3.2 Functional interpretation of DEGs

Identified DEGs were annotated according to results of BLAST searches against the NCBI nt, NCBI nr, Swiss-Prot, and TAIR databases. These revealed that 98.9, 75.3, 95.7, 89.2 and 84.9% of the DEGs (92, 70, 89, 83, and 79 genes) had significant hits in the NCBI nt, IWGCS, NCBI nr, Swiss-Prot, and TAIR databases, respectively (Table S3).

The DEGs were assigned to 10 statistically significant biological process categories (p-value ≤ 0.05) (Figure 2), most frequently: "response to water deprivation", "response to cold", "response to abscisic acid", "defence response to bacterium" and "response to salt stress" (7, 6, 6, 6 and 4 genes, respectively) (Figure 2). Three genes were also assigned to each of the remaining categories: "leaf development", "cellular response to phosphate starvation", "response to osmotic stress", "response to heat" and "lignin biosynthetic process" (Figure 2). These results indicate that most identified enriched DEGs are involved in abiotic stress responses.

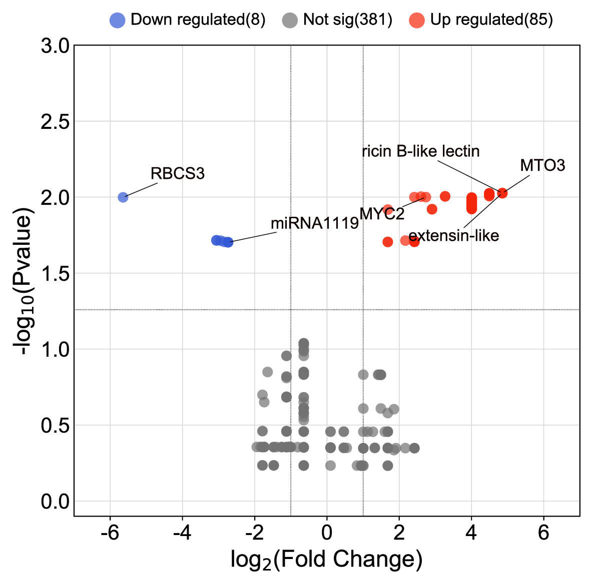

FIGURE 1
Volcano plot of DEGs between roots of drought-stressed and non-stressed wheat plants. Genes with the highest expression changes, the identified miRNA, and predicted target gene are shown.

3.3 Computational identification of miRNA-target modules among DEGs

Only one DEG met the key criteria for consideration as a potential miRNA-encoding gene. Accordingly, miRNA identified on contig 113

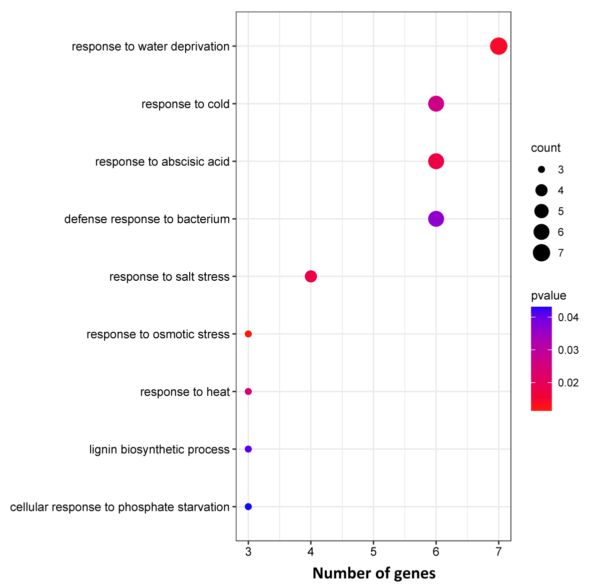

FIGURE 2
Results of enrichment analysis of identified DEGs based on biological processes using the GeneCodis tool.

with 207 bp in length had an MFEI index of -1.16. The mature miRNA was located at 42 to 65 nt of contig 113, with 24 bp lengths (Figure 3A), and significantly matched an entry in the miRbase database (*T. aestivum* L. *miR1119*) with no mismatch (Figure 3B).

Using the psRNATarget tool, one target gene among the DEGs was identified for *miR1119* with an expectation value of 1.5 and UPE value of 38.08. Moreover, psRNATarget predicted that *miR1119* regulates expression of its target gene by cleavage. The identified target gene corresponded to contig 247, encoding MYC2 protein. The binding region of *miR1119* to the target gene is shown in Figure 3C.

Based on EST sequences analysis, the expression profile of the identified miRNA and its target gene was as expected, with downregulation of the identified miRNA and upregulation of the predicted target gene under drought conditions (Figure 1).

3.4 Pathway analysis

As shown in Figure 4A, identified DEGs, including *miR1119* and *MYC2*, were enriched in six significant pathways (p-value ≤ 0.05), including ABA (10 genes), NaCl response (8 genes), Jasmonic acid (5 genes), Salicylate (4 genes), Nitric oxide (NO) (3 genes) and D-mannitol (3 genes). Hierarchical relationships among genes, small molecules, functional classes, and cell processes incorporated in significant pathways are shown in Figure 4B. This visualization revealed that various TFs and protein kinases (PKs) involved in ABA, JA, salicylate, and NO signalling pathways are recruited in drought responses. Moreover, it revealed that processes controlled by these signalling pathways, including photosynthesis, root development and morphology, redox homeostasis, lipid peroxidation, cell death, and activation of antioxidant enzymes, play key roles in wheat's drought responses. The identified module also seems to be an important upstream component in the predicted network, corroborating the importance of the *miR1119-MYC2* module in these responses.

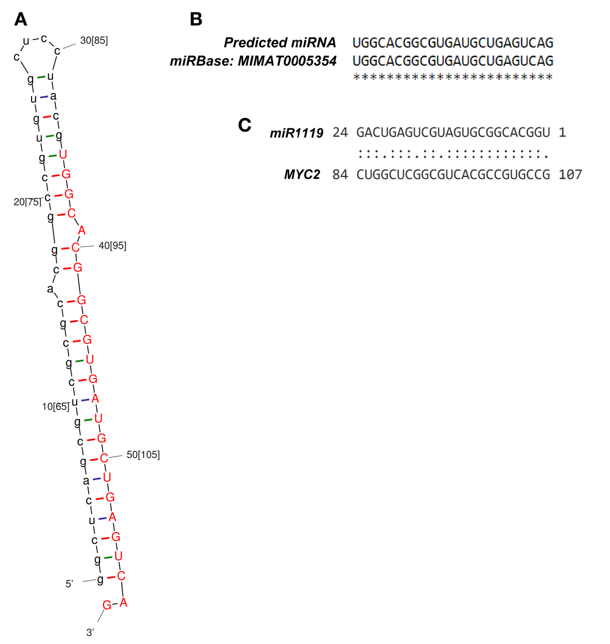

FIGURE 3
Structural characteristics of the identified miRNA-target module. **(A)** Stem-loop structure of identified wheat *miR1119*, with the 24-nt mature miRNA shown in red. **(B)** Alignment of the identified *miR1119* and *T. aestivum* L. *miR1119* from miRBase. **(C)** Binding site of the *miR1119-MYC2* module.

3.5 qPCR assay of identified miRNA-target module under drought conditions

The qPCR results revealed that *miR1119* expression significantly differed between the wheat genotypes and under the two treatments (Figure 5A). Its expression was significantly lower (about 2-fold) at the 3 h time point under drought than under the non-stressed conditions in the susceptible genotype Moghan3 (Figure 5A). However, there were no significant differences in its expression at the later (24 and 48 h) time points in Moghan3 (Figure 5A). In contrast, in roots of the tolerant genotype Sirvan. *miR1119* was dramatically downregulated (about 4.5-fold) at 3 h under the drought treatment and although its expression subsequently increased it remained significantly lower under drought stress than under control conditions

In both genotypes, *MYC2* was conversely expressed relative to *miR1119*, suggesting that miR1119 regulates the target gene through a cleavage mechanism. *MYC2* was slightly, but significantly, upregulated under drought stress at 3 h in Moghan3, but there was no significant between-treatment difference in its expression in Moghan3 at the 24 and 48 h time points (Figure 5B). In contrast, *MYC2* expression was significantly higher under drought than under control conditions at all time points in the tolerant genotype, Sirvan (Figure 5B). Furthermore, *MYC2* expression was significantly higher in Sirvan than in Moghan3 under the drought treatment at all time points (Figure 5B). Maximum upregulation (up to 3-fold) of *MYC2* was also observed at the 3 h time point (Figure 5B), and MYC2 transcript levels were consistently higher in the tolerant genotype than in the susceptible genotype under drought stress.

Taken together, the computational findings and expression patterns strongly indicate that *miR1119* may regulate expression of the *MYC2* gene.

3.6 Effects of drought on root ABA content

A considerable difference between the contrasting wheat genotypes was observed in root ABA content. In the tolerant genotype Sirvan it was significantly elevated after 3 h of the drought treatment (Figure 6) and its level did not significantly change from then until the end of the experiment (Figure 6). There was no significant between-treatment in the ABA content of roots of the susceptible wheat genotype Moghan3 at the 3 h time point (Figure 6). It was significantly higher under drought stress than in control conditions at the 24 and 48 h time points in Mogan3 roots, but still significantly lower than in Sirvan roots under drought at all time points (Figure 6).

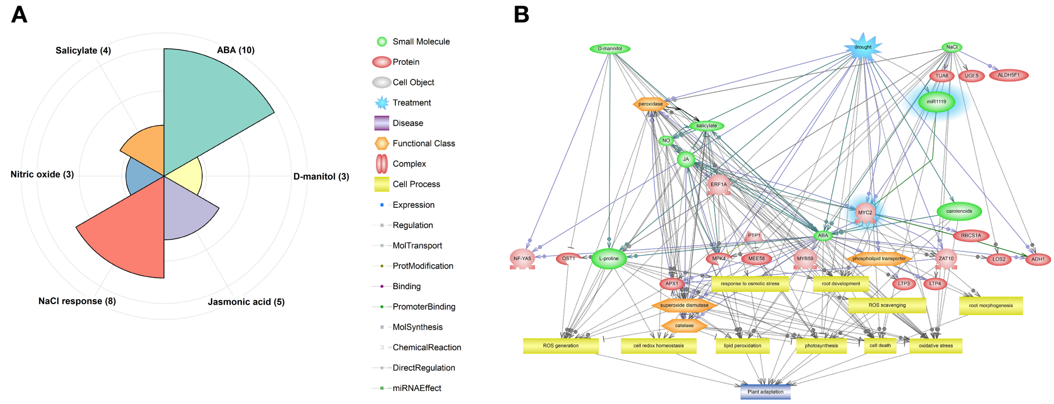

FIGURE 4
Results of pathway analysis of the identified DEGs, including the predicted *miR1119-MYC2* module. **(A)** Enriched pathways according to the hypergeometric test (*p*-value ≤ 0.05). **(B)** Hierarchical network of components of the enriched pathways, with the *miR1119-MYC2* module highlighted in blue.

3.7 Effects of drought on photosynthetic parameters

The drought treatment significantly reduced the photosynthetic rate (A_N) in both wheat genotypes. However, the reduction was considerably stronger in Moghan3 than in Sirvan (Table 1). In both genotypes, the E and g_s were also significantly reduced, especially at the 48 h time point, under the drought treatment (Table 1). However, the reduction were significantly greater in Sirvan than in Moghan3 (Table 1). The SPAD value significantly decreased under drought conditions in both wheat genotypes and the tolerant genotype had substantially higher SPAD values than the susceptible genotype (Table 1). In all time points of drought treatment, WUEi in Sirvan was significantly higher than in Moghan 3. The difference was more considerable at the 48 h time point, under the drought treatment (Figure S1).

3.8 Effects of drought on antioxidant enzyme activities

The drought treatment resulted in significant increases in activities of all three studied antioxidant enzymes (SOD, POD,

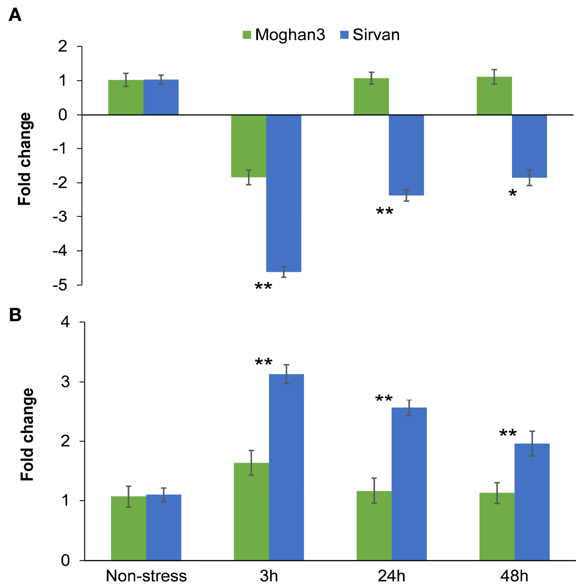

FIGURE 5
Expression profiles of the *miR1119-MYC2* module in roots of the Sirvan and Moghan3 wheat genotypes under drought conditions: **(A)** *miR1119* and **(B)** *MYC2*. The values are means ± SE (n = 5 biological replicates, each consisting of one plant with two technical replicates). Significant differences between the two genotypes according to Duncan's multiple range test (p-value ≤ 0.01) are indicated by asterisks: *p ≤ 0.05, **p ≤ 0.01.

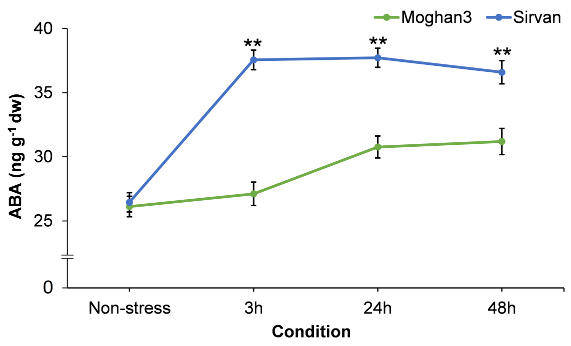

FIGURE 6
ABA contents in roots of the Sirvan and Moghan3 wheat genotypes measured under drought conditions. The values are means ± SE (*n* = 5 biological replicates). Significant differences between the genotypes according to Duncan's multiple range test (*p*-value ≤ 0.01) are indicated by asterisks: **p* ≤ 0.05, ***p* ≤ 0.01.

and CAT) in the Sirvan roots at all time points (except CAT at the 3 h time point) (Figure 7). Moreover, activities of all three enzymes increased with time in response to drought in this genotype. In contrast, no significant between-treatment differences were observed in antioxidant enzyme activities in Moghan3 roots at 3 h (Figure 7). In addition, although SOD, POD and CAT activities were significantly increased at later (24 and 48 h) time points of drought stress, their activities were considerably lower in Moghan3 than in Sirvan (Figure 7).

3.9 Physiochemical changes in response to drought

No significant changes were detected in the RWC of either wheat genotype after 3 h of the drought treatment (Table 2). At the 24 and 48 h time points, RWC was lower in both genotypes, but significantly higher in Sirvan than in Moghan3 (Table 2). The drought treatment also increased the proline content of roots of both wheat genotypes (Table 2), but it was higher in Sirvan than in Moghan3 at all time points except 3 h (Table 2). In both genotypes it was highest after 48 h of drought stress. H_2O_2 accumulated in both wheat genotypes under drought stress, but the accumulation was weaker in Sirvan than in Moghan3 at all time points (Table 2). The drought treatment significantly increased EL% in both genotypes, but there was less stress-derived damages (as reflected in EL%) in Sirvan than in Moghan3 (Table 2).

3.10 Relationships among measured characteristics

A biplot of the first two principal components obtained in the PCA is shown in Figure 8. A biplot of the first two principal components obtained in the PCA is shown in Figure 8. Based on locations of drought-tolerant and drought-susceptible genotypes in the biplot it was divided into an upper 'stress-tolerance' (green) region and lower 'stress-susceptibility' (yellow) region where Sirvan and Moghan3 were located, respectively (Figure 8). *MYC2* expression, ABA content, activities of antioxidant enzymes, proline content, RWC, A_N and SPAD were located in the stress-tolerance region, indicating that high levels of these characteristics are important for drought tolerance in wheat. In contrast, *miR1119* expression, H_2O_2 content, EL%, *E*, and g_s were located in the stress-susceptibility region, indicating that high levels of these traits in drought conditions contribute to high susceptibility.

4 Discussion

Regulatory miRNA-target modules are essential contributors to plant stress responses and tolerance and provide an effective tool for manipulating crops' stress tolerance (Zhang et al., 2022a). Systems biology approaches facilitate the identification of such regulatory modules in complex networks by combining computational and experimental methods (Cramer et al., 2011; Shamloo-Dashtpagerdi et al., 2022a). Thus, we adopted such an approach by combining analysis of EST sequences of wheat roots and physiochemical characteristics of wheat plants under drought and control conditions. The results provided new insights into drought responses of wheat, including the apparent involvement of a *miR1119-MYC2* module.

The first report on the responsiveness of *miR1119* of wheat to abiotic stresses was presented by Lu et al. (2011). A subsequent detailed investigation by Shi et al. (2018) revealed that under drought stress tobacco lines overexpressing wheat *miR1119* exhibited more drought tolerance than wild-type plants. They

TABLE 1 Photosynthetic parameters (photosynthetic rate as net CO_2 assimilation (A_N), transpiration rate (*E*), stomatal conductance (g_s) and Chlorophyll (Chl) content (SPAD)) of the Sirvan and Moghan3 wheat genotypes measured under non-stressed and drought conditions.

	A_N ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)		*E* ($mmol\ H_2O\ m^{-2}\ s^{-1}$)		g_s ($mol\ H_2O\ m^{-2}\ s^{-1}$)		SPAD	
	Sirvan	Moghan3	Sirvan	Moghan3	Sirvan	Moghan3	Sirvan	Moghan3
Non-stress	16.310^a	16.134^a	3.805^a	3.785^a	0.097^a	0.097^a	33.397^a	33.435^a
3 h	15.842^b	15.050^c	2.696^c	3.089^b	0.09^c	0.093^b	32.761^b	32.554^b
24 h	13.314^d	12.321^e	2.070^e	2.421^d	0.081^e	0.086^d	30.875^c	29.130^d
48 h	10.349^f	8.641^g	1.014^g	1.402^f	0.056^g	0.064^f	27.284^e	24.467^f

Different letters indicate significant differences in means based on the Duncan test (p-value ≤ 0.01).

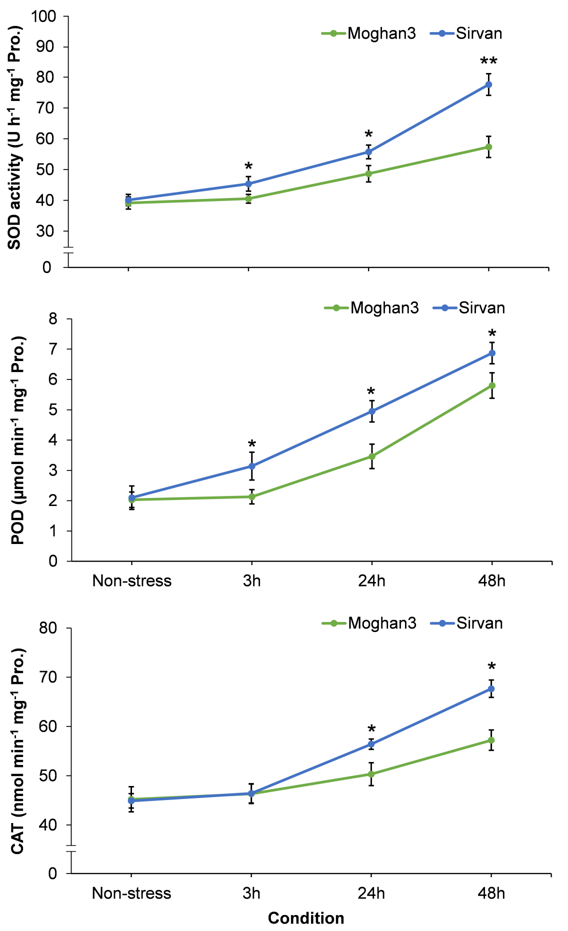

FIGURE 7
Activities of antioxidant enzymes (SOD, POD, and CAT) in roots of the Sirvan and Moghan3 wheat genotypes under drought conditions. The values are means ± SE (*n* = 5 biological replicates). Significant differences between the genotypes according to Duncan's multiple range test (*p*-value ≤ 0.01) are indicated by asterisks: *$p \leq 0.05$, **$p \leq 0.01$.

also showed that *miR1119* plays important roles in drought responses of both the monocot wheat and eudicot tobacco (Shi et al., 2018). Another study showed that *miR1119* is highly expressed in wheat roots and targets several *phenylalanine ammonia-lyase* (*PAL*) genes in wheat, which play key roles in plants' adaptation, growth, and development (Rasool et al., 2021). However, despite the insights provided by such studies, many questions about *miR1119*'s function in wheat responses to drought and underlying physiochemical processes remained to be answered.

In wheat, numerous miRNAs are known to regulate expression of their targeted TFs, thus playing key roles in its drought tolerance mechanisms (Budak et al., 2015). Some of these miRNAs and their targets (shown in parentheses), which are differentially expressed in leaves of contrasting wheat genotypes under dehydration stress (Ma et al., 2015), included *miR159a,b* (*MYB3*), *miR156k* (*SBP*), *miR444c.1* (*MADS-box*), *miR160a* (*ARF*), *miR164b* (*NAC*), *miR166h* (*HD-ZIP4*), *miR169d* (*CCAAT-box TF*), *miR393b,i* (*TIR1*), *miR396a,c,g* (*GRF*), *miR444d* (*IF3*) and *miR827-5p* (*finger-like protein*). These miRNA-TF modules are involved in regulation of diverse drought tolerance pathways. For example, *miR159a,b*-MYB functions in auxin signalling and oligopeptide transport, *miR164b*-NAC participates in oxidative stress responses, *miR169d*-CCAAT-box regulates ABA-responsive transcription and miR397-Ice1 modulates responses to water deprivation (Budak et al., 2015). However, despite the versatility and importance of MYC TFs in plants' growth, development, and environmental responses, there have been few reports regarding their functions in crop plants, especially economically important monocot crops, including wheat. In two previous studies, Chen et al. (2019) and Bai et al. (2019) respectively identified 26 and 27 non-redundant MYC TFs in the whole wheat genome. Specifically, expression profiling of identified *MYC* genes revealed that, like their dicot counterparts, the *MYC2* gene is differentially expressed in responses to differences in light quality, diverse hormone stimuli, and a wide array of abiotic stresses in wheat (Bai et al., 2019; Chen et al., 2019). These results clearly indicate that this gene participates in wheat's response to abiotic stresses, including drought. However, to our knowledge no previously published studies provide information on miRNA-based regulation of MYC2 expression under abiotic stress conditions in any plants, except for suggestions by (Guo et al., 2017) that the *MYC2* gene may be a *miR172g-3p* target in phytohormone signalling in Oolong Tea (*Camellia sinensis* (L.) Kuntze). Thus, we believe that our study provides the first information on the relationship between *miR1119* and *MYC2* in wheat and the *miR1119-MYC2* module's role under drought stress. Our computational analysis indicates with high confidence (expectation value 1.5) that *MYC2* is a potential target of *miR1119*, because with a stringent cut-off threshold (expectation

TABLE 2 Physiochemical properties of the Sirv$_{an}$ and Moghan3 wheat genotypes measured under non-stressed and drought conditions.

	RWC%		Proline (µg g^{-1} fw)		H_2O_2 (µmol g^{-1} fw)		EL%	
	Sirvan	Moghan3	Sirvan	Moghan3	Sirvan	Moghan3	Sirvan	Moghan3
Non-stress	93.82^{a}	93.74^{a}	2.1^{f}	2.05^{f}	2.31^{g}	2.23^{g}	4.63^{g}	4.67^{g}
3 h	93.05^{b}	92.89^{b}	3.14^{e}	3.08^{e}	2.98^{f}	3.42^{e}	5.09^{f}	5.84^{e}
24 h	87.87^{c}	83.65^{d}	7.95^{c}	6.64^{d}	3.62^{d}	4.78^{c}	9.47^{d}	11.09^{c}
48 h	79.57^{e}	70.35^{f}	13.14^{a}	10.32^{b}	5.17^{b}	8.67^{a}	15.73^{b}	19.35^{a}

Different letters indicate significant differences in means according to the Duncan test (p-value ≤ 0.01).
Relative Water Content (RWC) was evaluated in their leaves while proline content, H_2O_2 content, and electrolyte leakage (EL) were measured in the roots..

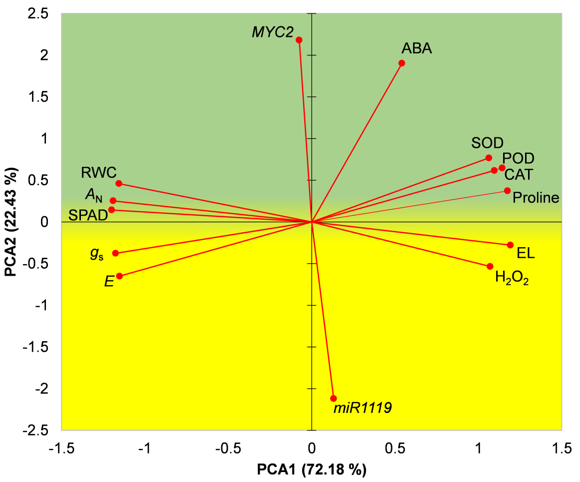

FIGURE 8
PCA biplot of measured characteristics of the Sirvan and Moghan3 wheat genotypes under drought conditions. The green and yellow regions correspond to drought stress tolerance and drought stress susceptibility, respectively. PCA1, first principal component; PCA2, second principal component.

≤ 2), psRNATarget can identify target genes with high accuracy (up to 82%) and low false positive prediction rates (Dai and Zhao, 2011). Furthermore, studying the expression profile of miRNAs and their potential target genes might provide evidence about their functions. Our results show that the identified wheat *miR1119* and its predicted target gene *MYC2* were expressed differentially in response to drought treatments. Moreover, their expression differed between wheat genotypes with different degrees of drought tolerance, and there was a consistent negative correlation between the expression of *miR1119* and *MYC2* in both wheat genotypes. These findings strengthen computational predictions concerning *MYC2* as a putative target gene of *miR1119* and the hypothesis that *miR1119* and *MYC2* play important roles in wheat's drought tolerance.

The transcription factor *MYC2* is a known master regulator of stress signalling (Song et al., 2022) that finetunes crosstalk between JA signalling pathways and those of other phytohormones, such as ABA, salicylic acid (SA), gibberellins (GAs), and auxin (IAA) (Kazan and Manners, 2013). *MYC2* participates in multiple complex processes in plants, including the regulation of biotic and abiotic stress responses, and their growth and development (Luo et al., 2023). Some biological processes related to drought responses of wheat in which *MYC2* is involved are shown in the gene network based on pathway analysis (Figure 4). *MYC2* targets a wide range of downstream genes (Kazan and Manners, 2013; López-Vidriero et al., 2021), so has expected involvement in several drought response processes. For example, *ADH1* (*ALCOHOL DEHYDROGENASE 1*), one of the *MYC2* target genes (Abe et al., 2003) that was present in the identified DEGs and predicted gene network (Figure 4), is reportedly involved in tolerance of biotic and abiotic stresses, including drought (Shi et al., 2017; Su et al., 2020). Our pathway analysis, physiochemical assays, and statistical analysis illuminate how the *miR1119-MYC2* module may contribute to wheat's drought tolerance. PCA indicated that *miR1119* and *MYC2* expression is negatively and positively associated with ABA content, respectively. Moreover, ABA accumulation was faster and stronger under drought in the tolerant wheat genotype, Sirvan, than in the susceptible genotype, Moghan3. This is consistent with expectations, as regulation of ABA biosynthesis is one of the fastest responses of plants to abiotic stresses such as drought and salinity (Peleg and Blumwald, 2011), and upregulation of ABA biosynthetic genes in drought stress conditions has been observed in several plant species (Vishwakarma et al., 2017). It has also been demonstrated that *MYC2* positively regulates ABA biosynthesis and signalling (Verma et al., 2020), and that drought priming treatments induce drought tolerance in wheat by upregulating genes involved in ABA biosynthesis, including *MYC2* (Wang et al., 2021). Furthermore, *MYC2* positively regulates the transcription of *ABA2* and *NCED3* genes encoding two key enzymes in the ABA biosynthesis cascade (Verma et al., 2020). These observations, along with our results, support the hypothesis that *MYC2* is involved in wheat's ABA biosynthesis and hence drought stress responses and tolerance. However, further research is needed to elucidate fully the participants in regulatory mechanisms of ABA biosynthesis in wheat roots involving *MYC2* and their precise roles.

ABA fundamentally regulates plants' morphological and physiological activities in response to drought stress (Mukarram et al., 2021). We found that ABA content was positively associated with RWC, and negatively associated with g_s, and E in wheat. In addition, RWC was significantly higher (but g_s and E lower) in Sirvan than in Moghan3. Under short-term water deficit, as in our experiment, ABA regulates stomatal closure and modulates mesophyll conductance without restraining CO_2 fixation (Sorrentino et al., 2016; Brunetti et al., 2019). Accordingly, higher RWC during drought conditions leads to efficient photosynthesis activity (Aliakbari et al., 2021), which is reflected in more A_N in the tolerant genotype. This finding was further confirmed as our results showed that the tolerant genotype Sirvan maintained higher WUEi, i.e., higher ratios of A_N to g_s. Improving WUE is an important subject to alleviate the deleterious effects of drought stress and to maintain a higher grain yield of wheat (Ahmed et al., 2019). Therefore, unsurprisingly, water uptake, transport, and loss seem to play key roles in wheat's drought tolerance. Besides the role of ABA in leaf stomata aperture, which regulates WUE and energy partitioning of crops (Yu et al., 2004), previous studies have also shown that ABA regulates root hydraulic conductance (*Lp*), resulting in adjustments of plants' water relations and increases in WUE during drought (Kuromori et al., 2018). Moreover, increases in levels of endogenous ABA in roots of plants under drought stress boost adaptive responses, including increases in root elongation and reductions in lateral root formation, that increase root water uptake efficiency (Kuromori et al., 2018; Maurel and Nacry, 2020). Altogether, it can be postulated that the upregulation of *MYC2* due to the downregulation of *miR1119* in roots, and consequently greater ABA accumulation, contribute to increases in WUE and drought tolerance of wheat.

Responses to drought include ROS bursts induced by osmotic stress, which cause membrane oxidation and damage (Sachdev et al., 2021; Mansoor et al., 2022). Both enzymatic mechanisms (involving

SOD, POD and CAT) and non-enzymatic mechanisms (involving proline and various antioxidants) detoxify ROS, maintain cellular ROS homeostasis (to varying genotype- and stress level-dependent degrees), and contribute to plants' overall drought tolerance (Laxa et al., 2019; Shamloo-Dashtpagerdi et al., 2022; Shamloo-Dashtpagerdi et al., 2022a). ABA plays a major role in the induction of such adaptive responses in wheat to stresses, including drought (Bano et al., 2012; Yu et al., 2020; Wang et al., 2021). Activities of SOD, POD, and CAT enzymes and the accumulation of proline were all located in the tolerant region of the PCA biplot, and positively correlated to both *MYC2* expression and ABA content. In addition, the SPAD, EL% and H_2O_2 measurements indicate that chlorophyll degradation and membrane damage levels were lower in Sirvan than in Moghan3 under drought stress. Thus, Sirvan has more potent antioxidative activities than Moghan3 in drought conditions. Under salinity stress, *MYC2-like* overexpression reportedly enhances salt tolerance in transformants through several physiochemical responses, including increases in proline accumulation and SOD, POD, and CAT activities (Liu et al., 2022). Similarly, *TaMYC2* responds to abiotic (cold, drought, alkali and salt) stresses and increases tolerance in Caucasian Clover (*Trifolium ambiguum* M. Bieb.) by increasing antioxidant enzymes' activities (Zhao et al., 2022). It has also found that *TaMYC2* overexpression significantly improved drought and cold tolerance of transgenic tobacco by increasing these activities. Similarly, MeJA, NaCl, and PEG treatments significantly increase *SmMYC2* expression in *Salvia miltiorrhiza* Bunge (Deng et al., 2022). Furthermore, overexpression of *SmMYC2* in transgenic Arabidopsis plants increased their SOD, POD, CAT enzyme activities, and proline content, but reduced their malondialdehyde (MDA) levels and ROS accumulation, indicating that it could enhance their slat tolerance (Deng et al., 2022). Accordingly, our results show that *MYC2* also confers protection against ROS accumulation by regulating antioxidant enzymes' activities and proline accumulation in wheat roots and indicate that genotype-dependent increases in *MYC2* expression linked to the decrease in *mir1119* expression may contribute to wheat's drought tolerance.

5 Conclusion

We have used a combination of computational, statistical, and experimental methods to investigate the potential miRNA-target gene modules involved in wheat's drought responses and tolerance. Accordingly, we found that *miR1119* significantly contributes to wheat responses to drought stress. We also found clear associations between the expression patterns of the *miR1119-MYC2* module and a wide array of physiochemical changes of wheat under drought stress conditions. The module is differentially regulated in two contrasting wheat genotypes. It may contribute to wheat plants' drought tolerance by adjusting their water relations and alleviating oxidative stress damage by regulating activities of antioxidant enzymes and proline content. Therefore, it may be a key biomarker of levels of wheat genotypes' drought tolerance. Further functional characterization of *miR1119* and its target gene would provide more concrete evidence of the roles of these modules in regulating drought tolerance.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

RS-D conceived and planned the study, performed computational transcriptome analysis, statistically analysed the results and wrote the manuscript draft and discussion, AGS and AT carried out the drought experiment and wrote some parts of the manuscript. RRV supported the project and developed the methodology. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1161245/full#supplementary-material

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Effect of high salinity and of priming of non-germinated seeds by UV-C light on photosynthesis of lettuce plants grown in a controlled soilless system

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High salinity results in a decrease in plant photosynthesis and crop productivity. The aim of the present study was to evaluate the effect of UV-C priming treatments of lettuce seeds on photosynthesis of plants grown at high salinity. Non-primed and primed seeds were grown in an hydroponic system, with a standard nutrient solution, either supplemented with 100 mM NaCl (high salinity), or not (control). Considering that leaf and root K^+ concentrations remained constant and that chlorophyll fluorescence parameters and root growth were not affected negatively in the high salinity treatment, we conclude that the latter was at the origin of a moderate stress only. A substantial decrease in leaf net photosynthetic assimilation (A_{net}) was however observed as a consequence of stomatal and non-stomatal limitations in the high salinity treatment. This decrease in A_{net} translated into a decrease in growth parameters; it may be attributed partially to the high salinity-associated increase in leaf concentration in abscisic acid and decrease in stomatal conductance. Priming by UV-C light resulted in an increase in total photosynthetic electron transport rate and A_{net} in the leaves of plants grown at high salinity. The increase of the latter translated into a moderate increase in growth parameters. It is hypothesized that the positive effect of UV-C priming on A_{net} and growth of the aerial part of lettuce plants grown at high salinity, is mainly due to its stimulating effect on leaf concentration in salicylic acid. Even though leaf cytokinins' concentration was higher in plants from primed seeds, maintenance of the cytokinins-to-abscisic acid ratio also supports the idea that UV-C priming resulted in protection of plants exposed to high salinity.

KEYWORDS

chlorophyll fluorescence, growth, leaf gas exchange, lettuce, salinity stress, seed priming, phytohormone balance, UV-C

1 Introduction

The world's population is expected to reach 9.8 billion in 2050 (FAO, 1999). An increase in food production is thus essential to feed this increasing population. Food security depends on several factors, including abundance and quality of water resources. Salinity is considered as one of the main environmental stresses that reduces agricultural productivity around the world (Läuchli and Grattan, 2007). Twenty percent (45 million ha) of the current 230 million ha of irrigated land is already salinized. Furthermore, the area of saline soil is expected to increase worldwide, reaching 50% in 2050 (FAO, 2008).

Salt stress-related effects on agricultural yield are extremely harmful. Under salinity, crops exhibit slower growth rates and significantly reduced reproductive development (Munns and Tester, 2008; Roy et al., 2014). The negative salinity effects are first related to osmotic stress followed by salt toxicity (Munns, 2002), but also impaired nutritional balance and oxidative stress (Chaves et al., 2009; Isayenkov and Maathuis, 2019; Rasheed et al., 2020). Osmotic stress causes a rapid inhibition of the rate of expansion of young leaves and a significant decrease in stomatal conductance of older leaves (Munns and Tester, 2008). Salt toxicity occurs over time as very high Na^+ concentrations accumulate within plants. Toxic concentrations of salt inhibit the synthesis of proteins and metabolites that are essential for plant growth. These perturbations affect the carbon balance mainly through the reduction of photosynthesis (Ashraf and Harris, 2013), as well as sugar metabolism, phloem loading, sucrose translocation from source to sink organs (Pattanagul and Thitisaksakul, 2008; Lemoine et al., 2013; Penella et al., 2016), resulting in reduced plant growth and yield. In the chloroplast, salt stress induces the production of ROS, which can be deleterious if the antioxidant mechanisms are not able to eliminate them. The generated ROS not only induce photoinhibition by disrupting electron flow, but also affect PSII repair mechanism. ROS may notably inhibit the synthesis of protein D1 (Nath et al., 2013; Gururani et al., 2015).

Substantial changes in hormone concentrations and balance are associated with salinity stress (Fahad et al., 2015; Yu et al., 2020). These changes are behind the negative effects of high salinity on photosynthesis and other functions related to growth and development, but the same changes are also involved in adaptative processes aiming at increasing tolerance against high salinity. The view can be reversed. Changes in hormone concentrations and balance result from the triggering of adaptative processes, which may also result in decreased photosynthesis and growth. For instance, rapid production of abscisic acid (ABA) is essential for plant survival in conditions of drought and high salinity because of its role as a long-distance signal controlling stomatal conductance (g_s); xylem ABA concentration indeed controls g_s (Zhang et al., 2006), therefore preventing excessive losses of water that threaten plant survival in conditions of drought or high salinity, i.e. of restricted water availability. But substantial decreases in g_s come at the price of reduced net photosynthetic assimilation rate (A_{net}). Reduced A_{net} not only translates into reduced growth but also increased risk of photooxidative stress, which is associated to excess of energy absorbed by photosystems under the form of photons relative to the quantity of energy used by photochemistry, and the resulting production of reactive oxygen species (ROS) by the photosynthetic machinery. ABA itself regulates, along with other hormones (auxin, salicylic acid, cytokinins), ROS scavenging processes (Chaves et al., 2009). Auxin mediates numerous responses to stress *via* a nuclear pathway that can activate or repress several genes by recruiting specific DNA-binding transcription factors called ARFs (auxin response factors) (Roosjen et al., 2018; Verma et al., 2022). Downregulation of ARF4 in tomato results in increased root growth and leaf chlorophyll content, and upregulation of CAT1 (catalase 1), Cu/ZnSOD (superoxide dismutase), MDHAR (monodehydroascorbate reductase) in conditions of drought (Bouzroud et al., 2020). ABA and auxin pathways interact antagonistically in plant tolerance mechanisms. Chen et al. (2021) showed that ARF4 knockout resulted in upregulated ABA signaling by activation of SCL3 (SCARECROW-LIKE 3) transcription factor. The antagonist interaction between ABA and auxin is attested in a spectacular way by the observation that auxin can promote stomatal opening and reverse stomatal closure induced by ABA (Levitt et al., 1987).

Salicylic acid (SA) occupies a prominent place among plant hormones thanks to its pivotal role in both resistance against biotrophic and semi-biotrophic pathogens and tolerance against abiotic stress (Urban et al., 2022). SA receptor, NPR1 (non-expresser of pathogenesis-related protein 1), is indeed believed to be the master regulatory protein of defense and tolerance responses by being a transcriptional co-activator not only of PR genes (Jayakannan et al., 2015), but also of genes of tolerance against abiotic stress (Iglesias et al., 2022). The role of SA signaling and NPR1 is believed essential for controlling Na^+ entry into roots and K^+ accumulation in shoots in conditions of high salinity (Rasheed et al., 2020). SA not only exerts a positive effect on photosynthesis and growth in stressed plants (Urban et al., 2022), it also stimulates antioxidant activity, as shown on salt-stressed *Triticum aestivum* (Li et al., 2012) and tomato (Csiszár et al., 2014) plants. See also the review of Rasheed et al. (2020).

Cytokinins (CKs) have recognized roles in plant growth and development, senescence delay, as well as tolerance to biotic and abiotic stress (Kieber and Schaller, 2018; Cortleven et al., 2019). Stress conditions may lead to an increase in CKs concentrations in plants, as it was observed in maize and tobacco (Alvarez et al., 2008; Dobra et al., 2010). Nishiyama et al. (2011) observed that high salinity triggers up-regulation of ADENOSINE ISOPENTENYL TRANSFERASES (IPTs), which are key genes for CKs synthesis. Overproduction of endogenous CKs levels enhances drought stress tolerance in many plants but reduced levels also have been found to exert positive effects (Mandal et al., 2022). Generally speaking, the role in CKs in stress tolerance must be thought in conjunction with the other hormones. Considering salinity stress, CKs enhance the expression of genes involved in antioxidant activities (Arif et al., 2020), but they also activate genes involved in the type-A response, which suppresses expression of ABSCISIC ACID INSENSITIVE 1 (AB15). It ultimately leads to the downregulation of genes involved in ABA signaling. Genes like CYTOKININ RESPONSE 1 (CRE-1) and transcription factor ARR-B are downregulated under salinity

(Rao et al., 2016; Wang et al., 2020). CKs could eliminate tolerance to high salinity in plants by interacting antagonistically with ABA (Javid et al., 2011). Conversely, accumulation of stress-induced ABA downregulates CKs production *via* MYB2 transcription factor (Li et al., 2016). By contrast, CKs are credited with a positive interaction with SA (Jiang et al., 2013).

Improving the ability of plants to maintain growth and productivity in saline soils represents a major challenge to ensure food safety in the future. Possible ways and techniques to increase salinity tolerance have been the subject of several studies. The most common technique used for this purpose is genetic methods (conventional breeding programs, genetic engineering, interspecific hybridization, etc.). However, the genetic complexity of this trait reduces the efficiency of this technique. Consequently, the successful use of this technique to produce a salt-tolerant cultivar is very low (Flowers, 2004; Munns, 2005; Jisha et al., 2013; Cabello et al., 2014). Clearly there is the need to find ways to increase salt tolerance by non-genetic approaches, such as seed priming (Jisha et al., 2013).

Priming is defined as “a physiological process by which a plant prepares to respond to imminent abiotic stress more quickly or aggressively” (Thomas and Puthur, 2017; Dutta, 2018). Seed priming is a presowing approach for influencing seedling development by stimulating pregermination metabolic activities prior to the emergence of radicles and thus improving the germination rate, performance of the plants, and the plant tolerance to biotic and abiotic stresses (Afzal et al., 2016; Araújo et al., 2016). The enhanced tolerance of plants to abiotic stress by priming can be attributed to the activation of different stress-related metabolic processes (Thomas and Puthur, 2017).

Conventional seed priming consists of partially hydrating the seed to a point where germination processes are begun but not completed (Bilalis et al., 2012; Farooq et al., 2019). To enhance the growth and yield of many crop species under salinity stress, many seed priming agents have been reported in different articles, notably hydropriming (Srivastava et al., 2010; Amooaghaie, 2011), osmopriming (Sivritepe et al., 2005; Ahmed et al., 2017), nutriopriming (Harris et al., 2007; Harris et al., 2008; Khan et al., 2019; Moatter, 2020), and hormonal priming (Zhang et al., 2007; Afzal et al., 2011; Afzal et al., 2013). These conventional methods are accompanied by several drawbacks, such as a long treatment duration, high labor costs and, in the case of hormonal priming, negative environmental impacts. It is therefore necessary to develop fast, effective and more environmentally friendly priming protocols to replace these conventional techniques (Araújo et al., 2016; Dutta, 2018; Farooq et al., 2019).

Physical treatment can be considered as an alternative to conventional methods (Afzal et al., 2016). Among physical treatments, seed priming by UV-C light arouses increasing interest. UV-C radiation was initially explored for its germicidal effects, mainly in the food industry and for its potential for improving the post-harvest physiology of fruits and vegetables (Jagadeesh et al., 2011; Bravo et al., 2012). Recently, it has been observed that low doses of UV-C light (hormetic doses), applied to growing plants, can stimulate plant defenses against fungal diseases (Ouhibi, 2015; Urban et al., 2016; Vàsquez et al., 2017; Aarrouf and Urban, 2020; Forges et al., 2020; Vàsquez et al., 2020). UV-C seed priming effects have been reported by several researchers who have clearly demonstrated its positive impact on germination, seedling growth, and final yield. Additionally, UV-C seed priming can increase plant disease resistance. Cabbage seed treatment with UV-C light reduces the incidence of *Xanthomonas campestris* pv. campestris on plants by 75% (Brown et al., 2001). Siddiqui et al. (2011) observed reductions in disease development caused by Fusarium spp., *Rhizoctonia solani* and *Macrophomina phaseolina* on mung bean and groundnut plants. Recently, Scott et al. (2019) reported reductions in the incidence and progression of *Botrytis cinerea* and *Fusarium oxysporum* in tomato plants.

However, to our knowledge, there is only one study about dry seed treatment with UV-C light (Ouhibi et al., 2014). The latter observed a strong positive effect in lettuce plants grown at high salinity. They moreover observed that the positive effect of seed priming by UV-C light was associated with an increase in leaf antioxidant activity and in the content of phenolic compounds, but they did not investigate the way growth components were affected, notably leaf net photosynthetic assimilation (A_{net}), nor did they investigate the possible role of a change in the hormonal balance.

The major objective of the present study was to explore the effects of UV-C seed priming on photosynthesis of lettuce plants grown at high salinity. Special care was taken to control NaCl concentration in the nutrient solution. In addition to photosynthesis and growth parameters, we investigated the effects of UV-C seed priming on leaf concentrations in SA, ABA, auxin and CKs.

2 Materials and methods

2.1 Seed priming with UV-C radiation

Lettuce seeds (*Lactuca sativa* L. cv. Eden), provided by Nova Genetic (Longué-Jumelle, France), were treated using UV-C amalgam lamps at 254 nm supplying 50 mW cm^{-2} (UV Boosting, Boulogne-Billancourt, France). UV-C intensity was measured using a radiometer (RM-12. Opsytec Dr, Groebel. Germany) fitted with a 254 nm sensor. Preliminary experiments were carried out to test the effects of many UV-C doses ranging from 1 to 1000 kJ/m^2 on plant growth. Results of these experiments (not shown here) allowed to identify the most effective dose of UV-C radiation: 200 kJ m^{-2}. Seeds were exposed to UV-C treatment for 6 minutes 40 seconds. Pr and NPr abbreviations are used to refer to lettuce plants from primed seeds with 200 kJ m^{-2} and control plants issued from nonprimed seeds, respectively.

2.2 Growth conditions and experimental design

The experiment was performed in the greenhouse of Avignon University (France). Mean air temperature was 22.1°C (min: 11.9°C; max: 25.9°C) (Figure 1), and mean relative humidity 28% during the

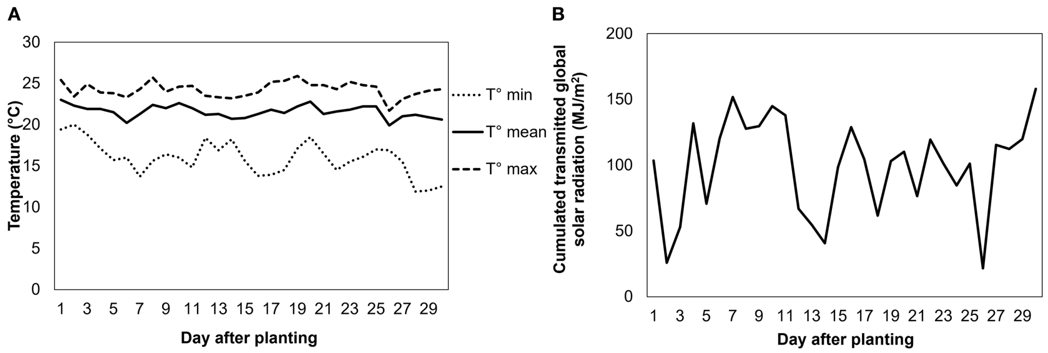

FIGURE 1
Daily mean, maximum, and minimum temperatures **(A)**, and daily cumulated transmitted global solar radiation at experiment **(B)**. Greenhouse of Avignon university (France) 43°54′43.3″N 4°53′21.0″E.

day and 78% during the night. Photosynthetically active radiation (PAR) of 450 ± 50 µmol photons $m^{-2}s^{-1}$ was provided by high-pressure sodium (HPS) lamps (400 Watts, Hortilux Schréder b.v., The Netherlands). Primed and nonprimed seeds were directly sown in rockwool cubes, where the seedlings were grown. Three weeks later, 96 plants were transferred to individual pots (7 cm in diameter) filled with clay pebbles and placed in a randomized block design on a hydroponic system specially designed to avoid the build-up of ionic gradients while maintaining a high level of oxygenation in the root environment (Figure 2). The system consisted of two plastic tanks (2 m^2) located next to each other, filled with the nutrient solution and without NaCl for the first one, and the same nutrient solution plus 100 mM NaCl for the second one, the so-called high salinity treatment. The hydroponic nutrient solution was comprised of 7% N, 4% P_2O_5, 5% K_2O, 0.036% Fe, 0.01% Mn, and 0.004% Zn. The electrical conductivity (EC) of the nutrient solution was set at 2.6 ± 0.3 dS m^{-1} in the control tank. In the presence of NaCl solution, EC reached ca. 10 ± 1 dS m^{-1}. NaCl was supplied at the time the plants were transferred to the plastic tank. In each tank, an air pump (32 W, >0.02 MPa, 60 l min^{-1}; HAILEA, China) with 6 immersed outlets was operated continuously to keep the concentrations of the minerals homogeneous in the nutrient solution and to supply oxygen to the roots.

The nutrient solution was completely changed every 10 days. pH was monitored daily and maintained at 6.0 ± 0.4 using a portable pH/EC/temperature meter (HANNA instruments, Portugal).

Prior to the onset of the trial, light and temperature measurements were performed at different times of the day to check that there were no climate differences between the two tanks. UV-C-treated (Pr) and control plants (NPr) were randomly distributed in each tank (control and with NaCl).

2.3 Growth and yield parameters

Twenty-one days after planting in the hydroponic system, the plants were harvested. The fresh mass of the leaves and roots was measured. For dry mass determination, the plant material was kept at 80°C in an oven for 48 h. Individual and total leaf areas were determined using ImageJ® (https://imagej.nih.gov/ij/) prior to the dry mass measurement.

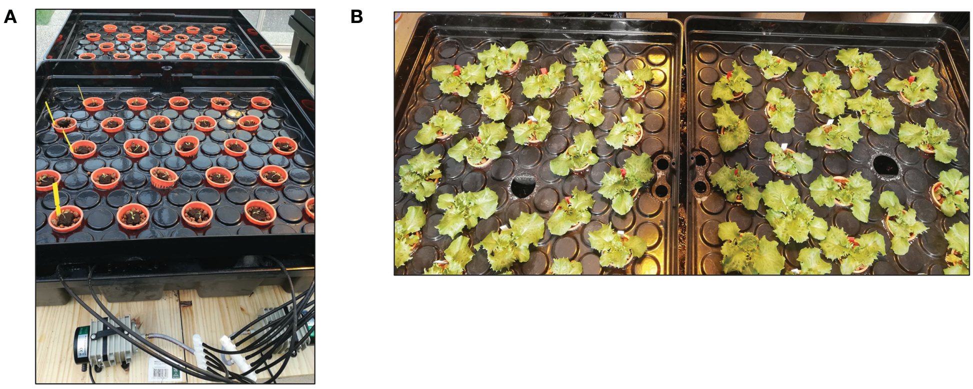

FIGURE 2
The hydroponic system at the time of planting **(A)**, and lettuce plants one week after planting **(B)**.

2.4 Determination of Na^+ and K^+ concentrations

Concentrations of Na^+ and K^+ were determined in the dry plant material and expressed on a dry mass basis. Roots were rinsed three times in cold distilled water after harvest. Cation extraction was realized with HNO_3 (0.5%) and estimated by standard flame photometry (PFP7, JENWAY, UK).

2.5 Leaf gas exchange measurements

Seven and 14 days after planting in the hydroponics system, leaf gas exchange measurements were carried out with an infrared CO_2/H_2O gas analyzer and leaf chamber system with an external light source in the 400-700 nm range (LI 6800, Licor, Lincoln, USA). The net CO_2 assimilation rate (A_{net}) and leaf conductance of water vapor (g_s) were measured between 10 a.m. on young, fully expanded leaves, with a level of PAR set at 400 µmol photons $m^{-2}s^{-1}$ and a partial pressure of ambient CO_2 (Ca) at 40 Pa. At the end of the A_{net} measurements, the light source was turned off (PAR = 0 µmol photons m^{-2} s^{-1}) for 3 min, and the dark respiration rate (R_d) was measured.

Photosynthetic capacity is commonly assessed through measurements of V_{cmax}, the maximum carboxylation rate of Rubisco, and J_{max}, the light-saturated electron transport rate. Their values can be derived from so-called A-C_i curves (Urban et al., 2017). Low g_s at day 7, and even more at day 14 (Figure 3B) prevented us to obtain reliable A-C_i curves. The photosynthetic capacity was therefore simply estimated as A_{max}, the maximal rate of net photosynthesis under saturating conditions. PAR was set at 2000 µmol photons m^{-2} s^{-1} and Ca at 2000 Pa. A_{max} was measured 7 and 14 days after transplanting the plants.

2.6 SPAD values

Seven and 14 days after planting, the leaf chlorophyll content was estimated by taking the mean of three readings with a portable Chlorophyll meter (Minolta Co. Ltd, Osaka, Japan).

2.7 Chlorophyll fluorescence measurements and an analysis of fluorescence transients using ChF induction curves

On the same day as the leaf gas exchange measurements (7 and 14 days after salinity application), chlorophyll fluorescence (ChlF) transients were measured before 10 a.m. with a portable

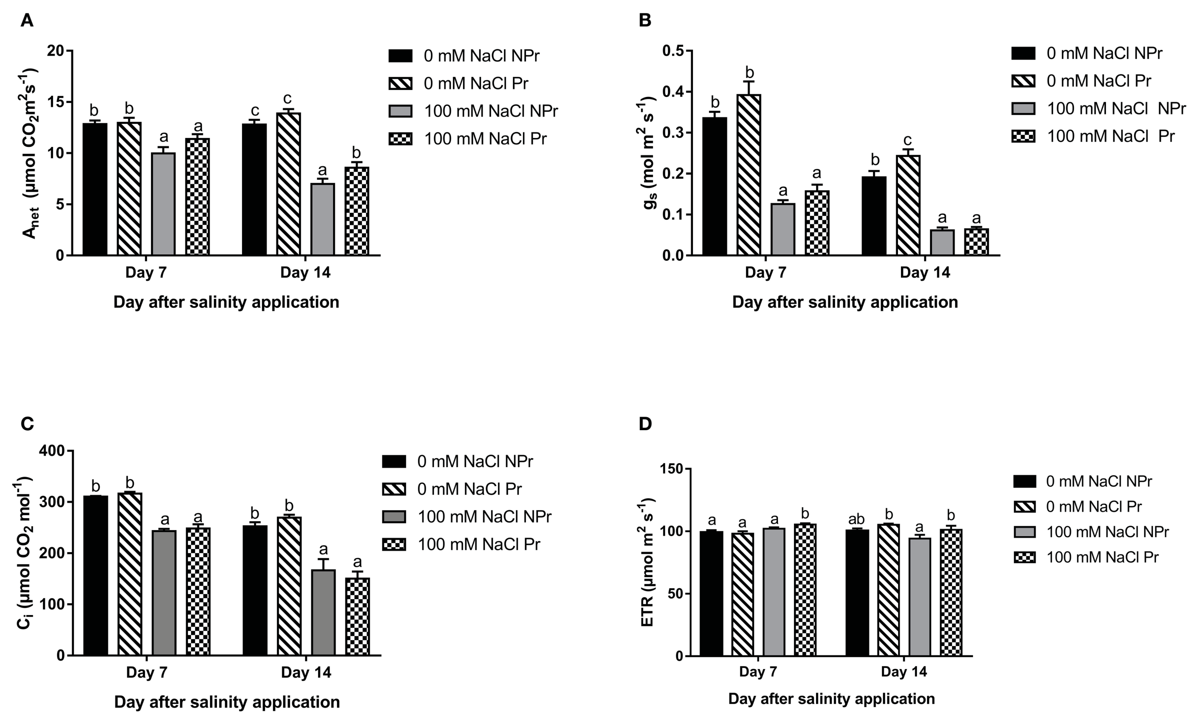

FIGURE 3
Effects of lettuce seed priming with UV-C radiation on leaf net photosynthesis of plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). Plants derived from seeds primed are designed by Pr, and non-primed by NPr. A_{net} represents net carbon assimilation rate **(A)**, g_s stomatal conductance **(B)**, Ci intercellular CO_2 concentration **(C)** and ETR electron transport rate **(D)**. Error bars represent the standard errors (n=10). Different letters indicate significant differences among means for each measurement date after salinity application at p<0.05 according to Dunn's test.

Pocket PEA chlorophyll fluorimeter (Hansatech Instruments, King's Lynn, Norfolk, United Kingdom). Leaves were dark adapted for 20 min with a lightweight plastic leaf clip prior to measurement and then exposed for 1 s to 3500 µmol photons m^{-2} s^{-1} (637 nm peak wavelength). The chlorophyll fluorescence intensity at t = 50 µs was considered F_0 (Strasser and Strasser, 1995). The fast ChlF kinetics (from F_0 to F_m, where F_0 and F_m are, respectively, the minimum and maximum measured ChlF of photosystem II (PSII) in the dark-adapted state) were recorded from 10 µs to 1 s. The ratio of variable ChlF (F_v) to F_m (F_v/F_m), i.e. the maximum quantum yield of PSII ($\varphi P_0 = TR_0/ABS$), the performance index (PI), a plant vitality indicator and its components (F_v/F_0, the variable to minimum ChlF ratio; RC/ABS, which represents the density of reaction centers expressed on an absorbed photon flux basis; $(1–V_j)/V_j$), where V_j is the relative variable ChlF at t = 2 ms) were calculated automatically (Strasser et al., 2000; Campa et al., 2017). We also calculated the dissipated energy flux on an absorbed photon flux basis (DI_0/ABS), an indicator of the importance of processes other than trapping, and the electron transport fluxes from Q_A to Q_B (ET_0) and from Q_B to PSI acceptors (RE_0), expressed as quantum yields ($\varphi E_0 = ET_0/ABS$ and $\varphi R_0 = R_0/ABS$). The latter parameter is arguably related to cyclic electron transport (CET) activity (Ripoll et al., 2016). Changes in CET activity play a major role in plant adaptations to stress. Moreover, we calculated the following parameters, which are indicators of potential damage: F_0, F_v/F_m, V_k/V_j, and S_m (Ripoll et al., 2016; Urban et al., 2017). V_k/V_j represents the ratio of variable ChlF at 300 µs (K-step) to variable ChlF at 2 ms (J-step), and S_m is the normalized area above the ChlF induction curve.

2.8 Determination of endogenous concentrations of phytohormones

Harvested lettuce leaves were immediately frozen in liquid nitrogen before being lyophilized. Endogenous phytohormone (abscisic acid, auxin, salicylic acid, and cytokinin) concentrations were determined by UPLC-XEVO in the Chemistry and Metabolomics platform of Jean-Pierre Bourgin Institute, INRAE Versailles (Grignon, France) following the method of Chauffour et al. (2019).

2.9 Statistical analysis

A randomized experimental design was used in this study. All data were analyzed using the Kruskal–Wallis nonparametric test and Dunn's *post hoc* test with Bonferroni correction. Analyzed data correspond to the mean values (± standard error) of growth parameters (n=24), SPAD (n=24), water content (n=24), Na^+ and K^+ contents (n=10), photosynthetic components (n=10), fluorescence parameters (n=48), WUE (n=10), and phytohormone content (n=6). All statistical analyses were performed using R software.

3 Results

3.1 Effects of high salinity and UV-C seed priming on growth

Growth parameters for plants derived from UV-C primed (Pr) and nonprimed (NPr) seeds under control (0 mM NaCl) and salinity (100 mM NaCl) conditions are presented in Figure 4.

Salinity significantly reduced all plant growth parameters, with the exception of the root dry mass. Total plant fresh mass (yield), plant leaf number, total leaf area, average individual leaf area, and leaf dry mass were reduced by 66% (Figure 4A), 48% (Figure 4B), 11% (Figure 4C), 65% (Figure 4D), and 74% (Figure 4E), respectively, in the NaCl-treated plants compared with the control.

Seed priming also resulted in an increase in leaf dry mass of 38% in the plants grown without NaCl.

In the plants grown at high salinity, UV-C priming resulted in an increase in yield (+20%, Figure 4A), leaf number of the plants (+8%, Figure 4B), total leaf area (+15%, Figure 4C) and individual leaf area (+17%, Figure 4D) compared to unprimed seeds.

3.2 Effects of high salinity and UV-C seed priming on plant sodium and potassium contents

High salinity resulted in a heavy accumulation of Na^+ ions in both leaves and roots (Figures 5A, B), and since there was no change in the leaf K^+ content (Figures 5C, D), there was a strong imbalance in the Na^+/K^+ ratio as a consequence (Figures 5E, F). There were no differences between plants from primed seeds and control plants, regardless of the presence of NaCl in the nutrient solution (Figure 5).

3.3 Effects of high salinity and UV-C seed priming on chlorophyll content (SPAD values)

Plant chlorophyll contents were estimated by SPAD measurements 7 and 14 days after NaCl application. SPAD values were increased by 22% at Day 7 and by 19% at Day 14 in the NaCl-treated plants compared with the control (Figure 6A). UV-C priming of seeds did not modify the chlorophyll content of the plants grown under high salinity conditions and did not modify the chlorophyll content of the control plants (Figure 6A).

3.4 Effects of high salinity and UV-C seed priming on water content

High salinity caused a decrease in leaf tissue hydration status. There were no significant differences between the water content in the leaves of lettuce plants issued from primed seeds (Pr) and the control (NPr) (Figure 6B).

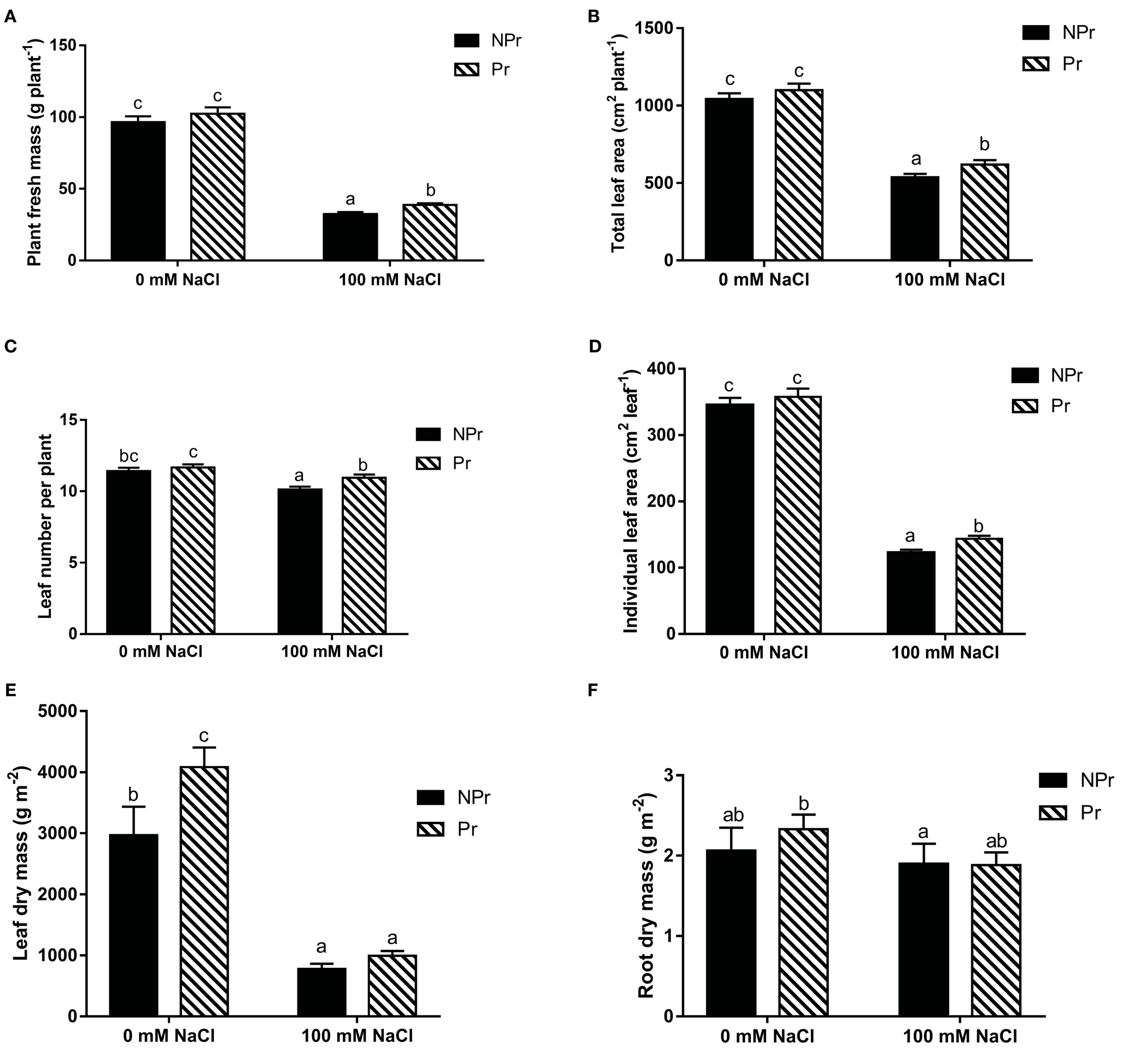

FIGURE 4
Effects of lettuce seed priming with UV-C radiation on growth and yield parameters of plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl(0 mM). Plants derived from seeds primed are designed by Pr,and non-primed by NPr. **(A)** Plant fresh mass (g plant^{-1}), **(B)** total leaf area per plant, **(C)** leaf number per plant, **(D)** individual leaf area, **(E)** leaf dry mass, **(F)** root dry mass.Error bars represent the standard errors (n=24). Different letters indicate significant differences at p<0.05 according to Dunn's test.

3.5 Effects of high salinity and UV-C seed priming on plant gas exchange parameters

High salinity resulted in a substantial decrease in A_{net}, gs and C_i 7 and 14 days after the onset of the NaCl treatment (Figures 3A–C). A_{net} was decreased by only 23% in Pr plants vs. 45% in NPr plants at Day 14 (Figure 3A). On that day, the photosynthetic electron transport rate (ETR) was also 7% higher in the Pr plants than in the NPr plants in the high salinity treatment (Figure 3D).

In NPr plants, high salinity resulted in a 19% increase in A_{max} at Day 7, followed by a 28% decrease (Figure 7). UV-C priming of seeds did not impact A_{max} or ETR in either the NaCl treatment or the control, regardless of the date.

Water use efficiency (WUE) was calculated as the ratio of leaf net photosynthesis (A_{net}) and leaf transpiration (E). Under high salinity, the WUE was 38% and 81% higher at Day 7 and Day 14, respectively (Figure 8). Priming did not modify the WUE at either time.

There was an increase in R_d in NPr but not Pr plants as a consequence of high salinity at Day 7 (Figure 9). A decrease in R_d was observed in Pr plants, i.e., as a consequence of UV-C priming of seeds in both NaCl-treated and control plants at Day 7. Such differences were no longer apparent at Day 14 (Figure 9).

3.6 Effects of high salinity and UV-C seed priming on chlorophyll fluorescence parameters

The parameters derived from the induction curves of maximal chlorophyll fluorescence, which are believed to be interpretable in

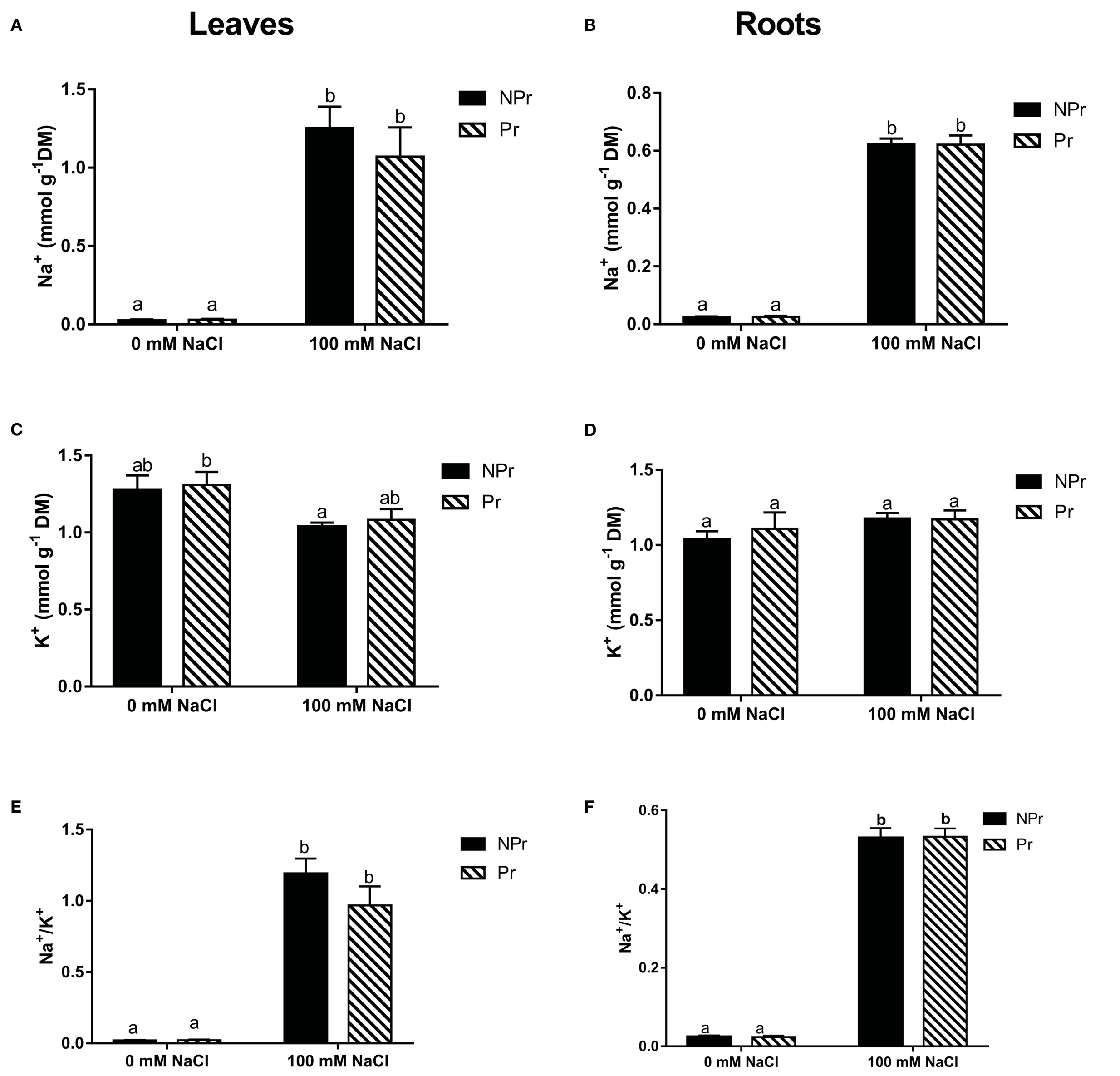

FIGURE 5
Effects of lettuce seed priming (Pr) with UV-C radiation on Na^+ and K^+ contents in leaves **(A, C, E)** and roots **(B, D, F)** at lettuce plants grown in high salinity condition (NaCl 100 mM) or in the absence of NaCl (0 mM). Plants derived from seeds primed are designed by Pr, and non-primed by NPr. Error bars represent the standard errors (n=10). Different letters indicate significant differences at p<0.05 according to Dunn's test.

terms of damage, are presented in Table 1 and Figure 10. At Days 7 and 14, there was a 5% increase in F_0 as a consequence of high salinity in both Pr and NPr plants. This increase in F_0 did not translate into any decrease in F_v/F_m. High salinity also resulted in a decrease in V_k/V_j by 19% and 24% at Day 7 and Day 14, respectively. S_m was 13 and 29% higher in high salinity-grown plants than in controls at Day 7 and Day 14, respectively.

Parameters of the quantum yields of electron transport and energy fluxes derived from the induction curves of maximal chlorophyll fluorescence are presented in Table 2 and Figure 10. There was an impact of high salinity on two parameters, namely, φE_0 and φR_0. Both parameters were 13% higher as a consequence of high salinity at Day 14. Priming of seeds by UV-C light had no effect and did not modify the responses to high salinity. In addition, high salinity and priming treatments had no impact on φP_0 or DI_0/ABS.

The results related to the fluorescence parameters contributing to the performance index (PI) are summarized in Table 3 and Figure 10. High salinity resulted in a significant increase in PI_{ABS} values at both Day 7 and Day 14 (+17% at Day 7 and +71% at Day 14 for NPr plants). PI_{tot} was also 69% higher at Day 14 as a consequence of high salinity in the NPr plants. Similarly, higher RC/ABS values were observed as a consequence of high salinity in NPr plants at both dates (+22% at Day 7 and +31% at Day 14). In contrast, F_v/F_0 was not affected. $(1\text{-}V_j)/V_j$ was 25% lower in NPr plants at Day 14. The UV-C

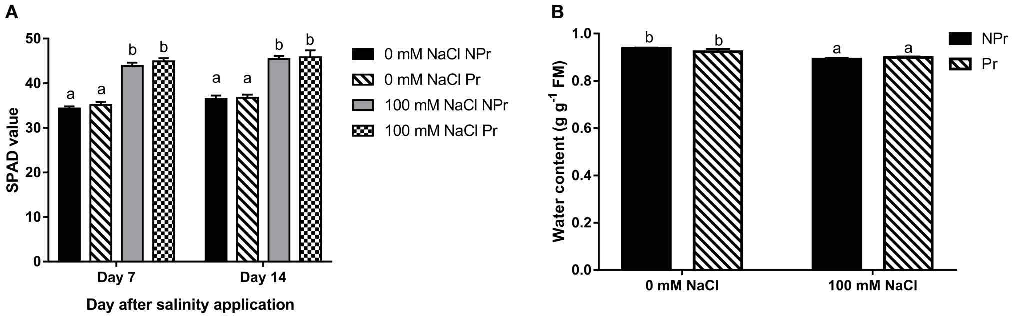

FIGURE 6
Effects of lettuce seed priming with UV-C radiation in chlorophyll content estimated by SPAD measurements **(A)**, and water content **(B)** of plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). Plants derived from seeds primed are designed by Pr, and non-primed by NPr. Error bars represent the standard errors (n=24). Different letters indicate significant differences at p<0.05 according to Dunn's test.

priming treatment did not impact the response to high salinity of these chlorophyll fluorescence parameters.

3.7 Effects of high salinity and UV-C seed priming on phytohormones

High salinity caused many changes in the phytohormonal status of lettuce leaves. In NPr plants, it resulted in a 38% decrease in auxin content (Figure 11A). For other hormones, an increase of 101% of ABA (Figure 11B), 194% of SA (Figure 11C) and 44% of CKs (Figure 11D) were obtained at 100 mM NaCl. The ABA/CKs ratio (Figure 11E) also increased by 37% in NPr plants.

Seed priming by UV-C light maintained the levels of ABA and auxin compared to NPr plants under NaCl conditions (Figures 11A, B). Seed priming induced a 31% increase in the SA concentration (Figure 11C) and a 21% increase in the CKs concentration (Figure 11D) compared to NPr plants under salinity conditions. The ratio of ABA to CKs remained unchanged in plants grown in the absence of NaCl (Figure 11E).

4 Discussion

4.1 Features of salinity stress in our trial

High salinity is known to impact plant photosynthesis and growth negatively, primarily through osmotic stress and then salt toxicity (Munns, 2002). Tolerance to high salinity is generally due to the selectivity of K^+ absorption to maintain Na^+/K^+ homeostasis in salt stress conditions (Tester and Davenport, 2003; Gupta and Huang, 2014). In our trial, we found, as expected, a substantial increase in Na^+ content in leaves and roots that was however not associated to a decrease in either leaf or root K^+ content of the lettuce plants submitted to high salinity. There are important differences with the observations of Ouhibi et al. (2014). Firstly, Na^+ leaf content reached only 1.2 mmol g^{-1} DM in our trial (Figure 5A) vs. 2.0 mmol g^{-1} DM in their trial. The difference was even more marked for Na^+ root content (Figure 5B). Moreover, K^+ leaf and root contents were not decreased as a consequence of high salinity in our trial (Figures 5C, D), by contrast to the observations of Ouhibi et al. (2014).

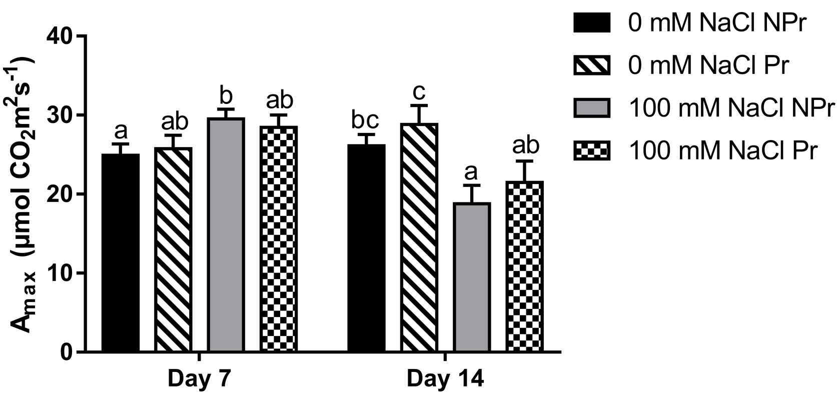

FIGURE 7
Effects of lettuce seed pnm1ng with UV-C radiation on leaf maximum photosynthesis A_{max} of plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). Plants derived from seeds primed are designed by Pr, and non-primed by NPr. Error bars represent the standard errors (n; 10). Different letters Indicate significant differences among means for each mesurment date after salinity Application at p<0.05 according to Dunn's test.

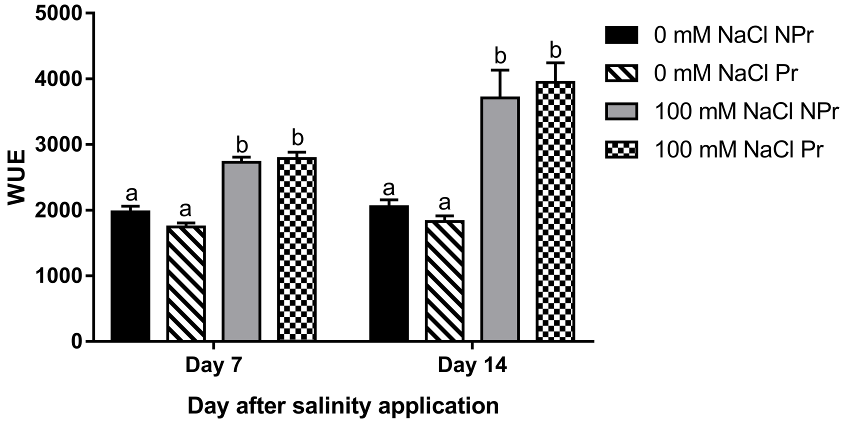

FIGURE 8
Effects of lettuce seed priming (Pr) with UV-C radiation on water use efficiency (WUE = A_{net}/Ei) of plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). Plants derived from seeds primed are designed by Pr, and non-primed by NPr. Error bars represent the standard errors (n=10). Different letters indicate significant differences among means for each measurement date, after salinity application at p<0.05 according to Dunn's test.

These differences are surprising in the first place since the same concentration of 100 mM NaCl was applied in both trials. But then, plants were grown in pots, in a conventional way by Ouhibi et al. (2014), whereas plants in our trial were grown in a full hydroponic system, where roots developed in a nutrient solution constantly oxygenated by bubbling. In such a system, ion convection is high because of bubbling, which prevents very effectively the formation of ion gradients in the root zone. Such gradients form naturally for all ions that are in excess in the supply relative to the uptake by plants. They form in traditional soilless systems based on the use of growing media, because the latter have no or little cation exchange capacity and because ion diffusion is too slow a process to homogenize ion concentrations across the nutrient solution (Urban and Urban, 2010). In commercial greenhouses equipped for soilless culture in growing media, ion gradients are routinely reduced by the supply of nutrient solution in excess, which leads to the well-known practice of "drainage" (Urban and Urban, 2010). Interestingly, Cabrera and Perdomo (2003) challenged the classification of rose plants as salinity-sensitive on the basis of observations they made in soilless systems with drainage. We formulate the hypothesis that we maintained 100 mM in the root zone of lettuce plants in our trial thanks to bubbling, as was our intention, whereas the NaCl concentration increased uncontrolled in the trial of Ouhibi et al. (2014) in the absence of a strategy of irrigation with drainage. In other words, the differences in Na^+ and K^+ contents between both trials reflect differences in intensity of salinity stress. Even though Na^+ contents increased in both leaves and roots as a consequence of high salinity in our trial, K^+

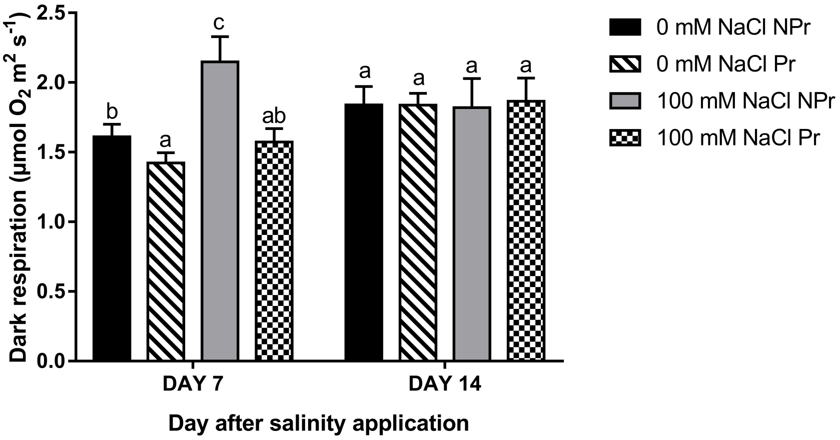

FIGURE 9
Effects of lettuce seed priming with UV-C radiation on the dark respiration rate (R_d) of plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). Plants derived from seeds primed are designed by Pr, and non-primed by NPr. Error bars represent the standard errors (n=10). Different letters indicate significant differences among means for each mesurement date after salinity application at p<0.05 according to Dunn's test.

TABLE 1 Effects of seed priming with UV-C radiation on damage parameters derived from induction curves of maximal chlorophyll fluorescence.

Date of measurement	Salinity stress	F_0		F_v/F_m		V_k/V_j		S_m	
		NPr	Pr	NPr	Pr	NPr	Pr	NPr	Pr
Day 7	0 mM	4689.587[a]	4718.891[a]	0.824[a]	0.823[a]	0.464b	0.468b	18.146[a]	18.372[a]
	100 mM	4922.375[b]	4896.087[b]	0.820[a]	0.822[a]	0.377[a]	0.375[a]	20.576[b]	21.013[b]
Day 14	0 mM	4446.479[a]	4390.109[a]	0.837[a]	0.836[a]	0.480[b]	0.474[b]	19.846[a]	20.016[a]
	100 mM	4655.979[b]	4600.935[b]	0.836[a]	0.837[a]	0.366[a]	0.365[a]	25.551[b]	24.678[b]

Plants from seeds primed are designed by Pr, and non-primed by NPr. Plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). F0 is a minimum of fluorescence, Fv/Fm is a maximum quantum yield of primary PSII chemistry, Vk/Vj is an indicator of inactivation of the oxygen evolving complex, and Sm the normalized area above the OJIP curve. Data presented are means of 48 values. Different letters indicate significant differences among means for each measurement date after salinity application at p<0.05 according to Dunn's test.

homeostasis suggests that K^+ transport systems were not mobilized (Lu et al., 2020).

Consistent with the idea that the salt toxicity component of salt stress was moderate or even inexistent in our trial is the absence of visual symptoms of damage. Our ChlF data moreover suggest that stimulation of photorespiration, possibly in addition to other alternative routes for electrons in excess and ROS scavenging processes, proved efficient in preventing damage to the photosynthetic machinery. The hypothesis that photorespiration was increased as a consequence of high salinity in our trial, is substantiated by the fact that the photosynthetic electron rate (ETR) was maintained while A_{net} decreased on both Days 7 and 14 (Figures 3A, D). Concerning ChlF data, on both Day 7 and Day 14, we found a moderate increase in F_0, which did not result in a decrease in F_v/F_m, whereas V_k/V_j was found to decrease and S_m to increase under conditions of high salinity (Table 1). According to Ripoll et al. (2016) and Urban et al. (2017), such changes do not support the hypothesis of a damaging effect on the photomachinery (Ripoll et al., 2016), and they therefore confirm that salt stress was moderate in this trial.

4.2 Hormonal changes associated with high salinity

Protection of the components of the photosynthetic electron transfer chain plays an important role in the adaptative response of plants to high salinity (Evelin et al., 2019). Such protective mechanisms may be attributed to SA, which was found to be dramatically increased in leaves of plants grown at 100 mM NaCl compared to control leaves (Figure 11C). One major hypothesis is that SA exerts a protective effect against stress by stimulating antioxidant responses (Figure 12). There is indeed a wealth of scientific evidence that SA stimulates the activity of antioxidant enzymes (superoxide dismutase, peroxidases, catalase) or enzymes of antioxidant systems, such as glutathione reductase. See, for instance, Dong et al. (2014) on this topic. Consistent with the idea that SA increase exerted a protective effect on the photosynthetic machinery and its functioning in plants grown at high salinity is the maintenance of nearly all ChlF parameters at the same levels than in the control (Tables 1–3). There was even an increase in S_m and a decrease in V_k/V_j on both Days 7 and 14

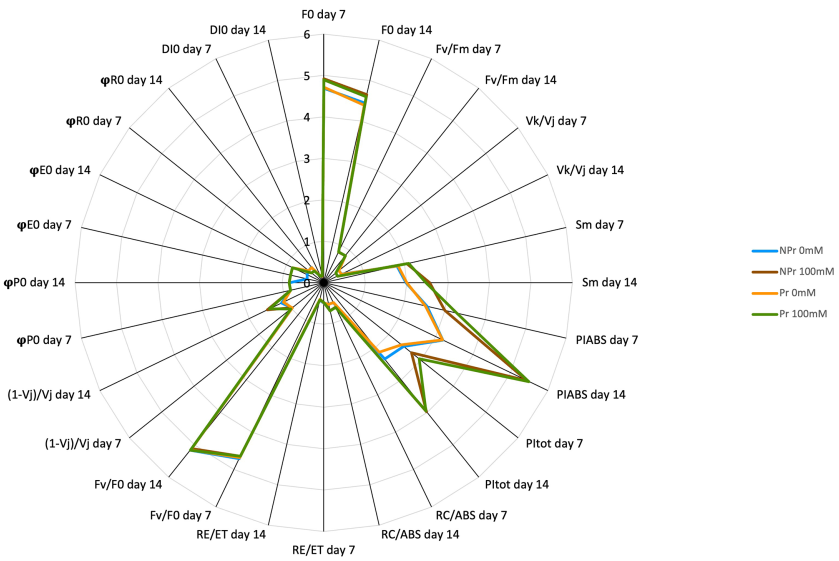

FIGURE 10
Spider plot of the parameters derived from maximal chlorophyll fluorescence induction curves. See also Tables 1–3. F0 and Sm values were divided by 1000 and 10, respectively, for the sake of legibility.

TABLE 2 Effects of seed priming (Pr) with UV-C radiation on field parameters derived from induction curves of maximal chlorophyll fluorescence.

Date of measurement	Salinity stress	$\varphi P_0 = (TR_0/ABS)$		$\varphi E_0 = (ET_0/ABS)$		$\varphi R_0 = (RE_0/ABS)$		DI_0/ABS	
		NPr	Pr	NPr	Pr	NPr	Pr	NPr	Pr
Day 7	0 mM	0.824[a]	0.823[a]	0.410[a]	0.409[a]	0.200[a]	0.198[a]	0.176[a]	0.177[a]
	100mM	0.820[a]	0.822[a]	0.406[a]	0.419[a]	0.192[a]	0.197[a]	0.180[a]	0.178[a]
Day 14	0 mM	0.837[a]	0.836[a]	0.447[a]	0.441[a]	0.188[a]	0.176[a]	0.163[a]	0.164[a]
	100mM	0.836[a]	0.837[a]	0.504[b]	0.499[b]	0.213[b]	0.208[b]	0.164[a]	0.163[a]

Plants from seeds primed are designed by Pr, and non-primed by NPr. Plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). TR_0/ABS maximum trapped exciton flux, ET_0/ABS Quantum yield of electron transport from QA- to PQ, RE_0/ABS Quantum yield of electron transport from QA- to final PSI acceptors, and DI_0/ABS dissipated energy flux per PSII. Data presented are means of 48 values. Different letters indicate significant differences among means for each measurement date after salinity application at p<0.05 according to Dunn's test.

(Table 1), and an increase in φE_0 and φR_0 at Day 14 (Table 2), and PI, the latter attributable to increases in RC/ABS and $(1-V_j)/V_j$ at Day 14 (Table 3). These changes indicate not only that the photosynthetic machinery was not damaged but that its functioning was improved. Our data more specifically suggest that quantum efficiency of electron transport from Q_A to PQ and to PSI acceptors was improved. Our observations confirm the ones of Lotfi et al. (2020) who also found a bettering of ChlF parameters, attested notably by an increase in the area derived from analysis of maximal ChlF induction curves, in *Vicia radiata* plants treated with SA and grown at high salinity.

The high levels of SA in leaves of lettuce plants grown at 100 mM NaCl were associated with an increase in leaf ABA and cytokinins, and a decrease in auxin concentrations (Figure 11), which confirms a majority of observations about the effects of high salinity on hormone concentrations in plants and also about interactions between hormones in plant tolerance against stress.

The high ABA endogenous concentration in leaves of lettuce plants grown at 100 mM NaCl (Figure 11B) is probably associated with the substantial decrease in g_s we observed in this trial (Figure 3B) (Jones et al., 1980; Cowan, 1982; Davies and Zhang, 1991). It may also have played a role in homeostasis or even improvement of ChlF fluorescence data, considering the role of ABA in antioxidant activities (Zhang et al., 2006; Chaves et al., 2009). It may not be excluded that high SA reinforced ABA. Szepesi et al. (2009) found indeed that exogeneous applications of SA increased ABA accumulation and improved stress tolerance on tomato plants grown under high salinity conditions.

Auxins are known to play a key role in growth and development in plants, notably root differentiation (Bali et al., 2017). We observed that reduced growth of the aerial part under high salinity conditions (Figure 4) was associated with a decrease in leaf auxin endogenous concentration (Figure 11A), which is consistent with previous observations in other species such as maize (Ribaut and Pilet, 1991; Ribaut and Pilet, 1994) and tomato (Dunlap, 1996). Interestingly, salinity stress resulted in a strong reduction of root growth in the trial of Ouhibi et al. (2014), but not in our trial (Figure 4F). We may attribute this difference to the fact that auxin depletion was relatively moderate in our trial, i.e. sufficient to limit leaf but not root development, another argument in favor of the idea that salinity stress was moderate. ABA and auxin pathways interact antagonistically in plant tolerance mechanisms. Auxin can promote stomatal opening and reverse stomatal closure induced by ABA (Levitt et al., 1987). This clearly did not happen for plants grown at high salinity in this trial (Figure 3B), arguably because high SA interfered with auxin signaling in an antagonist way (Iglesias et al., 2011). MicroRNA miR393, the main regulator of auxin receptors TIR1, AFB2 and AFB, was shown to be stimulated under salinity stress (Sunkar et al., 2007). Navarro et al. (2006)

TABLE 3 Effects of seed priming (Pr) with UV-C radiation on the fluorescence parameters contributing to the performance index (PI).

Date of measurement	Salinity stress	PI_{ABS}		PI_{tot}		RC/ABS		RE/ET		F_v/F_0		$(1-V_j)/V_j$	
		NPr	Pr	NPr	Pr	NPr	Pr	NPr	Pr	NPr	Pr	NPr	Pr
Day 7	0 mM	2.557[a]	2.515[a]	2.464[ab]	2.397[a]	0.535[a]	0.530[a]	0.493[a]	0.487[a]	4.708[a]	4.680[a]	0.971[a]	0.971[a]
	100mM	2.998[b]	3.294[b]	2.707[bc]	2.943[c]	0.655[b]	0.662[b]	0.475[a]	0.472[a]	4.568[a]	4.644[a]	0.963[a]	1.018[a]
Day 14	0 mM	3.190[a]	3.180[a]	2.355[a]	2.144[a]	0.526[a]	0.534[a]	0.424[a]	0.404[a]	5.167[a]	5.156[a]	1.132[b]	1.077[b]
	100mM	5.439[b]	5.493[b]	3.978[b]	3.950[b]	0.688[b]	0.695[b]	0.421[a]	0.417[a]	5.114[a]	5.167[a]	1.508[a]	1.451[a]

Plants from seeds primed are designed by Pr, and non-primed by NPr. Plants grown in high salinity conditions (100 mM NaCl) or in the absence of NaCl (0 mM). PI_{ABS} the performance index for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors, PI_{tot} the performance index for energy conservation from photons absorbed by PSII antenna until the reduction of PSI acceptors, RC/ABS the density of active PSII reaction centers expressed on the base of the quantity of light absorbed by the antenna, RE/ET the electron transport flux from Q_B to PSI acceptors, F_v/F_0 the contribution to the PI of the light reactions for primary photochemistry, and $(1-V_j)/V_j$ the performance due to the conversion of excitation energy to photosynthetic electron transport, and Data presented are means of 48 values. Different letters indicate significant differences among means for each measurment date after salinity application at p<0.05 according to Dunn's test.

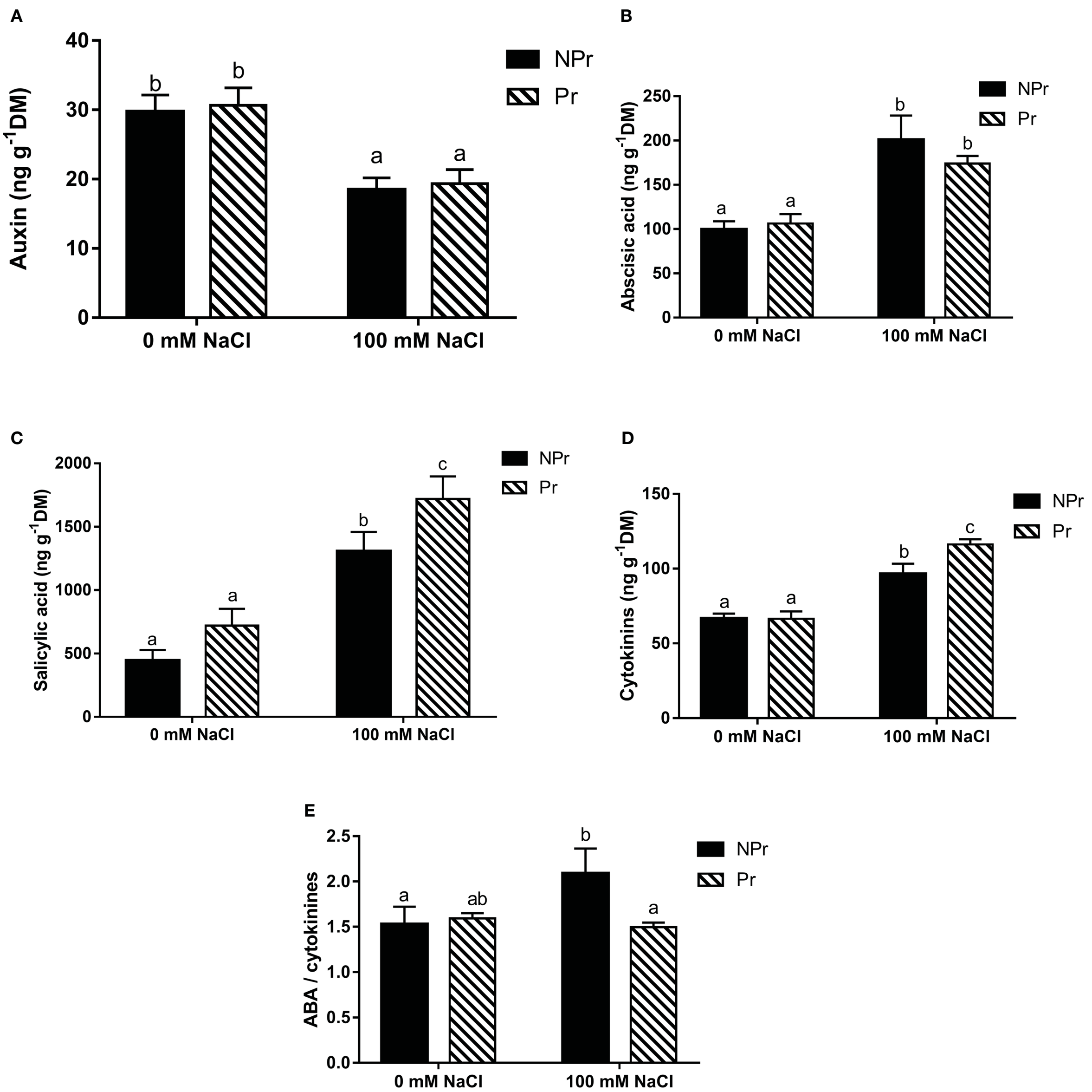

FIGURE 11
Effects of lettuce seed priming (Pr) with UV-C radiatoi n on phytohormones endogenous concentrations of plants grown in high salinity conditions (100 mM NaCl). auxin **(A)**, abscisic acid (ABA) **(B)**, sailcylic acid **(C)**, cytokinins **(D)**, and ABA/cytokinins ratio **(E)**. Error bars represent the standard errors (n=6). Different letters indicate significant dfiferences among means for each mesurment date after salinity application at p<0.05 according to Dunn's test.

found that stress tolerance was associated with miR393-triggered repression of auxin receptors. Observations by (Iglesias et al., 2011) on Arabidopsis mutants have shown that tolerance against salinity stress increases in double tir1 and afb2 mutants, whereas SA increases PR1 genes in these mutants. Such a mechanism is part of an integrated mechanism allowing plants to deal with multiple stresses (Agtuca et al., 2014).

Leaf endogenous concentration of CKs were higher in plants grown at high salinity than in the control (Figure 11D), which is consistent with observations in such species as *Arabidopsis thaliana* (Prerostova et al., 2017), apple (Keshishian et al., 2018), rice (Joshi et al., 2018) or tomato (Feng et al., 2019), but stays in contrasts with others like wheat (Avalbaev et al., 2016) or maize (Zhang et al., 2018). In fact, the way CKs are associated with tolerance to high salinity does not seem to result only from the specific responses of the different species but also to duration of stress and the way hormone signaling systems interact in the presence of stress (Mandal et al., 2022). Within this view, we may hypothesize that the decrease in the CKs/ABA ratio in the plants grown at high salinity (Figure 11E) is attributable to downregulation of CKs production by stress-induced ABA accumulation (Li et al., 2016). Even if CKs synthesis was downregulated, leaf CKs concentrations increased all the same in the plants grown at high salinity. This may have contributed to the protection of the photosynthetic machinery (Cortleven et al., 2014; Cortleven

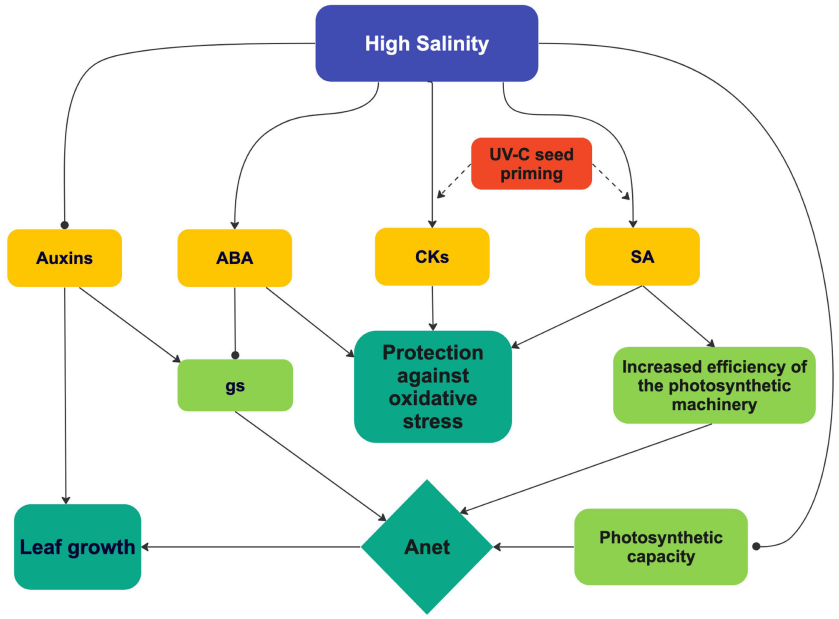

FIGURE 12
A tentative and integrated view of the effect of high salinity and UV-C priming on hormone concentrations, photosynthesis and leaf growth. See text for details. UV-C priming resulted in a reinforced effect of high salinity on SA and CKs concentrations.

and Schmülling, 2015), as the near-homeostasis of ChlF parameters would suggest (Tables 1–3).

4.3 Stomatal and non-stomatal limitations of photosynthesis in high salinity conditions-consequences on growth parameters

There was a strong decrease in A_{net} at Days 7 and 14 (Figure 3A). This decrease is consistent with similar observations made on lettuce plants grown under conditions of high salinity (De Pascale and Barbieri, 1995; Ekinci et al., 2012; Huang et al., 2019; Shin et al., 2020). The high salinity-associated decrease in A_{net} may be attributed to a decrease in g_s and C_i at Days 7 and 14 (Figures 3B, C), and moreover to an increase in mitochondrial respiration at Day 7 (Figure 9) and a decrease in photosynthetic capacity (A_{max}) at Day 14 (Figure 7).

The decrease of g_s in the leaves of lettuce plants grown at high salinity may be attributable to the increase in ABA content (Figure 11B). ABA signaling from roots to stomata is sensed by guard cells responsible for stomatal closure (Karimi et al., 2021). This phenomenon is very important to limit water losses through leaf transpiration (Niu et al., 2018). Our results showed a decrease in g_s and an increase in photosynthetic water-use efficiency (WUE = A_{net}/E), with E representing transpiration. Increases in WUE under high salinity conditions have been reported in many studies (Ashraf, 2001; Omamt et al., 2006) and they represent a normal response to moderately reduced g_s levels (Damour et al., 2010).

Photosynthetic capacity may be related to the chlorophyll content expressed on a leaf area basis, which determines the maximal rate of photosynthetic electron transport (Lawlor and Tezara, 2009). Our SPAD data do not support the view that the chlorophyll content was decreased by high salinity conditions (Figure 6A). In contrast, there was an increase in SPAD values, consistent with previous studies about the stimulating effect of high salinity on leaf chlorophyll content (Ashraf, 2004; Ashraf et al., 2008; Ekinci et al., 2012). Higher SPAD values are not attributable to a decrease in leaf water content in our trial, since the latter decreased only moderately as consequence of high salinity (Figure 6B), and therefore may rather reflect plant adaptation through modifications of cell and tissue anatomy that result in a higher chloroplast density per unit leaf area (Acosta-Motos et al., 2017; Adhikari et al., 2019). At Day 7, the higher SPAD values were correlated with higher A_{max} values in the leaves of the plants grown at salinity (Figures 6A, 7); but at day 14, A_{max} was lower, suggesting that factors other than leaf chlorophyll content were responsible of downregulation of photosynthetic capacity. The fact that A_{max} was lower at Day 14 only, suggests that high salinity-induced non stomatal limitations of photosynthesis may take some time before expressing themselves (Brugnoli and Björkman, 1992; Sarabi et al., 2019; Pan et al., 2021).

An increase in mitochondrial respiration, as the one we observed on Day 7 (Figure 9), is a common feature of adaptation to high salinity, even though there are contradictory observations (Jacoby et al., 2011). On Day 14, this increase was no more visible in our trial. Interpretation is not easy because the role of respiration in plant adaptation is a complex one, but it can at least be said that it is a negative feature when considering leaf A_{net}, the plant carbon budget and the resulting crop performance (Jacoby et al., 2011). On Day 7, we can state than higher respiration contributed to lower A_{net} in plants grown at high salinity.

The decrease in A_{net} we observed in plants grown in high salinity conditions accounts for the decreases in growth parameters (Figure 4). The latter are consistent with observations made on lettuce (Barassi et al., 2006; Ünlükara et al., 2008; Al-Maskri et al., 2010; Ekinci et al., 2012; Turhan et al., 2014; Ahmed et al., 2019), and other plant species, such as sorghum, rice and tomato (Gill et al., 2001; Minh et al., 2016; Rodríguez-Ortega et al., 2019) under high salinity conditions.

4.4 Effect of seed priming by UV-C light

The mitigating effect of UV-C seed priming on plant growth and yield in conditions of high salinity (Figures 4A–D) on Day 14 is consistent with other observations about the positive effect of UV-C seed priming. Brown et al. (2001) observed that UV-C light at 3.6 kJ m^{-2} results in a larger cabbage head diameter. A similar positive effect was observed in groundnut and mungo bean for seeds submitted to 30 minutes of UV-C radiation (Siddiqui et al., 2011). Compared to the control, mungo bean plants from Pr seeds exhibited leaves with a higher fresh mass (+100%) and leaf area (+40%). The same trend was observed in groundnut plants: +20% and +85% for the fresh mass and leaf area, respectively (Siddiqui et al., 2011).

Our observations confirm partly the ones of Ouhibi et al. (2014). For example, that study showed a sizable increase in the leaf area of plants issued from primed seeds compared to control plants at 0.85 kJ m^{-2}. This enhancement, evaluated at +50%, was very close to our observations (+48%). However, the mitigating effect of priming by UV-C light on growth of lettuce plants at high salinity was globally less pronounced in our trial than in theirs. We attribute this difference to the fact that we controlled NaCl concentration in the root zone of plants with our bubbling system, as exposed above.

The positive effect of UV-C seed priming on growth parameters of plants at high salinity can be explained by its effect on A_{net} (Figure 12), though this effect was statistically significant only at Day 14 (Figure 3A). The increase in A_{net} at Day 14 in our study was associated with an increase in ETR (Figure 3D) but not in SPAD values, gs, C_i or A_{max} (Figures 6, 3B, C, 7A), nor with a decrease in R_d (Figure 8). ETR data notably suggest that priming seeds by UV-C light improves the efficiency of light use or photochemical quenching, which both have been suggested as important tolerance mechanisms in plants submitted to high salinity (Pan et al., 2021).

The positive effect of seed priming by UV-C light on photosynthesis may be attributed to the effect of priming on SA and CKs leaf concentrations (Figures 11C, E) and the roles played by them on photosynthesis (Figure 12). There is evidence notably that SA stimulates Rubisco activity (Janda et al., 2014; Poór et al., 2019), whereas CKs increase the expression of photosynthetic genes encoding proteins of PSI, PSII, and the cytochrome b6f (Cytb6f) complex, which are involved in the photosynthetic electron transport chain (Rivero et al., 2007; Rivero et al., 2009; Rivero et al., 2010). Such a positive effect of CKs on the photosynthetic electron transport chain could translate into a positive effect on ETR. Indeed, this what was observable in plants from primed seeds grown at high salinity, at Days 7 and 14 (Figure 3D). Interestingly, priming resulted in the maintenance of the CKs/ABA ratio at Day 14 in the plants grown at high salinity (Figure 11E), which supports the idea that stress was less marked in plants from primed seeds and therefore that priming reduced the negative effect of high salinity.

The fact that leaf SA and CKs concentrations were higher in growing plants from UV-C-treated non-germinated seeds is intriguing and raises questions about UV-C light perception by seed coat and also about the mechanisms behind priming that lead to such increases. Considering that UV-C light can be at the origin of epigenetic responses (Molinier, 2017; de Oliveira et al., 2020), it would be interesting to analyze specific molecular markers, such as the ones associated with histone methylation (Müller-Xing et al., 2014).

5 Conclusion

Our observations were made under the conditions of strict control of NaCl concentration in the nutrient solution. Under such conditions, salinity stress may be considered as moderate and probably involves only osmotic stress and not toxicity due to excess Na^+ accumulation. We hypothesize that this may account for the less marked mitigating effect of seed priming by UV-C light on growth parameters we found in our trial compared to the one of Ouhibi et al. (2014). As a practical consequence, seed priming by UV-C light appears more recommendable for conditions of severe than moderate salinity stress. The dynamic of ions is very different in soil than in soilless conditions. Transpiration rates also are different in field than in controlled conditions. High transpiration may notably exacerbate the negative effect of high salinity in sandy soils with low cationic exchange capacity. We therefore recommend field trials to be conducted across a broad range of climatic and soil conditions with the objective of assessing the agronomic benefits of seed priming by UV-C light.

SA and cytokinins accumulation probably played an important role in the positive effects exerted by seed priming on photosynthesis and growth of lettuce plants grown in conditions of high salinity (Figure 12). The pivotal role played by SA in conjunction with other hormones in tolerance mechanisms against abiotic stress, as it emerges in the current literature, represents a strong incentive for investigating the mechanisms that lead SA leaf concentration to increase in lettuce plants from UV-C-treated non-germinated seeds. Besides the question of UV-C light perception by non-germinated seeds, the epigenetic hypothesis behind priming certainly deserves to be studied.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SF, conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing - original draft, writing - review and editing, and visualization. JA, conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing - original draft, writing - review & editing, visualization, supervision, project administration, and funding acquisition. FL-L, methodology, validation, writing - review and editing, and visualization. YL, methodology and data curation. FP, conceptualization, resources, and funding acquisition. LU, conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing - original draft, writing - review & editing, visualization, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Use of *Trichoderma* culture filtrates as a sustainable approach to mitigate early blight disease of tomato and their influence on plant biomarkers and antioxidants production

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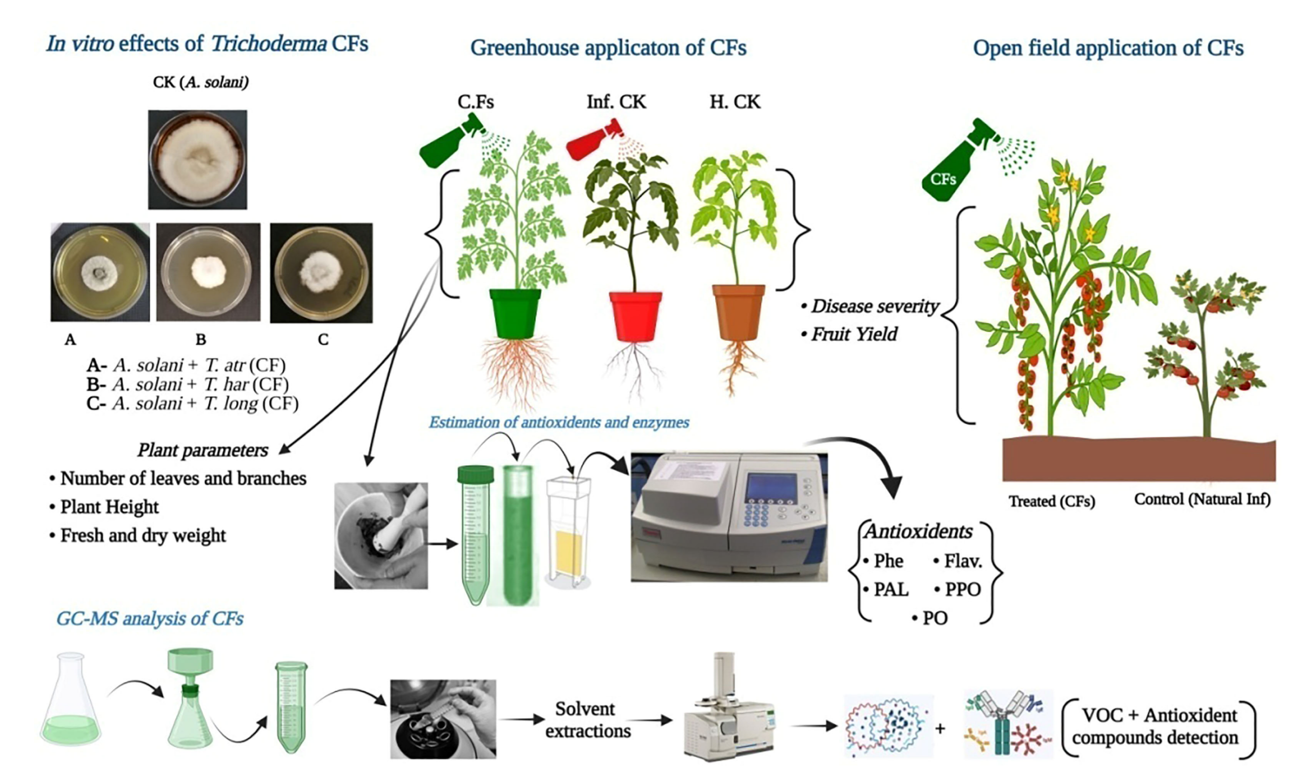
Introduction: *Alternaria solani* is a challenging pathogen in the tomato crop globally. Chemical control is a rapid approach, but emerging fungicide resistance has become a severe threat. The present study investigates the use of culture filtrates (CFs) of three species of *Trichoderma* spp. to control this disease.

Methods: Highly virulent *A. solani* strain and three *Trichoderma* fungal strains viz., *T. harzianum* (Accession No: MW590687), *T. atroviride* (Accession No: MW590689) and *T. longibrachiatum* (Accession No: MW590688) previously isolated by authors were used in this study. The efficacy of culture filtrates (CFs) to mitigate early blight disease were tested under greenhouse and field conditions, experiments were conducted in different seasons of 2020 using a tomato variety "doucen".

Results and discussion: The CFs of *T. harzianum*, *T. longibrachiatum*, and *T. atroviride* significantly inhibited the *in vitro* mycelial growth of *A. solani* (62.5%, 48.73%, and 57.82%, respectively, followed by control 100%). In the GC–MS analysis of *Trichoderma* CF volatile compounds viz., harzianic acid (61.86%) in *T. harzianum*, linoleic acid (70.02%) in *T. atroviride*, and hydroxymethylfurfural (68.08%) in the CFs of *T. longibrachiatum*, were abundantly present. Foliar application of CFs in the greenhouse considerably reduced the disease severity (%) in all treatments, viz., *T. harzianum* (18.03%), *T. longibrachiatum* (31.91%), and *T. atroviride* (23.33%), followed by infected control (86.91%), and positively affected the plant biomarkers. In the greenhouse, the plants treated with CFs demonstrated higher flavonoids after 6 days of inoculation, whereas phenolic compounds increased after 2 days. The CF-treated plants demonstrated higher antioxidant enzymes, i.e., phenylalanine ammonia-lyase (PAL) and peroxidase (POD), after 4 days, whereas polyphenol oxidase (PPO) was higher after 6 days of inoculation, followed by healthy and infected controls. In open field conditions, disease severity in CF-treated plants was reduced in both seasons as compared to naturally infected plants, whereas CF-treated plants exhibited a higher fruit yield than controls. The present results conclude that CFs can be a potential biocontrol candidate and a promising alternative to the early blight pathogen for sustainable production.

KEYWORDS

Alternaria solani, culture filtrates, antioxidants, volatile metabolites, biocontrol



GRAPHICAL ABSTRACT

1 Introduction

Tomato (*Solanum lycopersicum* L.) is a very significant crop that is widely grown worldwide, including Saudi Arabia (Imran et al., 2021), and is considered a major contributor to the fruit and vegetable diet of humans (Kapsiya et al., 2015). Tomato plants are vulnerable to various biotic factors, including viruses, nematodes, fungus, and bacteria (Imran et al., 2022a), under favorable growth conditions in this region, but the growth yield and production of tomatoes are mainly affected by fungal phytopathogens as various fungal diseases on plants have been reported during different growth stages, which leads to significant pre- and post-harvest yield losses (Gondal et al., 2012; Kolomiets et al., 2017; Tomazoni et al., 2017; González-Fernández et al., 2021; Abo-Elyousr et al., 2022). Among the fungal diseases of tomatoes, early blight disease caused by a pathogen called *Alternaria solani* is one of the most destructive diseases reported worldwide (Koley et al., 2015; Camlica and Tozlu, 2019; Mazrou et al., 2020; Zhang et al., 2020a; Imran et al., 2022b). Due to its adverse effects, this pathogen has drawn great attention over the years due to extensive yield losses in crops (Bauske et al., 2017). Various fungicides of different modes of action have been commonly used to control this disease, but in severe disease outbreaks, multiple applications of fungicides with a higher dose rate are required, which leads to the development of resistance in fungal pathogens (Pasche et al., 2004; Zhang et al., 2017; Zhang et al., 2020b) and ultimately may increase the toxicity of soil, which affects the microbiota population. The development of resistance in fungal pathogens is mainly associated with the detection of point mutations (Senthil et al., 2008; Edin et al., 2019) in the genetic material of fungal pathogens, which aids endurance (Sriwati et al., 2019; Wang W et al., 2021; Zhang et al., 2021). Except for emerging resistance, the multiple applications also pollute the environment, which has a negative impact on human health. Therefore, alternative approaches must be adopted to overcome the resistance problems in the pathogen of early blight disease in tomatoes.

Most recent studies reported the use of various endophytic microorganisms isolated from the rhizospheric zone of plants and screened for their inhibitory antagonistic potential against various fungal pathogens, including *A. solani* (Imran et al., 2022a; Imran et al., 2022b). Among the fungal bioagents, *Trichoderma harzianum*, *T. atroviride*, *T. longibrachiatum*, *T. gamsii*, and *T. asperellum* has been widely reported and used as the best biocontrol candidates for potential antagonistic activity against a variety of phytopathogens (Mazrou et al., 2020; Stracquadanio et al., 2020; Castro-Restrepo et al., 2022; Imran et al., 2022b). *Trichoderma* species as cell suspensions or culture filtrates (by acting as protective barriers) can effectively assist the plant to survive microbial competition as well as environmental stresses (Rahman et al., 2018). The results of a study by Alka et al. represent a significant *in vitro* and *in vivo* reduction of a fungal pathogen, *Rhizopus oryzae*, causing Rhizopus rot of tomatoes (Alka and Prajapati, 2017), when culture filtrates of *Trichoderma* species were applied. Additionally, the culture filtrates of various *Trichoderma* species as biofungicides against various fungal pathogens, viz., *Colletotrichum gloeosporioides* (Nurbailis et al., 2019), against anthracnose of great millet (Manzar et al., 2021), *Pythium* species, and *Phytophthora* species, demonstrated inordinate antifungal potential (Ben M'henni et al., 2022). The use of various *Trichoderma* species has widely been reported as an eco-friendly and safe approach to the control of plant diseases (Alka and Prajapati, 2017; Nurbailis et al., 2019; Manzar et al., 2022). The application of culture filtrates (CFs) of *Trichoderma* species as biocontrol not only acts as biostimulants against the inhibition of pathogens but also induces systemic or localized resistance in plants to biotic stresses and, ultimately, as a growth regulator, increases plant biomass (Abbas et al., 2019; Guzmán-Guzmán et al., 2019). The inhibition of fungal pathogens by *Trichoderma* species implicates various mechanisms, including specific metabolite and phenolic compound production, fibrolytic enzymes, various antimicrobial substances, direct parasitization, and competition for food by nutrients (Cavallo et al., 2020; Dini and Laneri, 2021; Dini et al., 2021a; Iannaccone et al., 2022). Moreover, *Trichoderma* species stimulate the production of phenolic compounds, which increase the nutraceutical value and defense system of plants (Cavallo et al., 2020; Dini and Laneri, 2021), causing the degradation of polysaccharides, chlorophenols, hydrocarbons, and xenobiotic pesticides (Zafra and Cortés-Espinosa, 2015; Dini et al., 2021b). During infection by a pathogen, *Trichoderma* species activate systemic resistance through multiple hormonal signaling pathways, which act as the primary barrier to the plant defense system (Mendoza-Mendoza et al., 2018). In the defense system of plants, the activation of defense enzymes, viz., polyphenol oxidase (PPO), peroxidase (POD), phenylalanine ammonia lyase (PAL), chitinase, β-1,3-glucanase, and various antioxidants such as catalase, flavonoids, and phenolics, has a significant role in inducing resistance (Anand et al., 2007; Almagro et al., 2009), along with microbial volatile elicitor compounds (Kashyap et al., 2022). Various studies reported a significant increase in the production of defense enzymes following the application of culture filtrates and/or cell suspensions of *Trichoderma* species to plants (Akram et al., 2021; Heflish et al., 2021; Mahmoud et al., 2021; Tripathi et al., 2021; Vukelić et al., 2021). Phenolic compounds are well known as they contain a wide group of chemicals (phenocarbonic acids, flavonoids, phenolic acids, lignans, polymeric lignans, and stilbenes) and, as plant growth regulators, modulate physiological processes such as vesicle trafficking, membrane permeability, signal transduction, and gene transcription in plants (Quideau et al., 2011; Babenko et al., 2019). Generally, phenylalanine ammonia lyase (PAL) has an imperative role in the biosynthesis of phenolic compounds as it catalyzes the non-oxidative eradication of the -NH_2 group from L-phenylalanine (Phe) to construct trans-cinnamate, which is a starting molecule for the synthesis of other phenolic compounds (Pereira et al., 2009; Jun et al., 2018). While polyphenol oxidase (PPO) catalyzes the O_2-dependent oxidation of ortho (o)-phenolics to o-quinones, which condensed the nutritive importance of protein (Constabel and Barbehenn, 2008). Peroxidase (POD) utilizes O_2 or H_2O_2 to oxidize various molecules, which are used in diagnosis and immune assays (Yoshida et al., 2003). The induction of defense enzymes, antioxidants, phenols, and flavonoids is mainly responsible for the activation of the defense system in plants, which protects them from the infection of pathogens (Schulz-Bohm et al., 2017; Zehra et al., 2017; Kaur et al., 2022). Previously, the effect of foliar application of *Trichoderma* as cell suspension on disease severity and fruit yield under greenhouse and open field conditions was studied (Imran et al., 2022a; Imran et al., 2022b). Considering the emerging fungicide resistance risks and their impact on the environment, some eco-friendly approaches are needed to adopt for the control of early blight pathogen, and to the author's knowledge, no study so far has been conducted to use the culture filtrates (CFs) of *Trichoderma* for the control of early blight disease in this region. Therefore, the aim of the present study was to investigate the effect of foliar application of *Trichoderma* culture filtrates (CFs) in greenhouses on disease severity and plant biomass; to study the effect of CFs on defense enzymes and antioxidant production in tomato plants; and to study the effect of CFs in open fields under natural infection conditions and their influence on fruit yield.

2 Materials and methods

2.1 Collection of the fungal pathogen, bioagents, and growth conditions

A highly virulent *A. solani* strain was previously isolated, screened, and identified by Imran et al. (2022b) was collected from the fungal stock culture of the Laboratory of Plant Pathology, Department of Agriculture, King Abdulaziz University, Jeddah, Saudi Arabia. The pathogenic strain was subcultured on a potato dextrose agar (PDA) medium plate at 27°C for 5–7 consecutive days, and the actively grown strain was preserved at 4°C for further use.

Three *Trichoderma* fungal strains, viz., *T. harzianum* (Accession No.: MW590687), *T. atroviride* (Accession No.: MW590689), and *T. longibrachiatum* (Accession No.: MW590688) previously isolated and identified by Imran et al. (2022a), were obtained from the fungal stock culture of the Laboratory of Plant Pathology, Department of Agriculture, King Abdulaziz University, Jeddah, Saudi Arabia. The *Trichoderma* strains were subsequently subcultured on PDA medium plates at 27°C for 3 days, and the active colony culture was preserved at 4°C.

2.2 *In vitro* assay

2.2.1 Preparation of culture filtrates and mycelial growth inhibition

Culture filtrates of *Trichoderma* strains were initially prepared by transferring 5-mm mycelial discs (3-day-old previously grown on PDA) to 50 ml sterile conical flasks containing 25 ml potato dextrose broth (PDA lacking agar). Mycelial discs of uniform diameter (8–10 discs/flask) were carefully placed (in floating positions) in PDB containing flasks that were subsequently incubated at 27°C in a shaker (125×*g*) for 7–10 days. The resulting suspension was centrifuged (10,000×*g* for 10 min at 4°C) and filtered through Whatman No. 1 filter paper (Sigma-Aldrich, USA), followed by a final filtration through a Manifolds vacuum filtration unit (MFA-1S SS316L, 100 µm; Bioevopeak, Shandong, China) for the purity of culture filtrates. The obtained supernatant (100% purity) was preserved at 4°C.

To assess *in vitro* mycelial growth inhibition, previously prepared culture filtrates (CFs) of *Trichoderma* strains were supplemented with PDA medium to evaluate their effect on *A. solani*. Briefly, PDA plates supplemented with culture filtrate (CF) suspension containing 20 ml of PDA/petri plate (1 ml of CF suspension was thoroughly mixed with 9 ml of PDA) were inoculated with a 5-mm mycelial disc (placed face-down in the middle) excised from the edge of a 5-day-old *A. solani* culture previously grown on PDA. Four replicates were used for each strain, whereas six plates were used as a replicate. The plates lacking culture filtrate suspension were used as a control. Plates were incubated at 27°C for 7–10 days until the completion of the control plate with the growth of the pathogen. Then, the colony diameter was measured on each plate and compared with the control.

2.3 Assessment and characterization of volatile compounds in fungal bioagents by gas chromatography–mass spectrometry analysis

2.3.1 Extraction of metabolites from culture filtrates

Metabolites from the culture filtrates were extracted with the method described by Stracquadanio et al. (2020). Briefly, the culture filtrates of *Trichoderma* strains were extracted three times by using ethyl acetate solvent (1:1). To dry the combined organic fraction, magnesium sulfate ($MgSO_4$) was used and evaporated at 35°C under reduced pressure. The residues having a red-brown color were recovered and dissolved in 10% methanol (CH_3OH) or dimethylsulfoxide (DMSO), and extracts were stored at −20°C to perform further analysis.

2.3.2 GC–MS analysis

The metabolites obtained from the culture filtrates of selected fungal bioagents were used to perform GC–MS analysis for the detection of active biomolecule components. The volatile compounds in fungal metabolites were identified with a single quadrupole mass spectrometer (GC–MS) detector (Stoppacher et al., 2010; Siddiquee et al., 2012). The identification of the volatile compounds in the culture filtrate of *Trichoderma* species was conducted by GC–MS analysis. This chemical identification of the compounds was determined by injecting the standard compounds into GC–MS or by comparison to library mass spectra from the NIST database. The energy for electron impact was 70eV, whereas the ion source temperature was adjusted to 250°C. The electron impact (EI) mass scan (m/s) range was 40–450 Da in fully scan acquisition mode (Hernádez-Rodríguez et al., 2008; Khan et al., 2021). The spectra of the identified compounds were compared with the available spectra of compounds in the GC–MS of the National Institute of Standards and Technology (NIST) database. More than 90% resemblance was considered a threshold for detection.

2.4 In planta assay

2.4.1 Effect of culture filtrates on disease severity

To study the efficacy of culture filtrates (CFs) to mitigate early blight disease, experiments were conducted in different seasons of 2020 under the greenhouse using the tomato variety “doucen.” Briefly, tomato seedlings were grown in 18-cm plastic pots containing peat moss (1:3), and at three- to four-leaf stage, seedlings were transplanted to the new pots containing an identical amount of growth medium. Previously prepared culture filtrates (100%) of *Trichoderma* strains were used as foliar application after twelve days of transplanting (30 ml plant^{-1}), whereas, after 24 h, the cell suspension of a virulent pathogenic strain (previously grown on PDA for 7 days adjusted with a hemocytometer) was sprayed (10^4 spores/ml; 30 ml plant^{-1}). Plants sprayed with sterile distilled water were treated as healthy controls whereas plants inoculated with the suspension of pathogens were treated as infected controls. Plants were covered with sterile polythene bags for three days to retain humidity for the initiation of infection by pathogens. The humidity (75%–80%) and optimal temperature (27 ± 1°C) inside the greenhouse were maintained. The experiment was performed with six replicates for each treatment, and six plants were subjected to each replicate. The experiment was repeated twice, and disease severity was measured with a reported 0–5 disease rating scale as follows: 0—[no infection on leaves]; 1—[0%–5% infection on leaves]; 2—[6%–20% infection on leaves]; 3—[21%–40% infection on leaves]; 4—[41%–70% infection on leaves], and 5—[>70% infection on leaves] (Gondal et al., 2012). Disease severity (%) was recorded as: disease severity = Σ (no. of infected plants × no. scale)/total no. of plants × higher no. scale × 100 (Akhtar et al., 2016).

2.4.2 Effect of culture filtrates on plant biomass

The biomass of tomato plants, viz., plant height and fresh and dry weight of roots and shoots, were measured after the determination of disease severity. Plant height was calculated inside the greenhouse within the pots, and subsequently, plants were harvested to measure the fresh weight. Then, the plants were placed in a moisture dryer chamber at 60°C for 5 days to ensure the complete drying of the moisture contents. The dry weight of the plants was calculated, and the means were compared among the treatments.

2.5 Effect of culture filtrates on secondary metabolite production

2.5.1 Preparation of leaf aliquot and estimation of secondary metabolites

In the greenhouse experiment, leaf samples from each treatment were randomly collected from randomly selected replicates at 0, 2, 4, 6, and 8 days after inoculation. Samples were immersed in liquid nitrogen to obtain the fine powder that was stored at −80°C to measure the secondary metabolites. viz., total phenol and flavonoids contents.

2.5.1.1 Total phenol contents

Total phenol contents in the samples were assayed by using the Folin–Ciocalteu reagent with the method of Meda et al. (2005) with trivial modifications. Briefly, 1 g of powdered sample was dissolved in 5 ml of methanol (80%). The aliquot was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred to new 2 ml tubes, followed by storage at −80°C before further analysis. Subsequently, 100 µl of the methanol-extracted sample was assorted with 750 µl of 1 N Folin–Ciocolteu reagent (1:10), followed by incubation for 5 min at room temperature. Thereafter, 60 µl of sodium carbonate (Na_2CO_3) (7.5%) was added, and the mixture was incubated for 30 min at room temperature. The absorbance of the mixture was recorded at 750 nm using a UV–Vis spectrophotometer. The phenolic contents in the reaction mixture were determined by using a standard curve obtained from gallic acid (0–5 mg) and expressed as mg/g plant material. Six replicates were used for each treatment.

2.5.1.2 Flavonoid contents

Flavonoid contents in the sample were determined according to the method of Chang et al. (2002) using an aluminum chloride ($AlCl_3$) reagent. Briefly, 250 µl of the methanol-extracted samples were mixed into 75 µl sodium nitrite (5%) followed by the addition of 1,250 µl distilled H_2O followed by incubation for 5 min at room temperature. Afterward, 150 µl aluminum chloride (10%), 500 µl sodium hydroxide (1M), and 275 µl distilled H_2O were added to the reaction mixture. The mixture was incubated for 5 min at room temperature, and the absorbance was recorded at 510 nm and expressed as mg/g plant material. Six replicates were used for each treatment.

2.6 Antioxidant enzyme assay

2.6.1 Protein assays

The concentration of protein in leaf extract was determined by Bradford's (1976) method using bovine serum albumin (BSA) as a standard. Protein contents were determined by a UV–Vis spectrophotometer (Thermo Fisher Scientific™) at an absorbance of 595 nm, and a standard curve was developed. The standard curve was used to determine secondary metabolites and antioxidants in leaf extract.

2.6.1.1 Preparation of crude enzyme

To prepare crude enzymes, previously ground (powder) leaf tissues were used to estimate antioxidant enzymes, viz., phenylalanine ammonia-lyase (PAL), peroxidase (POD), and polyphenol oxidase (PPO). Briefly, 500 mg of fine powder from each replicate was suspended in 2 ml of extraction buffer containing 20 ml of phenylmethyl sulfonyl fluoride (1 mM) and polyvinylpyrrolidone (0.1%). To determine PAL activity, sodium borate buffer (0.1 M, pH 8.7) was used, while for PO and PPO activity, sodium phosphate buffer (0.1 M, pH 7.0) was used. The extraction was conducted at 4°C, and samples were centrifuged at 12,000×*g* for 20 min at 4°C. The supernatant (the crude enzyme source) was transferred to a new centrifuge tube and preserved at −80°C to determine the enzymatic assay.

2.6.1.2 Phenylalanine ammonia-lyase assay

Phenylalanine ammonia-lyase (PAL) activity in treated tomato leaves was determined according to the method of Dickerson et al. (1984). Briefly, the reaction containing 200 µl of crude enzyme extract, 1,300 µl sodium borate buffer (0.1 M, pH 8.7) and 500 µl of L-phenyl alanine (12 mM) was incubated at 37°C in a water bath for 30 min. Subsequently, the PAL activity was determined at 290 nm absorbance with a UV–Vis spectrophotometer, whereas cinnamic acid (0–5 mg, nM) was used as a standard, and the PAL activity was expressed as min/g protein. Six replicates were used for each treatment.

2.6.1.3 Peroxidase assay

Peroxidase (POD) activity in treated tomato leaves was assayed using the method of Hemeda and Klein (1990). Briefly, the substrate was prepared by dissolving 5 ml of H_2O_2 (0.3%) in 5 ml of guaiacol (1%) and then adding 50 ml of sodium phosphate buffer (0.05 M, pH 6.5). The reaction mixture was prepared by suspending 1,475 µl of the substrate in 25 µl of crude enzyme extract. The change in absorption was recorded at the absorbance of 470 nm, and PO activity was calculated by the increase in absorbance caused by the oxidation of guaiacol. PO activity was expressed as µmol/min/mg of protein (E = 26.6/mM/cm).

2.6.1.4 Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was determined using the method of Kumar and Khan (1982). The reaction mixture containing 250 µl crude enzyme extract, 1,000 µl sodium phosphate buffer (0.1 M, pH 6.5), and 500 µl catechol (0.1 M) was incubated at room temperature for 10 min. Then, 500 µl of H_2SO_4 (2.5N) was added to stop the reaction. The purpurogallin-formed absorption was observed at an absorbance of 495 nm. While the initial addition of H_2SO_4 to the reaction mixture was used as a blank, PPO activity was calculated and expressed as U/min/mg protein (U = change in 0.1 absorbance/min/mg protein).

2.7 Open field trails

The efficiency of *Trichoderma* culture filtrates in two consecutive seasons [early (January–March) and late (September–December)] of 2020 was determined in open fields under natural infection conditions. The experimental site was prepared at the "Hada Al Sham" field station of King Abdulaziz University. In the early season, seedlings of the tomato variety "Doucen" were grown in a plastic seedling growing tray (50 holes) containing peat moss (1:3). At the three- to four-leaf stage, seedlings were transplanted in an agricultural field previously prepared (maintaining a 60-cm distance between rows and 45 cm within plants) (Supplementary Figure 1). Culture filtrates of fungal bioagents (previously prepared) were used as foliar applications. After one week of transplanting, the suspension of culture filtrates (100%) was applied as a foliar spray (50 ml/plant) to tomato plants. For healthy control, the plants were sprayed with sterile distilled water, whereas the untreated plants (lacking any of the treatment) were subjected to infected control. All treatments were applied in the evening, and plants were left for natural infection in an open field. Plants were irrigated properly as per requirements with a drip irrigation system, and standard agronomic practices were carried out throughout the experiment. For each treatment, including control, plants were randomly selected, and disease severity was recorded with a reported disease rating scale as previously mentioned (Gondal et al., 2012; Ismail et al., 2016). Fruit yield was recorded by harvesting the ripened fruit regularly from all replicates of all treatments, and the total yield for each treatment was calculated and compared.

The experiment was conducted in a completely randomized block design with six replicates, each containing nine plants. All recommended agronomic practices were adopted in the experimental field, and each treatment was applied randomly to plants. An experiment with identical parameters was performed in the late season. Disease severity and fruit yield in both seasons were recorded and compared with controls to observe the efficacy of culture filtrates against the natural infection of the early blight pathogen.

2.8 Statistical analysis

All *in vitro* experiments were conducted in triplicate, while field experiments were conducted in quadruplicate. Experiments were performed in a complete randomized design, and all collected data was analyzed by using Statistix 8.1 (Analytical Software, Statistix; Tallahassee, FL, USA, 1985–2003). The data from disease severity was transformed into arcsine values, and a one-way analysis of variance (ANOVA) was performed. The means of replicates in all treatments were compared using Fisher's least significant difference test at $p = 0.05$ (Steel et al., 1996).

3 Results

3.1 *In vitro* mycelial growth inhibition by culture filtrates

The *in vitro* application of *Trichoderma* culture filtrates (CFs) demonstrated significant ($p = 0.05$) mycelial growth suppression of the *A. solani* pathogen. However, the CFs of *T. harzianum* suppressed (62.5%) mycelial growth as lower (31.5 mm) mycelial growth was recorded, followed by the control (84 mm). Meanwhile, mycelial growth inhibited by CFs of *T. longibrachiatum* and *T. atroviride* (48.7% and 57.8%, respectively) was relatively lower than that of *T. harzianum* followed by the control (Figure 1A). Apparently, the *in vitro* application of all *Trichoderma* CFs significantly altered the growth pattern of the *A. solani* colony, followed by the control (Figure 1B). The results herein presented indicate that even lower volumes (1 ml of CFs to 9 ml of PDA) of CFs of these *Trichoderma* strains showed better growth inhibition of the *A. solani* pathogen.

3.2 GC–MS analysis of *Trichoderma* culture filtrates

The GC–MS chromatogram of *T. harzianum* demonstrated the presence of 10 peaks (Figure 2A) with 27 volatile compounds (Table 1A), whereas 25 volatile compounds (Table 1B) with eight peaks (Figure 2B) in *T. atroviride* and nine peaks (Figure 2C) with 32 volatile compounds (Table 1C) were detected in *T. longibrachiatum*.

In the CFs of *T. harzianum*, various groups of chemical compounds, viz., aldehydes, hydroxyls, different acids, glycerin, esters, and many hydroxyl groups containing compounds, were detected. The most abundant compound (61.86%) present in CFs of *T. harzianum* was harzianic acid ($C_{19}H_{27}NO_6$), detected at a retention time (Rt) of 12.58 (Table 1A; Figure 2A). Glycerin was also observed in a higher amount (9.98%), followed by other compounds that ranged between 5% and 8%. The other prominent constituents in the CFs of *T. harzianum* were dl-glyceraldehyde (8.21%), 2-Propanone, 1,3-dihydroxy (6.23%), 3-Deoxy-d-mannoic lactone (5.34%), 1,2,3-Propanetriol, monoacetate (5.10%), 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- (4.94%), and D-Alanine, N-propargyloxycarbonyl-,isohexyl ester (4.41%), whereas other detected compounds were in lower amounts (%).

Various groups of chemical compounds, including methyl, esters, sugar, glycerin, compounds containing an acetyl group, and compounds with a hydroxyl group, were detected in the CFs analysis of *T. atroviride*. The most significant and abundant compound (70.02%) in *T. atroviride* was 9,12-octadecadienoic

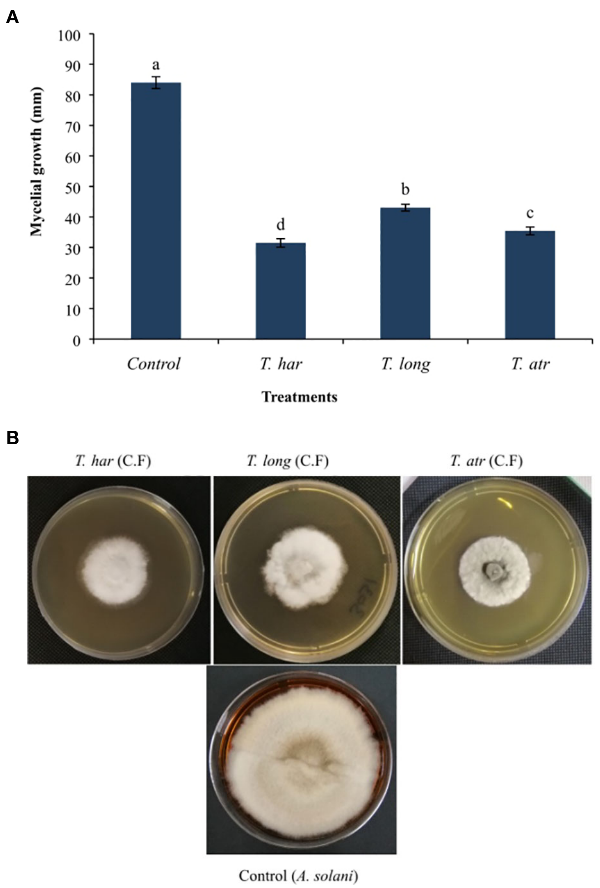


FIGURE 1
(A) *In vitro* effect of *Trichoderma* culture filtrates (CFs) on the mycelial growth of the *A. solani* pathogen. Values followed by different letters indicate that means are significantly different from each other according to Fisher's least significant difference at p = 0.05. *T. har*, *Trichoderma harzianum*; *T. long*, *Trichoderma longibrachiatum*; *T. atr*, *Trichoderma atroviride*. **(B)** The *in vitro* effect of *Trichoderma* culture filtrates on mycelial growth inhibition of the *A. solani* pathogen on PDA medium.

acid (Z,Z)—which is commonly known as "linoleic acid" at the retention time (Rt) of 12.61 (Table 1B; Figure 2B). The chemical compounds 3-deoxy-d-mannoic lactone (10.15%), 2-propanone, 1,3-dihydroxy- (8.66%), dl-glyceraldehyde (8.67%), and glycerin (7.2%) were present, whereas the other chemical compounds were present in a lower amount <5%.

The results of GC–MS analysis of *T. longibrachiatum* culture filtrates (CFs) demonstrated the presence of various groups, viz., nitrile, methyl, hydroxyl groups, monosaccharides, glycosides, alcohols, ketoses, and some unknown compounds. The compound 2-furancarboxaldehyde,5-(hydroxymethyl)—commonly known as HMF or 5-(hydroxymethyl)-2—was detected at a higher level (68.08%) at the retention time (Rt) of 12.65 (Table 1C; Figure 2C). The compounds 3-deoxy-d-mannoic lactone (18.94%) and à-D-glucopyranoside,O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl (5.78%) were present in higher amounts, whereas other compounds were also detected in subordinate amounts (<5%). In these results, the CFs of these *Trichoderma* demonstrated the presence of various chemical compounds in higher amounts, including some common hydroxyl group-containing compounds (Tables 1A–C).

3.3 *In vivo* planta assay

3.3.1 Effect of *Trichoderma* CFs on disease severity

The foliar application of *Trichoderma* CFs demonstrated favorable effects on the suppression of early blight disease under greenhouse conditions. The disease severity in *T. harzianum* CF-treated plants was relatively lower (18.03%) than in *T. longibrachiatum* (31.91%) and *T. atroviride* (23.33%)-treated plants, followed by control (86.91%) (Figure 3). These results indicate that the foliar application of *Trichoderma* CFs drastically protected the plants from the infection of early blight pathogens under greenhouse conditions, as significant protection (79.25%) was observed in *T. harzianum* CF-treated plants, which was relatively higher than *T. longibrachiatum* (63.28%) and *T. atroviride* (73.15%) treated plants. These results illustrate that the foliar application of *T. harzianum* CFs remarkably protected the plants more than *T. longibrachiatum* and *T. atroviride* under greenhouse conditions, even after the artificial inoculation of *A. solani*. These results clearly indicate that the application of *Trichoderma* CFs has a transpicuous ability to attenuate the *A. solani* infection, and even a lower volume of CFs provided better control of the early blight pathogen.

3.3.2 Effect of *Trichoderma* CFs on plant biomass

Foliar application of *Trichoderma* CFs not only alleviates the early blight infection on plants but also increases biomass, viz., the fresh and dry weight of roots and shoots. In all CF treatments, a considerable increase in the fresh and dry weight of plants was recorded. However, *T. harzianum* CFs instigated a more significant increase in fresh weight, branches, and leaves followed than other treatments (Table 2). In these results, *T. harzianum* CFs were found to be more responsive than *T. longibrachiatum* and *T. atroviride*. Thus, increased plant height allows more branches to emerge, which results in an increase in the weight of the plant and ultimately confers the plant growth promoter potential of these *Trichoderma* strains. Whereas no significant disparity between *T. longibrachiatum* and *T. atroviride* was recorded, although these treatments also demonstrated an increase in the growth parameters, followed by infected and healthy controls. In the present results, a significant reduction in the plant height, weight, branches, and number of leaves was recorded in the infected control (Table 2), which clearly demonstrates the catastrophic behavior of the early blight pathogen on plants. Results revealed that CFs of *T. harzianum* not only increase the plant height, weight, branches, and leaves of plants but also enhance the root development, which strengthens the plants to combat.

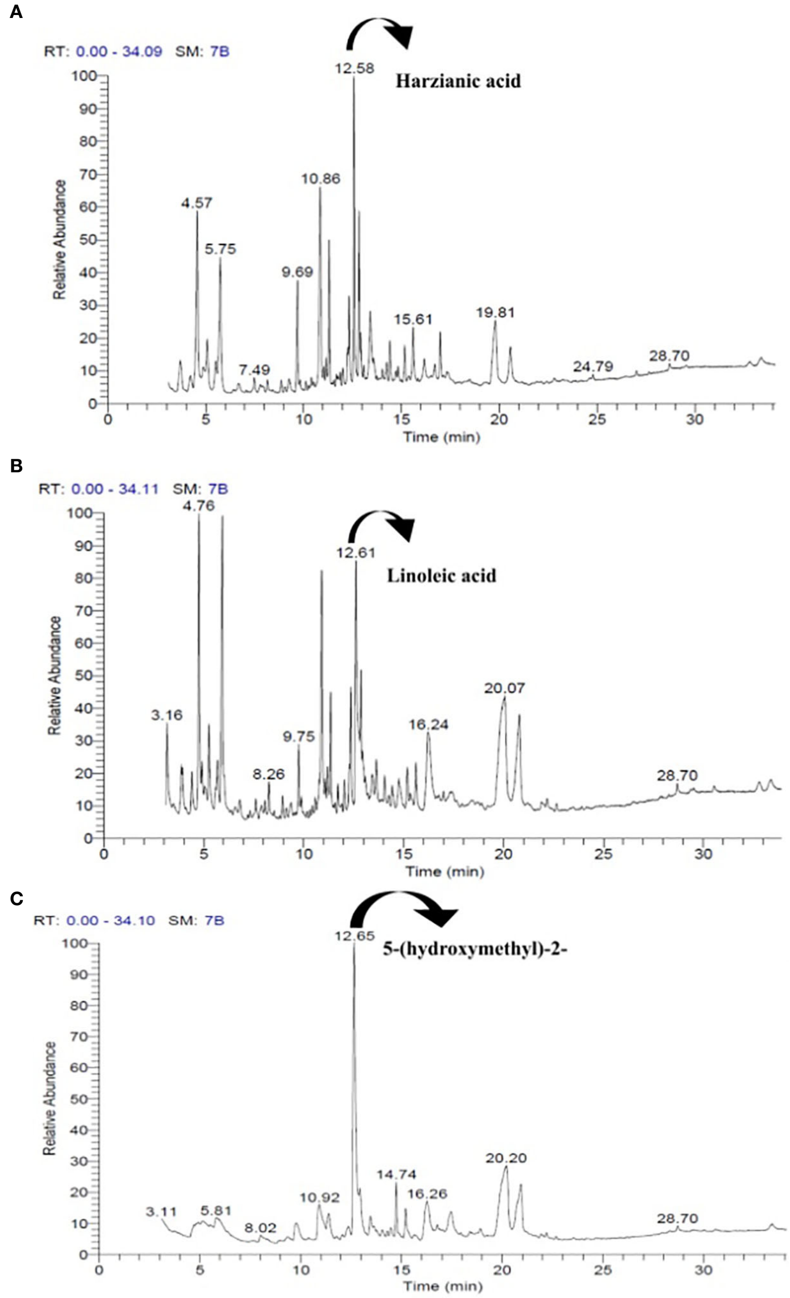


FIGURE 2
The standard GC–MS chromatograms of *T. harzianum* **(A)**, *T. atroviride* **(B)**, and *T. longibrachiatum* **(C)** strains and the peaks of volatile organic compounds (VOCs) detected from their culture filtrates.

3.4 Effect of culture filtrates on phenolic compounds

3.4.1 Total phenol contents

Initially, the total phenol contents (TPCs) in all treatments were constant and slightly increased after 2 days in *Trichoderma* CF-treated plants. However, this increase in TPC remained constant (0.77 mg g^{-1} plant material) until 6 days, which decreased to 0.41 mg g^{-1} plant material after 8 days of inoculation. In these results, the TPCs in *T. harzianum* CF-treated plants were significantly higher than those in *T. longibrachiatum* and *T. atroviride* (Figure 4A). Comparatively, the increase in TPCs in *T. harzianum* treatments (0.73–0.77 mg g^{-1} plant material) was recorded, which remained superior to other *Trichoderma* treatments. However, lower TPC in infected (0.39 mg g^{-1} plant material) and healthy control (0.35 mg g^{-1} plant material) plants were observed, which slightly increased (0.49 and 0.46 mg g^{-1} plant material, respectively) after 6 days and then declined after 8 days (0.26 mg g^{-1} plant material). These results indicate that the foliar application of *Trichoderma* CFs, particularly *T. harzianum* (in this

TABLE 1A Volatile organic compounds (VOCs) obtained from the culture filtrates of *T. harzianum* strain identified by GC–MS analysis. The bold letters indicates the compound which is abundantly present in the culture filtrates of *T. harzianum.*

Retention time (RT)	Compound name	Structure	MW	Abundance (%)
4.56	dl-Glyceraldehyde	$C_3H_6O_3$	90	8.21
4.87	Pilocarpine	$C_{11}H_{16}N_2O_2$	208	0.43
5.08	2-Furanmethanol	$C_5H_6O_2$	98	1.99
5.51	o-Acetyl-L-serine	$C_5H_9NO_4$	147	0.98
5.75	2-Propanone, 1,3-dihydroxy	$C_3H_6O_3$	90	6.23
6.69	Octadecanedioic acid	$C_{18}H_{34}O_4$	314	0.55
7.49	2-Furancarboxaldehyde, 5-methyl-	$C_6H_6O_2$	110	0.76
9.69	D-Alanine, N-propargyloxycarbonyl-,isohexyl ester	$C_{13}H_{21}NO_4$	255	4.41
10.12	9,12,15-Octadecatrienoic acid,2,3-dihydroxypropyl ester, (Z,Z,Z)-	$C_{21}H_{36}O_4$	352	0.34
10.41	5-Octadecenal	$C_{18}H_{34}O$	266	0.50
10.86	Glycerin	$C_3H_8O_3$	92	9.98
11.16	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.70
11.32	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	4.94
11.68	2-Cyclohexylpiperidine	$C_{11}H_{21}N$	167	0.41
12.33	2-Pentenoic acid, 3-methyl-, methylester	$C_7H_{12}O_2$	128	3.87
12.58	**Harzianic acid**	$\mathbf{C_{19}H_{27}NO_6}$	**365.40**	**61.86**
12.84	1,2,3-Propanetriol, monoacetate	$C_5H_{10}O_4$	134	5.10
12.92	Maltol	$C_6H_6O_3$	126	1.14
13.41	Stevioside	$C_{38}H_{60}O_{18}$	804	3.20
14.03	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	484	0.39
15.61	4-Amino-1,5-pentandioic acid	$C_7H_{13}NO_4$	175	2.28
16.99	7-Oxo-2-oxa-7-thiatricyclo[4.4.0.0(3,8)]decan-4-ol	$C_8H_{12}O_3S$	188	1.98
19.81	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	162	5.34
20.58	9-Octadecenoic acid (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	$C_{28}H_{44}O_4$	444	1.89
24.79	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.23
28.70	Hexadecanoic acid,1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	568	0.33
30.05	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.03

The compound in the bold letter was detected at the highest percentage. Mol, molecular; (n = 27).

TABLE 1B Volatile organic compounds (VOCs) obtained from the culture filtrates of *T. atroviride* strain identified by GC–MS analysis. The bold letters indicates the compound which is abundantly present in the culture filtrates of *T. atroviride*.

Retention time (RT)	Compound name	Structure	MW	Abundance (%)
3.16	2-Propanone, 1-hydroxy-	$C_3H_6O_2$	74	2.37
3.51	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.25
3.88	1-Butanol, 2-nitro-	$C_4H_9NO_3$	119	1.06
3.94	Butanedioic acid, 2,3-bis(acetyloxy)-,[R-(R*,R*)]	$C_8H_{10}O_8$	234	0.92
4.76	dl-Glyceraldehyde	$C_3H_6O_3$	90	8.67
5.06	Cyclopropanetetradecanoic acid,2-octyl-, methyl ester	$C_{26}H_{50}O_2$	394	0.48
5.26	2-Furanmethanol	$C_5H_6O_2$	98	2.35

(Continued)

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TABLE 1B Continued

Retention time (RT)	Compound name	Structure	MW	Abundance (%)
5.59	Deoxyspergualin	$C_{17}H_{37}N_7O_3$	387	0.43
5.69	o-Acetyl-L-serine	$C_5H_9NO_4$	147	1.80
5.93	2-Propanone, 1,3-dihydroxy-	$C_3H_6O_3$	90	8.66
7.90	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.51
8.26	Cyclopropanetetradecanoic acid,2-octyl-, methyl ester	$C_{26}H_{50}O_2$	394	0.93
8.80	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	484	0.07
9.75	D-Alanine, N-propargyloxycarbonyl-,isohexyl ester	$C_{13}H_{21}NO_4$	255	2.09
10.44	Dodecanoic acid, 3-hydroxy-	$C_{12}H_{24}O_3$	216	0.26
10.91	Glycerin	$C_3H_8O_3$	92	7.22
11.36	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	2.83
11.72	2-Cyclohexylpiperidine	$C_{11}H_{21}N$	167	0.92
12.36	2-Pentenoic acid, 3-methyl-, methyl ester	$C_7H_{12}O_2$	128	3.52
12.61	**9,12-Octadecadienoic acid (Z,Z)-**	$\mathbf{C_{18}H_{32}O_2}$	**280**	**70.02**
13.09	O-à-D-glucopyranosyl-(1➔3)-á-D-fructofuranosyl	$C_{18}H_{32}O_{16}$	504	0.24
16.21	d-Mannose	$C_6H_{12}O_6$	180	3.82
20.07	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	162	10.15
28.70	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.33
30.04	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.01

The compound in the bold letter was detected at the highest percentage. Mol, molecular; (n = 25).

TABLE 1C Volatile organic compounds (VOCs) obtained from the culture filtrates of *T. longibrachiatum* strain were identified by GC–MS analysis. The bold letters indicates the compound which is abundantly present in the culture filtrates of *T. longibrachiatum*.

Retention time (RT)	Compound name	Structure	MW	Abundance (%)
3.11	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.07
3.32	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.01
4.05	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.02
4.70	D-Streptamine,O-2-amino-2-deoxy-à-D-glucopyranos yl-(14)-O-[O-2,6-diamino-2,6-dideoxy -á-L-idopyranosyl-(13)-á-D-ribofurano syl-(15)]-2-deoxy-	$C_{23}H_{45}N_5O_{14}$	615	1.02
5.12	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.65
5.81	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	484	3.23
6.43	Deoxyspergualin	$C_{17}H_{37}N_7O_3$	387	0.10
7.02	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	484	0.00
7.18	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.00
7.57	Tetraacetyl-d-xylonic nitrile	$C_{14}H_{17}NO_9$	343	0.08
8.02	2-Hexadecanol	$C_{16}H_{34}O$	242	0.51
8.21	Dodecanoic acid, 3-hydroxy-	$C_{12}H_{24}O_3$	216	0.02
9.09	10-Octadecenal	$C_{18}H_{34}O$	266	0.01
9.76	4,5-Diamino-2-hydroxypyrimidine	$C_4H_6N_4O$	126	3.12
10.15	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.00

(Continued)

TABLE 1C Continued

Retention time (RT)	Compound name	Structure	MW	Abundance (%)
10.39	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.17
10. 92	DL-Arabinose	$C_5H_{10}O_5$	150	4.68
11.40	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	2.23
12.37	Hexadecane, 1,1-bis(dodecyloxy)-	$C_{40}H_{82}O_2$	594	1.25
12.65	**2-Furancarboxaldehyde,5-(hydroxymethyl)-**	$\mathbf{C_6H_6O_3}$	**126**	**68.08**
12.95	6-Acetyl-á-d-mannose	$C_8H_{14}O_7$	222	1.82
13.46	à-D-Glucopyranoside, O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl	$C_{18}H_{32}O_{16}$	504	1.55
14.74	Methyl 4-nitrohexanoate	$C_7H_{13}NO_4$	175	3.69
15.04	Dodecanoic acid, 3-hydroxy-	$C_{12}H_{24}O_3$	216	0.02
15.	64 Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.27
16.26	à-D-Glucopyranoside,O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl	$C_{18}H_{32}O_{16}$	504	5.78
17.46	Stevioside	$C_{38}H_{60}O_{18}$	804	2.85
18.40	Dodecanoic acid, 3-hydroxy-	$C_{12}H_{24}O_3$	216	0.49
20.21	3-Deoxy-d-mannoic lactone	$C_6H_{10}O_5$	162	18.94
20.94	á-D-Glucopyranose, 4-O-á-D-galactopyranosyl-	$C_{12}H_{22}O_{11}$	342	3.70
28.70	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.38
30.00	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.11

The compound in the bold letter was detected at the highest percentage. Mol, molecular; (n = 32).

study), escalated the phenolic compound production in tomato leaves, which perhaps activated the defense system of plants to combat *A. solani* infection.

3.4.2 Flavonoids contents

Initially, the flavonoids contents were lower and constant in all treatments, including control, which showed a slight increase after 2 days (Figure 4B) in *Trichoderma* CF-treated plants, but no elevation in infected or healthy control plants was recorded. A continuous increase in flavonoids up to 6 days was observed, while higher flavonoids production in *T. harzianum*, *T. atroviride*, and *T. longibrachiatum*-treated plants (0.84, 0.74, and 0.71 mg g^{-1} plant material, respectively) was recorded that gradually declined after 6 days. Comparatively, *T. harzianum* demonstrated a higher and more

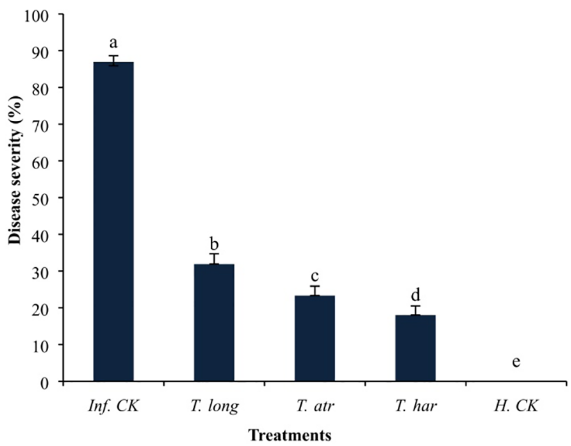

FIGURE 3
The effect of foliar application of *Trichoderma* culture filtrates (CFs) on the disease severity of *A. solani* on tomato plants in a greenhouse. Values followed by different letters indicate that means are significantly different from each other according to Fisher's least significant difference at p = 0.05. *T. har*, *Trichoderma harzianum*; *T. long*, *Trichoderma longibrachiatum*; *T. atr*, *Trichoderma atroviride*; Inf. CK, infected control (pathogen inoculation); H.CK, healthy control (distilled water inoculation).

TABLE 2 Greenhouse effect of *Trichoderma* culture filtrates (CFs) on plant biomarkers post-treatment with *A. solani*. *T. atr, Trichoderma atroviride*; *T. har, Trichoderma harzianum*; *T. long, Trichoderma longibrachiatum*; Inf. CK, infected control (pathogen); H.CK, healthy control (water).

Treatment	No. of leaves	No. of branches	Height (cm)	Shoot weight (g)		Root weight (g)	
				Fresh	Dry	Fresh	Dry
T. atr	23.91 ± 1.50b	10.83 ± 1.50a	50.35 ± 2.94b	26.75 ± 1.85b	4.67 ± 0.19b	19.91 ± 0.47b	4.40 ± 0.65b
T. har	32.08 ± 1.10a	11.16 ± 1.10a	63.85 ± 2.20a	31.65 ± 0.47a	4.61 ± 0.22a	25.16 ± 1.10a	5.51 ± 0.37a
T. long	23.08 ± 1.40b	9.38 ± 1.40a	51.42 ± 2.64b	21.57 ± 0.92c	3.75 ± 0.47b	15.98 ± 2.52c	4.36 ± 0.20b
Inf. CK	15. 0 ± 0.33c	5.15 ± 0.33 b	28.42 ± 2.73d	12.82 ± 1.37e	1.68 ± 0.21d	10.98 ± 0.61d	2.31 ± 0.15d
H. CK	17.33 ± 1.23c	7.87 ± 1.23 b	34.40 ± 1.37c	16.52 ± 0.79d	2.94 ± 0.34c	12.23 ± 0.74d	3.81 ± 0.11c

significant increase (0.84 mg g^{-1} plant material) in flavonoids after 6 days, followed by *T. atroviride* and *T. longibrachiatum* (Figure 4B). Overall, *Trichoderma* treatments remained supercilious, which evidently confers the positive response of these *Trichoderma* strains on flavonoid production in tomato plants.

3.5 Effect of culture filtrates on antioxidant enzyme

3.5.1 Phenylalanine ammonia-lyase assay

Initially, in all treated plants, the phenylalanine ammonia-lyase (PAL) activity remained lower and consistent. Subsequently, a gradual increase in PAL activity was recorded after 2 days, which reached its maximum after 4 days in *T. longibrachiatum*, *T. atroviride*, and *T. harzianum* (0.53, 0.52, and 0.47 nmol of cinnamic acid min^{-1} g^{-1} protein, respectively) treated plants (Figure 5A). Though the PAL activity in infected and healthy control plants demonstrated a considerable increase after 4 days (0.21 and 0.22 nmol of cinnamic acid min^{-1} g^{-1} protein, respectively), this increase was not significant compared to *Trichoderma* CF-treated plants. While no significant difference between *T. longibrachiatum* and *T. atroviride*-treated plants was recorded (Figure 5A). On the other hand, the results herein presented concluded that the PAL activity in *T. longibrachiatum* and *T. atroviride*-treated plants was considerably higher than that in *T. harzianum*, and these *Trichoderma* strains positively affected the PAL activity in tomato plants.

3.5.2 Peroxidase activity assay

Peroxidase (POD) activity in all treatments, including healthy and infected control plants, was relatively lower, and a slight increase in POD activity in *Trichoderma* CF-treated plants was observed after 2 days of inoculation (Figure 5B). However, the momentous increase in *Trichoderma* CF-treated plants after 4 days was recorded and remained higher in *T. longibrachiatum* (1.06 µmol min^{-1} mg^{-1} protein), followed by *T. harzianum* (0.95 µmol $min^{-1}mg^{-1}$ protein) and *T. atroviride* (0.96 µmol $min^{-1}mg^{-1}$ protein). Generally, no significant increase in POD activity in control plants was observed, though a persistent decrease was recorded. Comparatively, the POD activity in healthy and infected controls was lower than in *Trichoderma* treatments, whereas, among *Trichoderma* strains, *T. longibrachiatum* demonstrated a promising increase, which confers the positive effect of *T. longibrachiatum* on POD induction.

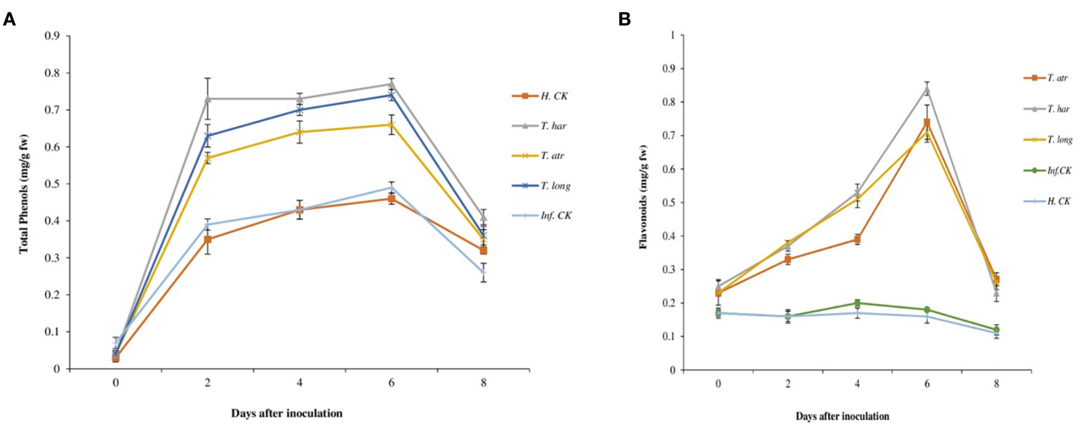

FIGURE 4
Effect of Trichoderma culture filtrates on **(A)** total phenol contents (mg gallic acid/g plant material), **(B)** flavonoids contents (mg/g plant material) in treated tomato leaves collected from greenhouse. *T. har, Trichoderma harzianum*; *T. atr, Trichoderma atroviride*; *T. long, Trichoderma longibrachiatum*; Inf. CK, infected control (plants treated with *A. solani* pathogen); H.CK, healthy control (plants treated with sterilized distilled water). Error bars on each graph represent the mean ± SE according to Fisher's least significant difference (LSD) test at p = 0.05.

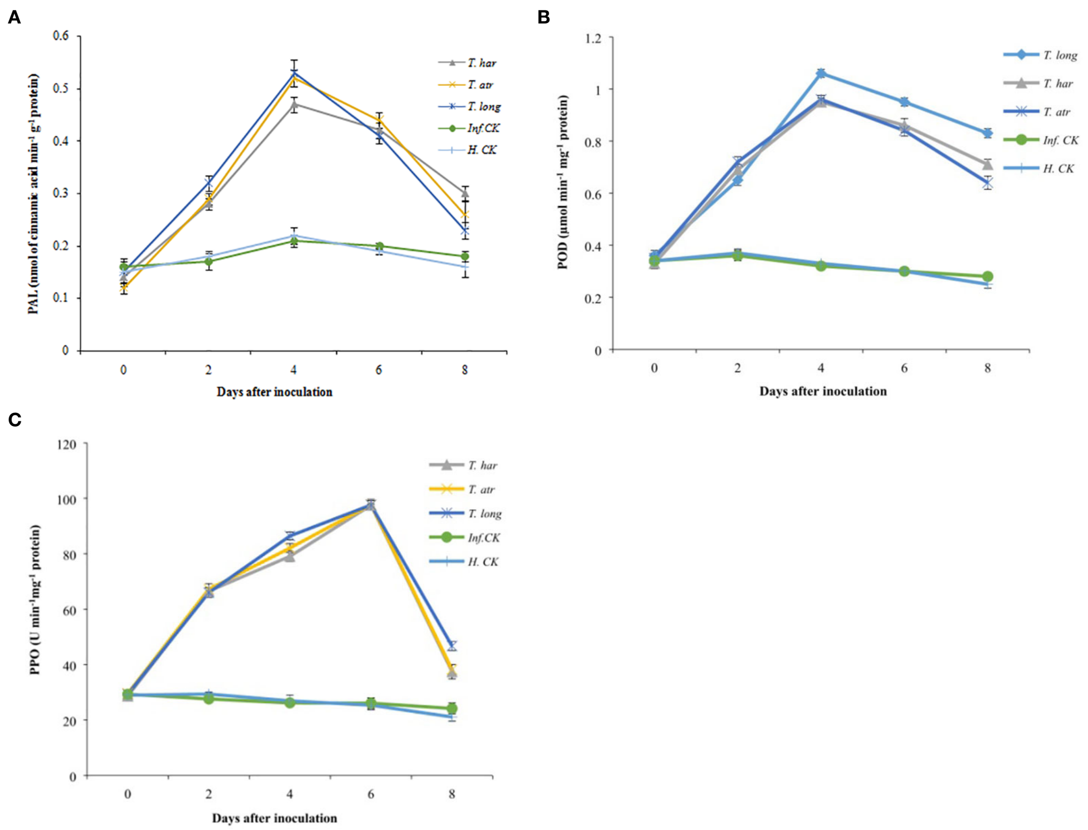

FIGURE 5
Effect of Trichoderma culture filtrates on **(A)** phenylalanine ammonia-lyase (nmol of cinnamic acid min^{-1} g^{-1} protein) activity, **(B)** peroxidase activity (µmol min^{-1} mg^{-1} protein), **(C)** polyphenol oxidase activity (U min^{-1} mg^{-1} protein) in inoculated tomato leaves collected from greenhouse. *T. har*, Trichoderma harzianum; *T. atr*, Trichoderma atroviride; *T. long*, Trichoderma longibrachiatum; *Inf. CK*, infected control (plants treated with A. solani pathogen); *H.CK*, healthy control (plants treated with sterilized distilled water). Error bars on each graph represent the mean ± SE according to Fisher's least significant difference (LSD) test at p = 0.05.

3.5.3 Polyphenol oxidase activity

Polyphenol oxidase (PPO) activity in all treatments was lower, primarily increasing after 2 days of inoculation, and no significant difference was observed among *Trichoderma* treatments as PPO activity remained uniform (66–67.24 U min^{-1} mg^{-1} protein) but higher than healthy (29.39 U min^{-1} mg^{-1} protein) and infected control (27.62 U min^{-1} mg^{-1} protein). A significant increase in PPO activity after 4 days was recorded, with a significantly higher value after 6 days (97 ± 0.76 U min^{-1} mg^{-1} protein) and a decline after 8 days (Figure 5C). PPO activity in infected and healthy control plants was relatively lower than in *Trichoderma*-treated plants. In these results, all *Trichoderma* strains positively responded to significant PPO production in plants.

3.6 Open field trails

3.6.1 Effect of culture filtrates on disease severity

The efficacy of *Trichoderma* culture filtrates (CFs) to mitigate the early blight disease under natural infection conditions was monitored, and the field application of *Trichoderma* CFs significantly reduced the early blight infection in the open field. In these results, significant reduction in disease severity (%) after the application of *T. harzianum* (SI-12.18%; SII-12.56%), followed by naturally infected control plants (SI-37.87%; SII-36.83%) in both seasons (Figure 6A). However, the application of *T. atroviride* and *T. longibrachiatum* also demonstrated vital control of early blight infection in both seasons (SI-21.17%; SII-20.75%) and (SI-16.62%; SII-19.12%), respectively. In the present study, the field application of *Trichoderma* CFs exhibited considerable protection to tomato plants even after natural infection with the early blight pathogen. Foliar application of *T. harzianum* CFs substantially protected the tomato plants from the natural infection of early blight disease in both seasons (SI-67.83%; SII-65.89%), while the application of *T. longibrachiatum* and *T. atroviride* CFs also provided significant protection. These results revealed that the applications of these *Trichoderma* CFs are capable of diminishing the early blight infection in open fields, and even lower volumes (50 ml/plant) can effectively control the early blight pathogen in open fields. Thus, it can be predicted that the increased volume of these *Trichoderma* CFs may provide exceptional management of this pathogen in fields. Additionally, multiple applications of CFs at different plant growth stages in a season may also help to reduce early blight infection. As in the present study, the application of these *Trichoderma* CFs in open fields provided a significant reduction in early blight.

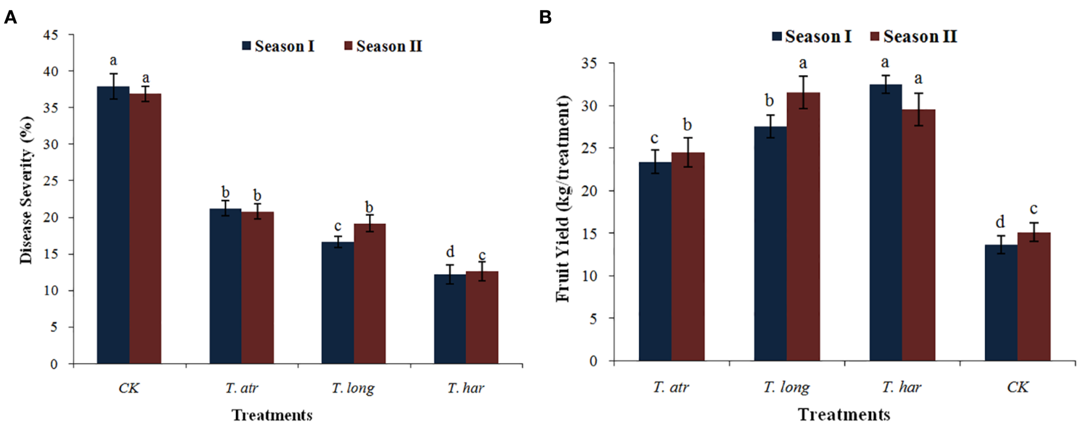

FIGURE 6
The effect of foliar application of *Trichoderma* culture filtrates (CF) on **(A)** disease severity (%) of early blight and **(B)** fruit yield in an open field under natural infection. Values followed by different letters indicate that means are significantly different from each other according to Fisher's least significant difference at p = 0.05. *T. har, Trichoderma harzianum*; *T. long, Trichoderma longibrachiatum*; *T. atr, Trichoderma atroviride*; CK, naturally infected control (untreated plants).

3.6.2 Effect of culture filtrates on fruit yield

The foliar application of *Trichoderma* CFs in field conditions not only curtailed the early blight disease severity but also improved the fruit yield in both seasons, as a significant increase in yield was observed. The fruit yield in *T. atroviride*, *T. longibrachiatum*, and *T. harzianum* was considerably higher than that in untreated/naturally infected control plants during season I, which increased in season II (Figure 6B). The *Trichoderma* treatments, viz., *T. atroviride* and *T. longibrachiatum*, exhibited lower fruit production (23.26 kg and 27.54 kg, respectively) during season I (Figure 6B), which increased in season II (24.48 kg and 31.54 kg, respectively). In the field application of *T. harzianum* CFs, higher (32.29 kg) fruit production was recorded in season I, which was slightly reduced in season II (29.58 kg). These results illustrate that the application of CFs of these *Trichoderma* strains in open fields not only minimized early blight infection but also improved yield, which symbolizes the positive effect of *Trichoderma* culture filtrates.

4 Discussion

Biological control approaches, particularly using fungal and bacterial microorganisms as biocontrol agents, are being considered as an emerging tool to combat fungicide resistance challenges. A variety of *Bacillus* and *Trichoderma* species with strong biocontrol potential, along with salt compounds and nanoparticles, have extensively been used to control plant diseases, including early blight on tomatoes (Mazrou et al., 2020; Stracquadanio et al., 2020; Castro-Restrepo et al., 2022; Imran et al., 2022b; Narware et al., 2023). Presently, the use of culture filtrates (CFs) in agriculture systems is considered an emerging method to control plant diseases and is getting a lot of attention due to its effective potential to inhibit plant pathogens. In the present study, the *in vitro* application of *Trichoderma* culture filtrates (CFs) revealed significant suppression of the mycelial growth of *A. solani*. The CFs of *T. harzianum* demonstrated strong inhibition of mycelial growth as lower mycelial growth (31.5 mm) was recorded, followed by *T. longibrachiatum* (43.06 mm) and *T. atroviride* (35.43 mm). The results herein presented illustrate the strong inhibitory potential of *Trichoderma* CFs. In a most recent study, dominant mycelial growth inhibition of *Pythium undulatum* and *Phytophthora inundata* by the CFs of *T. simmonsii* and *T. asperellum* was recorded (Ben M'henni et al., 2022). In another study, the CFs of various *Trichoderma* species, including *T. harzianum* (29.88 *mm*) and *T. viride* (27.13 *mm*), demonstrated remarkable suppression of *R. oryzae* (Alka and Prajapati, 2017). Additionally, the CFs of five *Trichoderma* species (*T. viride, Trichoderma* PP3, *T. harzianum*, *Trichoderma* PP2, and *T. koningii*) as biofungicides exhibited strong hindrance to *C. gloesporioides* growth (Nurbailis et al., 2019). Various studies have reported the significant mycelial growth inhibition of various fungal pathogens (Filizola et al., 2019; Mulatu et al., 2022), which sturdily confers the biocontrol potential of *Trichoderma* CFs, and these evidently support the results of the present study. *Trichoderma* species as biocontrol agents against fungal pathogens mainly involve various mechanisms, i.e., competition for food, space, and nutrients, resulting in the production of various metabolites that depreciate the mycelium of fungal pathogens (Alabouvette et al., 2006). There are ample pieces of evidence that report the abundant metabolite production by *Trichoderma* species, which showed significant inhibition of a broad spectrum of fungal pathogens from different taxonomic groups (Abbas et al., 2019; Edin et al., 2019; Mazrou et al., 2020; Mahmoud et al., 2021). In the present study, GC–MS analysis of *Trichoderma* species CFs demonstrated the abundant production of volatile organic compounds containing various classes of compounds. The chemical compounds and metabolites in the CFs of *Trichoderma* species identified from the known spectra in the National Institute of Standards and Technology (NIST) database exhibited the presence of harzianic acid ($C_{19}H_{27}NO_6$) in *T. harzianum*, linoleic acid ($C_{18}H_{32}O_2$) as 9,12-

octadecadienoic acid (Z,Z)—in *T. atroviride* and hydroxymethylfurfural ($C_6H_6O_3$) as 2-furancarboxaldehyde,5-(hydroxymethyl)—in *T. longibrachiatum* as the main components that were observed in relatively higher abundance (%). The abundance (%) and quantity of active compounds/metabolites in *Trichoderma* CFs can differ depending upon the method of compound extraction and the species of *Trichoderma*. Various researchers reported identical compounds during the GC–MS analysis of *T. harzianum*, *T. atroviride*, and *T. longibrachiatum* culture filtrates (Reithner et al., 2007; Anita et al., 2012; Stracquadanio et al., 2020), while dissimilarity in active metabolic compounds, viz., 6-pentyl-α-pyrone (Yassin et al., 2022), harzianic acid (Xie et al., 2021), acetic acid (Yassin et al., 2021), glacial acetic acid, and ethanoic acid (Siddiquee et al., 2012), was reported in the CFs of *T. harzianum* in GC–MS analysis. In the greenhouse application of CFs, *T. harzianum* demonstrated a promising effect for the reduction of disease severity that might be due to the excessive production of harzianic acid because harzianic acid is an important secondary metabolite from *T. harzianum* that has been widely reported to have strong antimicrobial and plant growth promoter potential against various phytopathogens such as *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Pythium irregulare* and also capable of chelating soil iron ($Fe3^+$) in plants (Vinale et al., 2013; Vukelić et al., 2021; Xie et al., 2021). During GC–MS analysis, various volatile compounds, including esters, hydrocarbons, ethers, alcohols, aldehydes, ketones, and different acids, were found. Various studies reported the presence of similar compounds with considerable yield (%) differentiation in metabolites of *Trichoderma* CFs (Anita et al., 2012; Sarsaiya et al., 2020; Sornakili et al., 2020; Stracquadanio et al., 2020; Rajani et al., 2021). In greenhouses, the foliar application of *Trichoderma* CFs demonstrated a significant reduction in disease severity (%), as lower disease severity (%) was recorded in the plants inoculated with *T. harzianum* CFs (18.03%) as compared to *T. longibrachiatum* (31.91%) and *T. atroviride* (23.33%), followed by infected control (86.91%). Besides the alleviation of disease severity, the CFs of *Trichoderma* strains also exhibited a momentous increase in the biomass of plants, viz., plant leaves and branches, and the fresh and dry weight of roots and shoots, which confers the growth promoter potential of *Trichoderma* species. However, various studies reported the optimistic influence of *Trichoderma* species on plant biomass. The application of *T. harzianum* and *T. asperellum* positively increased the plant biomass of *Diplotaxis tenuifolia* (Caruso et al., 2020) and *Mentha spicata*, respectively (Castro-Restrepo et al., 2022). In another study, the combined application of *Trichoderma simmonsii* and *Aspergillus westerdijkiae* also exhibited an increase in the growth of apple trees (Ben M'henni et al., 2022). These studies sturdily confer the plant growth promoter potential of *Trichoderma* species, which strappingly supports the findings of the present study. Thus, an increase in plant biomass and reduction in disease after the application of synthetic pathogenic infection might be associated with the stimulation of antioxidants and the production of polyphenolic secondary metabolites in plants that ultimately induce resistance against pathogen infection. Secondary metabolites such as lignin and phenolic acid significantly medicate the defense activity by strengthening the rigidity of the cell wall, which eventually prevents the invasion of pathogens into plants (El-Khallal, 2007; Abo-Elyousr et al., 2008). Various pathogenesis-related (PR) proteins are coded by the host and have a significant role in the defense system of plants induced by the infection of various pathogens as well as abiotic stress (Jiang et al., 2015). In the present study, a significant increase in total phenol (TP) contents, flavonoids, phenylalanine ammonia lyase (PAL), peroxidases (POD), and polyphenol oxidase (PPO) in *Trichoderma*-treated plants was recorded that was relatively higher than untreated/control plants. However, a significant increase in TP contents was recorded after two days of inoculation, while flavonoids contents increased after 6 days of inoculation in *Trichoderma*-treated plants followed by healthy and infected controls. Flavonoids play a significant role in plants and act as detoxifying agents, phytoalexins, signal molecules, and allelochemical agents (Fawe et al., 1998; McNally et al., 2003; Peer and Murphy, 2006). The increase in TP and flavonoids in plants demonstrates that these *Trichoderma* species robustly induce systemic resistance by releasing not only proteins but also secondary metabolites. The results reported by EL-Tanany et al. (2018) and Mayo-Prieto et al. (2019) showed a considerable increase in flavonoids and total phenolic contents when the tomato plants were inoculated with *T. viride* or *T. hamatum* after the inoculation with *A. solani* and *T. velutinum* after the inoculation with *R. solani*, respectively, and these documented findings are in support of our results and strongly underpin these findings. In another study, the highest increase in enzyme activity was recorded when the tomato plants were inoculated with *T. harzianum* and *R. solani* (Youssef et al., 2016). In accordance with Mahmoud et al. (2021), tomato plants treated with *A. cerealis* and *T. harzianum* extensively increase the total phenol and flavonoid contents, and our results are in agreement with the previously reported findings and substantiate that *Trichoderma* species accumulate PR-proteins and phenolic compounds, which hinder the invasion of the *A. solani* pathogen in plants. Furthermore, tomato leaves contain solavetivone, flavonoids, lubimin, phytuberol, phytuberin, glutinosone, and rishitin, which are toxic and antimicrobial agents that act as rebellious compounds against various phytopathogens (Kim et al., 2019).

Phenylalanine ammonia-lyase (PAL), peroxidases (POD), and polyphenol oxidase (PPO) are the major enzymes for the induction of resistance in plants against biotic and abiotic stresses. Phenolic compounds are endogenous growth regulators, whereas PAL is a key enzyme for the biosynthesis of phenolic compounds from the shikimic acid pathway, which may amend the defense response by regulating the biosynthesis of phenolic compounds after different stresses (Caretto et al., 2015; Abo-Elyousr et al., 2020). The activation of PAL is mainly associated with distinct signal transduction pathways with respect to apoptotic cell death and oxidative burst (Sasabe et al., 2000), and the induction of PAL by pathogen-derived elicitors can provide significant insights into signal transduction mechanism that activates the plant response. Further, the stimulation of PAL may also enable a quantitative appraisal of both plant responses and the effectiveness of protein. Generally, tomato plants, not only fruits but roots, stems, and leaves, are also considered rich sources of antioxidants and phenolic compounds, and in this study, a significant increase in PAL contents in tomato leaves was recorded after 4 days of inoculation followed by healthy and infected control plants. A study by Kumar et al. documented that the application of *T. harzianum* and *T. viride* increased the induction of defense enzymes, including total phenol and PAL, after 48 h of inoculation against early blight infection (Kumar et al., 2022). Various studies reported a significant increase in PAL activity in tomato plants after inoculation with the suspension of *Trichoderma* species (Kashyap et al., 2020; Tripathi et al., 2021; Kumar et al., 2022), which confers the positive impact of *Trichoderma* species on plants, and these results strongly support our findings. Peroxidases (POD) widely contribute to physiological processes such as the formation of suberin and lignin phytoalexin synthesis, cross-linking of cell wall components, and/or also participate in reactive nitrogen species (RNS) and reactive oxygen species (ROS) metabolism, which activate the hypersensitive response by limiting the expansion of pathogens (Peer and Murphy, 2006). While PPO implicates the lignification of plant cells and the oxidation of polyphenols into quinines (antimicrobial compounds) during the infection of pathogens. In this study, a significant increase in POD and PPO activity after the application of *Trichoderma* CFs was recorded; thus, the increase in PPO and POD activity that restricted the pathogen growth may be due to the oxidation of phenolic compounds to quinone, which increases the antimicrobial activity and ultimately strengthens the defense system of the plant. Various researchers documented the identical findings, which showed an astronomical increase in phenolic contents in response to various treatments (Pawel et al., 2020), which affirm that the increase in phenolic compounds activates the defense system of plants against the fungal infection. It is to be believed that phytotoxic chemical compounds engender the overproduction of ROS in plants and ultimately elevate the level of phenolic compounds, which may act as sufficient antioxidants to prevent the formation of cellular deterioration due to oxidative stress. Thus, the higher activity of PPO, POD, and PAL increased the oxidation of phenolic compounds, which led to distortion of cell wall structure and restricted pathogen growth.

In the present study, the open field trials under natural infection of early blight revealed that the CFs of these *Trichoderma* strains not only mitigated the infection of the pathogen but also escalated the yield production, which epitomizes the positive effect of CFs and their capability to promote fruit production in open field conditions even during natural infection. A study by Soesanto et al. (2022) documented that the culture filtrates of *T. harzianum* not only improved the growth and yield components of tomato plants but also increased the phenolic compounds, number of fruits, and total yield under *in vivo* conditions (Soesanto et al., 2022). In another study, a significant increase in yield and growth-related parameters of *Celosia cristata* was recorded after the inoculation of *Trichoderma* strains (Wang et al., 2021). Similar findings were also reported by Alka and Prajapati (2017); Vukelić et al. (2021), and Sriwati et al. (2019) when the culture filtrates (CFs) of different *Trichoderma* strains were used against various fungal plant diseases of tomato, and these results are in agreement with the findings of the present study, which positively support these results. In this study, the beneficial effect of *Trichoderma* species was confirmed, and a safe approach using culture filtrates (CFs) to diminish the early blight disease in open fields in this region was deployed.

5 Conclusion

These results of the present study suggested that the foliar application of *Trichoderma* culture filtrates (CFs) considerably reduced the mycelia growth of *A. solani* under *in vitro* conditions, which confers the strong inhibitory potential of CFs against fungal pathogens. Moreover, the CFs of these *Trichoderma* strains not only decreased the early blight infection in greenhouses, but also improved plant growth-related parameters and, in field conditions, also promoted fruit production. The most important aspect of CFs is the induction of antioxidants in plants, which act as the first barrier against pathogen infection. Significantly increased antioxidant production firmly restricts the further invasion of pathogens. Therefore, an appropriate number of CFs at different growth stages of tomato plants may ameliorate plant vigor, disease severity, and fruit yield by acting as plant growth promoters. We believe that it would be of great interest to carry out identical practices in other regions to minimize the fungicide resistance risk of early blight pathogens, which are becoming more serious concerns in sustainable agricultural production.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

MI: Conceptualization, experimentation, methodology, formal analysis, and writing—original draft. KA-E: Supervision and review and editing. MM: Supervision, conceptualization, formal analysis, and review and editing. MS: Supervision, revision, and formal analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1192818/full#supplementary-material

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Drought stress in rice: morpho-physiological and molecular responses and marker-assisted breeding

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Rice (*Oryza Sativa* L.) is an essential constituent of the global food chain. Drought stress significantly diminished its productivity and threatened global food security. This review concisely discussed how drought stress negatively influenced the rice's optimal growth cycle and altered its morpho-physiological, biochemical, and molecular responses. To withstand adverse drought conditions, plants activate their inherent drought resistance mechanism (escape, avoidance, tolerance, and recovery). Drought acclimation response is characterized by many notable responses, including redox homeostasis, osmotic modifications, balanced water relations, and restored metabolic activity. Drought tolerance is a complicated phenomenon, and conventional breeding strategies have only shown limited success. The application of molecular markers is a pragmatic technique to accelerate the ongoing breeding process, known as marker-assisted breeding. This review study compiled information about quantitative trait loci (QTLs) and genes associated with agronomic yield-related traits (grain size, grain yield, harvest index, etc.) under drought stress. It emphasized the significance of modern breeding techniques and marker-assisted selection (MAS) tools for introgressing the known QTLs/genes into elite rice lines to develop drought-tolerant rice varieties. Hence, this study will provide a solid foundation for understanding the complex phenomenon of drought stress and its utilization in future crop development programs. Though modern genetic markers are expensive, future crop development programs combined with conventional and MAS tools will help the breeders produce high-yielding and drought-tolerant rice varieties.

KEYWORDS

rice, drought, morpho-physiological responses, molecular responses, marker-assisted breeding, QTLs/genes

1 Introduction

Rice (*Oryza sativa* L.) is an important cereal crop consumed as a staple diet by half of the world's population. Though it is grown globally, more than 50% of rice production was contributed by Asian countries (FAO, 2015; Donde et al., 2019a). It belongs to the Poaceae family, genus *Oryza*, containing 24 species, among which 22 are wild and 2 cultivated species (Gouda et al., 2020). The *O. glaberrima* and *O. sativa* were two well-known cultivated rice species, and their germplasm mainly originated from Asia, Europe, US, and African countries (Morishima, 1984). The *O. sativa* is the most widely grown rice species based on geographical adaptability. It is subdivided into *japonica*, *indica*, and *javanica* cultivars (Morishima, 1984). The *indica* and *japonica* varieties are primarily grown in tropical, sub-tropical, and temperate regions, while the *javanica* is a rare variety grown in hot and humid climates.

Rapidly changing climatic patterns have affected normal agricultural productivity and threatened global food security (Food and Agricultural Organization, 2020). The increasing human population has increased food consumption; increasing food demands required grain yield enhancements in cereal crops (rice, wheat, barley, maize) (Gouda et al., 2020). It is the need of the hour to develop rice cultivars compatible with climatic variations and sustain grain yield while curtailing the negative impacts of abiotic stress. Among all the abiotic factors, drought is the most detrimental, limiting almost 50% of rice productivity yearly (Bouman et al., 2005; Nelson et al., 2014; Pandey and Shukla, 2015). Drought is a meteorological phenomenon indicating insufficient rainfall or higher evaporation rates that cause water deficit conditions (Rollins et al., 2013; Upadhyaya and Panda, 2019). It is estimated that about one-third of the world's total cropland suffers from drought stress; its intensity and severity are hard to predict as it depends on multiple factors such as rainfall frequency, evaporation rate, and soil moisture content (Rijsberman, 2006; Hao et al., 2018; Oladosu et al., 2019). Water availability below the optimum requirement compromised the actual yield potential of cultivars (Blum, 2011) by inhibiting active root growth and nutrient uptake (Todaka et al., 2017). Rice under drought stress exhibits poor growth and development due to inadequate morpho-physiological, biochemical, and molecular responses (Nahar et al., 2016; Kadam et al., 2017; Quinones et al., 2017). Breeding drought-tolerant varieties are a more sustainable and viable option for improving the rice's capacity to withstand drought conditions and maintain its productivity potential (Pandey and Shukla, 2015).

Rice productivity is generally predicted by its agronomic attributes, such as the number of productive tillers, number of grains per panicle, 1000-grain weight, plant height, panicle length, grain size, and weight (Hua et al., 2002). These agronomic attributes are inherited and regulated through multiple genetic expressions. Most of the modern cultivar's production potential remains stagnant because of the inability to cope with abiotic stress factors, i.e., drought, submergence, and salinity (Sabina et al., 2010). Plant breeders must develop genetically improved crop varieties that withstand biotic and abiotic stress conditions (Donde et al., 2019b; Gupta et al., 2019). Traditional breeding techniques are time-consuming and dependent on varying environmental conditions; breeding a new cultivar usually takes 8 to 12 years, and still, there are doubts about its authenticity (Sabina et al., 2010). Therefore, the breeder community is keenly interested in adopting modern breeding techniques and practices that simplify and speed up the breeding process. Pre-breeding techniques are employed for genetic mapping via the identification of genes/quantitative trait loci(s) (QTLs) that correspond to the phenotypic variation, after which these QTLs are introgressed into elite gene pools through marker-assisted selection (MAS). Modern technologies will speed up the varietal development procedure and generate diversified germplasm for future research. This article focused on the drought-induced implications and their mitigation by using MAS.

2 Responses to drought stress in rice

Rice productivity is extremely threatened under drought conditions. Drought-induced morpho-physiological damage and biochemical dysfunction are evident in rice plants, which curbs active plant growth and development. It is reported that drought stress affects rice yield by up to 90% depending on the intensity, duration, and crop growth stage (vegetative or reproductive) (Basnayake et al., 2006; Venuprasad et al., 2007). Crop plants tend to avoid, escape, tolerate, and recover from drought-induced implications; this phenomenon is collectively called drought resistance (Yue et al., 2006; Luo, 2010; Shanmugavadivel et al., 2019). We've provided a brief definition of these terms (drought avoidance, drought escape, drought tolerance, and drought recovery) here, even though they are interchangeably used in the context of drought resistance. Drought avoidance is characterized as the ability of plants to sustain high water potential and continue optimal plant growth under moisture-stress conditions (Kumar et al., 2017). Drought escape is the early completion of the plant growth cycle before the onset of local moisture deficit conditions (Manavalan et al., 2009). Drought tolerance refers to a plant's innate capacity to survive in water-deficit conditions by sustaining physiological and biochemical activities with minimal plant damage (Luo, 2010). And the ability of a plant to restore its metabolic activity and regain its vigour after being exposed to extremely high levels of drought stress and dehydration is known as drought recovery (Luo, 2010). This section will briefly discuss how rice plants perceive drought stress and how their morpho-physiological and biochemical adjustments benefit survival mechanisms and maintain productivity (Table 1).

2.1 Morphological and yield-associated responses

Unlike other cereals, rice is a water-loving plant susceptible to drought stress (Panda et al., 2021). According to Fukai and Cooper (1995), roots, shoots, and leaves' responses to drought vary depending on the plant growth stage (early seedling, vegetative, or

TABLE 1 Different drought resistance responses in rice under varying conditions of water-deficit stress.

Drought Responses	Escape	Avoidance	Tolerance	Recovery	References
Morphological	Quick growth Early flowering Early maturity Plasticity ↑	Leaf rolling & Glaucousness ↑ Deep rooting Limited vegetative growth Small sized leaves Waxy layer on leaf surfaces	Staying green for a longer duration Elasticity ↑ Primary & secondary root growth ↑	Temporary wilting of leaves Growth of new young leaves	(Srivalli et al., 2003; Basnayake et al., 2006; Yue et al., 2006; Luo, 2010; Du et al., 2011; Bennett et al., 2012; Rana et al., 2012; Upadhyaya et al., 2012; Devi and Kar, 2013; Das and Roychoudhury, 2014; Hu and Xiong, 2014; Kumar et al., 2017; Mangrauthia et al., 2017; Upadhyaya and Panda, 2019; Panda et al., 2021)
Physiological	Photosynthesis ↑ Respiration ↑	Stomatal closure Transpiration rate ↓ Water use efficiency ↑ Avoiding dehydration Water storage in plant organs ↑	Osmo-protectant accumulation ↑ Protoplasmic Resistance ↑ Stomatal conductance ↑ Sustaining photosynthetic activity Dehydrant enzymatic activity ↑ Hydraulic conductivity ↑ ROS scavenging	Temporary loss of turgidity ROS detoxification ↑ Antioxidant activity ↑ Water conductance resumed	
Biochemical	Leaf N level ↑		Osmotic adjustments Accumulation of soluble sugars ↑ Proline content ↑	Utilization of stored carbohydrates ↑	

Here "↑" indicates increasing rate, while "↓" shows decreasing rate.

reproductive), the intensity of drought stress (mild to severe), and other environmental conditions. Various morphological parameters have been used to monitor plant responses to drought stress (Zaher-Ara et al., 2016; Upadhyaya and Panda, 2019). Drought stress alters the anatomy and ultrastructure of the leaf (Upadhyaya and Panda, 2019). Drought-induced low-water potential limits leaf growth (Zhu et al., 2020); additionally, reduced leaf area, leaf rolling, wilting, thickened leaf size, early senescence, stomatal closure, and cutinized layer on the leaf surface are some of the morphological traits associated with drought stress (Mishra and Panda, 2017; Hussain et al., 2018; Panda et al., 2021).

Root system efficiency is vital in combating drought stress conditions (Panda et al., 2021). Comas et al. (2013) stated that root mass (dry) and length are used to forecast rice production under water stress. Rice cultivars with a deep and prolific root system perform better in drought conditions (Mishra et al., 2019; Kim et al., 2020). Extreme drought conditions limit secondary root growth, thicken primary root structures, disrupt water relations, and result in poor nutrient uptake, leading to poor or stunted plant growth (Hussain et al., 2018; Panda et al., 2021).

Rice has three sensitive growth stages concerning drought stress: early seedling, vegetative, and anthesis (reproductive) (Singh et al., 2012). Water scarcity in the early seedling stage reduces drought stress, leading to unbalanced and poor stand establishment (Vibhuti et al., 2015). Drought stress interrupted active seed germination, causing osmotic imbalance, membrane impairment, decreased respiration, and ATP production (Kadam et al., 2017). Water constraint during the vegetative period causes delayed panicle initiation, followed by late maturity (Lilley and Fukai, 1994; Singh et al., 2012), directly correlated with yield decline. The most damaging impact of drought stress on grain yield appears to be during the reproductive growth stage. However, plants tend to recover during the vegetative growth phase, but recovery from the drought stress during the flowering phase is more complicated (Pantuwan et al., 2002; Alam Khan, 2012; Xangsayasane et al., 2014). A short span of drought stress during the reproductive growth phase severely curbs the rice grain yield by diminishing panicle length, poor seed setting, reduced number of kernels per panicle, and poor spikelet development (Figures 1–3) (Sikuku, 2012; Wei et al., 2017). It has been reported that drought stress during flowering has a detrimental impact on pollination, resulting in poor seed setting and reduced grain size and grain number; in severe drought cases, flowers abortion takes place, leading to a 100% yield decline (Hsiao et al., 1976; International Rice Research Institute, 1995; Kumar et al., 2006; Davatgar et al., 2009). It is therefore established that any intensity of drought stress (mild or severe) during the reproductive growth phase lowers the final grain production; this is because the translocation of assimilates from leaves to reproductive organs (panicle, kernel) is interrupted (Rahman et al., 2002). Additionally, rice cultivars that recovered from temporary drought patches exhibited better yield responses than drought-sensitive cultivars (Singh et al., 2012).

2.2 Physiological and biochemical responses

Drought stress disrupts the normal physiological functioning of rice plants, followed by restricted growth and reduced productivity (Upadhyaya and Panda, 2019). Drought-induced malfunctioning of vital physiological processes includes diminished photosynthetic activity, decreased water use efficiency (WUE), low transpiration rate, poor stomatal conductance, reduced CO_2 concentration, imbalanced water relations and membrane impairment (Figure 4) (Dash et al., 2018; Zhu et al., 2020; Panda et al., 2021).

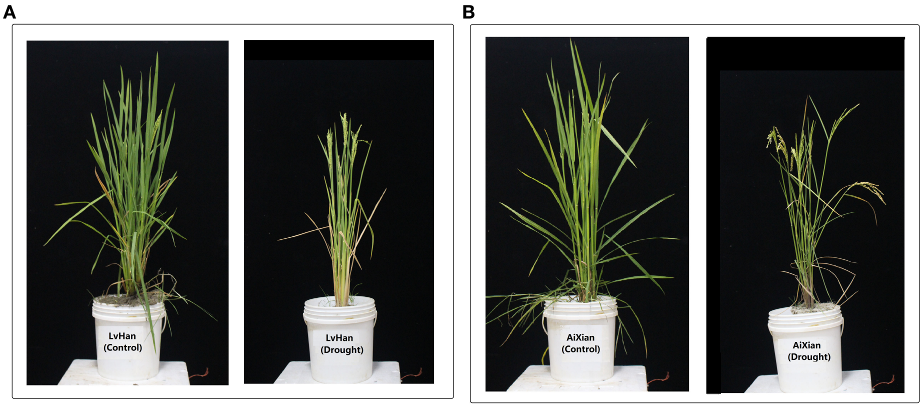

FIGURE 1
The field experiment exhibited the effects of drought stress on two rice cultivars, LvHan and AiXian (LvHan: drought tolerant and AiXian: drought sensitive), which are evident when compared to the control treatments. **(A)** LvHan (Drought) is subjected to drought stress at the early heading stage, and LvHan (Control) undergoes well-watered conditions throughout the growth cycle. **(B)** AiXian (Drought) is subjected to drought stress at the early heading stage, and AiXian (Control) undergoes well-watered conditions throughout the growth cycle. The experiment was grown in field conditions; drought stress was imposed at the heading stage. For drought treatments, irrigation stopped till leaves started wilting, and sprinkler irrigation resumed till harvesting maturity. Photos were taken after the flowering stage. (Unpublished: Own Experiment).

2.2.1 Water-relations

Plant water relations are attributed through various terminologies such as leaf water potential, relative water content (RWC), turgor pressure, WUE, and membrane stability index. The RWC and WUE are crucial metrics for determining rice's yield potential and performance in drought conditions (Farooq et al., 2009; Panda et al., 2021). Plant RWC is negatively impacted by moisture stress conditions, followed by osmotic imbalance, water exclusion, lipid peroxidation, membrane damage, and necrosis (Rao and Chaitanya, 2016). Drought-tolerant rice varieties can maintain adequate RWC and prevent membrane impairment. In a research investigation, Choudhary et al. (2009) exposed 28 days rice seedlings to water-scarce conditions for 72 hours. The results exhibited a uniform increase in RWC through osmotic adjustments (increased proline synthesis) and prevented membrane damage. The ability of crop plants to maintain membrane stability under drought-stress conditions is a vital feature of their tolerance mechanism (Pandey and Shukla, 2015). The Membrane stability index has been studied for its correlation with rice yield under drought conditions (Upadhyaya and Panda, 2019).

2.2.2 Photosynthesis

Photosynthesis (PS) is a vital physiological process that largely accounts for dry matter production in crop plants. Water-scarce conditions negatively influenced the status of RWC in plants; as water-saving tactics, stomatal closure occurs, reducing CO_2 influx, decreased transpiration rate, poor gaseous exchange, and electron transport. Drought stress lowers the efficiency of photosystems I & II (PSI and PSII), impairs rubisco activity and inhibits electron transport chain and ATP production (Farooq et al., 2009; Mishra

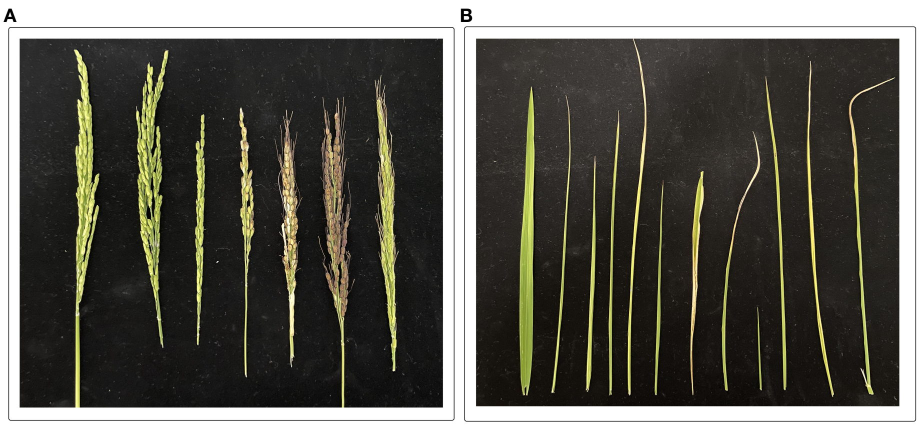

FIGURE 2
It is evident that the effects of drought stress on **(A)** panicles (starting from left side: two panicles are control treatments) and **(B)** flag leaves (starting from left: first leaf is control treatments) in various rice lines. The experiment was grown under field conditions; drought stress was imposed at the heading stage. For drought treatments, irrigation stopped till leaves started wilting, and sprinkler irrigation resumed till harvesting maturity. Photos were taken after the flowering stage. (Unpublished: Own Experiment).

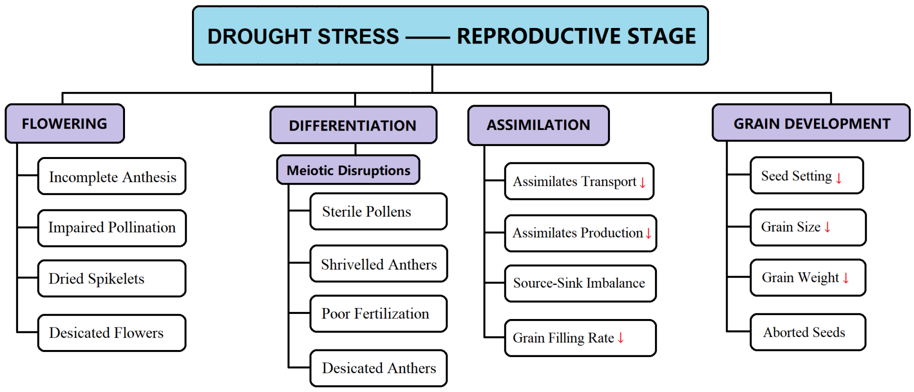

FIGURE 3
Drought-induced reproductive abnormalities are briefly illustrated for specific growth stages (e.g., flowering, differentiation, assimilate transport, grain filling, and so on). These disruptions in normal growth substantially decline the final paddy yield. [Here "↓" indicates decreasing rate].

and Panda, 2017; Upadhyaya and Panda, 2019). Photosynthetic pigments (i.e., chlorophyll, phycobilin, and carotenoids) showed lower efficiency under water-deficient conditions, resulting in inadequate light absorption, reduced light harvesting, and poor quality photoprotection (Jahan et al., 2013). Eventually causes limited photosynthesis and restricts photosynthates' production (Fahad et al., 2017; Gupta et al., 2020). Carotenoids also serve as a precursor for plant signaling under stress conditions, so a decrease in carotenoid contents has a negative impact on signal perception during drought stress (Ashraf and Harris, 2013; Panda et al., 2021).

2.2.3 Nitrogen metabolism

Nitrogen (N) metabolism is handy in combating water-deficient stress conditions, particularly in plants' tolerance response (Suresh

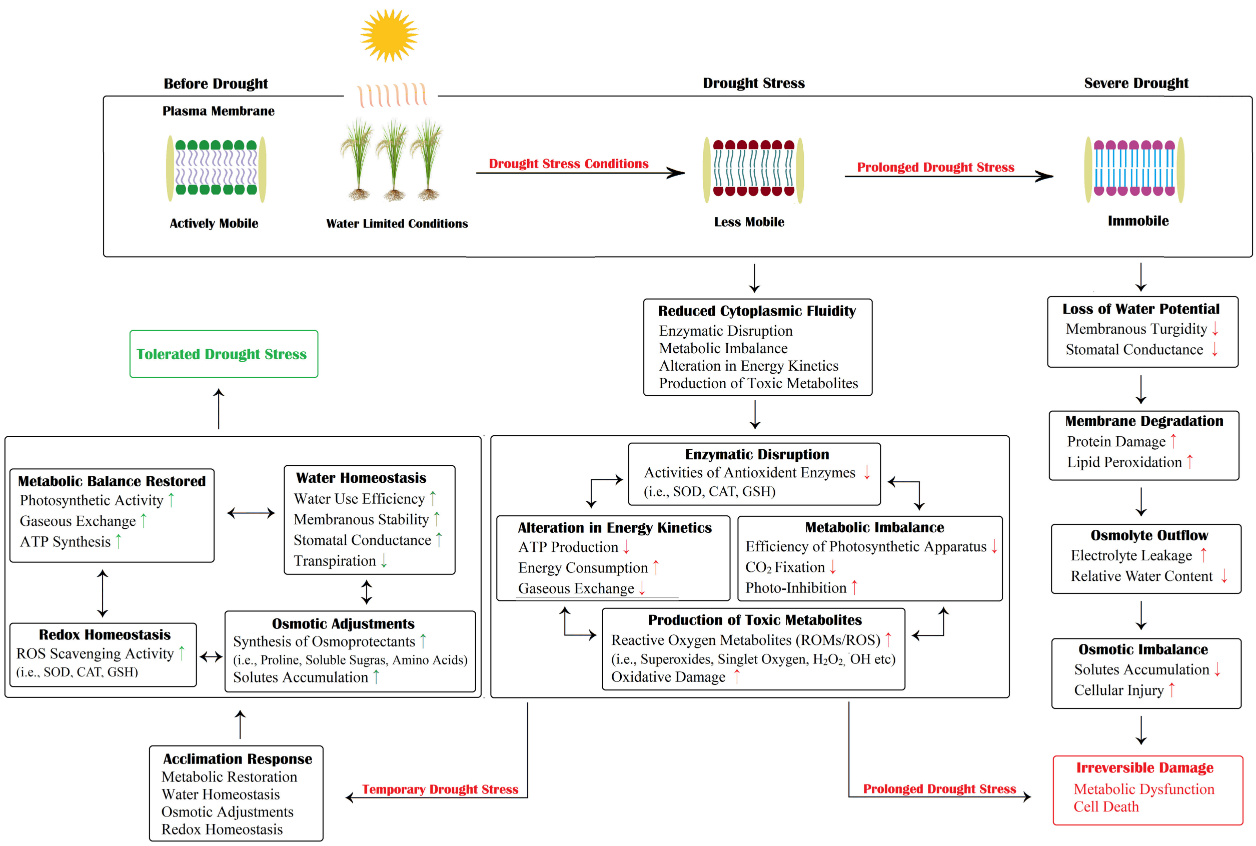

FIGURE 4
Schematic illustration of drought-induced damage and acclimation responses. The plant cell undergoes a number of osmotic, enzymatic, and metabolic changes. However, a severe and prolonged drought may cause irreparable damage to plant cells, eventually resulting in cell death. In contrast, short-duration drought stress can be tolerated. The plant resumes its normal growth and functioning. Here, "↑" and "↓" indicates a positive increase/decrease in a certain activity while "↑" and "↓" indicates a negative increase/decrease in a certain activity.

et al., 2015). Plants tend to regulate nitrogen metabolism under drought stress conditions by decreasing nitrate reductase activity (Xu et al., 2015). Glutamate dehydrogenase (GD), a stress-responsive enzyme, is vital in N metabolism and highly effective in detoxifying intracellular ammonia, synthesizing proline, and producing glutamate and soluble sugars (Zhou et al., 2015). Zhong et al. (2017) conducted an experiment in which rice plants were exposed to water deficit conditions with treatments of varying N levels. According to this study, high N levels increase rice's ability to adapt to water stress by reducing stomatal restrictions on photosynthesis, maintaining higher Rubisco activity, and enhancing the assimilation of nitrate and ammonia. Another experimental study conducted by Cao et al. (2018) confirmed that nitrogen fertilization in rice improved the rice's ability to withstand drought conditions by influencing photosynthetic activity, hormonal balance, carbohydrate assimilation, and distribution to other plant parts.

2.2.4 Mineral homeostasis

Plant drought tolerance responses are mediated by balanced mineral nutrition. These vital minerals, such as nitrogen, silicon, magnesium, calcium etc. are taken up by plants through their roots via water absorption. The uptake of these essential elements is pivotal in balancing a plant's mineral homeostasis and acclimation responses to abiotic stresses (Waraich et al., 2011; Zhong et al., 2017). Drought limits the active uptake of these essential minerals, resulting in stunted plant growth (Upadhyaya et al., 2013; Upadhyaya and Panda, 2019). It is reported that silicon fertilization increases the rate of photosynthesis, mineral absorption, and water use efficiency in rice, which helps counter the implications of drought stress more effectively (Chen et al., 2011; Cooke and Leishman, 2016). It has been reported that silicon and selenium effectively combat drought adversities by increasing the contents of amylase, phenolics, carbohydrates, and proteins, thereby increasing final grain yield (Emam et al., 2014; Suh et al., 2015). Li et al. (2017) reported the role of aquaporins in regulating hydraulic conductivity and its assisted by nitrate nutrition. Hydraulic conductivity is critical because of its role in facilitating plant nutrient transportation. Potassium (K) is also considered an essential nutrient having indispensable roles in plant physiology. It regulates the plant water potential and facilitates alleviating drought stress in tobacco and rice (Ahmad et al., 2016; Chen et al., 2017). Calcium (Ca) also effectively mitigates drought repercussions, particularly post-drought recovery responses (Devi and Kar, 2013). Zinc (Zn) nutrition is handy in ameliorating drought stress responses and post-drought recovery (Upadhyaya et al., 2017; Upadhyaya et al., 2020). It is directly and indirectly involved in various plant physiological activities, but any deviation from the optimum level results in toxicity and alterations in plant cell physiology, biochemistry, and anatomy (Alloway, 2013; Billard et al., 2015; Mattiello et al., 2015). The severe deficiency of Zn causes the disintegration of the cell membrane that hinders active plant growth and significantly dents the final grain yield; therefore, Zn fertilization is an effective way to overcome drought-induced complexities (Upadhyaya et al., 2013; Upadhyaya et al., 2017). Increased reactive oxygen species (ROS) accumulation under drought damages cellular structures because it interacts with lipids, proteins, nucleic acids, and pigments, impairing membrane function and causing lipid peroxidation that compromises cell viability. It can be avoided by increasing the scavenging response by antioxidant enzymes (Bartels and Sunkar, 2005; Pandey and Shukla, 2015). Under water-scarce conditions, the recommended fertilization of macronutrients (N, P, K, and Ca) and micronutrients (Si, Zn, and Mg) requires for activation of antioxidant defense mechanism and protection of plant cells from the harmful consequences of ROS accumulation (Dimkpa et al., 2017; Khan et al., 2017). Boron (B) is also thought to play a role in drought tolerance responses by promoting seed germination, mediating sugar transport, maintaining flower architecture, and developing pollen (Waraich et al., 2011). Recent research studies revealed that employing nano-fertilizers (particularly for micro-nutrients) in paddy fields will effectively tolerate the detrimental impacts of drought stress (Adhikari et al., 2016; Liu et al., 2016).

2.2.5 Osmotic adjustments

Low precipitation and dry conditions undermine plant turgidity. In water-deficit conditions, plants maintain their turgor by accumulating osmolytes, i.e., proline, soluble sugars (SS), amino-acids, and phenolics (Anjum et al., 2011); this phenomenon is called osmoregulation. Proline is a type of amino acid used as a protein building block in plants, considered a vital osmoprotectant (Hayat et al., 2012). Increased proline content was first observed in ryegrass under water-scarce conditions (Kemble and Macpherson, 1954). Mishra et al. (2018) found increased proline accumulation in rice under water deficit conditions compared to normal irrigated conditions. Increasing proline content is directly related to drought tolerance as it helps the plant continue stomatal conductance and maintain leaf turgidity (Kumar et al., 2017). Soluble sugars are critical for optimizing various physiological functions, notably photosynthesis and mitochondrial respiration (Gill and Tuteja, 2010a). Soluble sugar accumulation under drought protects cell membrane integrity and acts as an osmoprotectant (Upadhyaya and Panda, 2019; Hassan et al., 2021). There were rare studies conducted to investigate how soluble sugars relieve drought stress.

2.2.6 ROS accumulation and scavenging

Initially, ROS was found as a natural byproduct of aerobic metabolism; later also identified their role as a secondary signaling messenger under various environmental stresses (Panda et al., 2021). The excessive production of ROS and electrolytic leakage cause an imbalance in cellular homeostasis leading to oxidative damage to plant cells. Its severity may lead to cell death (Gill and Tuteja, 2010b). Thus, ROS is a double-edged sword since it causes oxidative damage when undergoing different abiotic stresses (i.e., drought, salinity, heavy metal stress, etc.). On the other hand, it acts as a signaling molecule in various physiological activities such as stomatal conductance, leaf senescence, and root hair growth (Das and Roychoudhury, 2014). ROS's dynamic equilibrium is needed to regulate active physiological functioning and optimal plant growth. Moisture stress in paddy fields causes ROS production and scavenging imbalance, leading to membrane impairments, degradation of biomolecules (such as proteins, lipids, and DNA), and disruption in physiological processes (Bartels and Sunkar, 2005; Gill and Tuteja, 2010b).

ROS scavenging has been accomplished by activating the antioxidant defence mechanism through the antioxidant enzymes and non-enzymatic antioxidant components (Das and Roychoudhury, 2014). The antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), dehydro ascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR). Non-enzymatic antioxidant components include ascorbic acid (AA), glutathione (GSH), α-tocopherol, carotenoids, flavonoids, and the osmolyte proline. The efficient antioxidant defence mechanism will be vital in countering the drought-induced repercussions of oxidative damage in rice (Mishra and Panda, 2017). SOD is found in almost all cellular organelles, and under drought-induced oxidative stress, increased SOD activity has been observed in rice plants (Melandri et al., 2020). CAT is found in mitochondria and peroxisomes, and its enhanced or diminished activity depends on stress intensity, it directly dismutases the H_2O_2 into H_2O and O_2 (Gill and Tuteja, 2010b). GPX is a well-known ROS scavenger and produces various related compounds, such as lignin, pyrogallol, and guaiacol, which act as scavenging donors for H_2O_2. Mishra and Panda, 2017 stated that rice's GPX level increased under drought conditions. There are also many non-enzymatic biochemicals (such as proline, glycine betaine, A-tocopherol, ascorbic acid, carotenoids, flavonoids, glutathione etc.) which protects plant cell from drought induced adverse impacts of oxidative stress via minimizing the harms of ROS (Hussain et al., 2019). Proline is known as prominent inhibitor against the programmed cell death due to oxidative stress (Aslam et al., 2022). Glycine betaine is considered as major osmolyte which maintains the membranous integrity under unfavorable environmental conditions. Similarly, tocopherols, ascorbic acid are present in the thylakoids of chloroplasts and meristematic cells, respectively, protects the lipid peroxidation and responsible for membranous stability through quenching ROS damaging effect (Hussain et al., 2019); an important metabolite, glutathione, is essential for scavenging ROS to prevent oxidative damage in all physiological compartments of plant cell (Yaqoob et al., 2022). Ascorbate-Glutathione (AsA-GSH) mechanism is vital in eliminating excessive H_2O_2 from rice plants (Wang et al., 2012; Bhattacharjee and Dey, 2018). Various studies elucidated the significance of the AsA-GSH cycle in countering the water-scarce-induced drought responses in rice (Qureshi et al., 2018; Melandri et al., 2020). According to Nahar et al. (2016), rice cultivars that are tolerant of drought stress produced more antioxidants, followed by activation of the antioxidant defence system, than those that were drought sensitive, leading to oxidative stress and plant death in severe cases.

2.3 Molecular responses

Rice exhibits a diversified molecular response to drought stress (Figure 5). The drought tolerance mechanism is initiated with signal sensing, followed by signal perception, transduction, genetic expressions, cellular regulation, and survival metabolic responses (Du et al., 2011; Oladosu et al., 2019). Drought is a multifaceted abiotic condition acclimated through regulating numerous genetic expressions (Kumar et al., 2017). Rice exposure to water-deficit stress exhibited multiple differential gene expressions, with about 5000 up-regulated and 6000 down-regulated gene expressions (Bin Rahman and Zhang, 2016; Joshi et al., 2016). These genes are categorized based on their localized functioning: (1) genes associated with membrane transport, (2) genes involved in signaling, and (3) transcriptional regulation (Kim et al., 2020). These genetic expressions are responsible for most rice plants' drought-induced physiological, biochemical, and molecular acclimation responses(Dash et al., 2018; Gupta et al., 2020). Transcriptomic and proteomic studies on rice have identified the transcriptomic factors (i.e., *MYB*, *DREB/CBF*, *AREB/ABF*, *NAC*, etc.) and their role in regulating the transcription of drought inducive gene expressions(Nahar et al., 2016; Zhang et al., 2016). Kumar et al. (2012) also reported the number of gene expressions and transcription factors (TFs) responsible for the drought tolerance response in rice. Previous research studies advocated that there are two main regulatory pathways for the induction of gene expression patterns for drought resistance mechanisms, known as (1) ABA-dependent and (2) ABA-independent regulation pathways (Du et al., 2011; Fu et al., 2017). The *MYB*, *NAC*, and *AREB/ABF* TFs drive the ABA-dependent pathway, while ABA-independent pathways are regulated via *DREB* TFs. Rabbani et al. (2003) stated that the exogenous application of ABA in rice effectively induces genetic expressions for combating the negative impacts of drought stress. A study on upland rice reported the role of drought-responsive genes in various signaling pathways (i.e., Ca^{2+}, ABA, and ethylene-accompanied proteins kinases and inducive factors), reducing oxidative damage, maintaining cellular homeostasis and osmoregulation (Rabello et al., 2008). The ABA-receptor complex regulates ABA-responsive transcription through *AREB/ABF*, and it involves *SnRK2*, which is integral for activating *ARB/ABF* by phosphorylation (Umezawa et al., 2010). The function of *SnRK2* indicates the significance of the plant's drought-responsive mechanism via swift adaptive action by plants under stress (Upadhyaya and Panda, 2019). As we mentioned earlier, ABA-independent pathways are governed by *DREB* TFs. Transcription of various genetic expressions in plant tissues is activated by the *DREB* TFs (Upadhyaya and Panda, 2019). The TF, *C2H2-type*, regulates stomatal closure upon exposure to water-deficit stress; this TF is also responsible for the induction of gene expression for quenching ROS and H_2O_2 and maintaining their dynamic balance under drought stress (Huang et al., 2009). Cominelli et al. (2005) stated that the expression of TFs, *AtMYB60* and *AtMYB60* primarily found in guard cells and controls the opening and closing of the stomatal aperture under drought tolerance responses.

3 Modern breeding with marker-assisted selection for drought management

Currently, many significant efforts are in progress to develop drought-resilient rice cultivars. Modern breeding techniques identify genes/QTLs regarding their phenotypic traits. After mapping, they are introduced into an elite gene pool, followed by

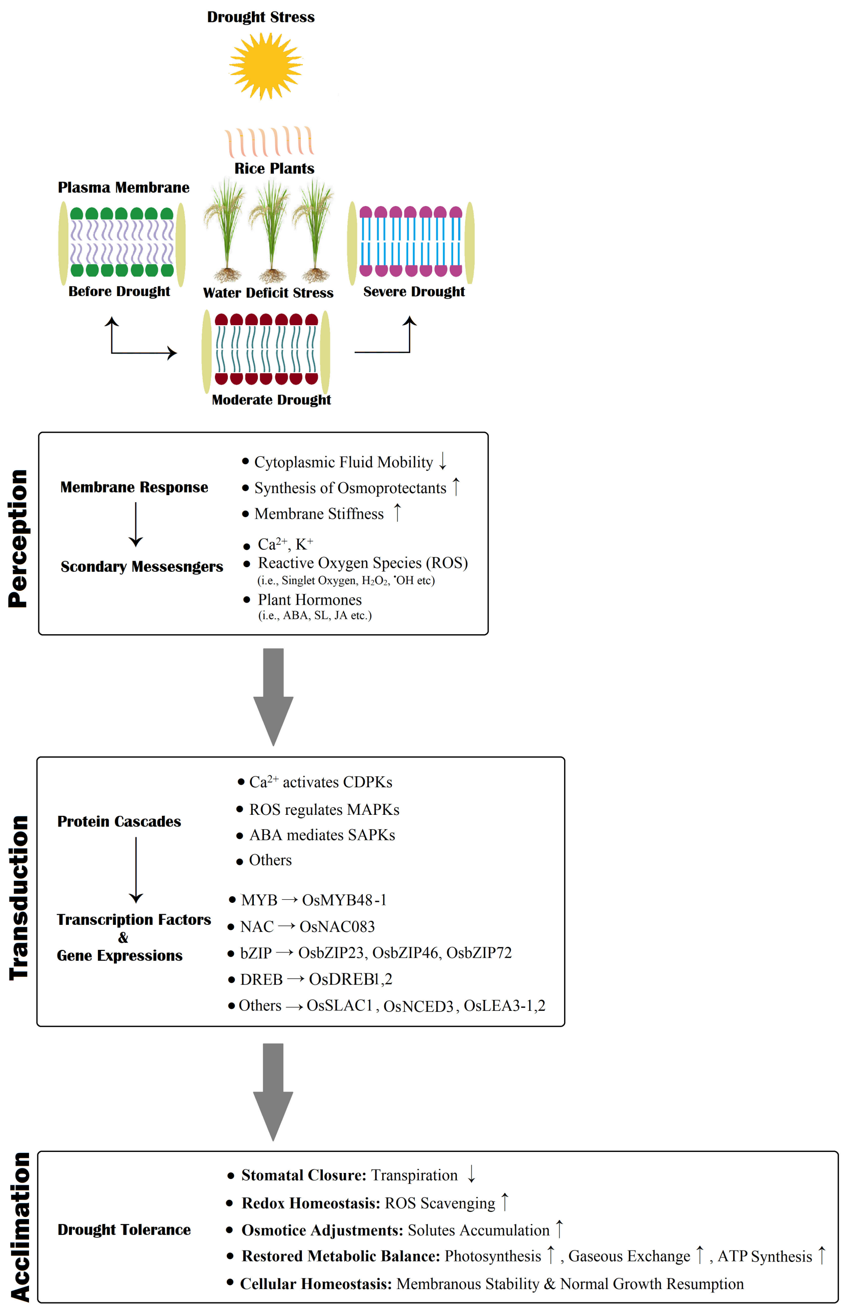

FIGURE 5
A schematic representation of rice drought perception, transduction, and final counter-response (acclimation). Drought stress is initially detected at the plasma membrane; subsequently followed by the influx of receptors, i.e., Ca 2+, ROS, Phytohormones, the initiation of protein kinase cascades, and protein cascade-driven up/downstream regulation generate gene expressions that assist drought tolerance. Here, "↑" indicates enhanced activity, and "↓" shows diminished activity.

MAS and the development of drought-tolerant varieties. The MAS is an indirect selection method in which the desired attributes are chosen based on markers (morphological, biochemical, or DNA/RNA variation) associated with a particular desired characteristic, i.e., high yield, drought resistance, tolerance (Lamont et al., 2014). Employing biomarkers and modern breeding approaches (i.e., molecular breeding and gene editing tools) is highly recommended to speed up the varietal development process (Donde et al., 2019b; Gouda et al., 2020). These modern approaches are very effective in discovering and better understanding the complex biological mechanisms in rice plants, i.e., drought tolerance, salt tolerance, cold tolerance etc. (Shakiba et al., 2017; Oladosu et al., 2019). Drought tolerance is a multifaceted mechanism operated through various genes and QTLs (Suh et al., 2015). These genetic variations can be utilized to develop drought-tolerant rice cultivars. In this section, we briefly discussed the MAS and its significance in prospective research studies regarding plant breeding.

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3.1 Marker-assisted crop breeding

In recent times, marker-assisted breeding approaches have been widely utilized, far better than conventional breeding techniques. It comprises multiple breeding techniques, such as identifying and mapping QTLs/genes and direct/indirect selection of genetic materials (Singh and Singh, 2015). Plant biomarkers are categorized into two major groups (1) classical markers and (2) molecular markers. Classical markers include morphological, cytological, and biochemical markers, whereas molecular markers include polymerase chain reaction (PCR) and hybridization-based molecular markers (Figure 6) (Joshi et al., 2016; Nadeem et al., 2017). Each type of marker has its own set of advantages and disadvantages, briefly stated in Table 2.

3.2 Classical markers

Classical markers are employed in various plant-breeding approaches but are not widely used because of certain limitations. Here we briefly described their potential use and limitations.

3.2.1 Morphological markers

This kind of classical markers phenotypically distinguish plant growth and development characteristics such as plant height, flower color, seed structure, growth habit under abiotic stress, and other agronomic features (Karaköy et al., 2013). It is a cheaper and easy way to mark prominent characteristics of a specific crop plant. Though plant breeders use morphological markers in their breeding programs for cereal crops (i.e., rice, wheat, sugarcane), it has certain constraints as they are easily affected by different biotic/abiotic factors and crop growth stages (Eagles et al., 2001).

3.2.2 Cytological markers

Cytological markers correspond to variations in the number, size, shape, banding pattern, position, and order of chromosomes (Nadeem et al., 2017). These markers provide a solid foundation for creating genetic linkage mapping and identifying normal and mutated chromosomes (Jiang, 2013; Xu et al., 2015).

3.2.3 Biochemical markers

Multiple enzymes encoded by various genes and performing similar functions are called isozymes or biochemical markers (Bailey, 1983). These markers are used to compute the genetic frequencies of various genes and subsequently detect the genetic diversity in populations (Mateu-Andrés and De Paco, 2005). Like morphological markers, isozymes are also easy to use and cost-effective. Their efficiency is also influenced by environmental stress and different plant growth stage (Mondini et al., 2009).

3.2.4 Molecular markers

The DNA fragments or gene sequences representing a particular locality in the genome are termed molecular markers (Semagn et al., 2006). Molecular markers are a significant advancement in modern plant breeding (Kebriyaee et al., 2012). Molecular markers are classified on the basis of their pattern of gene identification and their response to certain related traits. They are

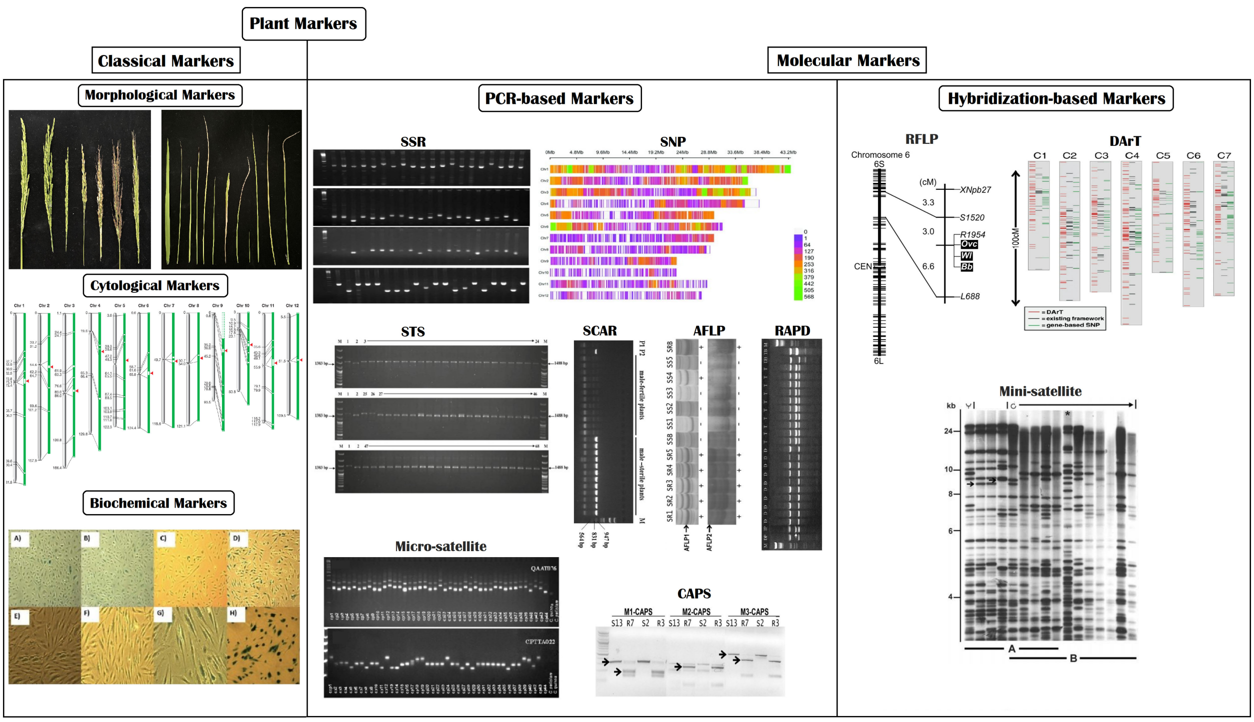

FIGURE 6
Pictorial illustration of commonly used plant markers.

TABLE 2 Merits and demerits of commonly used markers.

Markers	Merits	Demerits	References
(1) Classical Markers			
Morphological	Inexpensive & affordable Simple to use Visual observation	Lower polymorphic Influenced by abiotic factors Altered with varying growth phases	(Eagles et al., 2001)
Cytological	Useful in chromosome identification & genetic linkage-mapping	Expensive High skill required	(Jiang, 2013; Nadeem et al., 2017)
Biochemical	Economically affordable Simple to use	Lower polymorphic Influenced by abiotic factors	(Mondini et al., 2009)
(2) PCR-Based			
SSRs	Lesser DNA quantity needed Higher rate of reproducibility Co-Dominant marker	Expensive development Homoplasy risk Null alleles	(Kalia et al., 2011; Dhingani et al., 2015)
SNPs	Prior Sequencing not required Higher rate of reproducibility Co-Dominant marker	Expensive development	(Kundan et al., 2014)
STSs	Highly Robust Higher rate of reproducibility Transferable	Specie-specific marker development	(Cato et al., 2001)
AFLPs	Highly polymorphic Dependable(Eagles et al., 2001) Higher rate of reproducibility	Dominant marker Highly pure DNA with more quantity required Complex	(Ridout and Donini, 1999; Frisvad et al., 2005)
RAPDs	Simple in use Lesser DNA quantity required Highly polymorphic	Lower rate of reproducibility Dominant marker Pure DNA needed Non-specific locus	(Adhikari et al., 2017)
(3) Hybridization-Based			
RFLPs	Co-dominant marker Prior sequencing is not required Transferable Locus-specific	Higher DNA quantity required Costly & Time-consuming Restricted polymorphism	(Adhikari et al., 2017)
DArTs	Economically affordable Highly polymorphic Prior sequencing is not required Higher rate of reproducibility	Expensive development Dominant marker	(Wenzl et al., 2004)
Mini-satellite (VNTRs)	Highly polymorphic Easy to map	Non-transferable Complex	(Marwal et al., 2014)

categorized as PCR- and hybridization-based molecular markers (Joshi et al., 2016). The PCR-based molecular markers are further categorized into microsatellite or SSR (simple sequence repeat), SNP (single nucleotide polymorphism), STS (sequence-tagged site), SCAR (sequence characterized amplified region), AFLP (amplified fragment length polymorphism) and RAPD (randomly amplified polymorphic DNA), and CAPS (cleaved amplified polymorphic sequence). In contrast, markers based on hybridization consist of minisatellites, RFLP (restriction fragment length polymorphism), and DArT (diversity array technology) markers (Gouda et al., 2020; Adhikari et al., 2017). The most used molecular markers include AFLP, RFLP, RAPD, SSR, SNP, and other microsatellites. The use of molecular markers also differs from species to species. The five most critical factors for selecting a marker are reliability, high parental polymorphism, high-quality DNA, expert marker assay, and affordability (Mackill and Ni, 2008).

Currently, molecular markers are popular because of their ability to be used in any plant part and at any phase of development. Further, they have been easily created in large numbers, with no sensitivity due to environmental stimuli (Singh, 2017; Marwal and Gaur, 2020). Molecular markers are employed to investigate the genetic make-up of a particular plant at the molecular level. In each group of plants, markers and genes are discovered within proximity on the same chromosome (Grover and Sharma, 2016; Marwal and Gaur, 2020). To quantify the closeness, a genetic linkage map may be constructed based on how far the markers are from a particular gene. This genetic linkage map was used to investigate the associative relationships between significant traits and genes/QTLs, which enables to access desirable genes/QTLs through the MAS technique (Panda et al., 2021; Singh, 2017). As a substitute for the selection of phenotypic traits, MAS attributed DNA markers affiliated with the target locus. Through the application of MAS, it is possible to relate DNA markers to highly significant features including disease resistance, abiotic/biotic stress tolerance, and agronomic traits (Sahebi et al., 2018; Oladosu et al., 2019). Mas-ud et al. (2022) used molecular markers to test 110 rice genotypes for their ability to withstand drought. They discovered 20 genotypes with varying degrees of drought resistance, from extreme to moderate degrees. Similarly, Roy et al. (2021) evaluated 30 rice varieties, among which 27 were identified as tolerant to drought stress. In brief, MAS is considered the cheaper and speedy route to develop climate-resilient rice cultivars (Dixit et al., 2020; Adjah et al., 2022). Therefore, the role of molecular markers is very important in the determination of genetic diversity, gene mapping and their utilization in breeding programs for the sake of the development of new drought-resilient cultivars.

3.3 Major steps of MAS

MAS is carried out by employing various kinds of genetic/molecular markers. MAS consists of the following major steps: (1) selection of parent plants with diverse origins, (2) development of population for mapping purposes (recombinant inbred lines-RILs, nearly isogenic lines-NILs and backcrosses-BCs used for F_2 populations), (3) DNA extraction, (4) selection of suitable markers for QTL/gene mapping (i.e., RFLP, AFLP, SSR, RAPD, SNPs etc.), (5) phenotyping via correlation with morphological attributes, (6) construction of genetic-linkage pattern, (7) QTL/gene detection (genotyping), (8) validation, (9) and cloning. In this section, rather than detailing the earlier procedures, we thoroughly explored the various QTLs and genes associated with drought tolerance.

3.3.1 QTLs associated with drought tolerance

The QTL mapping for drought-related attributes has been broadly investigated in various cereal crops, including rice (Dixit et al., 2017; Dixit et al., 2020). Vikram et al. (2011) reported that identifying QTLs associated with tolerance expressions is very useful in the success of drought research screening programs. Until now, several QTLs have been identified which are directly or indirectly linked with morpho-physiological and growth parameters and are widely used for selecting tolerant genotypes for rice plants (Table 3) (Dixit et al., 2017; Vinod et al., 2019). Gomez et al. (2006) identified 24 QTLs linked with morpho-physiological attributes under drought stress. Those QTLs were found for the following growth and yield attributes: 1 for panicle length, 5 for leaf tolling, 4 for leaf drying, 3 for days to half flowering (50%), 5 for plant height, 1 for straw yield, and 3 for grain yield. Since *DTY1.1* (mapped on chromosome 1), for rice plant height, was the firstly documented QTL in rice with a consistent effect in drought-tolerant elite varieties for improving grain yield, therefore the *DTY* series of QTLs is well-known and of great significance (Vikram et al., 2011). The *DTY3.1* (mapped on chromosome 3) is responsible for the maximum number of filled panicles under drought stress, significantly impacting rice grain yield (Mohd Ikmal et al., 2023). Barik and his team mapped 5 QTLs associated with morpho-physiological attributes of rice under drought stress. It includes *LR9.1* (mapped on chromosome 9), which regulates the leaf rolling, *LD9.1* (mapped on chromosome 9) for leaf drying, *SF9.1* (mapped on chromosome 9) for spikelet fertility, *RWC9.1* (mapped on chromosome 9) for relative water content, and *HI9.1* (mapped on chromosome 9) for harvest index, respectively, under water-deficit drought stress at the reproductive stage (Barik et al., 2022). Bernier et al. (2009) examined the *QTL12.1* (mapped on chromosome 12) for its impact on rice grain yield in water-deficit

TABLE 3 Rice QTLs/Genes associated with drought tolerance.

QTLs	Trait/Function	Reference	Genes/TFs	Trait/Function	References
qLR8.1 ***qLR9.1*** ***qDLR8.1***	Leaf rolling	(Barik et al., 2019; Dixit et al., 2012; Lin et al., 2007)	*OsGRF6* *AtMYB60* *OsPYL*	Adjust shape and architecture Control stomatal aperture Regulate stomatal closure	(Cominelli et al., 2005; Kim et al., 2014; Kim et al., 2020; Zhou et al., 2010)
qLD9.1 ***qLD12.1***	Leaf drying	(Barik et al., 2019)	*OsTPS1*	Proline & soluble sugar accumulation	(Li et al., 2011)
qRWC9.1	Relative water content	(Barik et al., 2019)	*AtDREB1A*	Accumulation of osmoprotectants	(Ravikumar et al., 2014)
qDTR8	Transpiration rate	(Ramchander et al., 2016)	*DsM1*	ROS scavenging	(Ning et al., 2010)
qSF9.1	Spikelet fertility	(Barik et al., 2019)	*OsbZIP71* *AP37* *OsNAC1*	Enhance seed setting Increased seed filling Improved spikelet fertility	(Hu et al., 2006; C. Liu et al., 2014; Oh et al., 2009)
qPH1.1	Plant height	(Trijatmiko et al., 2014)	*OsbZIP23* *OsLEA3-1* *OsWRKY47*	Enhance grain yield Lower yield reduction	(Xiao et al., 2007; Xiang et al., 2008)
qPL-9 ***qGY3.1*** ***qDTY2.3*** ***qHGW1*** ***QHI9.1***	Panicle length & number Grain yield Total dry matter Harvest Index	(Lanceras et al., 2004; Sandhu et al., 2014; Sellamuthu et al., 2015; Barik et al., 2019)	*OsDRO1* *OsNAC10* *OsNAC5* *OsDREB2B*	Deep elongated roots Root diameter Number of roots	(Jeong et al., 2013; Uga et al., 2013)

conditions in India. They concluded that *QTL12.1* has no impact under well water conditions, but its expression augmented up to 40% with increased drought intensity. Numerous studies have investigated various QTLs associated with drought tolerance in different rice lines such as *DTY 2.1, DTY2.2, DTY12.1* (for rice grain yield under drought, located on chromosome 2) (Dixit et al., 2012; Dixit et al., 2014), *DTHI2.3* (for harvest index under drought, located on chromosome 2) (Lin et al., 2007), *DTY6.1* (for enhancing grain yield under drought, located on chromosome 6) (Venuprasad et al., 2007), *DRL2.1* (for root length, located on chromosome 2) (Iqbal et al., 2021), and *DTY6.3* (for grain yield, located on chromosome 6) (Yadav et al., 2019). *DRO1* (located on chromosome 1) is a QTL associated with deeper root penetration under drought stress and enhances the yield potential in rice crops (Uga et al., 2013). Shamsudin and their colleagues identified three drought-yield QTLs (i.e., *DTY2.2,3.1* and *12.1*) in Malaysian cultivar MR219 under drought conditions using the MAS-pyramiding technique (Shamsudin et al., 2016). Later successfully developed a high yielding drought tolerant rice variety with a potential of 1500 kg/ha. Past studies identified the number of QTLs having their role in various biochemical and physiological processes (Barik et al., 2022); but wasn't discovered associated genes with the same pace due to poor phenotypic impact and weaker mapping resolution (Singh et al., 2012; Bin Rahman and Zhang, 2016). Several studies have found a diverse range of molecular markers linked to these QTLs and used to screen new rice genotypes for drought tolerance.

3.3.2 Genes associated with drought tolerance

Under Drought stress, rice plants exhibited a number of up/down regulated genetic expressions. It includes 5000 up-regulated and 6000 down-regulated expressions (Joshi et al., 2016). These genetic expressions are related to drought stress signaling, membranous transport, and transcriptional control during cellular metabolism (Kim et al., 2020). Many genes expressed under drought lie in ABA-dependent or independent regulation mechanisms (Gupta et al., 2020). Fu et al. (2017) stated that *OsJAZ1* reduces drought tolerance via modulating ABA signaling, which coordinates plant responses to drought stress in rice. Root morphological responses to drought stress were carried through over-expressions of *OsDREB2B*, *CYP735A*, and *OsDREB1F* (Kim et al., 2020). Uga et al. (2013) revealed that *DRO1* is the responsible gene for root elongation and deeper penetration under water-limited conditions. Genetic expressions of *OsCPK9* increase plant tolerance to drought by enhancing stomatal closure and improving osmoregulation (Wei et al., 2017). Drought response at the vegetative stage is facilitated through the induction of *OsNAC10* (Jeong et al., 2013). The gene expressions of *OsMIOX* during severe drought stress prevent oxidative damage by activating ROS-scavenging enzymes (Duan et al., 2012). Table 3 Shown the list of such genes and their related functions for rice drought tolerance. Through MAS, these novel genotypes can be utilized in conventional and modern breeding programs to create rice varieties that are more compatible with drought conditions. The International Rice Research Institute has conducted most marker-assisted breeding trials in the last decade for developing drought-resistant rice varieties(Kumar et al., 2017; Sandhu and Kumar, 2017).

4 Conclusion and prospects

Rice plants exhibit critical behavior in stressful conditions, requiring proper management. Tolerance strategies, pathways, and mechanisms in drought have long been debated in scientific research. Different studies have been conducted to explore the causes of drought stress, and strategies have been devised to mediate the plant responses. We have elaborated on the rice-plant responses to drought stress at the morphological, physiological, biochemical, and molecular levels. Further, we discussed the role of modern breeding techniques (such as MAS) in increasing the pace of ongoing breeding programs for varietal development. The significance of available genetic resources (i.e., genotypes, QTLs, and other genetic make-ups) has been explained concerning their phenotypic characteristics under drought. Despite the number of studies, scientific research has not succeeded in figuring out the ideal plant responses in drought. The role of genes and QTLs is needed to be further explored because of their controlling potential in plant acclimation responses during stress. The discovery of potential genes/QTLs responsible for drought acclimation responses is a real quest for crop scientists in the current era of changing climate.

A broader understanding of the genetic basis of agricultural attributes in many crops has recently been accomplished because of advancements in molecular marker-assisted technologies. Modern research focuses on managing genotypes to demonstrate the effect on phenotype and introgressing them into high-yielding varieties. The importance of molecular markers and hereditary material needs to be further investigated in the near future to attain better opportunities to grow the plant under stressful environments. Detailed genome-wide association studies are needed to overcome the loopholes in markers-assisted breeding. In addition to this, modern molecular markers are quite expensive and required skilled persons for effective utilization. Along with the availability of low-cost molecular markers, it is critical to conduct training programs in research institutes to equip scientists and researchers with modern techniques and skills for efficient resource utilization for the development of climate-smart varieties. Economic conditions' favorability will stimulate the application of such gene-identifying/editing tools and techniques in a pragmatic way.

Author contributions

MH and WS conceived the concept of the review and prepared an outline of the review. MH and ZQ compiled the literature and wrote the different sections. YY and LY. aided in designing figures and arranging references. ND and TH provided technical assistance and editing support.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Genetic diversity for drought tolerance in the native forage grass *Trichloris crinita* and possible morpho-physiological mechanisms involved

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Introduction: The use of drought tolerant genotypes is one of the main strategies proposed for coping with the negative effects of global warming in dry lands. *Trichloris crinita* is a native forage grass occupying extensive arid and semi-arid regions in the American continent, and used for range grazing and revegetation of degraded lands.

Methods: To identify drought-tolerant genotypes and possible underlying physiological mechanisms, this study investigated drought tolerance in 21 genetically diverse *T. crinita* genotypes under natural field conditions. The accessions were grown under irrigated (control) and drought conditions for 84 days after initiation of the drought treatment (DAIDT), which coincided with flowering initiation. Various morpho-physiological traits were monitored, including total-, foliage-, and root biomass yield, dry matter partitioning to individual plant organs (roots, leaves, stems, and panicles), total leaf area, chlorophyll content, photochemical efficiency of photosystem II, stomatal conductance, and number of panicles per plant.

Results and discussion: Broad and significant variation ($p<0.001$) was found among the accessions for all the traits. Three highly tolerant and three very sensitive accessions were identified as the most contrasting materials, and their responses to drought stress were confirmed over two years of experiments. Under prolonged drought conditions (84 DAIDT), the tolerant accessions were generally more productive than the rest for all the biomass yield components analyzed, and this was associated with a postponed and more attenuated decrease in variables related to the plant photosynthetic activity, such as stomatal conductance, chlorophyll content, and photochemical efficiency. In contrast to previous findings, our data indicate no direct relationship between drought tolerance and the level of aridity in the accessions natural habitats, but rather suggest genetic heterogeneity and ample variation for drought tolerance in *T. crinita* natural populations derived from a particular location or environment. Also, having low total and forageable biomass yield, or increased biomass allocation to the roots (i.e., lower foliage/root ratio), under optimal water availability, were not associated with greater drought tolerance. The drought-tolerant accessions identified are of value for future genetic research and breeding programs, and as forage for range grazing and revegetation in arid regions.

KEYWORDS

Leptochloa crinita, drought stress, photoassimilates partitioning, biomass, stomatal conductance, photochemical efficiency

1 Introduction

Drylands cover ~41% of the Earth's land surface and will experience substantial expansion, degradation, and conversions among dryland subtypes under the predicted climate change scenarios (Reynolds et al., 2007; Yao et al., 2020). Such scenarios predict more frequent and intense drought periods in many regions, further aggravating the situation of arid lands (Overpeck, 2013; International Panel of Climatic Changes, 2014). One of the strategies to counteract land degradation problems is to reseed degraded areas with species capable of surviving in these ecosystems. Native species from arid environments are potentially valuable genetic resources for revegetation and rehabilitation of degraded drylands (Villagra et al., 2021). In this context, the selection of drought-tolerant species, and genotypes within a species, along with the use of adequate and sustainable management practices, are critical for a successful revegetation program with native species. *Trichloris crinita* (Lag.) Parodi [syn. *Leptochloa crinita* (Lag.) Peterson and Snow (Peterson et al., 2012; Peterson et al., 2015)] (Chloridoideae, Poaceae) is a perennial grass native to arid and semi-arid regions of North and South America (Quiroga et al., 2018). In these dry lands, range grazing is one of the few non-irrigated agricultural activities, with native grasses being the main forage resource (Busso and Fernández, 2018). Because of its good forage quality (Dominguez et al., 2022), drought tolerance, resistance to trampling and grazing, and rapid growth and competing aggressiveness among other native species (reviewed by Kozub et al., 2018b), *T. crinita* is widely promoted for range grazing and restoration of degraded rangelands in environments with low water availability (Passera et al., 1992; Cavagnaro and Trione, 2007; Guevara et al., 2009; Kozub et al., 2018b; USDA-NRCS, 2020). Despite the general drought tolerance attributed to *T. crinita*, as compared to other native forage grasses, it must be noted that this species grows naturally in vast geographical regions varying widely in precipitation regimes and overall water availability, with annual precipitations ranging from 150 to 1500 mm, and phenotypically-distinct ecotypes and populations can be found across these environments (Marinoni et al., 2015; Quiroga et al., 2018). This suggests that different adaptive mechanisms for water utilization and coping with drought stress may exist in the *T. crinita* germplasm. Such hypothesis has not been explored to date.

Because of its importance in arid regions, the Germplasm Bank of Native Grasses (GBNG) at the Argentine Institute for Research in Arid Regions (IADIZA) is dedicated to the collection, preservation, studying, and distribution of germplasm of native grasses from arid and semiarid regions of Argentina. In the last decades, representative plants from natural populations of *T. crinita* and other native grasses were collected from the Monte phytogeographical region, located in the west of Argentina, and evaluated at various levels. These studies revealed broad genetic diversity for this germplasm collection at the DNA level (using AFLP and SSR molecular markers), as well as for morphological, cytological, physiological, agronomic and forage-quality traits (Greco and Cavagnaro, 2005; Cavagnaro et al., 2006; Kozub et al., 2018a; Kozub et al., 2019; Dominguez et al., 2022). However, to date, no thorough evaluation of drought tolerance in the GBNG *T. crinita* collection has been performed. The characterization of these germplasm with regards to their drought-stress responses may reveal tolerant genotypes, of potential value for revegetation and forage grazing in extremely arid environments.

To cope with drought, plants of arid ecosystems have developed a range of physiological and morphological strategies, including the development of low specific leaf area (SLA), increased allocation of photoassimilates to the roots, reduced foliar biomass, reduced stomatal conductance, low photosynthetic rate, reduced transpiration rate and water loss, osmotic adjustment, low relative growth rate, and reduction of maximum quantum efficiency of photosystem II (Fv/Fm) (Poorter and Nagel, 2000; Chesson et al., 2004; Blum, 2005; Greco and Cavagnaro, 2005; Campanella and Bertiller, 2008; Manzoni et al., 2011; Acquaah, 2012; Poorter et al., 2012; Quiroga et al., 2013; Carrizo et al., 2020; Marinoni et al., 2020). Variations in some of these physiological traits have been shown to condition the growth and survival of various grass species when grown in arid environments.

Only a few studies have investigated drought tolerance in *T. crinita*. First, Greco and Cavagnaro (2003) evaluated three *T. crinita* genotypes under irrigated and drought conditions, reporting significant differences in drought tolerance among the genotypes, and suggested the development of higher root biomass as a possible adaptive trait associated with drought tolerance. Later, Quiroga et al. (2013) compared two ecotypes from contrasting environments varying in rainfall precipitations finding that, under drought conditions, the ecotype from the more-extreme arid site exhibited greater drought-tolerance than the ecotype from the region with greater water availability. Similarly, Marinoni et al. (2020) evaluated four ecotypes under hydroponics in a growth chamber, concluding that ecotypes of arid and semi-arid origins were more tolerant to drought than ecotypes from humid sites. Although these studies evidenced the existence of variation for drought tolerance in *T. crinita*, they were performed using very few (2-4) genotypes, under controlled conditions (in pots or hydroponics), in a single environment (i.e., one year and location), and analyzed a few morphological traits as response variables. Thus, a more comprehensive characterization of the drought-stress response – e.g., by means of analysis of various morphological and physiological traits- in a large and genetically diverse *T. crinita* collection, performed under replicated and natural field conditions, is required to: 1) have a robust assessment on the extent of genetic variation for drought tolerance in the *T. crinita* germplasm; 2) identify new drought-resistant genotypes that combine other desirable agronomic and forage quality traits; and 3) identify morpho-physiological mechanisms associated with drought tolerance in this species.

Based on the background information described above, we hypothesize that: 1) there is ample genetic variability for drought tolerance in the *T. crinita* germplasm, which will allow the identification and selection of drought-tolerant genotypes; 2) accessions from extremely arid regions have greater drought tolerance than accessions from less arid regions; and 3) variation in morpho-physiological traits explain the differences in drought tolerance found among *T. crinita* accessions. Thus, the present study characterized drought tolerance in a genetically diverse *T. crinita* germplasm collection, using a two-year partially replicated field experiment. To this end, 21 *T. crinita* accessions from the GBNG grown under irrigated and drought conditions were analyzed for morpho-physiological and productive (forage biomass) variables, with the ultimate goal of identifying highly-resistant genotypes and physiological mechanisms underlying drought tolerance in this grass species.

2 Materials and methods

2.1 Plant materials

Twenty-one *T. crinita* accessions from the GBNG, at IADIZA, were used. These materials were selected to maximize genetic diversity, from collections of representative plants from 48 natural populations of *T. crinita* dispersed throughout extensive arid and semi-arid regions (~350,000 km^2) in Argentina. The selected accessions are genetically and morphologically diverse, and they constitute a representative sample of the broad phenotypic variation observed naturally in the Monte phytogeographical region (Cavagnaro et al., 2006). The geographic locations and main climatic and edaphic characteristics at the collection sites of the accessions are presented in Table 1 and Figure 1. Noteworthy, these accessions are different from the plant materials used in previous studies by Quiroga et al. (2013) and Marinoni et al. (2020).

2.2 Experimental design

The field study consisted of a partially-replicated experiment, conducted during the 2017-2018 growing season (henceforth “2018”), in which 21 *T. crinita* accessions were evaluated. The experiment was repeated in the following season (2018-2019; henceforth “2019”) with the six most contrasting accessions with regards to their drought-tolerance performance in the previous year. The climatic conditions at the site of the experiment for 2018 and 2019 trials are presented in Supplementary Figure S1. For implantation, individual seeds were sown in 250 cm^3 pots with sterile soil, and plants were grown under greenhouse conditions until they had 5–6 leaves, after which the pots were placed outdoors for a period of rustication, and after one week they were transplanted to the experimental field of the Faculty of Agricultural Sciences, Nacional University of Cuyo (Mendoza, Argentina) (33° S, 68° 8’ O). In the field, the experimental design consisted of two plots, one for the irrigated (control) and one for the drought treatment, separated from each other by five meters, with each plot containing 252 *T. crinita* plants, for a total of 504 plants used in the experiment. All the plants in both treatment plots were drip-irrigated to field capacity during the first 80 days after the transplant, to ensure that the plants were fully established in the field. After the 80th day, and coincidently with flowering initiation, the drought treatment was imposed in one of the plots by completely eliminating irrigation, while the control plot continued with the same irrigation regime throughout the experiment. A 21 × 2 factorial design with divided plots (subplots) was used, being the two plots the drought and control treatments, and the 21 subplots the accessions within each plot. Each subplot consisted of 12 plants of each *T. crinita* accession, with a completely randomized distribution in the field (within each plot) with adjacent plants separated 80 cm from each other. To prevent any water from precipitations to infiltrate the ground, the soil of the entire experimental plot in both treatments, drought and irrigated, was covered with a 200-µm thick polyethylene, and covered with soil on top. By this means, water availability for the plants was completely controlled, and provided only by the drip irrigation system. Supplementary Figure S2 shows the field trial for evaluating drought tolerance in *T. crinita* accessions.

2.3 Sampling and measurements of response variables

Plants of all the accessions were harvested at four different sampling times during the vegetative cycle; at the beginning of the drought treatment (day 0), and 28, 56 and 84 days after initiation of

TABLE 1 *Trichloris crinita* accessions and main characteristics of their collection sites in Argentina.

Acc. ID	Province	Location	Latitude (° S)[ζ]	Longitude (° W)[ζ]	Altitude (m.a.s.l.)[ζ]	Annual mean temp (°C)[†]	Mean temp in GS (°C)[†]	Mean max temp in GS (°C)[†]	Mean min temp in GS (°C)[†]	Mean annual rainfall (mm)[¥]	Aridity index[β] A	Aridity index[β] B	Soil electrical conductivity (μmhos/cm)[ζ]	Climate classification (abbreviation)[λ]
1	Mendoza	Rivadavia, El Mirador	33.33	68.25	653	16.9	22.5	30.2	14.8	204	7.6	0.13	348	Warm desert (BWk)
3	Mendoza	Santa Rosa, Ñacuñan	34.04	67.90	540	16.9	22.6	30.5	14.7	327	12.2	0.15	264	Cold semi-arid (BSk)
4	Mendoza	Lavalle, Arroyito	32.79	67.37	540	19.3	24.6	32.0	17.1	190	6.5	0.16	296	Cold desert (BWh)
5	Mendoza	Luján de Cuyo, Ugarteche	33.21	68.95	950	16.2	21.5	28.8	14.2	247	9.4	0.14	1176	Warm desert (BWk)
6	Mendoza	Santa Rosa, Comte. Salas	33.83	68.00	530	16.5	22.2	30.2	14.2	327	12.3	0.15	227	Cold semi-arid (BSk)
7	Mendoza	Santa Rosa, Ñacuñán	34.04	67.90	540	16.9	22.6	30.5	14.7	327	12.2	0.15	361	Cold semi-arid (BSk)
8	Mendoza	San Carlos, Pareditas	33.96	69.04	940	14.4	19.4	27.7	11.0	334	13.7	0.20	342	Cold semi-arid (BSk)
9	Mendoza	Santa Rosa, Ñacuñán	34.04	67.90	540	16.9	22.6	30.5	14.7	327	12.2	0.15	281	Cold semi-arid (BSk)
10	San Juan	25 de mayo, El Encón	32.19	67.71	520	19.2	24.8	32.3	17.3	104	3.6	0.16	7825	Cold desert (BWh)
11	Mendoza	San Rafael, Guadales	34.49	67.83	606	16.3	21.9	30.2	13.5	334	12.7	0.16	293	Cold semi-arid (BSk)
12	Mendoza	Lavalle, El Retamo	32.51	67.41	525	19.4	24.7	32.0	17.4	190	6.5	0.16	2357	Cold desert (BWh)
13	Mendoza	Santa Rosa, Pichi Ciego	33.57	68.09	530	16.6	22.2	30.1	14.4	327	12.3	0.14	424	Cold semi-arid (BSk)
14	La Pampa	La Asturiana	37.83	65.36	260	15.2	20.5	29.1	11.9	519	20.6	0.20	1061	Cold semi-arid (BSk)

(Continued)

TABLE 1 Continued

Acc. ID	Province	Location	Latitude (° S)[ζ]	Longitude (° W)[ζ]	Altitude (m.a.s.l.)[ζ]	Annual mean temp (°C)[†]	Mean temp in GS (°C)[†]	Mean max temp in GS (°C)[†]	Mean min temp in GS (°C)[†]	Mean annual rainfall (mm)[¥]	Aridity index[β]		Soil electrical conductivity (µmhos/cm)[ζ]	Climate classification (abbreviation)[λ]
											A	B		
17	San Juan	25 de mayo, El Encón	32.15	67.51	520	19.3	24.8	32.3	17.3	104	3.6	0.18	1280	Cold desert (BWh)
18	La Pampa	Lihuel Calel, Sierra Chica	37.90	65.46	235	15.2	20.5	29.1	11.9	519	20.6	0.20	425	Cold semi-arid (BSk)
19	Mendoza	Luján de Cuyo, Agrelo	33.11	68.91	940	16.4	21.7	29.0	14.3	227	8.6	0.13	2050	Warm desert (BWk)
20	Mendoza	Santa Rosa, Pichi Ciego	33.57	68.09	530	16.6	22.2	30.1	14.4	327	12.3	0.14	424	Cold semi-arid (BSk)
21	San Juan	25 de mayo, El Encón	32.19	67.71	520	19.2	24.8	32.3	17.3	104	3.6	0.16	7825	Cold desert (BWh)
22	Mendoza	Lavalle, Arroyito	32.79	67.37	510	19.3	24.6	32.0	17.1	190	6.5	0.16	296	Cold desert (BWh)
23	Mendoza	Lavalle, El Retamo	32.47	67.42	525	19.5	24.8	32.1	17.4	190	6.4	0.17	3585	Cold desert (BWh)
24	Catamarca	Capayán, Miraflores	28.65	65.91	460	20.4	25.3	31.9	18.7	411	13.5	0.23	241	Warm semi-arid (BSh)

GS. growing season (spans the months of October to March, corresponding to spring-summer in the Southern hemisphere); m.a.s.l. meters above sea level.
[ζ] Data from accession passport data for soil analysis performed at the Germplasm Bank of Native Grasses (GBNG) at the Argentine Institute for Research in Arid Regions (IADIZA). [†] Data source: WorldClim v2.1 (https://www.worldclim.org/data/worldclim21.html#; Fick and Hijmans, 2017). Historical climate data (1970–2000) were extracted from layers of 30 arc-second of resolution (≈ 1 km). [¥] Data from closest weather station to the collection sites (<20 km). [β] Aridity indices were calculated according to De Martonne (1926) (A) and Zomer et al. (2022) (B). For both indices, greater values indicate lower aridity. [λ] According to the Köppen–Geiger climate classification.

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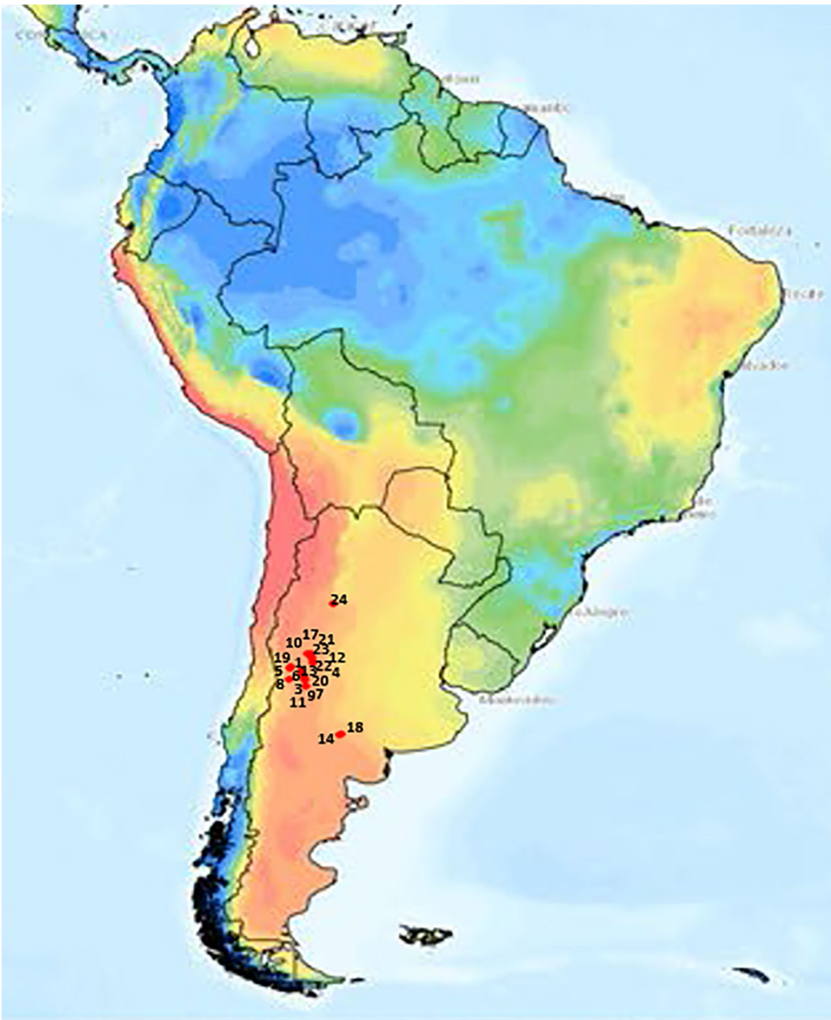

FIGURE 1
Geographical origin of 21 *Trichloris crinita* accessions (red dots) in South America, with Aridity Indices (Zomer et al., 2022) depicted in color.

the drought treatment (DAIDT). At each sampling date, three plants per accession were harvested in both the irrigated and drought plots. The aerial and underground parts of each plant were independently collected. The underground plant parts were obtained from a volume of soil of 0.064 m^3 (0.4 m × 0.4 m × 0.4 m) by excavation around the plant to a depth of 40 cm, considering that the majority of the root system in this species is found within this soil volume (Greco and Cavagnaro, 2005). The roots were then separated from the soil by rinsing with water and sieving through a 0.6 mm mesh screen, and dried in an oven at 60°C until constant weight, to obtain their dry matter (DM) content. The aerial part of the plant, henceforth referred to as "foliage", was subdivided into its components leaf blades (from the ligule to the leaf tip, henceforth referred to as "leaves"), 'stems+culms' (henceforth "stems"), and panicles, and oven-dried at 60°C until constant weight, to estimate their respective dry matter contents. Dry matter partitioning to each of the plant parts (i.e., leaves, stems, panicles, and roots) was calculated by dividing the DM of a particular plant part by the total DM of the plant, and expressed as percentage of the total plant biomass. The ratio of 'foliage DM/root DM', referred to as 'foliage/root ratio' (FRR), was calculated for each plant based on its DM contents in the foliage and roots. Total leaf area per plant was determined, prior to desiccation of the leaf samples in the oven, using a leaf area meter (LICOR, Model LI3000A. USA).

On a weekly basis and until the 84 DAIDT, the number of panicles per plant was recorded; and stomatal conductance, chlorophyll index, and maximum quantum efficiency of photosystem II (PSII) were determined on the fully expanded penultimate leaf of each plant, between 10 am and 2 pm. For each accession and treatment, three plants (i.e., three biological replicates) were measured at each time point. Stomatal conductance (g_s) was measured with a leaf porometer (Decagon Devices, Model SC-1, USA) on the surface of the abaxial side of the leaves, avoiding the midrib. Mean values of three readings per plant, for three plants per accession and treatment, at each time point, were considered to calculate mean g_s values. The chlorophyll index was determined using a chlorophyll meter (Minolta, SPAD 502 Plus, Japan). For each plant, the mean of three readings was obtained, and three plants per accession and treatment were analyzed at each time point. Maximum quantum efficiency of PSII (Fm/Fv) was determined by measurements of chlorophyll *a* fluorescence using a Plant Efficiency Analyzer (Pocket PEA; Hansatech Instruments, England). Prior to the measurements, the leaves were fully dark-adapted for 30 min to achieve the complete oxidation of the primary electron carriers. Chlorophyll fluorescence induction was prompted by a 3-s pulse of red light (peak wavelength of 627 nm) emitted from a LED lamp filtered by a NIR filter. This pulse was emitted at maximal saturation irradiance of 3500 µmol m^{-2} s^{-1}. The basal non-variable chlorophyll fluorescence (F_0) and the maximum fluorescence induction (Fm) were determined, and the variable fluorescence (Fv) was calculated as follows: Fv = Fm - F_0. Then, the maximum quantum efficiency of PSII (Fv/Fm) was estimated according to Maxwell and Johnson (2000).

In order to reflect the degree of change in the plant parameters analyzed due to the drought stress, the data for all the variables were expressed as percentage (%) relative to the values in the respective control treatments, according to the formula ($Value_{Drought}$/$Value_{Control}$) × 100, unless otherwise stated.

2.4 Statistical and principal component analyses

The data were analyzed using analysis of variance (ANOVA) by mixed linear models with a factorial structure, treating drought treatment, accession, and their interactions, as fixed effects, while biological replicates were treated as random effects. Different structures of residual variance were evaluated, and the best models were selected using the Akaike (AIC) and Schwarz (BIC) information criteria (Di Rienzo et al., 2017). Prior to the analysis, percentage values were transformed by the square root of the bow-sine function. All the statistical and graphic analyses were performed with the software InfoStat version 2020 (Di Rienzo et al., 2020) and R® version 3.5.3 (R Core Development Team). Means comparisons were performed using the DGC test (Di Rienzo et al., 2002). For all the variables, the data were expressed as mean value ± standard deviation and p-values < 0.05 were considered significant. Principal component analysis (PCA) was implemented in the InfoStat software to classify the accessions based on drought-response variables using a data matrix of 21 × 9, where the rows represent the 21 *T. crinita* accessions and the columns comprised the data for nine morpho-physiological traits.

3 Results

3.1 Total biomass yield

Total plant biomass (i.e., DM of the four plant parts analyzed combined), expressed as percentage relative to the total DM content in control plants, henceforth referred as "RTDM", was influenced by the accession, year of cultivation, sampling time, and their interactions (Table 2). In 2018, ample and significant differences (p<0.001) were found among *T. crinita* accessions and among sampling times (i.e., 0, 28, 56 and 84 DAIDT) for RTDM (Table 3). As expected, before initiation of the drought treatment

TABLE 2 Influence of the accession, sampling time, year of cultivation, and their interactions on the relative values (%) of total dry matter (RTDM), foliage dry matter (RFDM), roots dry matter (RRDM), foliage/root ratio (RFRR), leaf area (RLA), chlorophyll index (RCI), photochemical efficiency (RPE), stomatal conductance (Rg_s), and number of panicles per plant (RNPP) of *T. crinita* accessions grown under drought conditions in 2018 and 2019.

Year (N° accs)	Factor	RTDM (n=252)	RFDM (n=252)	RRDM (n=252)	RFRR (n=252)	RPE (n=819)	RLA (n=252)	RCI (n=819)	RPE (n=819)	Rg_s (n=819)	RNPP (n=819)
2018 (21 accs.)	Accession (A)	146.8***	1350.9***	916.2***	45.6***	47.8***	20.3***	66.3***	47.8***	47.8***	320.1***
	Sampling time (T)	2176.3***	142.6***	47.4***	63.0***	837.8***	2819.4***	2771***	837.8***	1734.2***	159.1***
	A x T	20.7***	20.8***	8.5***	10.5***	9.7***	9.27***	7.8***	9.7***	13.9***	18.3***
2018 and 2019 (6 accs.)		RTDM (n=144)	RFDM (n=144)	RRDM (n=144)	RFRR (n=144)	RPE (n=468)	RLA (n=144)	RCI (n=468)	RPE (n=468)	Rg_s (n= 468)	RNPP (n= 468)
	Year (Y)	66.3***	68.6***	ns	19.8***	21.5***	ns	64.2***	21.5***	13.0**	222.2***
	Accession (A)	257.8***	235.1***	54.2***	36.2***	468.5***	21.5***	310.3***	468.5***	142.6***	1563.1***
	Sampling time (T)	996.4***	773.0***	290.0***	8.9***	903.1***	1064.3***	1417.6***	903.1***	626.8***	321.2***
	Y x A	10.9***	10.9***	3.6**	7.00***	ns	4.1**	3.6**	ns	ns	148.5***
	Y x T	47.2***	33.7***	21.5***	ns	5.3***	5.6**	29.9***	5.3***	1.8*	5.4***
	A x T	69.6***	60.6***	15.5***	8.5***	72.1***	11.9***	15.8***	72.1***	38.9***	29.3***
	Y x A x T	7.3***	8.0***	ns	3.6***	2.3***	2.09*	4.7***	2.27***	ns	7.4***

Numbers are F values from ANOVA. Asterisks indicate statistically significant effects at p<0.05 (*), p<0.01 (**), and p<0.001 (***). ns, not significant. For the ANOVA, all the variables were expressed as percentage relative to the values in their respective irrigated controls. Twenty-one T. crinita accessions were evaluated in 2018, and the six most contrasting accessions with regards to drought-tolerance were re-evaluated in 2019 (i.e., the ANOVA of 2018 and 2019 comprised data for six accessions over two years).

TABLE 3 Time course variation for mean relative total dry matter (RTDM) content for *Trichloris crinita* accessions grown under drought conditions in 2018 and 2019.

Year	Accession	0 DAIDT	28 DAIDT	56 DAIDT	84 DAIDT
2018	1	95.4 ± 3.0 a	74.7 ± 6.4 c	79.6 ± 1.5 c	80.2 ± 0.5 c
	3	101.7 ± 2.5 a	72.0 ± 2.6 c	**81.8 ± 5.5 b**	**83.3 ± 1.6 b**
	4	98.9 ± 1.7 a	52.9 ± 1.2 f	56.9 ± 1.1 e	57.4 ± 2.3 e
	5	97.5 ± 9.6 a	***37.4 ± 0.8 g***	***27.6 ± 0.4 h***	***21.6 ± 0.6 i***
	6	97.8 ± 1.7 a	76.1 ± 1.8 c	69.6 ± 1.5 d	65.8 ± 4.6 d
	7	97.1 ± 2.8 a	76.3 ± 1.5 c	66.5 ± 1.4 d	68.3 ± 2.0 d
	8	103.3 ± 5.3 a	54.4 ± 3.1 f	50.0 ± 2.1 f	48.4 ± 2.0 f
	9	98.7 ± 2.4 a	68.0 ± 0.8 d	73.3 ± 1.2 c	77.8 ± 0.7 c
	10	101.6 ± 2.0 a	86.2 ± 0.7 b	65.8 ± 1.2 d	66.7 ± 1.2 d
	11	102.4 ± 3.6 a	86.5 ± 5.0 b	79.5 ± 3.4 c	76.5 ± 0.5 c
	12	97.2 ± 2.8 a	59.4 ± 1.5 e	52.4 ± 1.0 f	49.6 ± 3.0 f
	13	97.9 ± 2.1 a	65.0 ± 2.9 d	62.5 ± 0.7 d	63.7 ± 1.3 d
	14	98.0 ± 3.5 a	66.0 ± 1.0 d	66.4 ± 2.2 d	71.3 ± 1.6 c
	17	99.4 ± 2.1 a	71.4 ± 5.4 c	70.6 ± 2.7 c	71.2 ± 1.2 c
	18	101.7 ± 3.1 a	61.7 ± 2.2 d	52.9 ± 0.7 f	52.0 ± 2.8 f
	19	97.0 ± 2.0 a	78.4 ± 3.9 c	69.2 ± 1.3 d	68.4 ± 3.3 d
	20	96.7 ± 2.0 a	70.0 ± 1.6 d	65.3 ± 1.3 d	63.2 ± 2.2 d
	21	102.8 ± 2.9 a	**87.6 ± 1.2 b**	76.1 ± 1.8 c	74.7 ± 3.6 c
	22	96.6 ± 4.0 a	73.0 ± 1.9 c	62.5 ± 2.8 d	66.9 ± 1.7 d
	23	102.7 ± 3.9 a	86.4 ± 1.6 b	79.4 ± 0.9 c	79.0 ± 0.5 c
	24	98.4 ± 2.7 a	78.0 ± 3.2 c	73.5 ± 0.6 c	72.8 ± 2.6 c
2019	1	97.9 ± 1.2 a	61.3 ± 2.7 d	57.9 ± 2.4 e	84.2 ± 1.2 b
	3	99.4 ± 3.8 a	68.3 ± 1.5 c	**63.0 ± 2.6 d**	**90.1 ± 6.1 b**
	5	102.7 ± 4.7 a	***47.1 ± 5.9 f***	***19.2 ± 2.3 h***	***19.5 ± 2.7 h***
	9	97.1 ± 11.4 a	68.7 ± 3.6 c	53.0 ± 2.1 e	62.5 ± 1.2 d
	18	98.5 ± 1.0 a	70.9 ± 5.5 c	36.9 ± 1.4 g	45.6 ± 1.6 f
	22	101.5 ± 0.7 a	**82.1 ± 4.0 b**	42.7 ± 2.8 f	55.6 ± 1.8 e

RTDM values are the sum of DM of all the plant parts combined (i.e., roots, stems, leaves, and panicles) for plants in the drought treatment, expressed as percentage of the total DM content in the respective irrigated controls, according to the formula RTDM = $(TDM_{Drought}/TDM_{Irrigated}) \times 100$. Data for 2018 (21 accessions) and 2019 (6 accessions) were analyzed independently. Values with the same letter are not significantly different at $p \leq 0.05$. For each sampling time, the statistically most contrasting accessions were highlighted in color, depicting with dark and light blue the two statistical groups with the least affected accessions (i.e., the most drought-tolerant accessions), and with pink and red the statistical groups comprising the most affected accessions (i.e., the least tolerant accessions); whereas the greatest RTDM value is indicated in bold, and the lowest RDM in bold and italics. DAIDT, days after initiation of the drought treatment.

(0 DAIDT), all the accessions had values close to 100% and they were statistically comparable, indicating no substantial differences between the two treatment plots due to factors unrelated to the drought treatment. After the drought treatment was imposed, all the accessions were significantly and differentially affected by the drought stress at the second sampling date (28 DAIDT), as evidenced by the broad variation found for RTDM values, ranging from 37.4% (in acc. 5) to 87.6% (acc. 21). As the drought treatment progressed, RTDM values were further reduced, with mean values at the end of the drought treatment in the range of 21.6% (acc. 5) to 83.3% (acc. 3). For the last two sampling dates, eight statistically different groups of accessions were revealed for this trait (Table 3). Together, the nearly four-fold difference in RTDM between the least and the most affected accessions, and the large number of statistically different groups found for this trait, suggest broad genetic variation for drought tolerance in the *T. crinita* germplasm.

In general, RTDM was most-severely reduced in accessions 5, 8, 12, and 18, showing significantly lowest mean RTDM values at 56, and 84 DAIDT. In contrast, accessions 3, 1, 23, 9, 11, 21, 24, 14, and 17 exhibited the greatest mean RTDM values for these sampling times (Table 3). Although RTDM was significantly reduced in all the accessions at the first sampling time after the drought stress was imposed (i.e., 28 DAIDT), further variations for this trait differed, in rate and direction, for the different *T. crinita* accessions, as the drought treatment progressed. Thus, eleven accessions evidenced additional significant decreases in RTDM levels from 28 to 84 DAIDT, seven accessions did not vary significantly, and three accessions (accs. 1, 3, and 4) revealed significant increases in this time-frame (Table 3).

Based on these RTDM data, and results from the other drought-response variables analyzed in 2018 (described in sections below), the six most contrasting accessions with regard to drought tolerance were selected, considering accessions 1, 3, and 9 as the most drought-tolerant; and accessions 5, 18, and 22 as drought-sensitive. Accession 22, although its performance for RTDM was rather intermediate (Table 3), generally ranked among the most sensitive accessions for the other variables analyzed, along with accessions 5 and 18, therefore justifying its inclusion in the drought-sensitive group. These six selected accessions were re-evaluated in 2019, using the same methods and experimental procedures as in 2018.

Results for RTDM content in 2019 confirmed the data from the previous season. Thus, broad and significant variation ($p<0.001$) was found for this trait among the accessions, with accessions 1, 3, and 9 (considered as drought-tolerant) presenting significantly greater mean RTDM values than accessions 5, 18, and 22 (considered sensitive) for the last two time points of the drought treatment (Table 3). For these sampling times, and coincidently with results from 2018 using the complete set of 21 accessions, accessions 5 and 3 exhibited the lowest (19.5%) and greatest mean RTDM (90.1%) values, respectively.

3.2 Foliage/root ratio

The foliage/root ratio of *T. crinita* plants under drought stress, relative to this ratio in irrigated controls, referred to as 'relative foliage/root ratio' (RFRR), reflects whether the plant allocates more resources to the roots (RFRR > 100%) or the foliage (RFRR < 100%) under drought conditions, in comparison to controls. As depicted in Figure 2A, in 2018, highly variable and divergent responses were observed early in the drought treatment (28 DAIDT) among the accessions, with mean RFRR values varying from ~49% (indicating greater biomass allocation in the roots) to 168% (greater allocation in the foliage). In general, after this initial response, most of the accessions leveled-off or slightly inverted their trends as the drought stress continued but, in most cases, their RFRR values at 84 DAIDT did not vary much from the initial response at 28 DAIDT (i.e., the accessions that initially allocated more biomass to the roots finished the drought treatment with less than 100% RFRR, and the opposite occurred with accessions that initially allocated more biomass to the foliage). A group of six accessions (14, 6, 10, 11, 17, and 21) presented statistically greater RFRR values than the rest of the accessions at 84 DAIDT, and three of them (11, 21, 14) also ranked among the plant materials with greatest relative foliage biomass (Figure 3). Figure 2B presents RFRR data for year 2019 in the six selected contrasting accession. Accession 5 consistently allocated the greatest proportion of biomass to its roots, as indicated by the significantly lowest RFRR values found in this material for both years and all the time-points. On the drought-tolerant extreme, in 2019 accession 1 revealed the greatest mean RFRR value at the end of the experiment, but without reaching statistical differentiation with the rest of the accessions.

3.3 Photoassimilates partitioning

Figure 3 depicts time-course variation for relative biomass content in the foliage and roots of all the accessions grown under drought conditions, and expressed as percentage of the DM content in the foliage (RFDM) and roots (RRDM) of control plants. As expected, at 0 DAIDT, all the accessions had RFDM and RRDM values close to 100%, being not statistically different from each other (data not presented). After the drought stress was imposed, significant variation among the accessions was found for RFDM and RRDM at all the time points analyzed, with mean values for both traits generally decreasing in a genotype-dependent fashion as the drought conditions progressed. Thus, after 56 and 84 DAIDT, mean RFDM, which includes all the above-ground organs (stems, leaves, and panicles), varied from 19.6% to 87.4% across the accessions, whereas RRDM ranged from 26.2% to 89.7%. These data indicate broad genetic variation in the *T. crinita* germplasm with regard to the accessions ability to maintain root and foliage growth under drought stress.

For RFDM, accession 5 was the most affected plant material, and this was evidenced early during the drought stress (at 28 DAIDT), followed by accessions 8, 18, 12, and 4. Conversely, accessions 11, 3, and 1 were the least affected by drought at 56 DAIDT, with accessions 3 and 11 being also the least affected at 84 DAIDT. While most of the accessions either decreased or maintained statistically unaffected their mean RFDM values as the drought progressed, two accessions, namely accs. 3 and 9, significantly increased their RFDM values at 56 and 84 DAIDT compared to 28 DAIDT.

Variation for RRDM partially coincided with RFDM, as shown in Figure 3, and as indicated by the low to moderate -yet significant- correlation coefficient (r) values obtained between these two variables at 56 DAIDT ($r=0.30$, $p=0.0178$) and 84 DAIDT ($r=0.55$, $p<0.0001$), suggesting some level of independence between the two traits. Under prolonged drought conditions (56-84 DAIDT), mean RRDM was significantly lowest in accession 5; and greatest in accession 1 (at 84 DAIDT) and accessions 9, 13, 4, 24, and 23 (at 56 DAIDT). The majority of the accessions revealed significant decreases in –or maintained statistically unaffected- their mean RRDM values as the drought treatment progressed, with the exception of accessions 1 and 3, which increased their RRDM values at 84 DAIDT.

Results for the six contrasting accessions reevaluated in 2019 fully agreed with data from the previous season, clearly separating the drought-tolerant from the sensitive accessions (Supplementary Figure S3). Briefly, broad and significant variation ($p<0.001$) was found among the accessions for both RFDM and RRDM, with the range of variation increasing as the drought treatment progressed

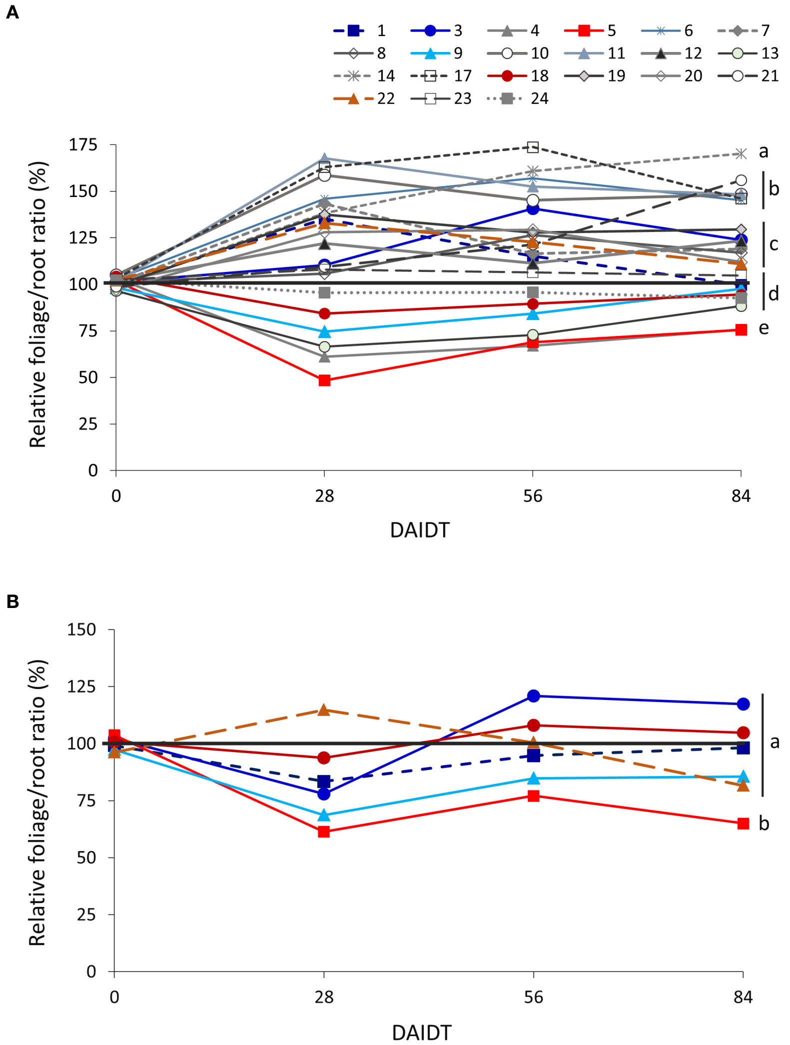

FIGURE 2
Time-course variation for relative foliage/root ratio (RFRR) for dry matter (DM) content in 21 *T. crinita* accessions grown under drought conditions in 2018 **(A)** and six contrasting accessions grown under the same conditions in 2019 **(B)**, over 84 days of drought stress. RFRR is expressed as percentage of the foliage/root ratio in the respective irrigated controls, as calculated by the formula: RFRR = $[(\text{FoliageDM/RootDM})_{\text{Drought}}/(\text{FoliageDM/RootDM})_{\text{Irrigated}}] \times 100$. For each time point, the black horizontal line separates accessions that, under drought stress, allocated relatively more biomass to the roots (RFRR<100%) or to the foliage (RFRR>100%) in comparison to controls. Data points with different letters on the right indicate significantly different mean values at the end of the drought treatment at p<0.05 (DGC test), according to the means comparison analyses for all the accessions, time-points, and years presented in Supplementary Table S3. DAIDT. Days after initiation of the drought treatment.

For the last two sampling times, all the tolerant accessions (accs. 1, 3, and 9) had significantly greater RFDM and RRDM values than the sensitive accessions (accs. 5, 18, and 22). At these time points, accessions 3 and 1 were the least affected, and accession 5 the most affected, for both variables.

Further analysis investigated DM partitioning to individual organs of the plant under irrigated and drought conditions. ANOVA for the percentage of the total DM allocated in roots, stems, leaves, and panicles revealed significant effects for the accession, the drought/irrigation treatment, the year of cultivation, the sampling time, and many of their interactions (Supplementary Tables S1, S2). Figure 4 depicts the variation found among the accessions for photoassimilates partitioning to these organs under irrigated and drought conditions. Nine accessions (accs. 6, 7, 10, 11, 14, 17, 19, 20, 22) consistently revealed significant decreases in DM partitioning to the roots for all the time points considered after the drought stress was imposed (i.e., 28, 56, and 84 DAIDT). Conversely, three other accessions (accs. 4, 5, and 13) consistently exhibited significantly greater biomass partitioning to the roots during the entire drought treatment. For the remaining eight accessions, no consistent variation was observed in photoassimilates partitioning to the roots. Overall, under drought conditions, biomass partitioning to the roots was greatest in accession 5, presenting 25-32% of the total plant DM allocated in its roots, whereas accession 21 had the lowest partitioning to this organ, accounting for 14-18% of the total DM.

Partitioning to individual above-ground organs of the plant varied significantly among the accessions, and between the drought and irrigated treatments within each accession (Figure 4). In some accessions, drought stress was associated with reduced biomass

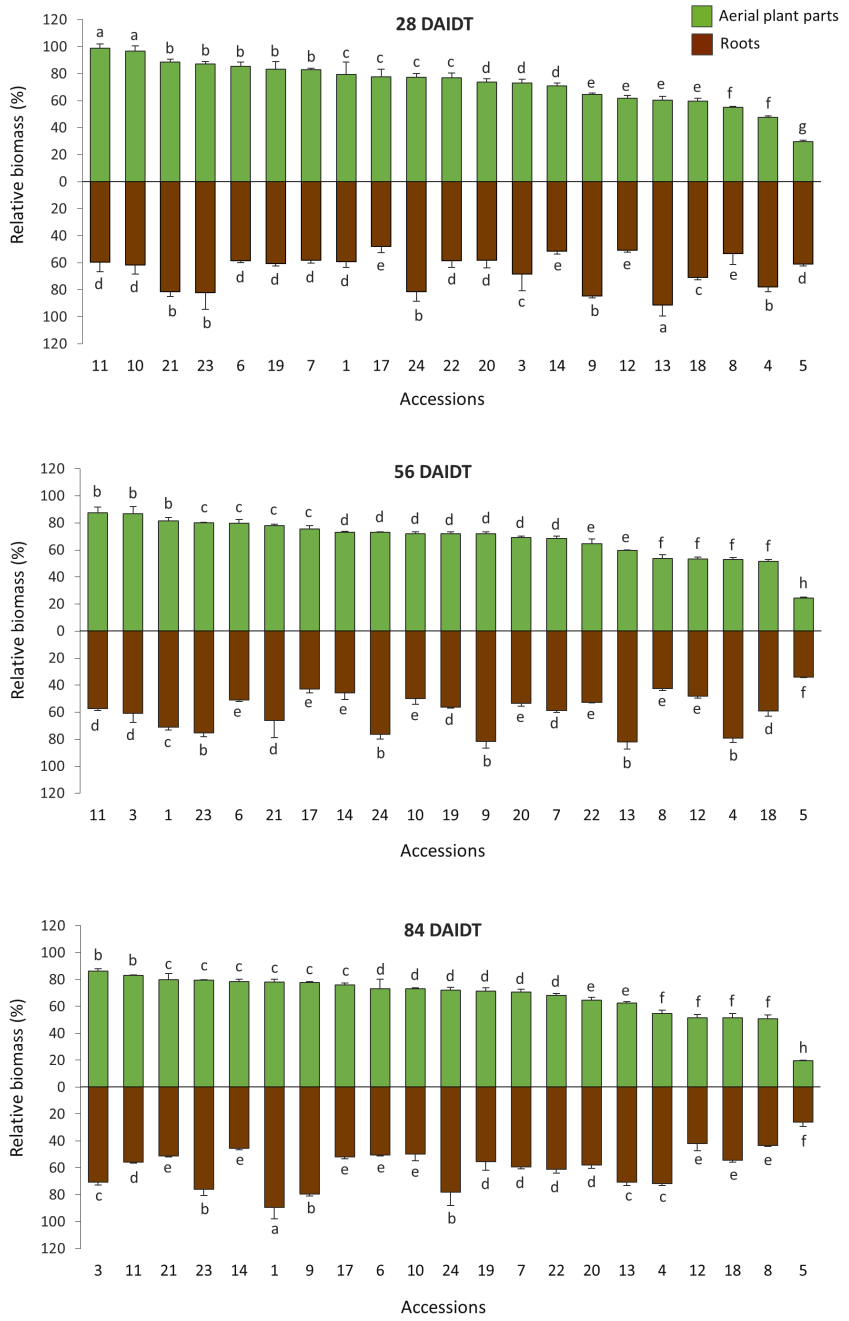

FIGURE 3
Time-course variation for relative foliage (RFDM) and roots dry matter (RRDM) content in 21 *T. crinita* accessions grown under drought conditions in 2018. Data are expressed as percentage of dry matter (DM) relative to the DM in these plant parts in their respective irrigated control plants, according to the formula ($DM_{Drought}/DM_{Irrigated}$) × 100. Foliage dry matter is the sum of DM values for stems, leaves, and panicles. Bars represent means of three biological replicates ± standard deviations. Data for 28, 56, and 84 days after initiation of the drought treatment (DAIDT) are presented, whereas baseline data (0 DAIDT) are not shown because all mean values were ~100% and not statistically different from each other. For each time point, data are presented in decreasing order based on the accessions RFDM levels. Bars not sharing a common letter are significantly different at $p<0.05$, DGC test.

partitioning to the leaves while increasing partitioning to the stems and panicles. This was more evident at the end of the drought treatment (84 DAIDT), when ten accessions exhibited significantly greater DM allocation in the stems and panicles, and nine accessions had reduced DM in leaves. Some exceptions to this trend were accessions 4, 5, and 18, which exhibited significantly lower partitioning to the stems consistently during all the drought-stress period (i.e., at 28, 56, and 84 DAIDT), and accession 7, showing the same performance for the last two time points. For the rest of the accessions, no consistent variations in DM allocation to specific above-ground organs were observed during the drought treatment.

Considering the six selected accessions, no direct association was found between the patterns of photoassimilates partitioning to

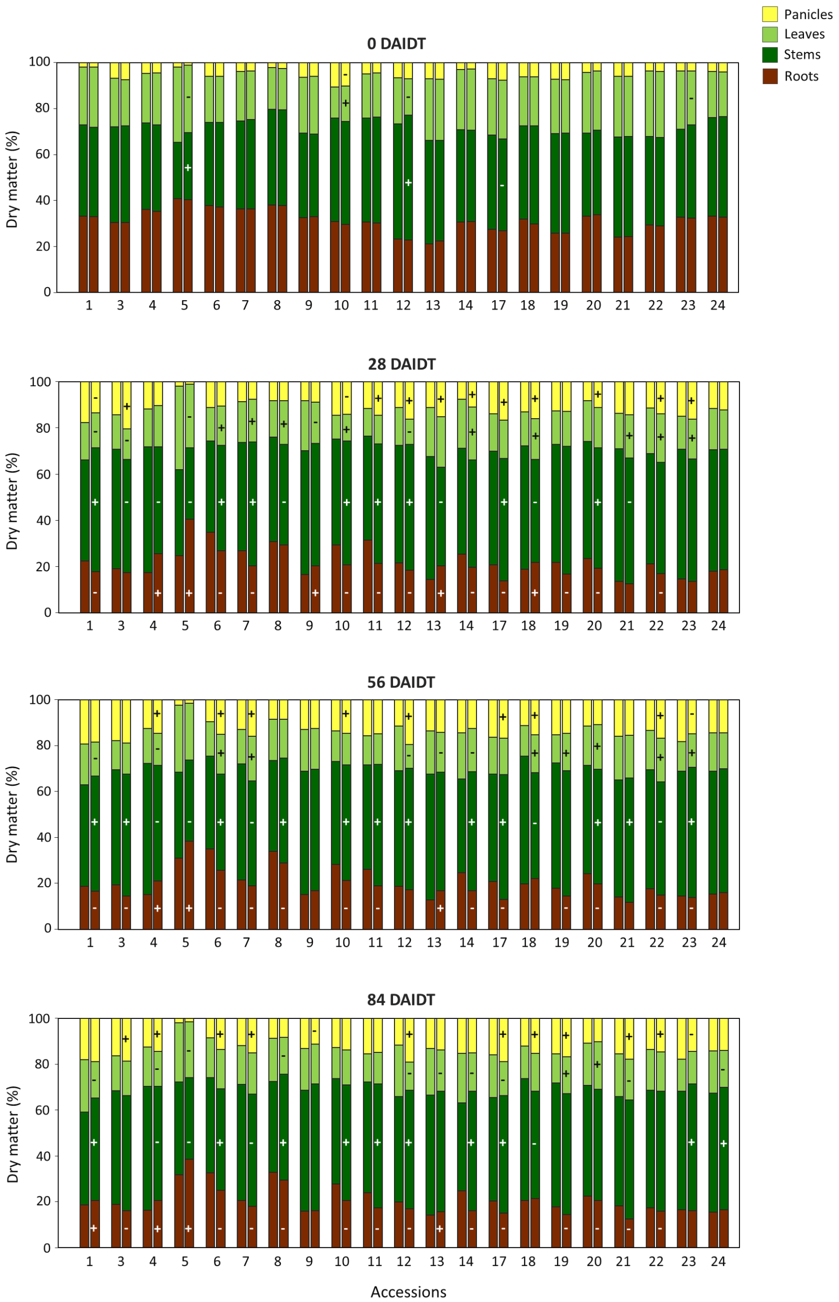

FIGURE 4
Percentage of the total dry matter (DM) per plant partitioned to different organs before the drought treatment (0 DAIDT), and 28, 56, and 84 days after initiation of the drought treatment (DAIDT) for 21 *T. crinita* accessions grown under irrigated (left bar) and drought conditions (right bar) in 2018. Plus (+) and minus (-) symbols in the drought treatment bar indicate significant increase and decrease in DM partitioning to a particular organ, respectively, relative to the irrigated (control) plants, at p<0.05, DGC test.

different plant organs under drought conditions with the two subclasses of accessions (i.e., tolerant *vs.* sensitive). For example, within the drought-sensitive group, accession 5 increased partitioning to the roots and decreased DM in the leaves, whereas accession 22 decreased DM allocation in the roots while increasing its content in panicles, and their performances were consistent throughout the drought treatment (Figure 4). In the 'tolerant' subgroup, accession 1 consistently revealed reduced DM in leaves while it was increased in stems, whereas no clear trend was observed for accessions 3 and 9.

In 2019, data for biomass partitioning to different plant parts generally agreed with results from the previous year (Supplementary Figure S4). Again, no clear association was found between DM partitioning patterns under drought conditions with the two subgroups of accessions. Coincidently with results from 2018, under drought stress, accession 5 consistently showed increased DM allocation in the roots and decreased DM in the stems, relative to its control, and presented the greatest percentage of DM allocation in the roots for all the accessions,

3.4 Leaf area

ANOVA for relative total leaf area (RLA) revealed significant effects for accession and sampling time (but not for year), and for all the interactions among accession, sampling time, and year (Table 2). Figure 5 depicts time-course variation for RLA for both years of experiments. In 2018, the drought treatment significantly reduced RLA in all of the accessions, as evidenced by the sudden and significant drop in mean RLA values from 28 DAIDT to the end of the experiment (Figure 5A). For most of the accessions, such decay in RLA further progressed during the drought treatment, reaching minimum values at 84 DAIDT. However, for a few accessions (namely, accs. 1, 3, 7, 9, 17, and 20), mean RLA values rapidly dropped at the beginning of the drought stress (28 DAIDT), along with most other accessions, but then they remained statistically invariable throughout the rest of the drought treatment. As result, these accessions had the greatest RLA at the end of the experiment (84 DAIDT), together with accession 23. Similar results were obtained in 2019 for the subset of six selected accessions, with the 'tolerant' accessions 1, 3, and 9 exhibiting significantly greater RLA than the 'sensitive' accessions 5, 18, and 22 at the end of the drought treatment (Figure 5B).

In order to integrate and compare the relative leaf area of the accessions during the entire drought treatment, the RLA data for the four time points analyzed were plotted in a graph (the one shown in Figure 5) and the 'area under the curve' (AUC) was calculated for each accession. Comparisons of the resulting mean AUCs revealed significant variation among the accessions ($p<0.05$), with accessions 21 and 6 showing the greatest AUC, and accessions 5, 12, and 4 the lowest AUC (Supplementary Figure S5A). In 2019, accession 9 had the greatest mean AUC and accession 5 the lowest, whereas the rest of the accessions (accs. 1, 3, 18, 22) were intermediate and statistically comparable (Supplementary Figure S5B).

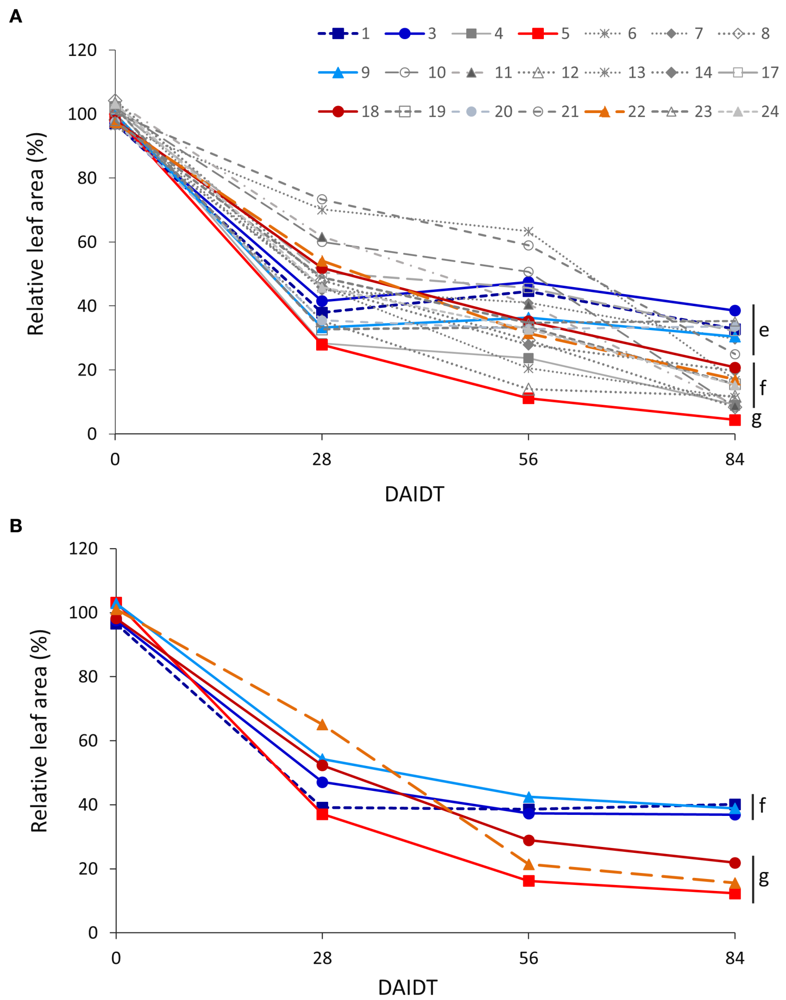

FIGURE 5
Time-course variation for relative leaf area (RLA) per plant for 21 *T. crinita* accessions grown under drought conditions in 2018 **(A)**. The experiment was partially replicated with six contrasting accessions in 2019 **(B)**. RLA is expressed as percentage of the total leaf area in the respective irrigated controls, as calculated by the formula: RLA = $(LA_{Drought}/LA_{Irrigated}) \times 100$. Data points with different letters on the right indicate significantly different mean values at the end of the drought treatment at $p<0.05$ (DGC test), according to the means comparison analyses for all the accessions, time-points, and years presented in Supplementary Table S4. DAIDT. Days after initiation of the drought treatment.

3.5 Leaf chlorophyll index

Mean leaf chlorophyll index (CI) in *T. crinita* plants grown under drought stress, relative to the CI of irrigated controls, referred to as 'relative CI' (RCI), varied significantly among the accessions and among time-points during the drought treatment (Figure 6). In 2018, little variation was observed among the accessions during the first 42 DAIDT, with most of the genotypes presenting CI values statistically comparable to their irrigated controls (data not presented), but as the drought stress progressed, the CI values of all but one of the accessions dropped, at varying rates (Figure 6A). A subgroup of nine accessions (accs. 5, 6, 7, 8, 18, 19, 20, 21 and 22) revealed the most rapid decay in mean RCI values, exhibiting these accessions the lowest RCI levels at the end of the drought treatment. The latter subgroup included accessions 5, 18, and 22, considered as drought-sensitive. These three materials, and accession 5 in particular, exhibited the lowest mean RCI values of all at 84 DAIDT. Conversely, accessions 9, 1, and 3 showed more gradual decays in RCI levels during the drought treatment, ending these accessions with the greatest RCI values of all at 84 DAIDT. Considering the entire germplasm collection, accession 9 was the least affected of all, exhibiting RCI values statistically comparable to the irrigated control until 63 DAIDT (data not shown), and then gradually decreased to end up with the greatest mean RCI value at 84 DAIDT, along with accessions 1 and 3. The rest of the accessions exhibited intermediate performances between these two sets of contrasting materials. Supplementary Figure S6A presents mean RCI values integrated throughout the drought treatment, expressed as AUC, for all the accessions in 2018. Such integrated analysis revealed eight significantly different groups, with accessions 5 and 9 being the plant materials with lowest and greatest mean AUC, respectively, in full agreement with their time-course performances shown in Figure 5A.

In the 2019 experiment, RCI data for the six selected accessions varied following a similar pattern as in 2018, with the drought-tolerant accessions (1, 3, and 9) presenting a delayed and more

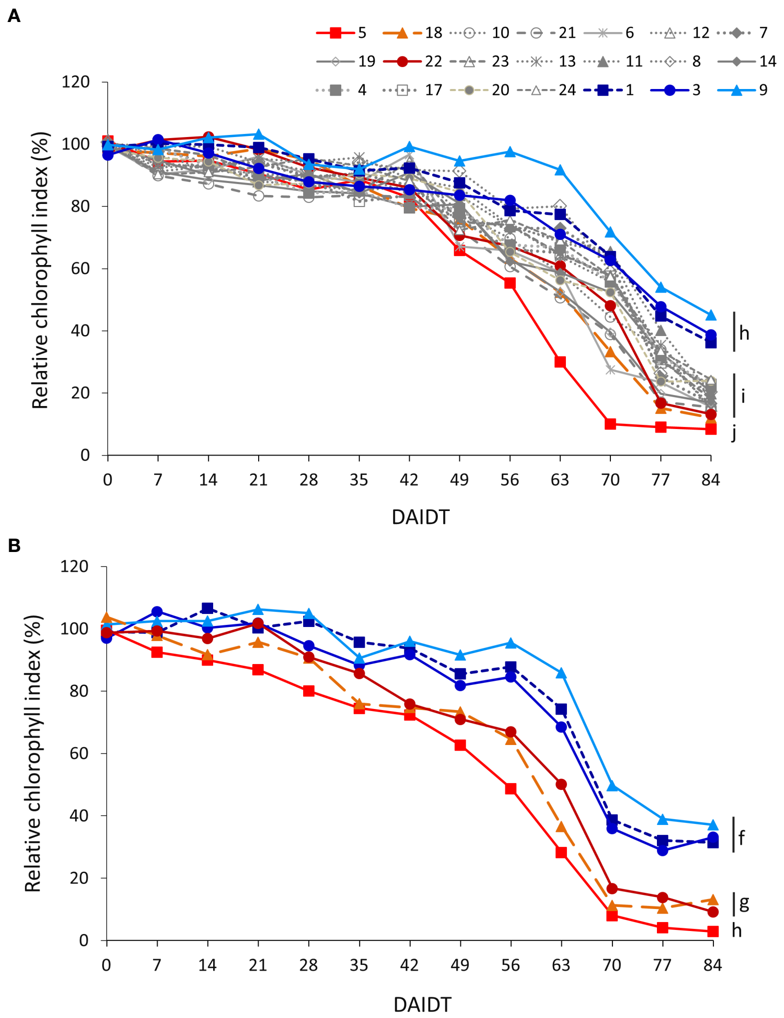

FIGURE 6
Time-course variation for relative chlorophyll index (RCI) (SPAD units) for 21 *T. crinita* accessions grown under drought conditions in 2018 **(A)**. The experiment was partially replicated with six contrasting accessions in 2019 **(B)**. RCI is expressed as percentage of the chlorophyll index in the respective irrigated controls, as calculated by the formula: RCI = $(CI_{Drought}/CI_{Irrigated}) \times 100$. Data points with different letters on the right indicate significantly different mean values at the end of the drought treatment at p<0.05 (DGC test), according to the means comparison analyses for all the accessions, time-points, and years presented in Supplementary Table S5. DAIDT. Days after initiation of the drought treatment.

gradual decay of their mean RCI values, as compared to the sensitive accessions (5, 18 y 22), resulting the formers in significantly greater RCI values at the end of the experiment (Figure 6B). Consequently, the AUC for this variable was also significantly greater in all the tolerant accessions as compared to the sensitive materials, with accessions 5 and 9 representing the most contrasting extremes (Supplementary Figure S6B).

3.6 Photochemical efficiency of photosystem II (Fv/Fm)

The photochemical efficiency (PE) of photosystem II (estimated by Fv/Fm ratio) in leaves of *T. crinita* plants grown under drought stress, relative to the PE of irrigated controls, referred to as 'relative PE' (RPE), was significantly influenced by the accession, sampling time, year of cultivation, and most of their interactions (Table 2). Figure 7 depicts the time-course variation for RPE in all the accessions during the drought treatment. In 2018, little variation was observed among the accessions during the first 35 DAIDT, with most of the accessions presenting PE values statistically comparable to their irrigated controls (i.e., mean RPE values were close to 100%), but as the drought stress progressed, RPE values of all the accessions began to decrease, at varying rates, with the most abrupt decay observed between 56 and 63 DAIDT for the accessions exhibiting the fastest decline in RPE levels (Figure 7A). From 56 DAIDT to the end of the experiment, the drought-stress response of the accessions became evidently and significantly different, varying from genotypes that maintained high levels of RPE (>70%) until the end of the drought treatment (e.g., accs. 3, 1, and 9) to genotypes

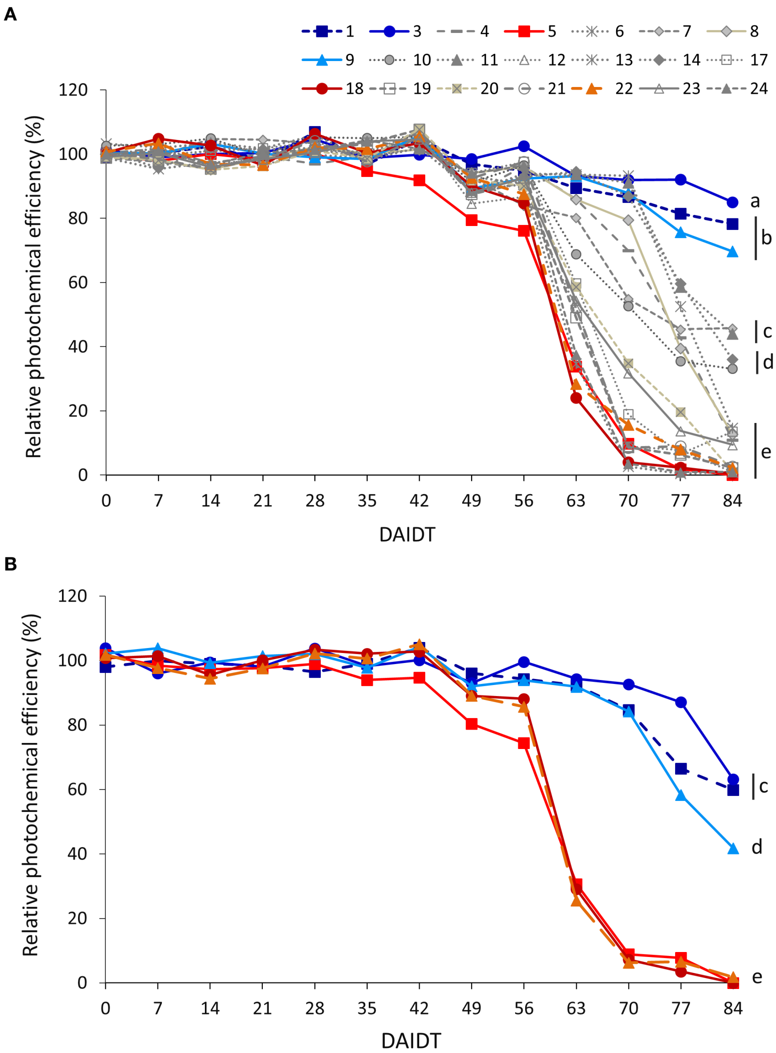

FIGURE 7
Time-course variation for relative intrinsic photochemical efficiency (RPE) of photosystem II, estimated by the ratio 'variable chlorophyll fluorescence/maximum chlorophyll fluorescence' (Fv/Fm), for 21 *T. crinita* accessions grown under drought conditions in 2018 **(A)**. The experiment was partially replicated with six contrasting accessions in 2019 **(B)**. RPE is expressed as percentage of the intrinsic photochemical efficiency (IPE) in the respective irrigated controls, as calculated by the formula: RPE = $(PE_{Drought}/IPE_{Irrigated}) \times 100$. Data points with different letters on the right indicate significantly different mean values at the end of the drought treatment at p<0.05 (DGC test), according to the means comparison analyses for all the accessions, time-points, and years presented in Supplementary Table S6. DAIDT. Days after initiation of the drought treatment.

that rapidly decreased their mean RPE, reaching minimum levels at 84 DAIDT close to 0% (e.g., accs. 5, 6, 18, 19, 21, 22, and 24). Supplementary Figure S7A presents integrated mean RPE values throughout the drought treatment and expressed as AUC for all the accessions. As depicted, accession 5 had significantly lowest mean AUC, whereas a group of six accessions (accs. 3, 1, 9, 11, 14, and 13) had the greatest AUC values.

In 2019, RPE data for the six selected accessions varied following a similar pattern as in 2018, with the drought-tolerant accessions (1, 3, and 9) presenting a delayed and more gradual decay of their mean RPE values, as compared to the sensitive accessions (5, 18, and 22), which exhibited an abrupt decay in RPE between 56 and 70 DAIDT, to reach minimums of less than 2% at the end of the experiment (Figure 7B). Coincidently with these results, the AUC for RPE in all the tolerant accessions was significantly greater than in the sensitive accessions (Supplementary Figure S7B). Altogether, the RPE data from both years suggest accessions 3, 1, and 9 as the most drought-tolerant genotypes, and accession 5 as the most sensitive one.

3.7 Stomatal conductance

Mean stomatal conductance (g_s) in *T. crinita* plants grown under drought stress, relative to the g_s of the irrigated control plants, referred to as 'relative g_s' (Rg_s), was significantly influenced by the accession, sampling time, year of cultivation, and most of their interactions (Table 2). In 2018, the effect of the drought stress on Rg_s was evidenced very early in the experiment, showing significant decreases -to varying extents depending on the genotype- for all 21 accessions in the first week of treatment (Figure 8A). In this short period, mean Rg_s values dropped from ~100%, before initiation of the drought treatment, to 24% in acc. 23, and up to 68% in acc. 22. In decreasing order, accessions 23, 24, 3, 1, 7, 11, 14, 9, and 10 were

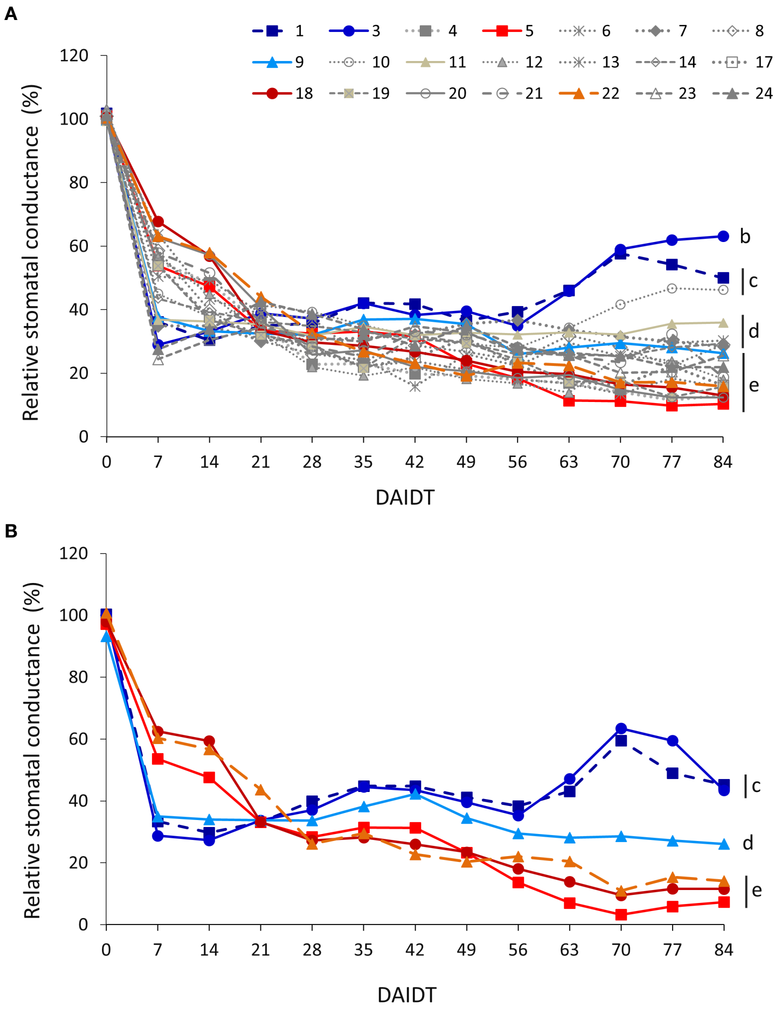

FIGURE 8
Time-course variation for relative stomatal conductance (Rg_s) (mmol m^2 s^{-1}) for 21 *T. crinita* accessions grown under drought conditions in 2018 **(A)**. The experiment was partially replicated with six contrasting accessions in 2019 **(B)**. Rg_s is expressed as percentage of the stomatal conductance (g_s) in the respective irrigated controls, as calculated by the formula: $Rg_s = (g_{sDrought}/g_{sIrrigated}) \times 100$. Data points with different letters on the right indicate significantly different mean values at the end of the drought treatment at p<0.05 (DGC test), according to the means comparison analyses for all the accessions, time-points, and years presented in Supplementary Table S7. DAIDT. Days after initiation of the drought treatment.

the most affected at this time point, exhibiting absolute stomatal conductance values in the range of 10.4-15.2 mmol m^2 s^{-1} (data not presented). However, after this sudden drop in mean Rg_s values, these accessions stabilized and –in some cases- even increased their Rg_s throughout the rest of the drought treatment, exhibiting at the end of the experiment significantly greater stomatal conductance than most of the other accessions. In contrast, the accessions that had a mild decrease in Rg_s at the beginning of the drought stress (7 DAIDT) (e.g., accs. 5, 18, 22, 20, and 24, among others), continued their decreasing trend throughout the rest of the experiment, reaching the lowest Rg_s values of all the accessions, with means in the range of 12.3-16.8% (6.4-9.2 mmol m^2 s^{-1}, in absolute g_s values), at the end of the drought period. Supplementary Figure S8A presents mean Rg_s values integrated throughout the drought treatment and expressed as AUC for all the accessions, showing accessions 3 and 1 with significantly greatest AUC, whereas eight accessions (accs. 4, 12, 13, 19, 17, 23, 5, and 20) comprised the lowest AUC group.

In 2019, Rg_s data for the six selected accessions varied following a similar pattern as in 2018. Thus, the drought-tolerant accessions 1, 3, and 9 exhibited a sudden and more profound decrease in mean Rg_s than the drought-sensitive genotypes (5, 18, and 22) at 7 DAIDT but, as observed in the previous year, the former group stabilized and –for accessions 1 and 3- increased their mean Rg_s levels, ending up with significantly greater Rg_s at 84 DAIDT (Figure 8B). Conversely, the drought-sensitive accessions continued to decrease their Rg_s during the rest of the drought treatment, reaching significantly lowest Rg_s values at the end of the experiment. Interestingly, accession 9 behaved as an intermediate material, being its mean Rg_s at the end of the drought period statistically lower than the Rg_s of accessions 1 and 3, and greater than that of the sensitive genotypes. Coincidently with these results, the AUC for Rgs in 2019 was significantly greatest in accessions 1 and 3, and lowest in accession 5, with the rest of the accessions being intermediate relative to the formers (Supplementary Figure S8B).

3.8 Number of panicles per plant

The number of panicles per plant (NPP) in *T. crinita* accessions grown under drought stress, relative to the NPP in their respective controls, referred to as 'relative NPP' (RNPP), was significantly influenced by the accession, the year of cultivation, the sampling time, and their interactions (Table 2). In 2018, an early response was observed for some of the accessions, either increasing (accs. 4, 7, 10, and 23) or decreasing their RNPP (e.g., accs. 5, 18, 19, 9, and 6) in the first 28-35 DAIDT (Figure 9A). All but one of these accessions stabilized their RNPP values as the drought conditions progressed, to end up with mean RNPP values in the range of 58-98% at 84 DAIDT. Accessions 6 and 10 were the only ones that maintained high RNPP values until the end of the trial, showing no statistical differences with their basal values at 0 DAIDT (i.e., at 84 DAIDT, their RNPP values were ~100%). Accession 5 was the most affected of all, exhibiting an abrupt decay in RNPP early during the drought treatment, which then slowly decreased until the end of the experiment to a final value of 19% RNPP. In 2019, RNPP data generally coincided with results from the previous year, with accession 5 revealing a very similar variation pattern as in 2018, atypical from the rest of the accessions, consistently presenting the lowest mean RNPP values throughout the entire drought treatment (Figure 8B). The rest of the accessions followed a similar variation pattern as in the previous year. Although time-course monitoring of this variable did not reveal an early separation of tolerant *versus* sensitive accessions, as observed for other variables, at the end of the drought treatment all the tolerant accessions had significantly greater RNPP values than the sensitive ones, with accessions 3 and 9 being the greater relative number of panicles per plant. Additional comparative analysis of RNPP, expressed as AUC, for both years of data, is presented in Supplementary Figure S9.

3.9 Relationships among drought response variables

Pairwise correlation coefficient values among all the variables at the end of the drought treatment, for both years, are presented in Table 4. In 2018, relative total (RTDM) and foliage biomass (RFDM), two of the most relevant variables reflecting forage yield under drought stress, were strongly correlated with each other ($r=0.98$, $p<0.0001$), and both traits were significantly and positively correlated with all the other variables (r=0.34-0.74, $p<0.01$). This suggest that, under prolonged drought conditions, the accessions with greater forage biomass (relative to irrigated controls) tend to have greater relative levels of total biomass production per plant, root biomass, foliage/root ratio, leaf area, chlorophyll content, stomatal conductance, photosynthetic performance, and inflorescences per plant. In 2019, all the variables were significantly and more-strongly correlated (r=0.55-1.00) than in the previous year, most likely reflecting a sampling bias due to the selection of the six most contrasting accessions used in the analysis. Nonetheless, the majority of the correlations found coincided -in significance and sign- between years, despite the observed differences in the strength of the associations.

3.10 Principal component analysis

Principal component analyses (PCA) with nine variables were conducted, independently, with data from years 2018 and 2019 (Figure 10). In the first year, two principal components (PC) explained, jointly, 74.9% of the total variation, with PC1 accounting for 53.4% of the variation. The variables that contributed most to PC1 were, in decreasing order, RTDM, RFDM, RRDM, RFRR, RLA, RCI, RPE, RGs, and RNPP. A group of nine accessions, located in the right half of the bi-plot, were the most representative of these variables, with accessions 3, 1 and 9 showing the strongest association, whereas most of the remaining accessions were, conversely, located in the left half of the bi-plot, with accession 5 being the one with strongest negative association with the variables. In the second year, considering only the selected six most contrasting accessions, the two main components

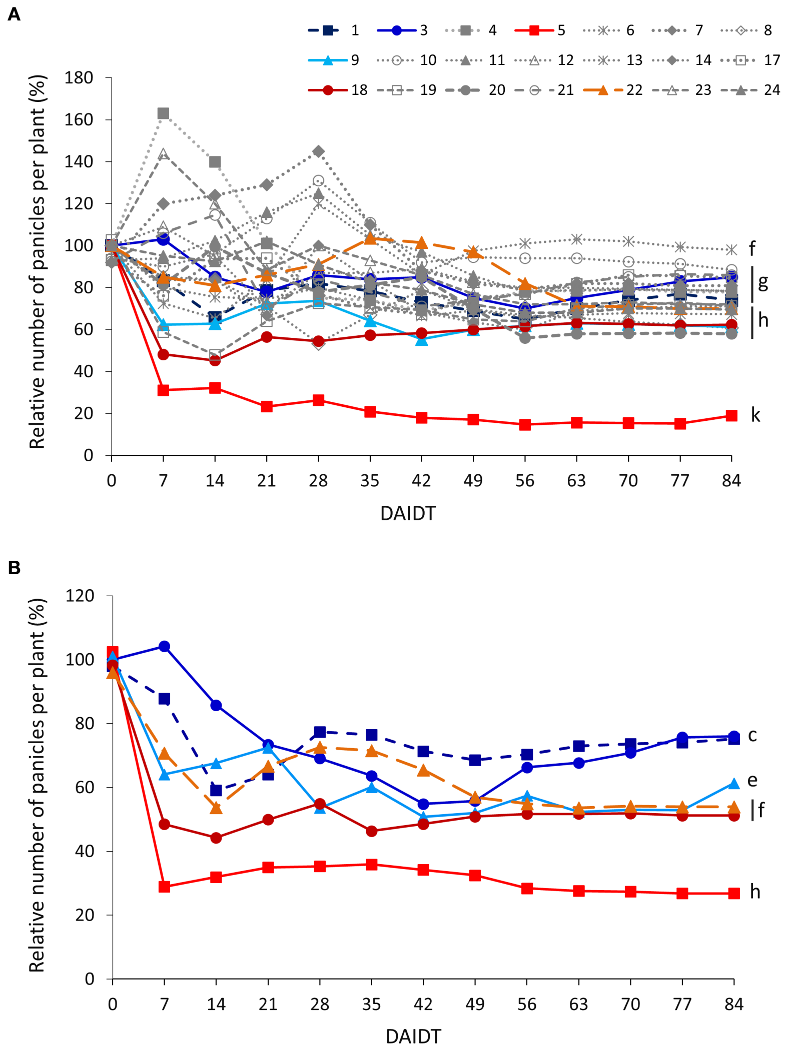

FIGURE 9
Time-course variation for relative number of panicles per plant (RNPP) for 21 *T. crinita* accessions grown under drought conditions in 2018 **(A)**. The experiment was partially replicated with six contrasting accessions in 2019 **(B)**. RNPP is expressed as percentage of the number of panicles per plant (NPP) in the respective irrigated controls, as calculated by the formula: RNPP = ($NPP_{Drought}/NPP_{Irrigated}$) × 100. Data points with different letters on the right indicate significantly different mean values at the end of the drought treatment at p<0.05 (DGC test), according to the means comparison analyses for all the accessions, time-points, and years presented in Supplementary Table S8. DAIDT. Days after initiation of the drought treatment.

accounted for 92.2% of the total variation, with PC1 explaining 86.6% and PC2 5.6%. In general, the same variables that were associated with PC1 in 2018, where also associated with this component in 2019. The drought tolerant and sensitive accessions were located on the left and right side of the bi-plot, respectively, with accessions 3 and 5 representing the most contrasting plant materials, as found in the previous year.

4 Discussion

The present work investigated variation for drought tolerance in a genetically-diverse germplasm collection of 21 *Trichloris crinita* accessions under natural field conditions by means of monitoring nine morpho-physiological traits associated with drought responses in plants during an 84-days drought treatment, using a partially-replicated two-year experiment. To the best of our knowledge, this is the most comprehensive study published to date concerning drought tolerance in this species, with regards to the number of genotypes, variables, and genetic environments (years) analyzed. Thus, although previous studies have generated valuable information, evidencing genetic variation for drought tolerance in this species, they evaluated relatively few germplasm (2-4 accessions or ecotypes) and traits under a single genetic environment (Greco and Cavagnaro, 2003; Quiroga et al., 2013; Marinoni et al., 2020). Considering that *T. crinita* is a native species of arid and semi-arid regions, covering extensive geographical areas, and promoted for range grazing and revegetation of degraded lands, a relevant aspect of this study is the fact that the experiments were carried out under field conditions, thereby facilitating extrapolation of the results to the species natural environment, whereas previous studies were conducted under controlled conditions, in pots (Greco and

TABLE 4 Pairwise Pearson correlation coefficient (r) values among nine morpho-physiological traits for *T. crinita* accessions after 84 days of drought stress, for years 2018 and 2019.

	RTDM	RFDM	RRDM	RFRR	RLA	RCI	RPE	RGs	RNPP
RTDM		0.98***	0.69***	0.34**	0.61***	0.55***	0.51***	0.45**	0.68***
RFDM	1.00***		0.53***	0.52***	0.55***	0.46**	0.47**	0.48**	0.74***
RRDM	0.95***	0.93***		-0.42**	0.51***	0.66***	0.42**	0.25*	0.30*
RFRR	0.75**	0.79**	-0.55*		0.03	-0.18	0.05	0.23	0.54***
RLA	0.77**	0.78**	0.71**	0.69**		0.46**	0.35**	0.17	0.30*
RCI	0.83***	0.83***	0.82***	0.62**	0.87***		0.64***	0.41**	0.16
RPE	0.86***	0.86***	0.78**	0.61**	0.75**	0.71**		0.68***	0.21
RGs	0.89***	0.88***	0.84***	0.61**	0.77**	0.71**	0.93***		0.41**
RNPP	0.99***	0.99***	0.94***	0.78**	0.81***	0.86***	0.84***	0.88***	

The diagonal gray boxes separate data for 21 accessions grown in 2018 (upper half) and six accessions in 2019 (lower half). RTDM, relative total dry matter; RFDM, relative foliage dry matter; RRDM, relative root dry matter; RFRR, relative foliage/root ratio; RLA, relative leaf area; RCI, relative chlorophyll index; RPE, relative photochemical efficiency of photosystem II; RGs, relative stomatal conductance; RNPP, relative number of panicles per plant. *, **, *** indicate significant correlation at p<0.05, p<0.01, and p<0.001, respectively.

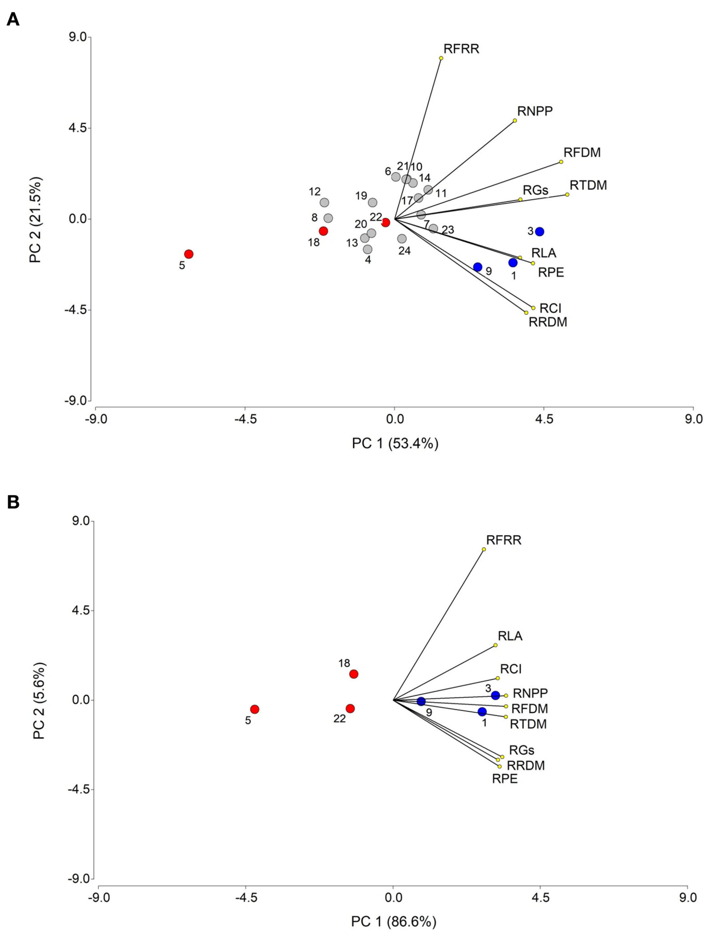

FIGURE 10
Principal component analysis (PCA) of nine biometric and physiological traits associated with drought tolerance, for 21 *T. crinita* accessions in 2018 **(A)** and 6 accessions in 2019 **(B)** grown under drought conditions for 84 days. The numbers of the accessions refer to the plant materials described in Table 1, indicating with blue and red circles the selected drought-tolerant and sensitive accessions, respectively, evaluated over two years, whereas the rest of the accessions are denoted in gray circles. Lines starting from the center point of the bi-plot depict the positive or negative association of the parameters with the two principal components (PC1 and PC2). RTDM, relative total dry matter; RFDM, relative foliage dry matter; RRDM, roots relative dry matter; RFRR, relative foliage/root ratio; RLA, relative leaf area; RCI, relative chlorophyll index; RPE, relative photochemical efficiency (RPE) of photosystem II, RGs, relative stomatal conductance; RNPP, relative number of panicles per plant.

Cavagnaro, 2003; Quiroga et al., 2013) or hydroponics in a growth chamber (Marinoni et al., 2020).

Previous evaluations of *T. crinita* germplasm for drought tolerance compared ecotypes or accessions collected from geographical sites varying in water availability [with ranges for mean annual precipitation in the collection sites of 104-324 mm (Greco and Cavagnaro, 2003), 326-625 mm (Quiroga et al., 2013), and 179-1142 mm (Marinoni et al., 2020)], generally finding positive associations between the level of aridity at the collection site and drought tolerance, as estimated by different plant growth and physiological parameters. For instance, Greco and Cavagnaro (2003) found that the accession from the site with greatest aridity was less affected by drought –as compared to irrigated control plants- than the other two accessions originated from less arid regions, reporting greater relative levels of total DM, shoot DM (calculated as the sum of DM in leaves, culms, and sheaths), and total leaf area associated with drought tolerance. Similarly, Marinoni et al. (2020) found that, under drought conditions, ecotypes from the driest collection sites had greater shoot and root biomass yield and greater number of tillers per plant (relative to irrigated controls) than ecotypes from less arid regions. Also, Quiroga et al. (2013) evaluated two ecotypes from different environments, finding that the one from the most arid region was less affected by drought than the one from humid origin, and the tolerant phenotype was associated with a slower extraction of water from the soil, lower leaf senescence rate, and greater leaf expansion rate.

In the present work, which included accessions derived from geographical sites varying in mean annual precipitation from 104 to 519 mm and mean annual temperature from 14.4 to 20.4 °C (Table 1), we found no significant correlations between aridity levels in the collection sites [estimated by the aridity indices of De Martonne (1926) and Zomer et al. (2022), and mean annual precipitation data] and the vast majority of the variables analyzed, for the last two sampling times in both years (Supplementary Table S9). Only RNPP, RPE, and RGs had significant negative correlations with some of the aridity estimates, but most of these associations were rather weak and marginally significant ($r = -0.25$ to -052, $p = 0.026$-0.049), and they were inconsistent across years and sampling times. These results suggest no generalized adaptive advantage for drought tolerance associated with the level of aridity in the native environments of the accessions. Furthermore, the two most contrasting materials, accessions 3 (most-tolerant) and 5 (most-sensitive), derived from environments with similar aridity levels, as indicated by comparisons of their mean annual precipitations (327 *vs.* 247 mm) and aridity indices by De Martonne (12.2 *vs.* 9.4) and Zomer et al. (0.15 *vs.* 0.14); whereas collection sites with relatively low (annual precipitation > 400 mm) and high aridity (annual precipitation < 220 mm) both included accessions varying broadly in their drought tolerance responses (Table 1). For example, accessions 1 (tolerant) and 22 (sensitive) derived from high-aridity environments, whereas accessions 14 (tolerant, based on total and foliage biomass yield) and 18 (sensitive) derived from La Pampa, the site with greatest annual precipitation (519 mm). Thus, conversely to previous studies suggesting an adaptive drought-tolerance advantage associated with the level of aridity in the accessions natural habitats (Greco and Cavagnaro, 2003; Quiroga et al., 2013; Marinoni et al., 2020), our results rather suggest that different genotypes varying in drought stress tolerance coexist in *T. crinita* natural populations derived from a particular location or environment, regardless of water availability, at least for the range of aridity conditions sampled in the present work. In full agreement with this hypothesis, we previously found that molecular marker (AFLP)-based genetic clustering of these same *T. crinita* accessions was not associated with geographical origin or habitat conditions, and suggested that *T. crinita* natural populations were genetically heterogeneous (Cavagnaro et al., 2006). Further support for genetic heterogeneity in *T. crinita* natural populations comes from studies -using this same germplasm collection- reporting lack of association between geographical origin or aridity levels in the accessions collection sites and various morphometric and quantitative agronomic traits, including forage productivity (Cavagnaro et al., 2006) and nutritional quality (Dominguez et al., 2022), as well as karyotype and cytogenetic characterizations (Kozub et al., 2019). Presumably, differences in the number and nature of the plant materials analyzed (2-4 ecotypes or populations *vs.* 21 accessions derived from single-plant descendants), and the range of aridity in the original habitats (e.g., range for mean annual precipitation was 179-1142 mm in Marinoni et al. (2020) *vs.* 104-519 mm in this work) may partially account for these discrepancies between previous works and the present study. It should be noted that the fact that the three aridity estimates used in our correlation analyses [i.e., the mean annual precipitation, and indices of De Martonne (1926) and Zomer et al. (2022)] yielded comparable results, including those from analysis using the mean annual precipitation (as used in previous studies), suggesting that these discrepancies are not due to differences in the choice of aridity estimators across studies.

Drought stress can strongly influence many plant growth, physiological, and biochemical parameters. In the present study, under prolonged drought stress (84 DAIDT), nearly all the accessions significantly reduced total-, foliage-, and root DM content, as well as total leaf area, chlorophyll content, photochemical efficiency, stomatal conductance, and number of panicles per plant, in comparison to their respective irrigated controls (Figures 3, 5-9). The very few exceptions were accessions 1 (for root DM), 3 (for photochemical efficiency), and 6 (for number of panicles per plant), which had levels of these variables that were statistically comparable to their controls (Figures 3, 7, 9). These data agree with our previous study reporting significantly reduced total-, aerial-, and root DM contents, and total leaf area in three *T. crinita* accessions grown under drought stress, as compared to their irrigated controls (Greco and Cavagnaro, 2003). Conversely, foliage/root ratio was the only trait in the present study for which divergently different responses were observed at the end of the drought stress, with some accessions exhibiting significant increases while others showed reduced or unaffected values relative to their controls (Figure 2). Interestingly, Greco and Cavagnaro (2003) found that shoot/root ratios did not vary significantly between drought-stressed and irrigated plants for the three accessions tested, whereas Marinoni et al. (2020) reported that, under drought, shoot/root ratios decreased in comparison to irrigated controls in the four

T. crinita ecotypes analyzed by them. Presumably, differences in the genotypes and number of plant materials used across these studies, as well as methodological differences (e.g., experiments conducted under field *vs.* pots conditions) may explain, at least partially, the different responses observed for this trait under drought stress.

Under drought stress, the productivity of a plant depends on some essential processes, such as the temporal distribution of biomass and the partitioning of photoassimilates (Anjum et al., 2017). The limited availability of assimilates, attributable to arrested photosynthesis and impaired partitioning of assimilates, determines the plant growth response under drought stress. The reorganization of assimilates partitioning under these conditions is generally accompanied by alterations in the expression of genes involved in the metabolism and transport of carbohydrates, and differs between drought-tolerant and sensitive genotypes (Aliche et al., 2020). For instance, some plants modify their assimilate distribution as a stress adaptation strategy and accumulate more soluble sugars in the leaf and root cells to concentrate the cytosol for osmotic adjustment, and reduce transport to the reproductive organs. This alteration in the partition of assimilates becomes inevitable for some plants during the limitation of water in the rhizosphere (reviewed by Chaves et al., 2002; Valliyodan and Nguyen, 2006). In contrast, the reduction in starch content in leaves and roots with decreased water supply is due to the conversion of starch to simple sugars for osmoregulation and the increased relationship between respiration and photosynthesis (Galmés et al., 2007). Plants often reallocate assimilates from shoot growth to root growth under drought conditions, increasing root spread into deeper soil layers (Rich and Watt, 2013). Conversely, other studies have reported decreased root growth in plants under drought stress (Tahere et al., 2000; Cui et al., 2008). Altogether, these studies suggest that the root growth response to drought stress depends on the genotype, the intensity and duration of drought stress, and the rate of stress development.

This work compared drought tolerance among the accessions by means of estimating their performances for nine morpho-physiological traits under drought conditions relative to their own irrigated controls, thereby expressing each variable as relative value (%). In crop breeding programs, one of the most commonly used criteria for selecting drought tolerant genotypes, is the use of indices that estimate yield lost under drought in comparison to normal (non-stressed) conditions (Mitra, 2001). Herein, the variables RTDM and RFDM estimate the loss of yield in total and foliage biomass due to drought, as compared to irrigated controls. Based on these variables, after prolonged drought conditions (84 DAIDT), accession 3 was the most tolerant material, consistently for both years, reducing its total plant biomass only 10-17% and its foliage biomass -a direct estimate of the total forageable biomass- 8-14% (Table 3, Figure 3, Supplementary Figure S3). Besides accession 3, other highly-productive accessions at 84 DAIDT were 1, 9, 11, 14, 17, 21, 23, and 24, which exhibited reductions in total and foliage biomass of 16-37% and 17-38% (data for both years), respectively. As comparison, accession 5, which represented the most drought-sensitive extreme, had a reduction of 78-81% and 80-83% of its total and foliage biomass, respectively, for the same time-frame and conditions. However, we observed that some members of the former group of high-biomass yielding materials –namely accessions 11, 14, 17, 21, 23, and 24- exhibited some dead plants and/or plants with a large proportion of dead tissue at the end of the drought treatment, also evidenced by their very low values for variables that reflect photosynthetic activity, such as RCI, RPE, and RGs (Figures 6-8, Supplementary Tables 5-7). Thus, based on these data, we took into consideration other variables, besides relative biomass yield, for the selection of the most tolerant plant materials. As result, accessions 1, 3, and 9 were selected as most tolerant because they consistently presented high relative biomass (estimated by the variables RTDM, RFDM, and RRDM), leaf area (RLA), and non-senescent functional leaves with photosynthetic activity (RCI, RPE, RGs) at the end of the drought treatment.

The most productive and tolerant accessions tended to have significantly greater mean values than the least productive and sensitive materials, for all the traits analyzed, at the end of the drought stress. This tendency was also reflected by the significant and positive correlations found between RTDM and RFDM with the rest of the variables in 2018 (r=0.34-0.74) and 2019 (r=0.75-0.99) (Table 4), and by the strong association between RTDM and RFDM with the most explanatory component in the PCAs of both years, along with most of the other variables (Figure 10). This suggest that under prolonged drought conditions, the tolerant accessions were able to sustain high relative biomass production in the aerial plant parts (predominantly in stems and panicles; Figure 4) and –to a lesser extent- in roots, resulting in greater relative foliage/root ratios and leaf area, exhibiting also greater chlorophyll content, stomatal conductance, and photochemical efficiencies than the drought-sensitive accessions. These results coincide with our previous findings showing that the most drought-tolerant accession had greater relative levels (expressed as a fraction of irrigated controls) of total leaf area, total DM, and shoot DM (equivalent to 'foliage DM' in the present study) than the other two –and more sensitive- accessions (Greco and Cavagnaro, 2003). They also agree with those of Marinoni et al. (2020) reporting that, under drought conditions, the two most tolerant ecotypes had greater relative levels of shoot DM, root DM, and tillers per plant than the more sensitive ecotypes.

Under drought conditions, *T. crinita* accessions varied widely with regards to the drought-response variables analyzed, and this may suggest mechanistic differences between the tolerant and sensitive genotypes in their ability to cope with such stress. For instance, the greater reduction in biomass yield components (RDTM, RFDM, RRDM, RLA) revealed in sensitive accessions could be a consequence of a greater reduction in their photosynthesis rate, presumably due to an earlier stomatal closure (lower RGs; Figure 8) triggered by higher abscisic acid (ABA) concentrations in response to the drought stress (Popova et al., 2000), and greater damage to the photosynthetic apparatus, as suggested by the earlier decay and much lower final RPE values observed –for these accessions- at the end of the drought stress (Figure 7). This drought stress-induced damage is generally accompanied by degradation of chlorophyll pigments, which is also suggested by the faster decay of RCI values in sensitive *vs.* tolerant accessions (Figure 6), as well as reduced concentration and enzymatic activity of Rubisco, weakened electron transport and photosynthesis photophosphorylation, and altered levels of relevant metabolites (Seleiman et al., 2021). In comparison, drought tolerant accessions seem to have some sort of protective mechanisms that postpone and/or attenuate the negative effects of drought stress on these physiological parameters, as indicated by the delayed and/or ameliorated decay in most of the drought-response variables analyzed (Table 1, Figures 3, 5-9). In line with this hypothesis, we previously found that the most drought-tolerant of three *T. crinita* accessions had –at any given time point during the drought treatment- greater leaf water potentials, suggesting a greater efficiency at minimizing the loss of water status in the plant, and the appearance of the first drought symptoms (folded leaves) were significantly delayed (14-28 days), as compared to the more sensitive genotypes (Greco and Cavagnaro, 2003). Additional studies with a few highly-contrasting (tolerant *vs.* sensitive) accessions examining a larger number of physiological parameters and paralleled with global gene-expression analysis (e.g., transcriptome profiling) may be necessary to fully understand the mechanisms underlying drought resistance in this species.

A general belief in plant ecophysiology is the trade-off between the capacity of a genotype to grow when resources are abundant, and its capacity to tolerate resource shortages (Chapin, 1980; Huston, 1994; Bazzaz, 1996). For arid environments, this paradigm predicts a negative association between the potential biomass yield under optimal water availability (i.e., under irrigation) and drought tolerance. Results from our previous work with three *T. crinita* accessions matched the predicted negative association between potential biomass yield and drought tolerance (i.e., the least productive accession under optimal water availability was the most drought-tolerant material, and viceversa) (Greco and Cavagnaro, 2003), thereby supporting the trade-off hypothesis. However, from our present data, using a much larger number of accessions, no performance trade-offs emerged between optimal growth and drought tolerance, as indicated by the absence of significant negative correlations between total and foliage biomass yield under irrigated conditions *versus* the majority of the drought-response variables analyzed [the only exception was RFRR which showed a weak association (r=-0.26 to -0.30) with the former variables in 2018 but not 2019]. In contrast, we found significant positive correlations between potential biomass yield and four major drought-response variables (RTDM, RFDM, RRDM, and RLA), with r values in the range of 0.26-0.61 (data not shown), suggesting that the most productive genotypes under optimal water availability also tend to be more productive under drought stress. In agreement with this line of evidence are two contrasting examples, namely accession 5, the least productive genotype under irrigation and yet the most drought-sensitive one; and accession 9, exhibiting high biomass yield under irrigation and high tolerance to drought. Altogether, our current data does not support the trade-off hypothesis. Instead, they coincide with more recent studies which have explicitly tested, and rejected, this hypothesis in several grass species (Fernandez and Reynolds, 2000; Couso et al., 2010; Jung et al., 2020).

We found broad genetic variation for drought tolerance in this *T. crinita* collection, as indicated by all the drought-response variables analyzed (Tables 2, 3, Figures 3-9). Overall, accessions 3 and 5 were the most extreme and contrasting genotypes in the entire germplasm collection for the majority of the traits analyzed in both years, with accession 3 being most tolerant for five of the nine traits considered (RTDM, RFDM, RLA, RPE, Rg_s) and accession 5 being the most sensitive one for eight traits (RTDM, RFDM, RRDM, RLA, RCI, RPE, Rg_s, and RNPP). More broadly, considering all the traits and years, accessions 1, 3, and 9 were selected as most drought-tolerant, whereas accessions 5, 18, and 22 were considered most sensitive. Considering these two subsets of contrasting accessions, it should be noted that, at the end of the drought treatment, all the tolerant materials had statistically superior ($p<0.05$) performances than the sensitive accessions for nearly all the variables, consistently for both years of data (Table 3, Figures 3, 5-9, Supplementary Tables S4-S8), and this was also reflected by their evident separation in the PCAs integrating all the variables (Figure 10). The only exception was RFRR, for which several tolerant and sensitive accessions overlapped and were statistically comparable at the end of the drought treatment in both years (Figure 4). These most-contrasting accessions will be instrumental for investigating the genetic basis underlying drought tolerance in *T. crinita*. For example, tolerant and sensitive genotypes can be intercrossed to produce F_1 progenies and, by self-pollination of the latter, F_2 segregating populations that can be used for mapping quantitative trait loci (QTL) and -combined with comparative transcriptome analysis of tolerant *versus* sensitive plants- identifying candidate genes for drought tolerance. Although the genome of *T. crinita* has not been sequenced, the increasingly widespread use of high-throughput NGS technologies, such as genotyping by sequencing (GBS) and RNA-Seq, can accelerate the construction of highly-saturated linkage maps with well-resolved QTLs, as well as transcriptome profiling, facilitating further strategies for candidate gene identification. Such approach has been successfully used in other grasses (Gelli et al., 2017; Kiranmayee et al., 2020; Pendergast et al., 2022).

The drought-tolerant accessions identified in this study are valuable materials for revegetation and range grazing in extremely arid regions that would otherwise be agriculturally unexploited. This becomes particularly relevant in the current context of climate change, predicting increased temperatures (e.g., a 2–4°C increase in mean diurnal temperature is predicted by the end of the century for the central West part of Argentina), changes in precipitation patterns, and increased desertification in some regions (International Panel of Climatic Changes, 2014). The drought-tolerant accessions 1 and 9 may be of particular interest in this context, considering their high nutritive value as forage, as indicated by recent findings showing that these two accessions were among the plant materials with greatest crude protein content in this same germplasm collection (Dominguez et al., 2022).

From a breeding perspective, it is desirable to combine drought tolerance with high forage biomass yield and nutritive value. Given that these *T. crinita* germplasm have already been characterized –finding broad and significant variation- for these traits (Cavagnaro et al., 2006; Dominguez et al., 2022; this work), and that the reproductive system of *T. crinita* was recently elucidated and classified as autogamous and self-compatible (Gutierrez et al., 2016; Kozub et al., 2017), it is now theoretically feasible to combine these –and other- traits of interest by sexually intercrossing these materials. For this purpose, it is worthwhile noticing that our 21 accessions represent individual genotypes, as they are single-plant descendants [i.e., each accession derives from seeds obtained from a single mother plant, selected as representative of a particular natural population sampled from the 'Monte' phytogeographical region (Cavagnaro et al., 2006)], thereby facilitating their rapid inclusion in breeding programs, as opposed to plant materials with more complex genetic structures, such as ecotypes (Marinoni et al., 2020) or natural populations (Quiroga et al., 2013), used in other studies.

5 Conclusions

A broad and genetically diverse *T. crinita* collection was characterized for drought tolerance on the basis of quantitative morpho-physiological parameters, revealing significant and ample variation among the accessions for all the traits. Highly-tolerant and sensitive accessions were identified, and they will be used in future studies to investigate the genetic basis underlying drought tolerance in this species. Under prolonged drought conditions, the tolerant accessions were more productive for all the biomass yield components analyzed, and this seemed to be associated with a postponed and more attenuated decrease in variables related to the plant photosynthetic activity, such as stomatal conductance, chlorophyll content, and photochemical efficiency. The tolerant materials identified will be incorporated in breeding programs aiming at developing new varieties that combine drought tolerance with other traits of interest, such as high forage biomass yield and nutritional value, facilitating their widespread use as forage and revegetation of degraded drylands. Altogether, these data provide a platform for future studies and breeding programs for one of the most widely distributed grass species in arid environments of the Americas.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

DD, JC, and PC conceived the project. DD and JR performed the experiments. DD and AL performed most of the analyses. DD and PC wrote the draft. JC, YC, and PC edited the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1235923/full#supplementary-material

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When drought meets heat – a plant omics perspective

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Changes in weather patterns with emerging drought risks and rising global temperature are widespread and negatively affect crop growth and productivity. In nature, plants are simultaneously exposed to multiple biotic and abiotic stresses, but most studies focus on individual stress conditions. However, the simultaneous occurrence of different stresses impacts plant growth and development differently than a single stress. Plants sense the different stress combinations in the same or in different tissues, which could induce specific systemic signalling and acclimation responses; impacting different stress-responsive transcripts, protein abundance and modifications, and metabolites. This mini-review focuses on the combination of drought and heat, two abiotic stress conditions that often occur together. Recent omics studies indicate common or independent regulators involved in heat or drought stress responses. Here, we summarize the current research results, highlight gaps in our knowledge, and flag potential future focus areas.

KEYWORDS

drought, heat, transcriptomics, proteomics, metabolomics

Introduction

Plants are sessile organisms that cannot escape from adverse conditions, and are thus at the mercy of biotic and abiotic environmental factors that strongly affect their growth, survival and performance (Suzuki et al., 2014; Zhang et al., 2022). Furthermore, climate change, especially the change of the limiting factors temperature and water availability, vastly reduces crop yields, which threatens productivity and ultimately food security (Bailey-Serres et al., 2019). These different environmental stresses can be perceived in the same or in different tissues with specific systemic signalling and acclimation responses. For example, plants generally recognize drought stress in the soil through the root system and transmit a signal to the shoot (Takahashi et al., 2018; Maurel and Nacry, 2020), while high temperature stress is predominantly perceived in the aboveground parts (Bita and Gerats, 2013; Hasanuzzaman et al., 2013; Quint et al., 2016). The response of plants to individual heat or drought stress and the underlying specific signalling pathways are well-studied (Quint et al., 2016; Vu et al., 2019b; Gupta et al., 2020), but in several cases these stresses coincide (Figure 1) and thus likely impact plants differently than the individual stresses

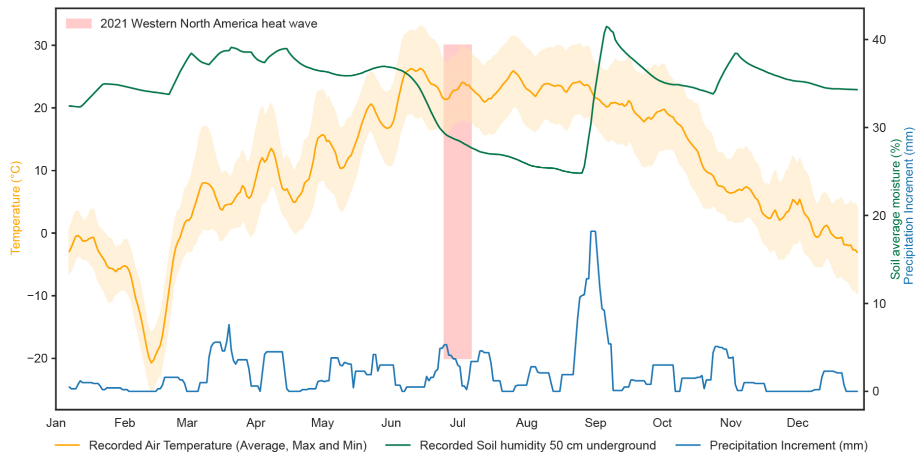

FIGURE 1
Representative meteorological data of high temperature and water availability stress co-occurring in field conditions from the Shagbark Hills (2068) station in Iowa - USA, sourced from SCAN (Soil Climate Analysis Network) (USDA Natural Resources Conservation Service, 2022). The 10-day moving average of air temperature anomalies (orange), soil humidity (%) at 50 cm depth (green) and precipitation increments (blue) were plotted for the year 2021 in the location. Soil average moisture remained stable along the seasonal fluctuations of air temperature, but sharply decreased after the temperature peaked and the 2021 Western North America heat wave (25/06/2021 - 07/07/2021) took place (highlighted in red) (Wikipedia contributors, 2023). The soil moisture level was only restored to its previous level around late August. The data suggest that, in the surrounding crop field areas, plants were exposed to moderate/high temperature stress prior to the exposure to drought.

(Suzuki et al., 2014; Zandalinas et al., 2020a; Zandalinas et al., 2020b). When exposed to combined stressors, different types of interactions, such as additive, synergistic, equalization, dominant, and antagonistic effects can occur, leading to the induction of diverse stress-responsive transcripts, proteins, and metabolites (Shaar-Moshe et al., 2017). Omics studies provide a holistic view on these change and can uncover complex regulatory pathways in which a large number of transcripts, proteins, and metabolites undergo similar or opposite changes, highlighting differences between combined and individual stresses.

Here, we will discuss recent findings related to combined drought and heat stress (referred to as combined stress). We will focus on how plants adapt to this stress combination through developmental and physiological processes and how different omics levels are regulated. Since the majority of omics data is on the aboveground parts of the plants, we mainly describe these (unless stated otherwise) (Supplementary Table 1).

Developmental and physiological responses to individual and combined heat and drought stress

As air temperature rises, the water content in soil tends to decrease, indicating that temperature and water availability stress are likely to co-occur in field conditions (Lobell and Gourdji, 2012; Konapala et al., 2020; Bevacqua et al., 2022; Coughlan de Perez et al., 2023). This outcome of probabilistic meteorological models that highlight the frequency of combined heat and drought events can already be observed in the field (Figure 1). Both drought and heat stress individually influence seed germination, cell division and expansion, photosynthesis, and yield (Avramova et al., 2015; Quint et al., 2016; Nelissen et al., 2018; Vu et al., 2019b; Gupta et al., 2020; Tiwari et al., 2022). Depending on the severity (water content in the soil) and the duration, drought stress can vary considerably. Mild drought stress slows down growth, resulting in a decrease in leaf area and reduction in biomass, and reduces yield (Verelst et al., 2013; Clauw et al., 2015). Severe drought stress has a far more devastating impact on plant physiology, causing growth to nearly cease, plants to wilt and ultimately resulting in plant death (Harb et al., 2010; Muller et al., 2011). Drought also leads to stomatal closure to reduce evaporation as a rapid defence against dehydration (Buckley, 2019; Gupta et al., 2020). Similarly, the impact of temperature stress also depends on the frequency, severity and duration of the stress (Zhu et al., 2022). Exposure to a mildly increased ambient temperature can induce various alterations in plant architecture to move sensitive parts away from high temperature and improve cooling capacity and trigger floral transition (Quint et al., 2016; Vu et al., 2019b). A further increased temperature and a high frequency and/or prolonged duration of high temperature can decrease germination rates, inhibit growth and floral transition, result in a reduction in yield and even lead to plant death (Gan et al., 2014; Chiu et al., 2016; Quint et al., 2016; Wu et al., 2017; Li et al., 2019; Vu et al., 2019b; Li et al., 2020; Zhu et al., 2021; Ying et al., 2022; Zhu et al., 2022). Elevated temperatures can have a positive effect on the photosynthetic rate and carbon assimilation in plants, but this beneficial effect is strongly suppressed once a certain threshold temperature is exceeded (Yamasaki et al., 2002; Shaar-Moshe et al., 2017; Yang et al., 2020). Finally, high temperatures lead to stomatal opening and an increased stomatal conductance associated with leaf cooling, and prolonged exposure to high temperature reduces the stomata number (Yamori et al., 2006; Crawford et al., 2012).

The simultaneous occurrence of high temperature and drought stress can further suppress plant growth and yield compared to their individual effects. When Arabidopsis and different food crops are exposed to combined stress, stems are shorter and leaves are less abundant, the fresh weight and viability of pollen are further decreased, the seed yield and fresh weight are also further decreased compared to control plants and/or to individual stress conditions (Mishra et al., 2018; Choukri et al., 2020; Demirel et al., 2020; Cohen et al., 2021; Lehretz et al., 2021; Li et al., 2022; Mahalingam et al., 2022; Rahman et al., 2022). In contrast, under combined stress, the transpiration response in Arabidopsis is dominantly promoted by heat stress compared to the individual stress, whereas in the individual stress, it is promoted by high temperature but repressed by drought stress compared with normal conditions (Rizhsky et al., 2004). In Arabidopsis and soybean, heat and drought also antagonistically regulate stomatal movement, but in this context drought dominantly decreases stomatal conductance and photosynthesis in combined heat and drought stress conditions (Rizhsky et al., 2004; Sinha et al., 2022).

To identify the molecular machinery involved in plant regulation and acclimation under combined stress conditions, a comprehensive analysis of the transcriptome, proteome, post-translational modifications (PTMs) and metabolome is essential.

Transcriptome responses to combined heat and drought

In Arabidopsis, drought stress significantly impacts gene expression in plants, primarily of genes associated with hormone-mediated growth regulation, response to osmotic stress, reactive oxygen species, salt stress, cell wall modification and cell growth (Clauw et al., 2015). High temperature predominantly induces an up-regulated transcriptional response to heat, protein folding and metabolic process in Arabidopsis (He et al., 2019). A 7-day individual heat or drought treatment leads to more differentially expressed genes (DEGs) compared to a 3-day treatment under individual stress in the barley flag leaf (Mikołajczak et al., 2023).

The biological processes associated with responses to a chemical, a stimulus, an oxygen-containing compound, and to stress are all highly enriched in drought, heat and combined stress treatments in lentil leaves (Hosseini et al., 2021), indicating overlapping signalling pathways. Combined stress induces differentially expressed genes in food crops associated with the ribosome pathway and with photosynthesis and chloroplast-related processes compared with control conditions, and uniquely up- and down-regulated genes enriched in metabolic and biosynthetic processes of the organonitrogen compound, peptide and amide, translation and cytoplasm-related terms (Hosseini et al., 2021; Tan et al., 2023). Restructuring of the transcriptome due to the simultaneous occurrence of heat and drought stress varies in different studies. The transcriptomic signature, such as the percentage of overlapping DEGs, indicates that either high temperature or drought plays a major regulatory role in the transcriptome under combined stress, such as the expression patterns of most *HEAT SHOCK TRANSCRIPTION FACTORS (HSFs)* are predominantly regulated by heat, while of most abscisic acid (ABA)-related genes are primarily regulated by drought (Rizhsky et al., 2004; Shaar-Moshe et al., 2017; Sinha et al., 2022; Mikołajczak et al., 2023; Sinha et al., 2023) (Figure 2). In addition, additive/synergistic transcriptional responses to combined stresses, often with a dominant impact of one stress, also occurs. For example, the expression of some *HEAT SHOCK PROTEINs* (*HSPs*) quickly responds to individual high temperature or drought stress with a significant increase, and the combination of high temperature and drought additionally increases their expression (Rizhsky et al., 2002; Rizhsky et al., 2004; Mahalingam et al., 2022; Rahman et al., 2022) (Figure 2). However, plants subjected to combined stress, also display different DEG response patterns compared to individual heat or drought stress, indicating a limited expression overlap among individual stresses and combined stresses (Rizhsky et al., 2004; Liu et al., 2018; Hosseini et al., 2021; Mikołajczak et al., 2023; Sinha et al., 2023; Tan et al., 2023). For example in wheat, combined stress induces specific alternative

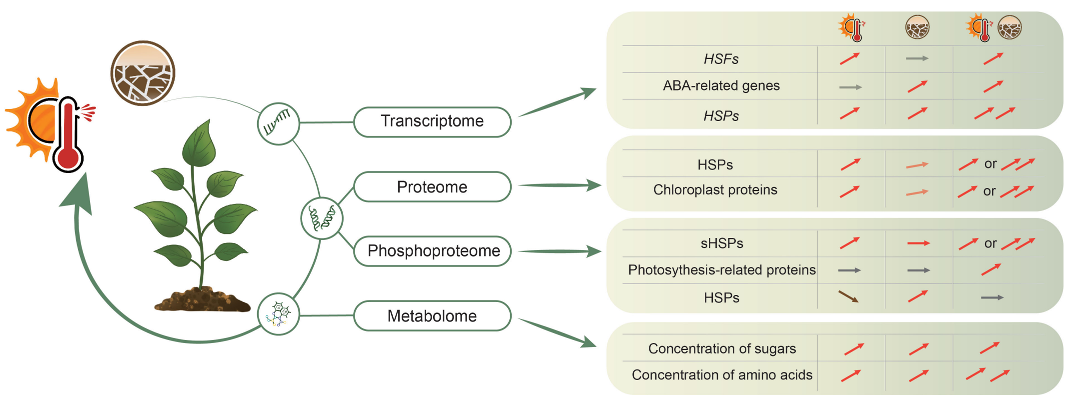

FIGURE 2
Combined heat and drought stress can differentially regulate plant transcriptome, proteome and PTMs (post-translational modification), and metabolome in plants to adapt to environmental changes. Different omics reveal varying regulatory patterns for several regulators under conditions of individual heat and drought or combined heat and drought stress. HSFs, Heat Shock Transcription Factors; HSPs, HEAT SHOCK PROTEINs; sHSPs, small HEAT SHOCK PROTEINs. The icon with the thermometer and sun indicates the heat stress and the cracked land indicates the drought stress. The arrows indicate increase, decrease or no change under indicated conditions.

splicing that is absent under individual stresses, and some of these alternatively spliced genes are associated with glutathione biosynthesis and DNA methylation (Liu et al., 2018). Furthermore, under combined stress, a significant number of transcripts are oppositely regulated compared to each individual stress, or distinct from the effect of the individual stress showing expression levels of untreated plants (Rizhsky et al., 2004; Sinha et al., 2023). However, among these unique transcripts induced by combined stress, a much lower overall similarity was found in different soybean organs (Sinha et al., 2023), indicating a unique transcriptional response in different plant organs, and emphasizing the importance of focused studies to understand tissue and organ-specific responses to combined stress conditions.

These different types of interactions of DEGs detected under combined stress occur in different processes and pathways, which are largely related to photosynthesis and encoding mitochondrial proteins (Rizhsky et al., 2004; Hosseini et al., 2021). GO terms associated with these unique DEGs also pinpoint the response to ABA and the metabolic and biosynthetic processes of organonitrogen (Rizhsky et al., 2004; Hosseini et al., 2021; Mikołajczak et al., 2023).

Proteome responses to combined heat and drought

Changes in gene expression will – to some extent – result in changes at the protein level (Greenbaum et al., 2003); and regulation of translation, protein abundance and protein activity through, for example, post-translational modifications, add another layer of regulation to the proteome.

Under drought conditions, there was a significant induction in the abundance of proteins in maize related to carbohydrate metabolism pathways, including glycolysis and the pentose phosphate pathway (Vu et al., 2016). Conversely, proteins involved in chromatin organization, including several histones, exhibited an overall decrease in expression levels (Vu et al., 2016). High-temperature stress significantly affects protein structure and stability in Arabidopsis, especially those involved in ribosomal proteins/nucleic acid binding, proteasomal proteins, and cytoskeletal proteins (Volkening et al., 2019). These affected processes of differentially expressed proteins (DEPs) also depend on the species and on the developmental stage (Liu et al., 2015; Lu et al., 2017; Zhang et al., 2017; Liu et al., 2021).

Based on a limited number of studies, the abundance of the differentially regulated crop proteins under combined stress is primarily associated with ribosomes, metabolic processes and photosynthesis (Rollins et al., 2013; Zhao et al., 2016; Tang et al., 2023). Combined stress, and either one or both individual stress conditions, share a large proportion of DEPs of enzymes in maize leaves, such as kinases, phosphatases, enzymes involved in phytohormone signalling, or other metabolic enzymes (Zhao et al., 2016). Some DEPs are predominantly regulated by a single stress and/or exhibit additional regulation under combined stress. For example, the abundance of the majority of identified HSPs or chloroplast proteins in sweet potatoes is up-regulated by heat stress and only slightly affected by drought stress, and under combined stress, an additional increase is observed (Tang et al., 2023) (Figure 2).

Post-translational modifications (PTMs) increase the functional diversity of the overall proteome. While several studies have explored PTMs under single drought or heat stress conditions (Scharf and Nover, 1982; Castro et al., 2012; Zhang et al., 2014; Vu et al., 2016; Botha et al., 2017; Chen et al., 2017; Benlloch and Lois, 2018; Vu et al., 2018; Morrell and Sadanandom, 2019; Xu and Xue, 2019; Pengyan et al., 2020; Zhang et al., 2020; Han et al., 2021; Han et al., 2022), there are only a few studies that have investigated phosphorylation, under combined stress conditions. Under drought conditions, the pathways primarily associated with sodium transport, immune response, and chromatin silencing of detected phosphorylated protein are affected in maize leaves (Vu et al., 2016). Differentially phosphorylated proteins upon mild heat in wheat leaves, compared to non-stress conditions, are enriched in biological processes associated with heat, protein folding, response to hydrogen peroxide and glucose transport (Vu et al., 2018). Combined stress differentially regulates protein phosphorylation in the maize leaf, and out of 282 phosphoproteins, 46 of them are common between individual stress and combined stress (Hu et al., 2015). The phosphorylation level of detected HSPs and small HSPs (sHSPs) in maize is mainly regulated in response to heat and combined stress, but does not significantly change under drought (Hu et al., 2015) (Figure 2). However, several phosphoproteins related to photosynthesis, carbon metabolism and protein processing are detected under combined high temperature and drought conditions (Hu et al., 2015) (Figure 2).

Other common PTMs, such as ubiquitination and sumoylation, targeting Lys residues, have also been studied under individual heat and drought conditions (Catala et al., 2007; Miura et al., 2009; Castro et al., 2012; Li et al., 2015; Wu et al., 2016; Xu and Xue, 2019; Pengyan et al., 2020; Andrási et al., 2021; Han et al., 2021). However, there is limited available data on the ubiquitinome and sumoylome in the context of combined stress. Nevertheless, high temperature or drought-induced ubiquitination regulates ABA signalling (Chiu et al., 2016; Yu et al., 2016; Xu and Xue, 2019), and ubiquitination regulates drought tolerance via the ABA signalling pathway (Seo et al., 2012; Lim et al., 2017; Xu and Xue, 2019; Tong et al., 2021; Singh et al., 2022). High temperatures inhibit Arabidopsis seed germination by dampening both protein ubiquitination and proteasome activity in an ABA-dependent manner (Chiu et al., 2016). The differently ubiquitinated proteins under high temperatures are enriched in a wide range of molecular functions (Li et al., 2015; Pengyan et al., 2020). Sumoylation can be stimulated in plants under heat (Miller et al., 2010; Miller et al., 2013; Hendriks and Vertegaal, 2016; Rytz et al., 2018; Han et al., 2021) or drought stress (Catala et al., 2007; Miura et al., 2009; Castro et al., 2012; Wu et al., 2016; Joo et al., 2022) and largely depends on SUMO E3 ligase SAP AND MIZ1 DOMAIN-CONTAINING LIGASE 1 (SIZ1). SIZ1 facilitates conjugation of SUMO to protein substrates, and positively regulates plant heat tolerance and acquired thermotolerance (Yoo et al., 2006; Kim et al., 2017; Rytz et al., 2018). Sumoylation occurring on chromatin is associated with gene expression in response to high temperature (Niskanen et al., 2015; Han et al., 2021). The transcripts that are differentially regulated by sumoylation are largely involved in responses to heat stress and development-related processes (Han et al., 2021). Some studies exhibit that SIZ1 has both positive and negative effects on drought tolerance (Catala et al., 2007; Miura et al., 2009; Vu et al., 2016; Benlloch and Lois, 2018; Xu and Xue, 2019). So far as we know, there are no omics data on sumoylation under drought or combined stress conditions, but overexpression of SIZ1 enhances photosynthesis performance and yield compared to the wild type under combined stress (Mishra et al., 2017). This suggests a complex regulation of sumoylation via SIZ1, which might be affected by the stress intensity and potentially plays an important role under drought or combined heat and drought stress.

Metabolome responses to combined heat and drought

Under combined stress, plants may experience a reduction in growth and yield as mentioned above. Under such conditions, the levels of biological markers of oxidative stress, such as malondialdehyde (MDA) and H_2O_2, further increase (Jin et al., 2016; Rahman et al., 2022).

To safeguard cells from stress-induced damage, plants adapt by reprogramming their metabolic pathways. While there are several metabolome datasets under individual drought and heat stress (Ye et al., 2016; Sun et al., 2019; Abdelrahman et al., 2020; Lecourieux et al., 2020; Guo et al., 2021; Lu et al., 2022; López et al., 2023; Xie et al., 2023), there are only a few studies with respect to combined stress (Zinta et al., 2018; Alhaithloul et al., 2019; Lawas et al., 2019; Qaseem et al., 2019; Xie et al., 2020; Yousaf et al., 2022; Ru et al., 2023). Under combined stress, the processes of carbohydrate metabolism, amino acid metabolism and organic acid are differentially affected compared with a non-stress condition (Zinta et al., 2018; Lawas et al., 2019). There is an increase in stress-responsive metabolites in flowering spikelets, particularly in terms of their abundance under severe combined heat and drought stress, compared to mild combined stress conditions (Lawas et al., 2019). Under combined stress, there is a significant and strong transient increase in the concentration of most soluble sugars, such as glucose, fructose, and raffinose (Figure 2), although the concentration of sucrose and starch decreased compared to a non-stress condition (Zinta et al., 2018; Alhaithloul et al., 2019). These soluble sugars increase the osmotic potential in the cell, drawing water into these cells to maintain the turgor pressure (Fàbregas and Fernie, 2019), and act as protectants to cope with rapid stress (Rosa et al., 2009; Du et al., 2019). Under combined stress, some results show that the total amount of soluble sugar further increased compared to individual heat and drought stress (Qaseem et al., 2019; Ru et al., 2023). However, this increased effect of soluble sugar exhibits variation across different varieties and species, in response to combined stress compared with individual stress or control conditions, providing a possible explanation for the variable tolerance observed among different varieties and species (Zhou et al., 2017; Qaseem et al., 2019; Alsamadany et al., 2023; Ru et al., 2023).

The concentrations of amino acids exhibit a distinct profile when subjected to combined stress (Zinta et al., 2018). Under combined stress, a strong transient increase of amino acids, such as histidine, isoleucine, leucine, methionine, and proline, are observed (Figure 2), while alanine, asparagine, and aspartate do not show a difference under these combined treatments (Zinta et al., 2018; Lawas et al., 2019; Xie et al., 2020; Yousaf et al., 2022).

The concentration of fatty acids is also differentially impacted by combined stress compared with a non-stress condition, and both saturated (SFA) and unsaturated fatty acids (UFA) exhibit specific temporal patterns. The concentration of mainly SFAs increases during exposure to stress (Figure 2), while mono- and poly-UFAs mostly decrease or remain unchanged during stress (Zinta et al., 2018). The increase in SFAs and decrease in UFAs following prolonged stress could potentially be linked to the adaptation of membranes in managing fluctuations in fluidity. The membrane plays an important role in signal perception and transduction (Inda et al., 2014; Niu and Xiang, 2018). For example, high temperature promotes membrane fluidization, while hyperosmotic stress can reduce membrane fluidity (Laroche et al., 2001; Los and Murata, 2004; Mansilla et al., 2004; Martinière et al., 2011; Leach and Cowen, 2014; Vu et al., 2019a). The pH value surrounding the cell membrane can affect its permeability and polarity, and drought stress can trigger cytoplasmic alkalinisation, thereby impacting membrane dynamics (Geilfus, 2017; Angelova et al., 2018). The different modifications of signal perception under combined stresses might lead the distinct signal transduction, which can also influence the activity of membrane-associated proteins and downstream targets (Niu and Xiang, 2018; Vu et al., 2019a).

Conclusion

Omics experiments under combined heat or/and drought stress, provide systems level knowledge on how plant growth and yield, and the associated physiological and biochemical responses, are regulated under stress (Figure 2). Heat and drought stress may exert distinct effects on various tissues or organs. However, due to the limited number of omics datasets, and the majority of studies being conducted on leaf or whole plants, the potential interplay between organs and signalling pathways remains largely unexplored. Although the initial sensing of these stresses likely occurs locally and specifically, the resulting biochemical signals can be quickly transferred to and perceived by other tissues and organs. This coordinated regulation between local and transferred signals might explain the different regulations observed under combined stress that are absent under a single stress. There are several conserved responses to both heat and drought stress, including those related to ABA signalling and heat shock proteins (Rizhsky et al., 2002; Rizhsky et al., 2004; Zhao et al., 2016; Marín-de la Rosa et al., 2019; Andrási et al., 2021; Tamang et al., 2021; Wang et al., 2021; Mahalingam et al., 2022; Tang et al., 2023).

Different omics reveal varying regulatory patterns for several regulators or metabolites under individual heat or drought stress and combined heat and drought conditions (Figure 2), such as the HSPs that are differentially regulated at the transcript, protein and phosphoprotein level as mentioned above (Rizhsky et al., 2002; Rizhsky et al., 2004; Hu et al., 2015; Zhao et al., 2016; Mahalingam et al., 2022; Rahman et al., 2022; Tang et al., 2023). The expression of HSPs and the HSP protein level is higher under combined stress compared to individual stress. However, the phosphorylation of HSPs is up-regulated under drought stress and down-regulated under heat stress, while no significant change in phosphorylation is observed under combined stress.

Breeding stress-tolerant crop varieties under increased temperature and drought is a fundamental way to help deal with climate change and to assure future food security. Understanding the underlying mechanisms of abiotic stress tolerance in crops is crucial to address how abiotic stress affects crop yield and quality effectively, and to provide useful markers and genes for genetic improvement. Selecting the crucial players under combined heat and drought stress, allows us to further understand how plants perceive different stresses and integrate these signals into various tissues and organs, which can be used for targeted breeding to improve plant tolerance under heat and drought stress. Despite the numerous transcriptomes, there have been relatively few studies on post-translational modifications (PTMs), such as phosphorylation, ubiquitination, and SUMOylation, under combined heat and drought stress. PTMs play an important role as rapid and reversible molecular switches, effectively regulating biological pathways and processes within cells (Blazek et al., 2015; Coll-Martínez and Crosas, 2019; Xu et al., 2019), and a comprehensive understanding of protein modification under combined stress is thus crucial to fully capture signalling mechanisms. Furthermore, the integration of other omics data, such as the translatome, and the integration of these multiple omics data is the next key step (Li et al., 2022; Tang et al., 2023).

Finally, we advocate for more omics studies under combined stress conditions, focusing on different species and different organs. In order to investigate plant responses under combined stress, it is crucial to consider the intensity, duration, and timing of the stress conditions. Optimal wet lab experimental setups should incorporate representative climate data to ensure accuracy. Additionally, studying responses directly in the field allows for the consideration of other environmental variables, providing more comprehensive results.

Author contributions

XX wrote the manuscript and generated Figure 2. CF generated Figure 1. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1250878/full#supplementary-material

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Morphological performance and seasonal pattern of water relations and gas exchange in *Pistacia lentiscus* plants subjected to salinity and water deficit

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Soil water deficit and salinity represent a major factor impacting plant survival and agricultural production. The frequency and severity of both abiotic stresses are expected to increase in a context of climate change, especially in arid and semi-arid regions. This work studied the growth pattern, biomass and mineral distribution and the seasonal pattern of water status, photosynthetic rate and stomatal conductance in plant of *Pistacia lentiscus* grown under different levels of water deficit and salinity. *P. lentiscus* plants growing under greenhouse conditions were subjected to four irrigation treatments during 11 months: control (C, 1 dS m^{-1}), moderate water deficit (MW, 1dS m^{-1}, 60% of the control), severe water deficit (SW, 1 dS m^{-1}, 40% of the control) and saline (S, 4dS m^{-1}). The results show that *Pistacia lentiscus* plants were more affected by deficit irrigation than salinity. Deficit irrigation and salinity inhibited plant height, with reductions of 20%, 22% and 35% for S, MW and SW, respectively. Total leaf area was not modified by effect of the treatments, with the result that plant compactness increased in MW. The salt stressed plants only showed lower relative growth rate at the end of the experiment. Plants responded to saline or drought stress by increasing their osmotic adjustment, which was more pronounced under salinity. Saline plants had the highest values in Na^+ and Cl^- ions and the lowest values for K^+/Na^+ and Ca^{2+}/Na^+ ratios in leaves and stems, which is correlated with a decrease in growth, stomatal conductance, photosynthesis and stem water potential, and can be used as a diagnostic tool to assess plant tolerance to salinity stress. As a measure of plant hydration, relative water content was more sensitive to deficit irrigation than salinity, being a good indicator of water stress. *P. lentiscus* plants subjected to both deficit irrigation treatments exhibited an increase in their intrinsic water use efficiency, which is an important adaptation for plants growing in environments with water scarcity.

KEYWORDS

biomass accumulation, gas exchange, ion uptake, irrigation, mineral distribution, osmotic adjustment, principal component analysis, water status

1 Introduction

The escalation of both the frequency and magnitude of water stress and salinity is foreseen to manifest in numerous global regions within the framework of ongoing climate change (Chaudhry and Sidhu, 2022; Soares et al., 2022). Rising temperatures and changing precipitation patterns are projected to exacerbate existing water scarcity issues, while sea level rise and changing groundwater dynamics are expected to increase the incidence of salinity intrusion in coastal areas (Asif et al., 2023). As water stress and salinity stress become more common the impacts on agriculture, ecosystems, and human well-being are likely to become more severe (Semeraro et al., 2023). In response to these challenges, there is a growing need to develop adaptive strategies to mitigate the impacts of water and salinity stresses. These strategies may include improving water use efficiency, developing crop varieties that are more resistant to drought and salinity, encouraging sustainable land management strategies, and allocating resources towards the development of water treatment and desalination technologies are essential steps in fostering sustainable water resource utilization (Acosta-Motos et al., 2020; Albatayneh, 2023; Patel et al., 2023).

Water stress occurs when plants experience a lack of water, either due to insufficient rainfall, high temperatures, or other factors that increase evaporation. This can lead to reduced plant growth, leaf wilting, and even death in severe cases. Plants respond to water stress by reducing their stomatal openings to conserve water, which can also affect the photosynthetic process and reduce their ability to grow (Arve et al., 2011; Bodner et al., 2015). Salinity stress occurs when plants are exposed to high levels of salt in the soil or water. High salinity can lead to reduced water uptake by plants, which in turn can lead to reduced plant growth, wilting, and even death. Plants respond to salinity stress by developing mechanisms to exclude or tolerate salt, such as increased root growth or salt secretion (Acosta-Motos et al., 2017; Acosta-Motos et al., 2020). Both water stress and salinity can have significant impacts on agricultural productivity and ecosystem health. In some cases, plant breeders have developed crop varieties that are more resistant to these stresses, but in other cases, it may be necessary to implement management practices such as irrigation scheduling, soil amendments, or alternative crops to mitigate their effects (Paranychianakis and Chartzoulakis, 2005; Phogat et al., 2020).

Pistacia lentiscus, also known as mastic tree or lentisk, has potential uses in a context of climate change. As a drought-tolerant species, it is likely to be more resilient to water stress than many other ornamental plants, making it a good choice for landscaping in regions prone to drought (Bussotti et al., 2014; Leotta et al., 2023). Also, its deep roots and ability to thrive in poor soils make it a potentially useful species for erosion control and land restoration projects (Ramón Vallejo et al., 2012). In addition to its potential uses in a context of water stress and drought, *Pistacia lentiscus* may also have applications in regions affected by salinity stress. The species is known for its ability to tolerate high levels of salt in the soil and can even grow in saline soils near the coast. In areas affected by salinity stress, the use of *Pistacia lentiscus* as an ornamental plant in landscaping projects may be particularly valuable (Álvarez et al., 2018; Castillo-Campohermoso et al., 2020). The plant's tolerance to salt and its ability to thrive in poor soils make it a good choice for revegetation and erosion control projects in coastal areas (Parraga-Aguado et al., 2013). By incorporating *Pistacia lentiscus* into agroforestry systems, farmers may be able to improve soil structure, reduce soil salinity, and improve water use efficiency, leading to higher crop yields and greater resilience in the face of climate change. Finally, the species has been successfully integrated into agroforestry systems in Mediterranean regions, where it has been used for shade, windbreaks, and as a source of animal fodder (Williams, 2002). It is well-known that analysing individual stresses, such as water supply restrictions and salt stress, rather than their combination, can provide several advantages when studying their effects on plants. Studying individual stresses allows to isolate and understand the specific effects of each stress factor. This can help in determining the unique physiological and morphological responses of *Pistacia lentiscus* plants to each stressor drought stress and salinity. The purpose of this work was to study growth pattern, biomass and mineral distribution and the seasonal pattern of water status, photosynthetic rate and stomatal conductance in plant of *Pistacia lentiscus* grown under deficit irrigation and salinityto determine the ability and response of plant to cope with water and saline stresses, which represents a major factor in plant growth and survival in a context of climate change.

2 Materials and methods

2.1 Plant and experimental conditions

Pistacia lentiscus L. plants (180) were grown in 15 x 15 x 20 cm pots (4L) filled with a substrate blend comprising coconut fibre, black peat mixed with sphagnum peat, and perlite of 5:4:1, enriched with Osmocote plus fertilizer (2 g L^{-1} substrate) containing a balanced formulation of nitrogen (N), phosphorus (P), potassium (K), and microelements (14:13:13N, P, K plus microelements). The experimental investigation took place within a controlled greenhouse environment, which was previously detailed in the methodology section in Álvarez et al. (2018). Temperature values (T^a) ranged between 2.5 and 37.9 °C and relative humidity (RH) values oscillated between 20.3 and 90.7%. A mean temperature of 20.3°C and relative humidity (RH) of 62.9% was registered during the experimental period. Before commencing the treatments, all plants were consistently irrigated to reach field capacity on a daily basis for a period of four weeks.

2.2 Water irrigation treatments

After acclimating the *P. lentiscus* plants to greenhouse conditions, (a total of 45 plants per treatment) were subjected to an experimental duration of up to 11 months, from December 2007 to November 2008. Four distinct irrigation treatments were implemented, including a control treatment where plants received watering up to 100% of the water holding capacity, with 15% leaching (v/v) using tap water of electrical conductivity (EC) measuring 1.0 dS m^{-1}. Additionally, a saline water treatment was employed, maintaining the water holding capacity at 100% while incorporating salt to attain a concentration of 44 mM Na Cl (4.0 dS m^{-1}). Furthermore, two deficit irrigation treatments were implemented, known as moderate deficit irrigation (MW) and severe deficit irrigation (SW). These treatments involved the application of 60% and 40% of the water quantity supplied in the control treatment, respectively. All the plants were watered subjected to daily watering throughout the experimental period.

2.3 Leaf mineral content

The inorganic solute concentrations were measured at the end of the experimental period in the same plants used for the biomass parameters calculation. The concentrations of Ca^{2+}, K^+ and Na^+, Mg^{2+}, P, S, Mn, B and Zn ions were measured using a digestion extract of 100 mg of tissue powder with 50 ml of a mix of HNO_3: HCl O_4 (2:1, v/v) by using an inductively coupled plasma optical emission spectrometer (ICP-OES-IRIS intrepid II XDL, Thermo Fisher Scientific Inc., Lough-borough, UK). The concentration of Cl^- ions was performed utilizing a chloride analyser (Model 926, Sherwood Scientific Ltd., Cambridge, UK).

2.4 Plant growth, ornamental traits

Upon reaching the conclusion of the experimental period, the substrate surrounding the roots of ten plants per treatment was meticulously rinsed away. The plants were then carefully separated into their constituent parts, including leaves, stem and roots. Subsequently, they were subjected to oven-drying at a temperature of 80°C until an invariable weight was achieved, enabling the measurement of their respective dry weights (DW). Leaf area was assessed in the identical set of plants, by employing a leaf area meter (Delta-T Devices Ltd., Cambridge, UK). Specific leaf area (SLA) was computed by dividing the leaf area by the corresponding leaf dry weight, while leaf area ratio (LAR) was determined by dividing the leaf area by the total dry weight. Plant height was periodically measured in a sample of 25 plants per treatment and was taken as the vertical distance from substrate to the highest leaf. Relative growth rate (RGR) was calculated by determining the rate of height increase per unit of initial plant height. Compactness, on the other hand, was calculated dividing the leaf area by the corresponding plant heights. Leaf colour measurements were conducted using a Minolta CR-10 colorimeter, which facilitated the assessment of colour coordinates such as lightness (L*), chroma (C*) and hue angle (h °) following the methodology established by McGuire (1992). A total of seven plants per treatment were utilized for this analysis.

2.5 Water status

During the course of the experiment, various physiological parameters related to leaf water status were assessed. These parameters included leaf water potential (Ψ_l) at predawn and midday, leaf turgor potential (Ψ_t), leaf osmotic potential (Ψ_o), leaf osmotic potential at maximum saturation (Ψ_{100s}), stem water potential (Ψ_s) at midday and relative water content (RWC) at predawn and midday. Eight plants per treatment were selected, and measurements were performed on mature leaves at midday. The estimation of Ψ_l was carried out using the method outlined by Scholander et al. (1965), employing a pressure chamber (Soil Moisture Equipment Co, Santa Barbara, CA, USA). Leaves were promptly placed in the chamber within 20 seconds of collection and pressurised at a rate of 0.02 MPa s^{-1} (Turner, 1988). The Ψ_t was calculated as the difference between leaf water and leaf osmotic potential. Osmotic potential was determined using a Wescor 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA). Freshly cut leaves were wrapped in aluminum foil, subjected to cell membrane rupture by liquid nitrogen, and stored at −30 °C. Thawed leaves were squeezed to extract a drop for osmotic potential measurement. To determine the osmotic potential at maximum saturation (Ψ_{100s}), the leaves were immersed in distilled water at 4°C in the dark for 24 hours until they reached maximum turgor. Following the removal of excess water with filter paper, the leaves were wrapped in aluminium foil and frozen in liquid nitrogen as described earlier for osmotic potential measurement (Gucci et al., 1991). Ψ_s was measured in leaves that had been covered with both a plastic sheet and aluminium foil for at least 2 hours before measurement to minimize leaf transpiration. This ensured that leaf water potential equalled stem water potential (Begg and Turner, 1970). The RWC of leaves was calculated following the method described by Barrs (1968).

2.6 Gas exchange and photosynthesis parameters

During the course of experiment, the fluctuations in leaf stomatal conductance (g_s) and net photosynthetic rate (P_n) were evaluated in mature leaves at midday. Measurements were performed on eight plants per treatment using a gas exchange system (LI-6400, LI-COR Inc., Lincoln, NE, USA). Leaf gas exchange was measured on young, fully expanded leaves, placed in a 2 cm^2 leaf cuvette. The CO_2 concentration in the cuvette was maintained at 400 µmol mol^{-1} (≈ ambient CO^2 concentration). Measurements were performed at a saturating light intensity subindice of 1200 µmol m^{-2} s^{-1} and at ambient temperature and relative humidity. The g_s reflects the rate at which stomata open and close, allowing the exchange of gases between the leaf and the atmosphere, while P_n represents the rate of carbon dioxide uptake during photosynthesis. Additionally, the P_n/g_s ratio, calculated as the ratio of net photosynthetic rate to stomatal conductance, was utilized as an indicator of the intrinsic water use efficiency, providing insights into the plant's ability to balance water loss and carbon assimilation.

2.7 Statistics

The plants were arranged in a randomized block design and placed on crop benches. Each of the four treatments, namely control (C), moderate irrigation treatment (MW), severe irrigation treatment (SW) and salt treatment (S) was divided into three blocks. Within each block, 15 plants were randomly allocated resulting in a total of 45 plants per treatment. Normality and homoscedasticity of variances were assessed for all variables through the Shapiro–Wilk and Bartlett tests, respectively. To compare the treatments, a one-way analysis of variance (ANOVA) was conducted, followed by a Tukey HSD *post hoc* test. Statistical significance was considered at a threshold of $P \leq 0.05$. Stars and rays graphs were generated where each axis represents a specific variable, and the intersection with a vertex of the polygon indicates the relative magnitude of that variable. Additionally, a principal component analysis (PCA) was performed to examine leaf nutrient data. PCA is a statistical method proposed by Pearson (1901) and Hotelling (1933), which aims to describe the variation observed in p random variables using a set of new variables called principal components. These components are uncorrelated with each other and obtained in order of importance. PC1 captures the maximum variation explained by the original variables, while PC2 explains the remaining unexplained variation while being uncorrelated with the PC1, and so on. The selection of principal components was based on eigenvalues greater than or equal to 1.0 (Supplemental Table 1 and Supplemental Figure 1). The statistical analyses were performed using StatGraphics Centurion XV software (StatPoint Technologies, Warrenton, VA, USA).

3 Results

3.1 Plant quality and mineral distribution throughout the plant

Upon completion of the experimental duration, no statistically significant disparities were observed in the dry matter accumulation of the *P. lentiscus* plants submitted to saline irrigation (S) and to moderate deficit irrigation (MW) (Table 1). However, the severe water deficit treatment (SW) significantly reduced total dry weight (DW) compared the rest of the treatments, while the number of leaves and leaf blade area were not affected by the water-deficit and salinity conditions of the substrate. Total DW of SW-plants was 67% of the control values. The biomass distribution was affected by the water-availability, increased Leaf/Total DW and decreased Stem/Total DW (Table 1). Both water deficit treatments significantly increased the leaf area ratio (LAR) compared with control, the effect being more pronounced in SW plants, while the specific leaf area (SLA) was increased in S and SW plants (Table 1). Plant height was significantly reduced by salinity and by both water deficit treatments, with reductions of 20%, 22% and 35% for S, MW and SW, respectively; however, total leaf area was not modified, with the result that plant compactness increased in MW (Table 1). As regards the evolution of relative growth rate, the relationship between relative growth rate (RGR) and plant height revealed the presence of two distinct growth periods throughout the growing season, specifically in the months of March-April and July-August. This pattern was observed across all plants, despite any other factors that may have influenced plant growth, although both water deficit treatments showed lower RGR in the first growth period and SW plants also in the second growth period, and especially in September (Figure 1). The salt stressed plants only showed a lower RGR than the control after the second growth period, at the end of the experiment.

To offer a more concise understanding of the effectiveness of each examined treatment, a diagram called a stars and rays graph was created utilizing the key morphological characteristics. To summarize, the greater the distance between the point where each trait's axis intersects with the outer boundary of the diagram and the center of the diagram itself, the greater the magnitude of that trait. This method aids in visually highlighting the variations between the different treatments. For example, Control treatment (C) followed by saline treatment (S) are seen as those treatments that best fulfil the morphological traits

TABLE 1 Growth parameters at the end of the experiment in *P. lentiscus* subjected to different irrigation treatments.

Parameters	Treatments				P
	C	S	MW	SW	
Total DW (g plant^{-1})	61.80±2.92b	62.35±7.12b	53.46±2.27b	41.18±1.53a	**
Leaf blade area (cm^2)	14.62±1.43	13.62±1.56	12.63±0.79	12.10±1.39	ns
Number of leaves per plant	78.70±7.29	92.00±15.63	103.70±8.84	88.86±6.66	ns
Leaf/total DW (g g^{-1})	26.51±1.42a	26.12±1.58a	32.72±1.36b	32.27±1.31b	**
Stem/total DW (g g^{-1})	0.38±2.17b	0.40±1.36b	0.31±1.84a	0.30±1.31a	***
Root/total DW (g g^{-1})	0.36±2.14	0.33±1.15	0.37±2.13	0.39±1.99	ns
SLA (cm^{-2} g^{-1})	49.80±3.84a	63.87±4.51b	58.92±2.76ab	76.59±3.82c	***
LAR (cm^{-2} g^{-1})	13.20±1.35a	16.88±1.11ab	18.63±1.33b	23.89±1.63c	***
Plant height (cm)	68.95±3.59c	55.40±3.12b	54.15±3.39b	45.40±1.83a	***
Compactness (cm^2 cm^{-1}) cm-1)	16.57±1.47a	22.12±3.36ab	26.06±1.53b	22.70±2.27ab	*

Values are the mean of ten plants, except in plant height, when values are the mean of 25 plants.
Means within a row without a common letter are significantly different according to Duncan 0.05 test.
(P; probability level, ns; non significance, *P<0.05, ** P ≤0.01, *** P ≤ 0.001).

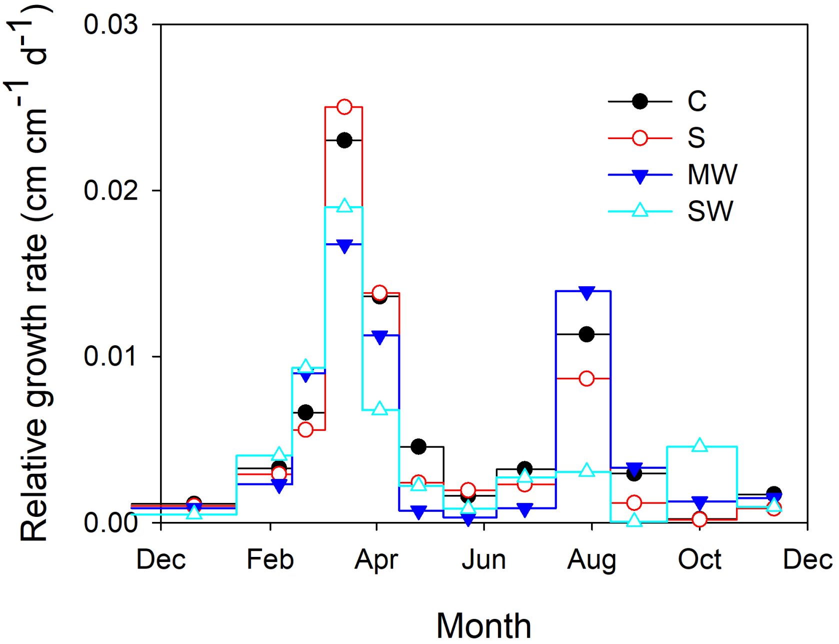

FIGURE 1
Relative growth rate of *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of 25 plants per treatment. Symbols represent the different treatments: Control (filled circles), S (open circles), MW (filled triangles) and SW (open triangles).

studied. On the other hand, the treatments MW and especially SW show small magnitudes for all of the parameters studied, which point the greater capacity of these treatments to respond with morphological changes to the stress imposed (Figure 2).

There were no discernible alterations in leaf color for MW and S plants when compared to the control group, as indicated in Figure 3. However, in plants subjected to SW, higher h° values and lower L* and C* values were recorded at the conclusion of the experiment, confirming a darker and less saturated green hue of the foliage compared to the control plants (Figure 3).

Figure 4 displays the concentrations of Na^+, Cl^-, K^+, and Ca^{2+} in the leaves, stems, and roots at the conclusion of the experimental period. Notably, no noticeable buildup of Na and Cl was detected in plants exposed to either water stress treatment. However, the concentration of both ions exhibited an upward trend in all plant components as salinity levels increased, as depicted in Figures 4A, B The K^+ concentration decreased to 70% in the leaves of S plants compared to control plants, whereas an increase was observed in the root of these plants (up to 22%). The K^+ concentration increased with severe water deficit in all parts of the plants (up to 55-60%) (Figure 4C). With respect to $Ca^{2+,}$ its concentration decreased in the leaves of S plants and increased by 40, 49 and 74% in stem of S, MW and SW plants (Figure 4D). Next, in all plant components, the ratios of K^+/Na^+ and Ca^{2+}/Na^+ were observed to decline due to saline treatment, while in severe water deficit treatment these ratios were higher than in control plants (Table 2). Correlation between different growth parameters (total DW, plant height, SLA and compactness) and Na^+ and Cl^- are shown in Table 3, the plant height reduction was significantly related with Na^+ and Cl^- concentration. The augmentation of specific leaf area (SLA) exhibited a strong positive correlation with both Na^+ and Cl^- concentrations, with a higher correlation coefficient (r) observed for Cl^-. No significant correlations were observed between any other parameters examined in the study (Table 3).

Furthermore, to assess the distinct separation of the various treatments based on leaf nutrient levels at the conclusion of the experiment, a principal component analysis (PCA) was performed. The scatter diagram (Figure 5A) and bigraphic (Figure 5B) figures were obtained by the principal component table. In addition, the average score for each of the four treatments was added (Supplemental Table 2A). The analysis in the scatter plot and biographic revealed a notable detach of the treatments using the two principal components (PCs). However, PC1, which accounts for 56.89% of the experimental variability, played a particularly significant role in the observed separation, was better, with a value of F = 89.69***, classifying the treatments into four clusters good separated from left to right (SW, MW, C and S) (Supplemental Tables 2B, C). Additionally, PC2, which accounts for 14.18% of the experimental variability, also demonstrated a substantial ability to separate the treatments. This was further supported by a statistically significant F value of 17.38***, classifying the treatments in two clusters (S and SW) and (MW and C) (Supplemental Tables 2D, E). For PC1, the variables or nutrients most important with more weight (variables with a higher absolute value) were: Mg^{2+} and Ca^{2+}. For PC2, the variables or nutrients most important with more weight were: Cl^-, Na^+ and B (Supplemental Table 3).

3.2 Physiological measurements: seasonal pattern of water status and gas exchange

As regards the seasonal pattern of leaf relative water content (RWC), for the majority of the experiment (first seven months), no

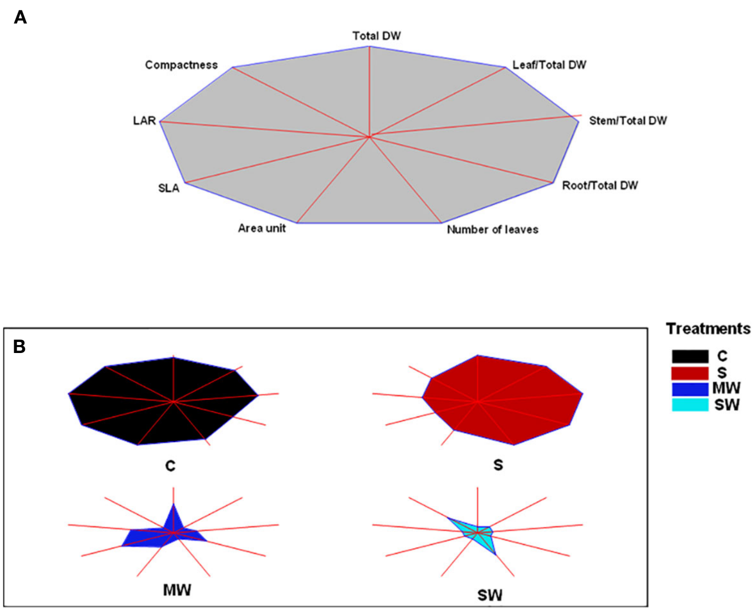

FIGURE 2
Stars and rays graphs displaying the differences between accessions for the morphological traits analyzed. Each axis represents one variable, and its intersection with a vertex of the polygon indicates the relative magnitude for that variable. **(A)** Reference polygon for variable identification. Leaf area ratio (LAR) and specific leaf area (SLA) are shown as their inverses to visually correlate higher magnitude with a positive trait. **(B)** Individual graphs for each accession. DM, dry matter (%).

significant differences were observed between the control plants and those subjected to saline treatment, as depicted in Figures 6A, B. However, towards the end of the experiment, the impact of salinity became evident as indicated by reduced values of relative water content, especially at midday. In both deficit irrigation plants RWC at midday was reduced in summer (Figure 6B), but was much more marked under SW, reaching minimum values in May and June (64%). After this period (as of July) RWC midday showed a recovery in both water deficit plants and they reached the control values at the end of the experiment. However, at predawn, both levels of deficit irrigation had opposite effects on RWC (May and June). SW led to a decrease in RWC in the early summer and moderate water deficit led to an increase in RWC compared with control (Figure 6A).

Additionally, this trend is evident in the seasonal variations of predawn leaf water potential (Ψ_l) values, as depicted in Figure 7A. Overall, the control plants exhibited higher leaf water potential values compared to the stressed treatments, indicating a noticeable difference in water status between the two groups, the SW treatment showing the lowest values in June, around -0.9 MPa. Throughout the course of the experiment, the plants subjected to the saline treatment displayed intermediate Ψ_l values at predawn between control and MW, suggesting that saline treatment induced a reduced level of osmotic stress than MW. The presence of salinity and deficit irrigation resulted in a decrease in leaf osmotic potential at predawn, with a more pronounced effect observed in the SW treatment, as depicted in Figure 7B. This, in turn, led to higher values of leaf pressure potential specifically in the SW treatment (Figure 7C) and occasionally in S plants. Leaf water potential at midday (Ψ_l) exhibited a declining trend across all treatments in response to increasing atmospheric evaporative demand. The highest Ψ_l values were recorded in December, while the lowest values occurred in August. Notably, the SW treatment demonstrated the most extreme reduction in Ψ_l, reaching values as low as approximately -3.25 MPa during August (Figure 7D).

The differences between treatments in Ψ_l midday values were consistently lower than the predawn values, primarily because of the influence of environmental factors (Figure 7D). Throughout the experimental period, no significant variations in Ψ_l levels were observed among the treatments, even though the substrate moisture was clearly different, as was reflected in the Ψ_l values at predawn. Only in August, plants of severe water deficit treatment showed Ψ_l values significantly lower than control (Figure 7D). Plants of the MW treatment showed Ψ_l values at predawn lower than control during most of the experiment, but these plants showed similar or even higher values than control at midday. Leaf osmotic potential at midday (Ψ_o) was decreased by salinity and by deficit irrigation (Figure 7E), which caused higher values of leaf pressure potential (Ψ_t) in saline and water stress treatments at midday, except in August, when it resulted in turgor loss in SW plants (Figure 7F).

Stem water potential (Ψ_s) values were notably higher in the control plants compared to the other treatments, and Ψ_s values decreased by salinity and water deficit (Figure 8A). The standard error associated with stem water potential (Ψ_s) measurements was

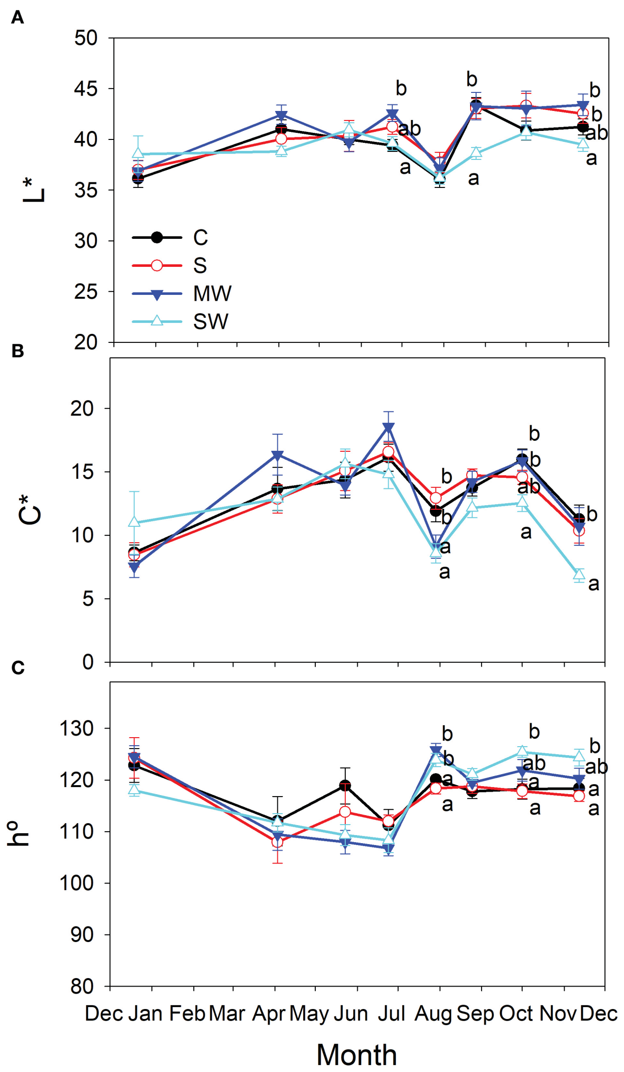

FIGURE 3
Evolution of leaf color parameters, Lightness [L*; **(A)**], Chroma [C*; **(B)**] and hue angle [h°, **(C)**] of *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of seven plants per treatment and vertical bars indicate SE. Symbols represent the different treatments: Control (filled circles), S (open circles), MW (filled triangles) and SW (open triangles). Different letters indicate significant differences between treatments according to Duncan t-test ($P \leq 0.05$).

considerably lower compared to that of leaf water potential (Ψ_l). Moreover, significant differences in Ψ_s levels were consistently observed between treatments throughout the entire experimental period. These findings indicate that Ψ_s served as a more sensitive parameter in identifying significant variations between the treatments than Ψ_l measured at midday. Furthermore, it was observed that significant differences between treatments were identified at earlier stages when considering Ψ_s compared to Ψ_l at predawn, in both salinity and water deficit (Figure 8). This indicates again that Ψ_s measurements were more effective in detecting treatment distinctions at an earlier time point during the experimental period. The maximum and minimum disparities observe between Ψ_s and Ψ_l measurements taken simultaneously from the same plant were found to correspond with the maximum and minimum values of g_s, respectively (Figure 8B). In general, the plants of saline and both water deficit treatments showed lower Ψ_{100s} values than control plants throughout the experimental period (Figure 9), The variation observed between the values obtained from the control plants and the stressed plants was considered as an estimate of the degree of adjustment (0.22, 0.31 and 0.5 MPa for MW, SW and S, respectively).

Salinity, and especially water deficit reduced stomatal conductance (g_s) from the beginning of the experiment (Figure 10A), the effect being more pronounced in SW plants in the early summer (May and June). After which the values of g_s for water deficit plants recovered, reaching the control values. In contrast, differences in the g_s values with respect to the control produced by salinity were more marked at the end of the experiment, reaching g_s values very low at this time (Figure 10A). Such reductions in photosynthesis levels were also evident, although the differences observed were less pronounced (Figure 10B). The P_n values fell earlier and stronger in the severe water stress than in the

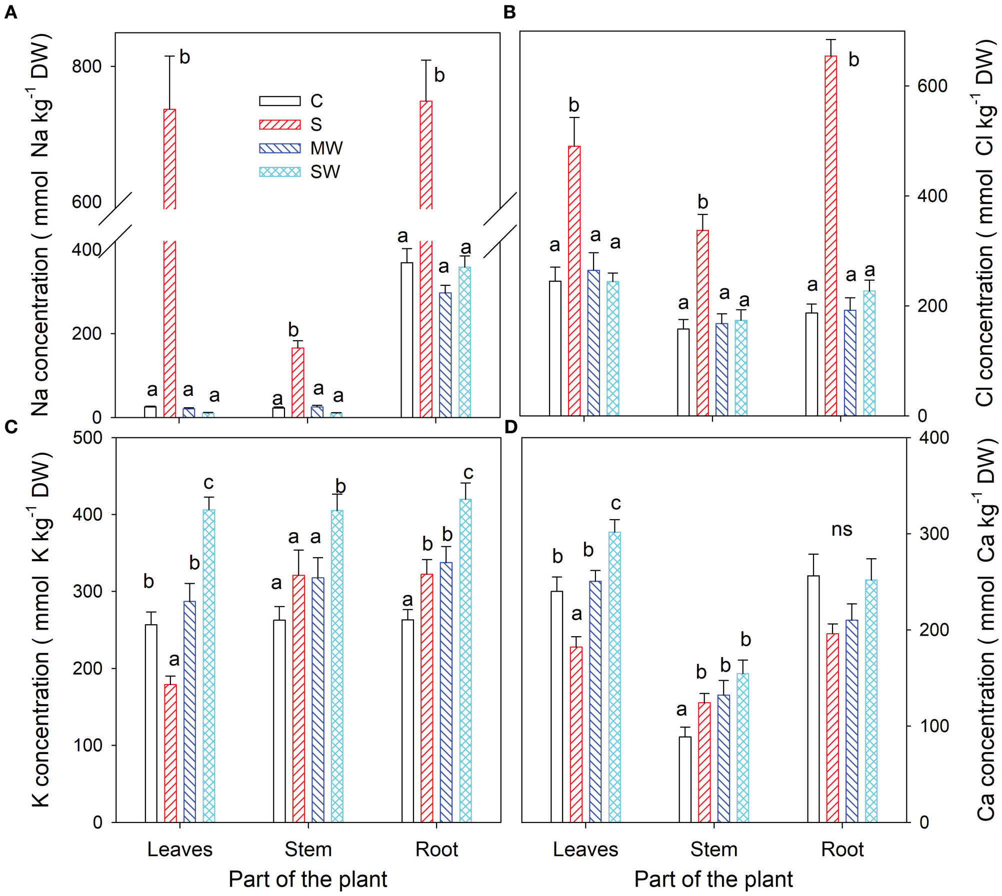

FIGURE 4
Concentrations of Na^+ **(A)**, Cl^- **(B)**, K^+ **(C)** and Ca^{2+} **(D)** at the end of the experiment in *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of ten plants per treatment and vertical bars indicate SE. Different letters within a part of the plant indicate significant differences between treatments according to Duncan t-test ($P \le 0.05$).

saline treatment, but by the conclusion of the experiment, there was a notable decrease observed in this parameter disappeared in the water stressed plants, and MW and SW plants had higher P_n values than control plants. In general, the plants subjected to water stress treatments exhibited higher P_n/g_s ratios, representing intrinsic water use efficiency, compared to the control plants throughout

TABLE 2 Leaf, stem and root K^+/Na^+ and Ca^{2+}/Na^+ ratios at the end of experimental period in *P. lentiscus* subjected to different irrigation treatments.

Parameters		Treatments				P
		C	S	MW	SW	
K^+/Na^+	Leaves	10.8±0.9bB	0.3±0.1aA	17.1±3.1bB	42.9±3.0cB	***
	Stem	13.2±1.8bB	2.1±0.4aB	14.8±1.8bB	44.1±3.9cB	***
	Root	0.8±0.1bA	0.5±0.1aA	1.2±0.1cA	1.2±0.1cA	***
		***	***	***	***	
Ca^{2+}/Na^+	Leaves	10.4±1.3bC	0.3±0.1 aA	14.2±1.9bC	32.2±2.6cC	***
	Stem	4.3±0.5bB	0.8±0.1aB	6.4±0.9bB	16.4±1.6cB	***
	Root	0.7±0.1bA	0.3±0.0aA	0.7±0.0bA	0.7±0.0bA	***
		***	***	***	***	

Values are the mean of ten plants.
Means within a row without a common lower case letter are significantly different according to Duncan 0.05 test. Means within a column without a common capital letter are significantly different according to Duncan 0.05 test.
(P; probability level, *** $P \le 0.001$).

TABLE 3 Correlation coefficients (r) between some growth parameters and Na+ and Cl_ concentration in the leaves.

Parameter	Na		Cl	
	r	P	r	P
Total dry weight	-0.122	0.60 ns	-0.014	0.9 ns
Plant height	-0.72	0.003 **	-0.595	0.005 **
SLA	0.64	0.002 **	0.71	0.0004 ***
Compactness	0.11	0.63 ns	0.107	0.65 ns

ns, non significance, **P<0.01, *** P ≤ 0.001.

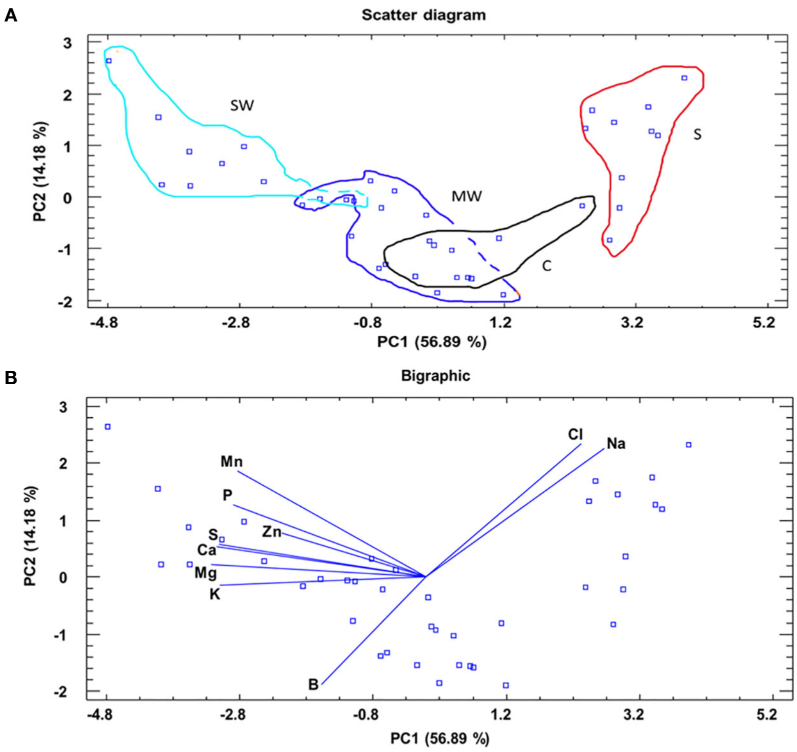

FIGURE 5
A principal component analysis applied to the different treatments (C, MW, SW and S). A scatter diagram and two principal components (PC1 and PC2) resulted in a model that explained 71.07% of the total variance **(A)**. Bigraphic representation of the principal components marking with lines for each variable and wit points for each score **(B)**.

the duration of the experiment, as depicted in Figure 10C, but no pronounced differences were found between control and S, in the later treatment P_n and g_s were proportionally reduced.

4 Discussion

Climate change is affecting Mediterranean regions with extreme weather events, such as prolonged droughts and increases in soil salinity. These conditions can affect the capacity for grow and survive in plants, even for species that are adapted to these regions. Species that grow in Mediterranean regions, such as *Pistacia lentiscus* provide important ecosystem services such as ornamental production, soil protection, and carbon capture (Romano et al., 2022). Therefore, it is essential to find ways to protect and improve their capacity to adjust to changing conditions. The growth response of species to stresses is a complex expression of physiological and biochemical parameters. Although the tolerance mechanisms for salt and drought stress are similar physiologically and sometimes, they overlap, some aspects of a plant's physiology and metabolism may change if the plant is under salinity or water stress (Álvarez et al., 2018). In our conditions, we found that *Pistacia lentiscus* plants were more affected by deficit irrigation than by saline water. Concretely, different stresses induced different growth responses in *Pistacia lentiscus*, in this sense, both deficit irrigation treatments showed significant differences compared to the control and salinity in leaf/total DW and stem/total DW. However, the severe irrigation deficit treatments had differences in variables such as total DW, SLA (as measure of the proportion of plant biomass invested in leaf area but

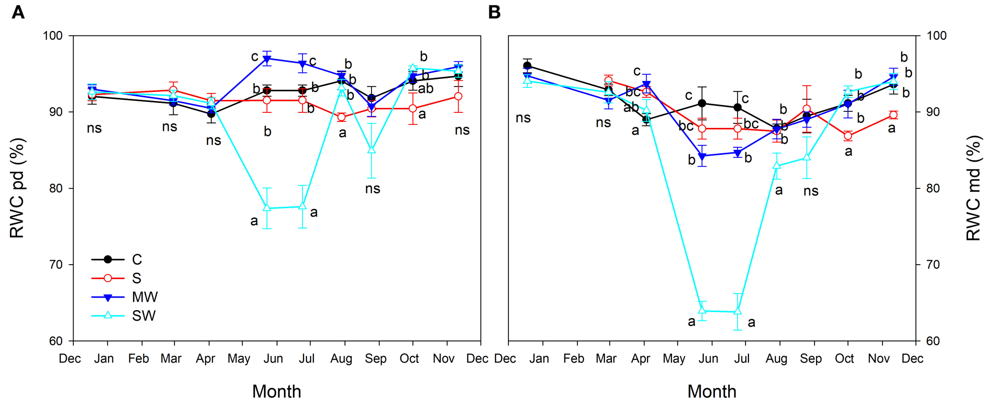

FIGURE 6
Evolution of leaf relative water content (RWC) at predawn **(A)** and midday **(B)** of *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of eight plants per treatment and vertical bars indicate SE. Symbols represent the different treatments: Control (filled circles), S (open circles), MW (filled triangles) and SW (open triangles). Different letters indicate significant differences between treatments according to Duncan t-test ($P \leq 0.05$). ns, non significance.

also of relative thickness, density or spread of leaves), LAR (as measure of photosynthetic surface relative to respiratory mass), or plant height (Wellstein et al., 2017; Gonzalez-Paleo and Ravetta, 2018).

Several variables and integrated them into a star and ray graph, which corroborated the differences between treatments indicated above were analysed. Aesthetically and commercially, customers appreciate a compact and architecturally balanced plant with increased foliage size in relation to plant height and with no alterations in leaf colour (Álvarez et al., 2013). Indeed, leaf colour is also an important attribute of ornamental plants, when used in gardening or landscaping projects, which influences its commercial value (Navarro et al., 2009). Customers associate yellow leaves with senescence, which is a negative trait that decrease the quality and attractiveness of the plant (Boutigny et al., 2020). In our conditions, no significant modifications in the leaf colour parameters were found and only treatment with moderate lack of irrigation had the highest value for the compactness variable. Therefore, the kind of stress should be taken into consideration as a key factor when using saline water and/or deficit irrigation as an irrigation strategy. This may affect the ornamental quality of the plants, assessed through growth parameters, to a greater or lesser extent (Koniarski and Matysiak, 2013; Sánchez-Blanco et al., 2019). Our results indicate that although both deficit irrigation and salinity reduced relative growth rate in *Pistacia lentiscus*, the time that each stress took to have an impact on plant growth differed significantly between salinity and water stress. Salinity affected growth much later than water stress, and such a reduction was only visible after a considerable amount of time had passed since the beginning of the irrigation treatments, as salts take time to accumulate inside plants before the concentrations reach dangerous levels and influence plant function (Munns and Tester, 2008). This underlines the fact that the duration of the water and salt stress is a crucial factor (Álvarez and Sánchez-Blanco, 2015; Vivaldi et al., 2021).

Sodium (Na^+) and chloride (Cl^-) are two ions that can accumulate in plant roots, particularly when plants are exposed to high levels of salts. When salt is present in the soil, the plant roots take up both water and salt. Sodium and chloride ions are particularly mobile and can easily move into the root cells. High concentrations of Na^+ and Cl^- in the root cells can create an osmotic imbalance, leading to reduced water uptake and increased water stress. Additionally, high levels of Na^+ in the plant can compete with other essential cations, such as potassium (K^+) or calcium (Ca^{2+}), for uptake and transport, leading to nutrient imbalances and potential toxicity (Acosta-Motos et al., 2016). A low K^+/Na^+ and Ca^{2+}/Na^+ ratios can cause several physiological changes in plants, such as reduced photosynthesis, nutrient imbalance, and oxidative stress, which can negatively affect plant growth and productivity. Sodium and potassium interact on two levels: interference with K^+ nutrition and replacement of Na^+ for K^+ (Haro et al., 2010). High Na^+ concentrations in plants cause K^+ shortage symptoms and impair several K^+-mediated physiological activities, including protein synthesis and enzymatic responses. Furthermore, membrane depolarization generated by Na^+ input into the cell impairs K^+ uptake through inward-rectifying K^+ channels, making it thermodynamically unfavourable, as well as increased K^+ outflow through outward-rectifying channels (Shabala et al., 2006; Coskun et al., 2013). Despite the controversy over whether NRT proteins transport NO^{3-}, K^+ or both, it is evident that Na^+ competes with K^+ in plant uptake specifically through High-Affinity K^+/K^+ uptake/K^+ Transporter, High-Affinity Potassium Transporters and Non-Selective Cation Channel (Kronzucker and Britto, 2011). High-Affinity Potassium transporters are required for K^+ uptake, root hair production and tolerance to abiotic stressors (Nieves-Cordones et al., 2014). They may, nevertheless, play a crucial function in facilitating Na^+ absorption. Indeed, several members of this family have been demonstrated to mediate high-affinity Na+ uptake.

As a result of salt stress, some species have developed mechanisms to maintain a high K^+/Na^+ and Ca^{2+}/Na^+ ratios. These mechanisms include the selective uptake of K^+ or Ca^{2+} over Na^+, the exclusion of Na^+ from uptake, the compartmentalization of Na^+ in vacuoles in the root cells, and the

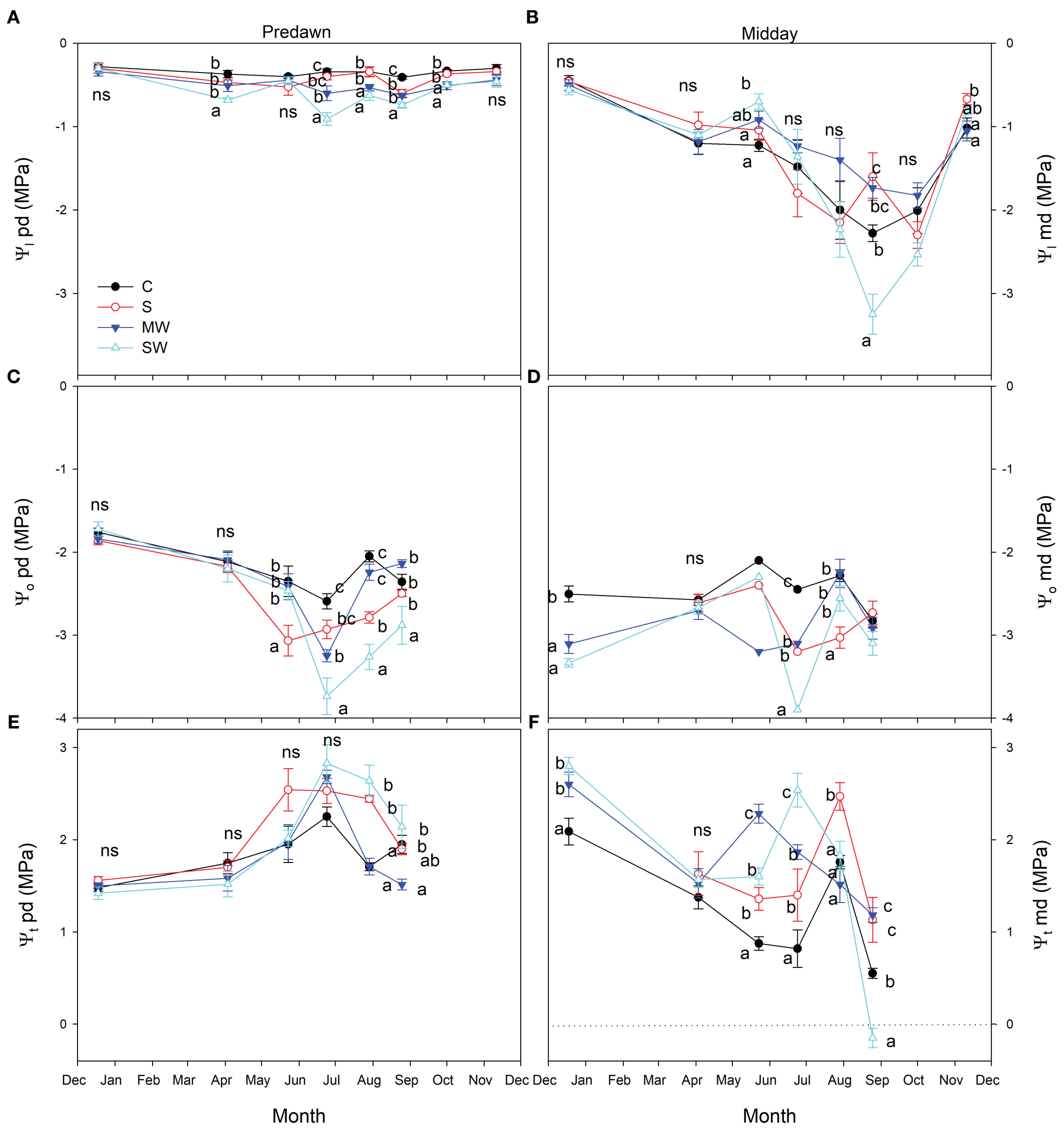

FIGURE 7
Evolution of the leaf water potential at predawn [Ψ_l pd, **(A)**], leaf osmotic potential at predawn [Ψ_o pd; **(B)**], leaf turgor potential at predawn [Ψ_t pd; **(C)**], leaf water potential at midday [Ψ_l md, **(D)**], leaf osmotic potential at midday [Ψ_o md, **(E)**] and leaf turgor potential at midday [Ψ_t md, **(F)**] of *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of eight plants per treatment and vertical bars indicate SE. Symbols represent the different treatments: Control (filled circles), S (open circles), MW (filled triangles) and SW (open triangles). Different letters indicate significant differences between treatments according to Duncan t-test ($P \leq 0.05$). ns, non significance.

transport of Na^+ back to the soil through the salt glands or leaf excretion (Parihar et al., 2015; Isayenkov and Maathuis, 2019; Acosta-Motos et al., 2020). Maintaining a high K^+/Na^+ and Ca^{2+}/Na^+ ratios in plant tissues can be critical for plant survival under salinity stress. Therefore, the K^+/Na^+ and Ca^{2+}/Na^+ ratios can be used as a diagnostic tool to assess plant tolerance to salinity stress. By measuring the K^+/Na^+ and Ca^{2+}/Na^+ ratios in plant tissues, growers and researchers can gain insights into the plant's response to salinity stress and develop strategies to enhance plant tolerance to salinity stress (Acosta-Motos et al., 2014; Liu et al., 2019; Bello et al., 2021). In our study, the saline treatment (S) shows the highest values in Na^+ and Cl^- ions and the lowest values for both ratios in leaves and stems. On the contrary, the severe deficit treatment (SW) showed the highest values in K^+ and Ca^{2+} ions and both ratios also in the same organs. Also, this nutritional behaviour associated with the saline treatment (S) and severe water deficit treatment (SW) made it possible to separate them very well, especially because of the importance of K^+ and Na^+ and other nutrients as Mg^{2+} and Ca^{2+} in PC1 and due to the Na^+ and Cl^- ions in PC2. Therefore, other nutrients in leaves, as Mg^{2+} or Ca^{2+}, also are important. Mg^{2+} can have a higher demand as a nutrient for chlorophyll synthesis (as it is part of its chemical structure), which is essential for carrying out a more efficient process of photosynthesis and increasing the water use efficiency (WUE) under stress situations (Dias et al., 2017; Tränkner et al., 2018; Ouled Youssef and Krouma, 2021). In our study, a higher concentration of Mg^{2+} in leaves could optimise the photosynthetic process despite increased stomata closure in the SW and MW treatments (especially in SW) contributing to an increase

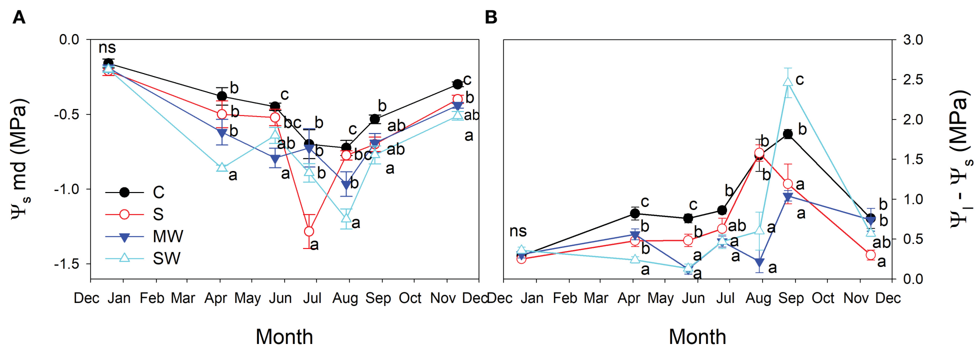

FIGURE 8
Evolution of the stem water potential [Ψ_s; **(A)**], and difference between stem and leaf water potential [Ψ_s-Ψ_l, **(B)**] of *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of eight plants per treatment and vertical bars indicate SE. Symbols represent the different treatments: Control (filled circles), S (open circles), MW (filled triangles) and SW (open triangles). Different letters indicate significant differences between treatments according to Duncan t-test ($P \leq 0.05$).

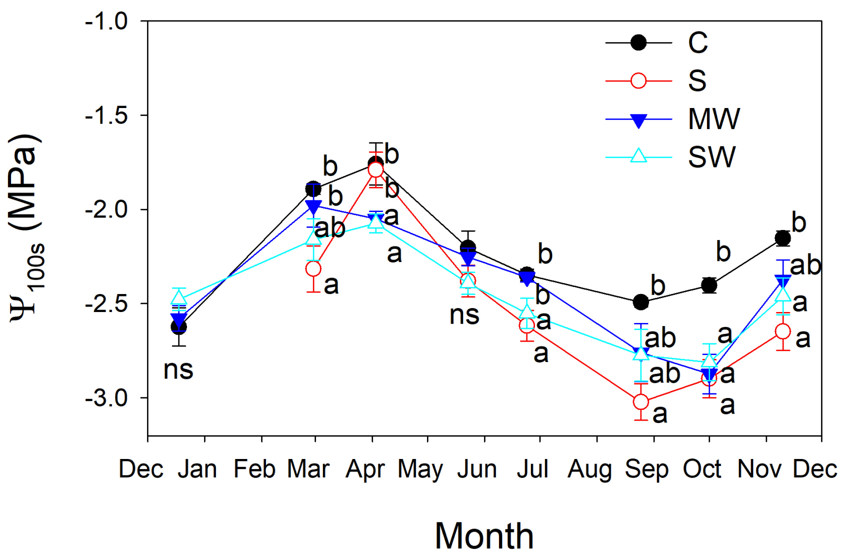

FIGURE 9
Evolution of the leaf osmotic potential at full turgor (Ψ_{100s}) of *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of eight plants per treatment and vertical bars indicate SE. Symbols represent the different treatments: Control (filled circles), S (open circles), MW (filled triangles) and SW (open triangles). Different letters indicate significant differences between treatments according to Duncan t-test ($P \leq 0.05$).

in WUE. Ca^{2+} is crucial to ensure the maintenance of cellular rigidity, as it plays a vital role in the stiffness and stability of cell walls (as it is part of their chemical composition). Ca^{2+} helps maintain the structural integrity of cells and prevents them from collapsing or being damaged under water stress conditions. Additionally, Ca^{2+}can improve cellular turgor or maintain proper cellular turgor even under severe deficit irrigation conditions contributing to cell survival and normal functioning (Ahanger et al., 2013; Kour et al., 2023). In our study, a higher concentration of Ca^{2+} in leaves could assume the same roles as indicated above in the SW and MW treatments (especially in SW).

The relative water content (RWC) at predawn and midday are commonly used time points to assess changes in plant water status. RWC at predawn reflects plant water potential and indicates the level of water stress. Since photosynthesis and transpiration haven't started yet, there is no additional water loss during this time. On the other hand, RWC at midday can indicate plant water use efficiency. A higher RWC at midday suggests the plant maintains good hydration despite transpiration, while a lower RWC indicates water stress (Gindaba et al., 2005). In our study, moderate water deficit led to higher RWC values at predawn during summer months compared to the control. RWC values at midday were more affected by irrigation restriction, with lower values in severe treatments. Saline treatment showed little difference from the control. RWC is more sensitive to water stress than salt stress due to its definition as a measure of plant hydration. Water stress reduces RWC, while salinity stress hampers water absorption. RWC is a more rapid and sensitive indicator of water stress compared to

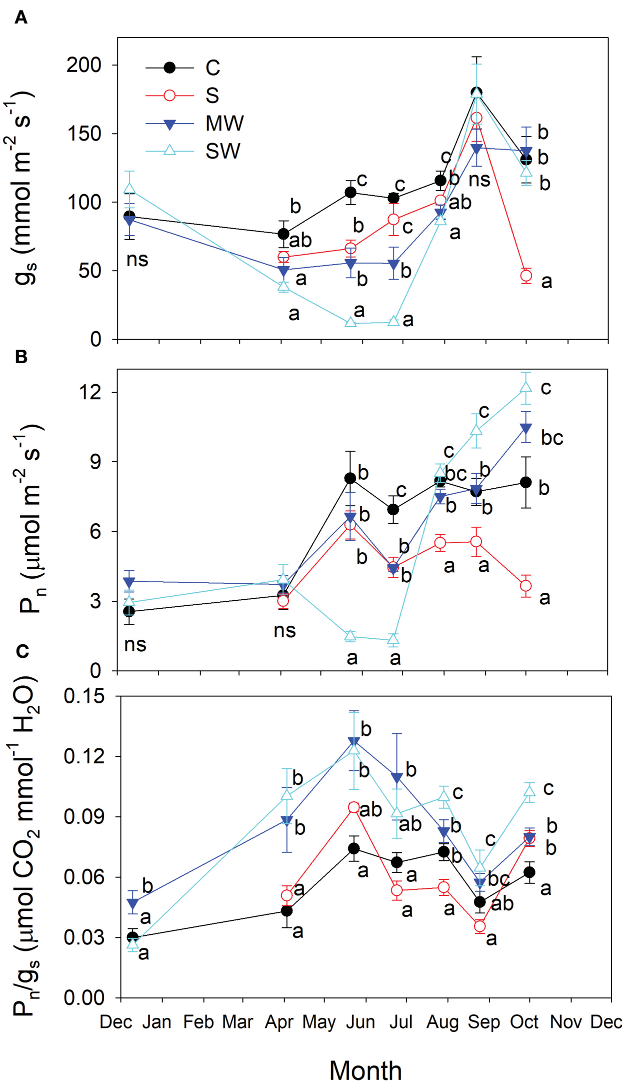

FIGURE 10
Evolution of stomatal conductance [g_s; **(A)**], net photosynthetic rate [P_n, **(B)**] and intrinsic water use efficiency [P_n/g_s; **(C)**] of *Pistacia lentiscus* plants subjected to saline and deficit irrigation treatments. Values are means of eight plants per treatment and vertical bars indicate SE. Symbols represent the different treatments: Control (filled circles), S (open circles), MW (filled triangles) and SW (open triangles). Different letters indicate significant differences between treatments according to Duncan t-test ($P \leq 0.05$).

plant growth and yield affected by salinity stress. RWC values in drought treatments recover after the hottest months, as cooler temperatures reduce water stress and enhance water uptake. Recovery depends on factors like severity and duration of stress, plant species, tolerance, and soil water availability (Toscano et al., 2019). During hot and dry months, water stress can cause a decline in RWC, potentially leading to plant damage or death (Alhaithloul, 2019). However, if the stress is not severe, the plant can recover RWC during cooler and wetter months (Galmés et al., 2007). Cooler temperatures reduce transpiration and slow water loss, maintaining or increasing RWC. They also increase soil water-holding capacity, supplying more water for plant uptake and aiding RWC recovery (Juenger and Verslues, 2023).

In a similar way to RWC, leaf water potential can vary throughout the day due to changes in plant water status, with different values typically observed at predawn and midday and it has also demonstrated usefulness as a stress index in a large number of species (Álvarez et al., 2019; Sánchez-Blanco et al., 2019; Álvarez et al., 2020). The difference between the leaf water potential at predawn and midday can be used to evaluate the plant's ability to maintain its water status throughout the day. A smaller difference between the two values indicates that the plant is able to maintain its water status during the day and that it has good water use efficiency, while a larger difference indicates that the plant is experiencing water stress during the day and that it may not be using water efficiently. In our study, as one would expect the changes in leaf water potential were more evident in predawn that in midday due to the influence of environmental factors as mentioned above and more pronounced in drought treatments during the summer months.

During drought and saline conditions, the availability of water in the soil decreases, and plants often experience water stress and as a result, the stem water potential of the plant also decreases (Pérez-Pérez et al., 2009; Yang et al., 2020). When water is readily available in the soil, the plant takes up water through its roots and this water is transported through the plant's vascular system to the leaves. This movement of water is driven by differences in water potential between the soil, the plant, and the atmosphere (Berry et al., 2019; Scharwies and Dinneny, 2019). When soil water availability decreases due to drought or salinity, the plant's ability to take up water is reduced, and water transport through the plant is also affected. As the plant attempts to cope with water or salt stress, it may reduce its transpiration rate and water use to conserve water (Bertolino et al., 2019). This could result in a decline in stem water potential as the plant attempts to maintain water balance and avoid tissue damage (Levy et al., 2013; Lugassi et al., 2019). In some cases, the stem water potential may decrease to a level that is so low that the plant wilts or even dies (Abideen et al., 2021). In our study, depending on the month analysed and especially in the summer months, sometimes it is the saline treatment or the severe deficit irrigation, the treatments that show the lowest values in stem water potential. To cope with low stem water potential, plants can develop various mechanisms to optimize water use and minimize water loss. Some of these mechanisms include stomatal regulation, root and leaf adaptations, dormancy and shedding, or cellular protection. In our study, *Pistacia lentiscus* plants regulated the opening and closing of stomata to control water loss through transpiration and plants subjected to saline and water deficit produced osmoprotectants and antioxidants in order to help protect cells from damage caused by dehydration and oxidative stress. These mechanisms collectively help plants survive and adapt to low stem water potential by reducing water loss, optimizing water uptake, and maintaining essential physiological processes as much as possible during challenging conditions.

Plants increase their osmotic adjustment in response to salt or drought stress, which results in the accumulation of osmolytes like soluble sugars, amino acids, and organic acids, in plant cells in response to a depletion of the available soil moisture (Callister et al., 2006; Singh et al., 2015; Khan et al., 2020). This allows the plant to maintain its turgor pressure and prevent wilting. Osmotic adjustment is closely related to the leaf water potential and relative water content of the plant. Maintaining high RWC at low leaf water potentials is primarily related to osmotic adjustment, which could be considered as a strategy to tolerate water stress (Pérez-Pérez et al., 2009; Banks and Hirons, 2019). In our study, *P. lentiscus* plants submitted to water stress and salinity showed osmotic adjustment, which was more pronounced under salinity than under both levels of water deficit. NaCl-induced osmotic adjustment is also a common response to salinity stress caused by high concentrations of salt in the soil or irrigation water (Zhang et al., 2022). To counteract the decrease in water availability due to high salt concentration, plants can adjust their osmotic potential by accumulating solutes, sugars, and amino acids, in their cells. However, the effectiveness of osmotic adjustment in alleviating the effects of salinity stress depends on the plant species and cultivars, as well as the concentration and duration of the salt stress (Liao et al., 2022; Egea et al., 2023). In our study, *Pistacia lentiscus* might exhibit some tolerance to a salinity level of 4 dS m^{-1}, but it's essential to remember that "tolerance" doesn't mean thriving. Even tolerant plants can show signs of stress and reduced growth under elevated salt conditions. *Pistacia lentiscus* is known to have some degree of salt tolerance, but its exact response to different salinity levels can vary. In our study, *P lentiscus* plants irrigated with salinity level of 4 dS m^{-1} during 11 months reduced growth, altered nutrient uptake, increased the osmotic adjustment and reduced gas exchange parameters.

The findings from our study indicate that the observed reduction in g_s is an adaptive and efficient mechanism used by the species to control transpiration (Hatfield and Dold, 2019). This mechanism helps the plant can withstand salinity and water stress, particularly during periods of high transpiration. In the case of plants under deficit irrigation conditions, this mechanism limits water loss (Lefi et al., 2023). For saline plants, the reduction in g_s helps to decrease the salt load on leaves, thus increasing longevity by keeping salts at subtoxic levels for a longer time (Izadi et al., 2022). The delay in the reduction of g_s in saline plants as compared to deficit irrigation plants is due to the time it takes for salts to accumulate inside the plant before reaching toxic levels that could affect plant functioning (Munns, 2011; Pirasteh-Anosheh et al., 2016). Similar changes in P_n were observed also in g_s between the treatments studied. The *P. lentiscus* plants that were subjected to both deficit irrigation treatments exhibited an increase in their intrinsic water use efficiency (P_n/g_s), particularly during periods of high-water demand. An increase in intrinsic water use efficiency (P_n/g_s) in plants indicates that they are able to photosynthesize and produce the same amount of biomass while using less water, which is an important adaptation for plants growing in environments with water scarcity and salt stress (Fernández-García et al., 2014; Liao et al., 2022). Plants face a constant dilemma: to die of thirst or to die of hunger. This is because plants need water to carry out photosynthesis and produce energy, but they also need nutrients such as nitrogen, phosphorus, and potassium to grow and develop. If plants do not receive enough water, their ability to photosynthesize is compromised, reducing their energy production capacity and ultimately leading to plant death by dehydration (Chaves et al., 2016). On the other hand, if plants do not receive enough nutrients, their growth is affected, and their energy production capacity is also reduced, leading to death by starvation. To survive, plants must find a balance between water and nutrient uptake and water loss through transpiration. This is achieved by regulating stomatal aperture, which controls the entry and exit of water and gases in the plant (Bhattacharya, 2021). While this balance may vary depending on environmental conditions and plant species, most plants have evolved to optimize their efficiency in using water and nutrients and to adapt to variable conditions in their environment.

It can be inferred that the application of moderate and severe deficit irrigation (60 and 40 % reductions with respect to the control) and the use of saline water with a determined level of salinity (around 4 dS m^{-1}) is feasible for growing this species. Based on its observed behaviour when applying deficit irrigation strategies and irrigation with low quality water, *Pistacia lentiscus* is proposed as a suitable species for gardening projects and landscaping in arid and saline areas

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

SA performed the experiment and drafted the manuscript. MS-B and SA designed and instructed the research work. SA and JA-M carried out statistical analysis. MS-B also coordinated the study and provided study material and facilities for the experiments. The three authors were involved in data interpretation, paper preparing and article writing. All authors have read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1237332/full#supplementary-material

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Elevated tropospheric ozone and crop production: potential negative effects and plant defense mechanisms

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Ozone (O_3) levels on Earth are increasing because of anthropogenic activities and natural processes. Ozone enters plants through the leaves, leading to the overgeneration of reactive oxygen species (ROS) in the mesophyll and guard cell walls. ROS can damage chloroplast ultrastructure and block photosynthetic electron transport. Ozone can lead to stomatal closure and alter stomatal conductance, thereby hindering carbon dioxide (CO_2) fixation. Ozone-induced leaf chlorosis is common. All of these factors lead to a reduction in photosynthesis under O_3 stress. Long-term exposure to high concentrations of O_3 disrupts plant physiological processes, including water and nutrient uptake, respiration, and translocation of assimilates and metabolites. As a result, plant growth and reproductive performance are negatively affected. Thus, reduction in crop yield and deterioration of crop quality are the greatest effects of O_3 stress on plants. Increased rates of hydrogen peroxide accumulation, lipid peroxidation, and ion leakage are the common indicators of oxidative damage in plants exposed to O_3 stress. Ozone disrupts the antioxidant defense system of plants by disturbing enzymatic activity and non-enzymatic antioxidant content. Improving photosynthetic pathways, various physiological processes, antioxidant defense, and phytohormone regulation, which can be achieved through various approaches, have been reported as vital strategies for improving O_3 stress tolerance in plants. In plants, O_3 stress can be mitigated in several ways. However, improvements in crop management practices, CO_2 fertilization, using chemical elicitors, nutrient management, and the selection of tolerant crop varieties have been documented to mitigate O_3 stress in different plant species. In this review, the responses of O_3-exposed plants are summarized, and different mitigation strategies to decrease O_3 stress-induced damage and crop losses are discussed. Further research should be conducted to determine methods to mitigate crop loss, enhance plant antioxidant defenses, modify physiological characteristics, and apply protectants.

KEYWORDS

abiotic stress, antioxidants, atmospheric pollutants, oxidative stress, photosynthesis, prooxidant, reactive oxygen species

1 Introduction

Ozone (O_3) is a major environmental stressor that affects crop production negatively. The concentration of O_3 is predicted to increase by 25% by 2050 and 60% by 2100 (Meehl et al., 2007; Jimenez-Montenegro et al., 2021). Ozone is present both in the stratosphere and the troposphere, where the stratospheric O_3 is known as the "good O_3" or "O_3 layer" because it absorbs the harmful ultraviolet (UV-B) rays of sunlight and acts as a UV-ray filter, preventing its harmful effects on living organisms on Earth including plants. Tropospheric or ground-level O_3 is responsible for promoting damaging effects on living cells, organs, and species; therefore, it is known as "bad O_3" (Xu, 2021). Tropospheric O_3, namely ground-level O_3, is an air pollutant and secondary air pollutant recommended by air quality guidelines in all countries. Although they are generated in the troposphere by sunlight-driven chemical processes that combine nitrogen oxides (NOx) and volatile organic carbons (VOCs), the use of fossil fuels by the industrial and transport sectors has significantly increased the level of O_3 in the troposphere, mainly due to the release upon combustion of several O_3 precursors (Zhang et al., 2019; Hasan et al., 2021). Consequently, the phytotoxic effects of increased tropospheric O_3 on many plant species, losses in global crop production, especially in industrial and urban areas, and damage to ecological health and environmental sustainability in the long term are well known (Monks et al., 2015; Ramya et al., 2023). However, the generation of O_3 occurs in the troposphere through photochemical reactions between precursors emitted by anthropogenic, natural, and agronomic sources, such as NOx, carbon monoxide, methane, VOCs, and peroxyacetyl nitrate (Vainonen and Kangasjärvi, 2015).

Plants take up O_3 through the stomata, and it is later converted into reactive oxygen species (ROS) in the apoplast. The accumulation of ROS damages the photosynthetic machinery, causes stomatal closure, and degrades ribulose-1,5-bis-phosphate carboxylase/oxygenase (RuBisCO) (Ren, 2021). However, the damage caused by O_3 to plants depends on the dose and exposure time. Ozone exposure can be categorized as acute or chronic. High doses of O_3 over short periods (acute damage) may lead to programmed cell death and leaf damage, particularly in sensitive plant species. However, a lower dose of O_3 for a longer duration (chronic damage) affects the photosynthetic rate, causing growth reduction and rapid leaf senescence, with or without visible damage to the leaves (Chen et al., 2018; Emberson et al., 2018). Increased O_3 exposure results in higher yield loss through foliar damage, suppression of photosynthesis with altered carbon translocation, and consequently earlier plant senescence occurs. Numerous distinct alterations in gene expression, metabolic profiles, and enzyme activity occur in plants exposed to O_3. Acute O_3 exposure in sensitive accessions led to increased cell death, lesion formation, and reduced photosynthesis (Morales et al., 2021). Owing to its high reactivity and instability, O_3 can cause oxidative stress in apoplast plant cells by chemically altering different components such as proteins to form short-lived ROS. Plants activate antioxidant defense mechanisms to scavenge ROS and prevent their negative effects. Ozone-tolerant plants have different characteristics that suppress cellular toxic elements, such as peroxidation, and maintain cell membrane stability, which is made possible by the activation of both enzymatic and non-enzymatic antioxidant components (Dhevagi et al., 2021; Ramya et al., 2021a). Thus, plants can become tolerant to O_3, which ultimately safeguards their yield under adverse circumstances.

The generation of O_3 has a strong relationship with different meteorological factors, being positively correlated with sunshine hours and negatively correlated with wind speed and relative humidity (Markovic and Markovic, 2005). Consequently, the level of O_3 changes over the season and even during the day, with high values in the day and dry months and low values in the night and wet months (Jain et al., 2005). It has been reported that the diurnal variation in O_3 coincides with the intensity of solar radiation and higher air temperatures. Nevertheless, ground-level O_3 concentrations remain low under nighttime conditions because there is no photolysis of nitrogen dioxide (NO_2) or photooxidation of O_3 precursors (Ma et al., 2021). Upon exposure to ultraviolet (UV) radiation, NO_2 dissociated into nitric oxide (NO) and oxygen (O). The short lifetime of O_3 is related to its ability to react with NO to produce NO_2 and O_2 again (Simon et al., 2014).

In this review, we explored the processes involved in O_3 uptake by plants and their perception of this pollutant. In this study, we investigated the multivariate effects of O_3 on plant growth, nutrition, physiology, yield, and oxidative stress. In addition, we discuss strategies for mitigating the phytotoxic effects of O_3 and enhancing the performance of O_3-affected plants. These strategies include crop management practices, carbon dioxide (CO_2) fertilization, using chemical elicitors, proper nutrient management, and the selection of tolerant crop varieties. Therefore, this review aims to provide a comprehensive understanding of O_3-induced damage in plants and techniques for improving O_3 tolerance, thereby shedding light on O_3-related research.

2 Ozone uptake by plants

Ozone pollution has been perceived mostly as a daytime problem because gas generation occurs through complex photolytic reactions, and the leaf stomata are open during this period, allowing O_3 uptake into plants. Nevertheless, O_3 uptake may also occur at night because the stomata are not completely closed (Dawson et al., 2007; Rannik et al., 2009). Furthermore, O_3 can enter the leaves by direct absorption through the leaf exterior surfaces, albeit less than the amount of O_3 entering the stomata (Pleijel et al., 2004). Besides the uptake of O_3 via the stomata, O_3 can also be deposited onto agricultural systems via non-stomatal pathways (e.g., soil and cuticular deposition) (Morales et al., 2021). Stomata are the first barrier to overcome the damage in plants caused by O_3 because stomatal aperture control is responsible for O_3 flux into the leaves (Fiscus et al., 2005). The size of the stomatal aperture is controlled by the activity of the guard cell ion channels and transporters responsible for the movement of osmolytes across the tonoplast and plasma membrane (Kollist et al., 2011; Hedrich, 2012). Ion channel regulation is controlled by reversible protein phosphorylation by protein kinases and phosphatases (Evans et al., 2005).

Three levels of O_3 concentration have been established: low (20–70 nL O_3 L^{-1}), moderate (70–150 nL O_3 L^{-1}), and high (>150 nL O_3 L^{-1}) (Grulke and Heath, 2020). According to European Environmental Legislation, the threshold O_3 concentration should not exceed 40 ppb to protect crops during the growing season (Proietti et al., 2021). The chronological and spatial sequences of these actions define how O_3 exposure affects plant physiology. First, O_3 enters the leaf apoplast via the stomata, where it is degraded into secondary ROS at two different locations (mesophyll and guard cell walls) by triggering specific calcium (Ca) signatures in the cytosol. High concentrations of Ca and ROS in the cytosol of guard cells result in stomatal closure and, consequently restriction of O_3. There is a high influx of O_3 in the apoplast of mesophyll cells, inducing excessive ROS accumulation that is not scavenged by apoplastic antioxidants, such as ascorbate (AsA) (Grulke and Heath, 2020). Higher accumulation of ROS in the apoplast triggers several downstream signaling pathways that work in parallel, in series, or both (Roychowdhury et al., 2019; Li et al., 2023). Reactive oxygen species signaling and apoplast propagation in the apoplast are related to the induction of respiratory burst oxidase homolog (RBOH) activity and type III peroxidases. The signaling process in the apoplast is then quickly relayed to the chloroplast, where the ROS signal is amplified by chloroplastic ROS formation. This process is regulated by heterotrimeric G proteins (Joo et al., 2005; Booker et al., 2012). The intracellular pathways promoted by ROS involve the activation of mitogen-activated protein kinase (MAPK) cascades, modification of intracellular redox homeostasis, and generation of NO (Morales et al., 2021).

The absence of α- or β-subunits of the G-protein in *gpa1* or *agb1* mutants resulted in the first early peak of ROS not being generated, while only the G α-subunit was required for the second peak of ROS accumulation (Joo et al., 2005; Vainonen and Kangasjärvi, 2015). These results indicate that ROS synthesis in the chloroplasts may be involved in apoplast signaling process (Shapiguzov et al., 2012; Sierla et al., 2013). Retrograde signaling from the chloroplasts to the nucleus may cause modifications in nuclear gene expression (Leister, 2012; Estavillo et al., 2013). This signaling process may be achieved via ABI4 (Koussevitzky et al., 2007), WHIRLY 1 (Isemer et al., 2012), and PTM (PHD-type transcription factor with transmembrane domains) transcription factors (Sun et al., 2011) as well as metabolites such as Mg-protoporphyrin IX (Strand et al., 2003), heme (Woodson et al., 2011), 3′-phosphoadenosine-5′-phosphate (PAP) (Xiao et al., 2012) and β-cyclocitral (Ramel et al., 2012).

3 Plant sensing and indication of O_3 stress

Plants respond differentially to ozone exposure. The response pattern of ozone-tolerant and sensitive plants has been reported in several studies. Ozone-responsive proteins and signaling molecules are primarily involved in ozone sensing. The mechanisms involved in O_3 sensing are as follows: a) recognition by an apoplastic receptor protein, which can be changed by the ROS synthesis related to O_3 breakdown, b) oxidation of plasma membrane lipids resulting in the generation of lipid-based signaling molecules that are further sensed, and c) modification in the redox homeostasis due to the participation of ascorbic acid (AsA), glutathione (GSH) or the ratio NAD(P)H/NAD(P) (nicotinamide adenine dinucleotide phosphate-with and without hydrolase) (Kangasjärvi et al., 2005).

Excess ROS production due to O_3 exposure causes cellular damage; however, ROS initially act as signaling molecules (Jaspers and Kangasjärvi, 2010). ROS can alter signal transduction proteins within membranes in response to O_3 stress (Rossard et al., 2006). Consequently, several cellular changes occur, such as a) depolarization and dysfunction of the membrane, b) modification of cell wall compounds, c) promotion of MAP kinase protein cascades to generate new proteins through transcription factor activation, d) ozonolysis of double bonds in the unsaturated fatty acids of cell membranes, and e) lipid peroxidation in the membrane (Evans et al., 2005; Iriti and Faoro, 2008; Sharma et al., 2012; Emberson et al., 2018).

Baier et al. (2005) noted that the signaling process of O_3 from the chemical reaction sites in the apoplast or plasma membrane to the cytosol can be associated with O_3 induced ROS production, particularly H_2O_2, which functions as a diffusible messenger (Laloi et al., 2004) and modulates cytosolic AsA and GSH (Gomez et al., 2004). Extracellular peroxidases (PRX) and plasma membrane-bound NADPH oxidases (RBOH) enhance ROS generation under O_3 stress (Kangasjärvi et al., 2005). In addition to H_2O_2, ethylene and salicylic acid (SA) have been reported as secondary or tertiary messenger molecules involved in O_3 sensing, because O_3 or O_3-derived ROS can activate them. Both ethylene and SA can increase oxidative signaling (Sandermann, 2000; Kangasjärvi et al., 2005).

Exposure of plants to O_3 also triggers SA-induced cell death because SA can inhibit the main H_2O_2-scavenging enzymes such as catalase (CAT) and ascorbate peroxidase (APX) (Faoro and Iriti, 2009). Under severe exposure to O_3, NO synthesis increased. This molecule plays a crucial role in the signaling of plants subjected to stress conditions. Moreover, NO can react with H_2O_2 and NO donors such as sodium nitroprusside (SNP), leading to the accumulation of H_2O_2 in plants (Astier et al., 2018). Another consequence of long-term exposure to O_3 is increased cell wall lignification in many plants, which helps decrease O_3 penetration (Cabané et al., 2012).

The physiological damage caused by O_3 exposure is related to photosynthetic reduction, ROS generation, increased dark respiration, and reduced crop yield (Cailleret et al., 2018). More accurately, the main effects of chronic exposure to O_3 are a reduction in the photosynthetic rate, growth reduction, and premature senescence without visible symptoms (Krupa, 2003). In contrast, acute exposure to O_3 results in cell death and other adverse effects (Kangasjärvi et al., 2005). Symptoms of O_3 exposure can be observed between the veins on the adaxial leaf surfaces of older and middle-aged leaves; however, symptoms can appear on both leaf surfaces (adaxial and abaxial) in some species if the damage is severe (Cho et al., 2011; Vaultier and Jolivet, 2015). Induced chlorosis and bronzing (several spots on the leaves) are the most frequent symptoms of chronic O_3 exposure, and acute exposure may result in a higher number of visible lesions (Kangasjärvi et al., 2005).

4 Ozone as a Prooxidant and O_3-induced oxidative stress

Ozone can directly generate ROS in the leaf mesophyll and guard cell walls after entering the stomata (Grulke and Heath, 2020). Higher exposure activates the pro-oxidant activity of O_3 by increasing ROS accumulation and decreasing the antioxidant machinery in plant cells (Dhevagi et al., 2021; Ramya et al., 2021a). Moreover, the overgeneration of ROS and the reaction between O_3 and plasma membrane lipids results in peak levels of superoxide and thiobarbituric acid (Marchica et al., 2019). This phenomenon confirmed the oxidative stress in plant cells upon O_3 exposure. Under O_3 exposure, plants suffer several physiological damages, resulting in a lower photosynthetic rate, which in turn results in growth reduction, premature senescence, and cell death, mainly due to the generation of ROS. To counteract these harmful effects, plants activate different antioxidant systems to scavenge the reactive molecules (Figure 1).

Several studies have investigated the pro-oxidant effects of O_3 and the respective changes in redox homeostasis. Rice (*Oryza sativa* L.) seedlings of three different cultivars, Nipponbare and BRRI dhan28 (sensitive to O_3), and L81 (O_3-tolerant introgression line), were grown from April to October 2016 in a controlled climate greenhouse. Plants were sprayed with 80 ppb O_3 for 7 hours daily, five weeks after transplantation to induce acute stress. Owing to tolerance differences between cultivars, malondialdehyde (MDA) content increased in sensitive cultivars but not in the tolerant line. However, the total leaf AsA did not show any significant differences among the cultivars (Ashrafuzzaman et al., 2017). Tobacco (*Nicotiana tabacum* L.) plants exposed to 300 nmol mol^{-1} for 4 h at midday showed clear symptoms of leaf necrosis and a reduced net photosynthetic rate. Oxidative stress caused by exposure to O_3 results in an increase in H_2O_2 and MDA contents, and ion leakage (Guo et al., 2009). In another experiment, two cultivars of bean (*Phaseolus vulgaris* L.) with different O_3 tolerance levels (O_3-sensitive "Cannellino" and O_3-tolerant "Top Crop") were subjected to a severe O_3 stress (165 nL L^{-1}). Biochemical characterization of different cultivars showed that exposure to this phytotoxic air pollutant increased superoxide dismutase (SOD) and CAT activities in both cultivars, which was more pronounced in the tolerant genotype (Guidi et al., 2010). Similarly, two cultivars of soybean (*Glycine max* L.) with opposed degrees of O_3 tolerance (O_3-sensitive "Mandarin (Ottawa)" and O_3-tolerant "Fiskeby III") were treated with 70 ppb for 4 days (7 h day^{-1}). Histochemical assays performed on these cultivars showed an accumulation of H_2O_2 via 3,3′-diaminobenzidine (DAB) in the sensitive cultivar, whereas no spots were detected in the tolerant cultivar. Nevertheless, superoxide anion ($O_2^{\bullet -}$) generation showed different trends in the two cultivars when tested by nitro blue tetrazolium chloride (NBT) reduction. There were no significant differences in SOD and glutathione reductase (GR) activities between the genotypes or O_3 treatments (Chutteang et al., 2016). Similarly, Szpunar-Krok et al. (2020) tested the effects of two doses of O_3 (5 and 10 ppm), different exposure times (2, 4, 8, 12, and 16 min), and two application periods (21 and 28 d after sowing) on potatoes (*Solanum tuberosum* L.). This experiment revealed a significant decrease in the total antioxidant activity based on ABTS$^{\bullet +}$ and DPPH$^{\bullet}$ radical assays. Table 1 includes additional information on studies that investigated the pro-oxidant effects of O_3 on different crops.

5 Plant responses to O_3

The response of plants to O_3 stress depends on the concentration and duration of exposure. Ozone may also be deposited in plant cells by non-stomatal channels, such as soil and cuticular deposits, in addition to being taken up by the stomata (Morales et al., 2021). Because stomatal opening and closing

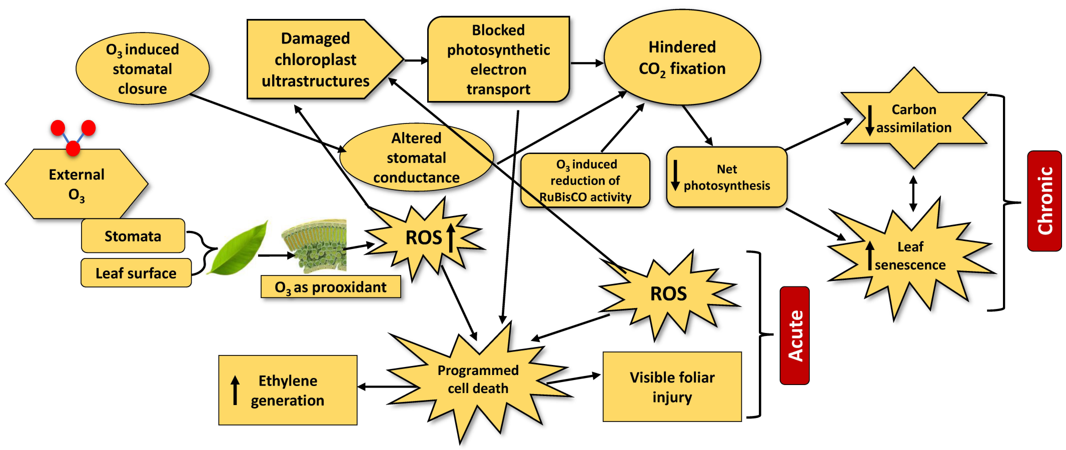

FIGURE 1
The major physiological processes and mechanisms involved in leaf injury under chronic and acute ozone exposure. The upward arrow inside boxes indicates increase and downward arrow indicates decrease.

regulate the flow of O_3 into the leaves, stomata act as an initial defense against O_3 damage in plants. However, the activities of transporters and ion channels in guard cells that move osmolytes through the tonoplast and plasma membrane regulate the size of the stomatal aperture. After accumulation in plant cells, O_3 induces short-term responses in plants depending on the frequency of stress exposure, such as the production of noticeable fine bronze or pale-yellow spots on the upper surface of the leaves. However, O_3-induced phytotoxicity begins when O_3 diffuses into the vacuolar space of the leaves through stomatal openings, promoting oxidative stress by encouraging the rapid production of ROS in the apoplast (Figure 2). Additionally, O_3 can directly diffuse into the cytosol through the cell membrane, generating ROS that alters stomatal conductance (g_s) (Ainsworth, 2017.) Numerous studies have used external O_3 to investigate the responses of plants to stress in terms of growth, biomass, reproduction, and yield (Figure 2).

5.1 Crop growth

The response of plants to O_3 varies depending on species, cultivar, and developmental stage (Table 2). A few varieties of wheat (*Triticum aestivum* L.) and rice show visible signs of foliage injury during their early growth stages (Ramya et al., 2021b). Ozone causes early leaf senescence and abscission, which can affect biomass and growth by allocating carbon to edible plant portions. Additionally, the O_3-induced reduction in root growth is greater than the reduction in shoot growth because of impaired carbohydrate partitioning in the roots (Witting et al., 2009). However, it adversely affects shoot growth. Yadav et al. (2021) observed significant effects on plant growth when experimenting with four Indian wheat cultivars (early-sown cultivars HUW468 and HD3086; late-sown cultivars HUW234 and HD3118). They reported a greater reduction (26%) in the aboveground biomass of early-sown cultivars than that of late-sown cultivars (21%) under ambient and elevated O_3 (ambient+20 ppb).

5.2 Plant physiology and metabolism

Ozone-induced phytotoxicity negatively affects plant physiology and metabolism, including photosynthesis, respiration, transpiration, relative water content, and secondary metabolite accumulation in various crop plants (Cho et al., 2011; Ainsworth et al., 2012; Hassan et al., 2017). In particular, evaluating physiological processes is a more accurate method for assessing intrinsic O_3-induced injuries in plants because physiological damage can start at lower O_3 concentrations and before the onset of visible impairment (Pandey et al., 2019). Several studies have evaluated the detrimental effects of ambient and elevated O_3 on the physiological processes in plants (Table 3). O_3-induced injuries are also related to reduced dry mass accumulation in leaves, lower leaf area-based antioxidant levels, and altered g_s. However, the O_3 uptake is linked to $g_{s,}$ which varies according to the absorption capacity of the cuticle and stem in different plant species, such as the cuticle and stem. For example, increasing the amount of O_3 leads to a reduction in g_s and stomatal pore area in tomatoes (*Solanum lycopersicum* L.) (Thwe et al., 2014). Moreover, Yendrek et al. (2015) reported that legume crops, such as peas (*Pisum sativum* L.), soybeans, and beans, display reduced net photosynthetic rates and leaf longevity. Cabbage (*Brassica oleracea* var. *capitata*) (cv. Tekila and Primero) plants showed decreased photosynthetic rates (71.2%), stomatal conductance (81.03%), and chlorophyll content (32.98%) (Ramakrishnan et al., 2023) when exposed to O_3 (200 ppb). Ozone-induced stomatal closure occurs because of the inhibition of carbon assimilation in chloroplasts, which leads to an accelerated internal carbon dioxide (CO_2) concentration (Ainsworth et al., 2012). Moreover, O_3 exposure directly impacted the net CO_2 assimilation rate and CO_2 fixation ability negatively. Total carbon sequestration and transpiration rates were also related to reduced $g_s$and photosynthetic rates. It has also been observed that O_3-induced alterations in the CO_2 assimilation rate further influence plant respiration, leading to reduced crop growth and productivity (Ainsworth, 2017). In addition to ROS production, the concentration of secondary metabolites in plant cells is altered by O_3-induced stress. Higher concentrations of O_3 activate the first enzyme of the phenylpropanoid pathway, leading to a higher accumulation of flavonoids, phenolic acids, monolignols, GSH, gamma-aminobutyric acid (GABA), terpenoids, and volatile organic compounds such as isopropanoids (Mikkelsen et al., 2015).

Ozone exposure also causes a nutrient imbalance in plant cells by altering the allocation of nutritional elements and their ratios in the belowground (root) and aboveground parts (stem and leaves) and disrupting other physiological activities. For example, the potassium (K), calcium (Ca), sodium (Na), iron (Fe), and zinc (Zn) contents in potato tubers are lower under elevated O_3 than under ambient conditions, which lowers tuber quality (Kumari and Agrawal, 2014). Similarly, Ghosh et al. (2020a) revealed that the concentrations of nitrogen (N), phosphorus (P), K, magnesium (Mg), and Ca were reduced in the leaves and shoots of wheat under ambient and elevated (ambient+20 ppb) O_3 stress from 2 weeks after germination to maturity for 4 h. However, carbon was enhanced under the same stressful conditions, which led to an increase in the C:N and C:K ratios in the leaves. Similarly, as O_3 alters the absorption and distribution of macronutrients owing to changes in organic matter mineralization, the uptake of other nutrients is also influenced. Under O_3 stress, copper (Cu) concentration was significantly reduced in the leaves, shoots, and roots, although the reduction was higher in the shoots than in the roots. Ozone-induced reduction in leaf N has also been observed in previous studies (Chen et al., 2015; Pandey et al., 2018).

5.3 Reproductive development

Reproductive development is the key determinant of plant productivity and species distribution. Increased O_3 has a detrimental effect on the reproductive system, primarily because it alters the allocation of carbon among tissues and directly affects plant reproductive processes (Gillespie et al., 2015). Several studies have demonstrated the effects of O_3 on flower initiation, floral development, pod formation, seed quality, seedling germination,

TABLE 1 Effects of ozone stress in crops and the different defense responses triggered in each species.

Plant species	O_3 levels	Stress period	Physiological and defense response		References
Brassica oleracea (L.)	70 ppb	3 days	CAT, APX, and POD activities	Enhanced	Rozpądek et al. (2013)
Capsicum baccatum (L.)	Average of 0.172 ppb	62 days (6 h day^{-1})	Lipid peroxidation and protein carboxylation in leaf	Increased	Bortolin et al. (2014)
			SOD, CAT and APX activities	Decreased	
Glycine max (L.)	200 ppb	4 h	Leaf AsA content	Increased	Gillespie et al. (2011)
			DHAR activity	Increased	
			GR activity	Reduced	
			MDHAR, APX, SOD and CAT activities	No change	
Oryza sativa (L.)	150 ppb	6 h	Production of $O_2^{\cdot-}$ and lipid peroxidation in leaf	Increased	Ueda et al. (2013)
O. sativa (L.)	51 ppb	30 days (7 h day^{-1})	MDA and proline contents	Increased	Ramya et al. (2021b)
Solanum tuberosum (L.)	Average of 50 ppb	60 days (6 h day^{-1})	MDA and H_2O_2 content; SOD, GR and APX activities	Increased	Kumari et al. (2015)
Triticum aestivum (L.)	80 ppb	30 days (8 h day^{-1})	Electrolyte leakage, lipid peroxidation, POD, and CAT activities	Increased	Zheng et al. (2011)
T. aestivum (L.)	Average of 66 ppb	5 months (8 h day^{-1})	Lipid peroxidation, MDA, H_2O_2, O_2 and OH^- content in leaves	Enhanced	Ghosh et al. (2020b)
Vigna unguiculata (L.)	40, 50, 60, 70 and 80 ppb	15 min, twice a day	AsA activity, proline content	Increased	Malaiyandi and Natarajan (2014)

(AsA, Ascorbate; DHAR, Dehydroascorbate reductase; GR, Glutathione reductase; MDHAR, Monodehydroascorbate reductase; APX, Ascorbate peroxidase; SOD, Superoxide dismutase; CAT, Catalase; $O_2^{\cdot-}$, Superoxide anion; H_2O_2, Hydrogen peroxide; GR, Glutathione reductase; O_2, Oxygen; OH^-, Hydroxyl radical).

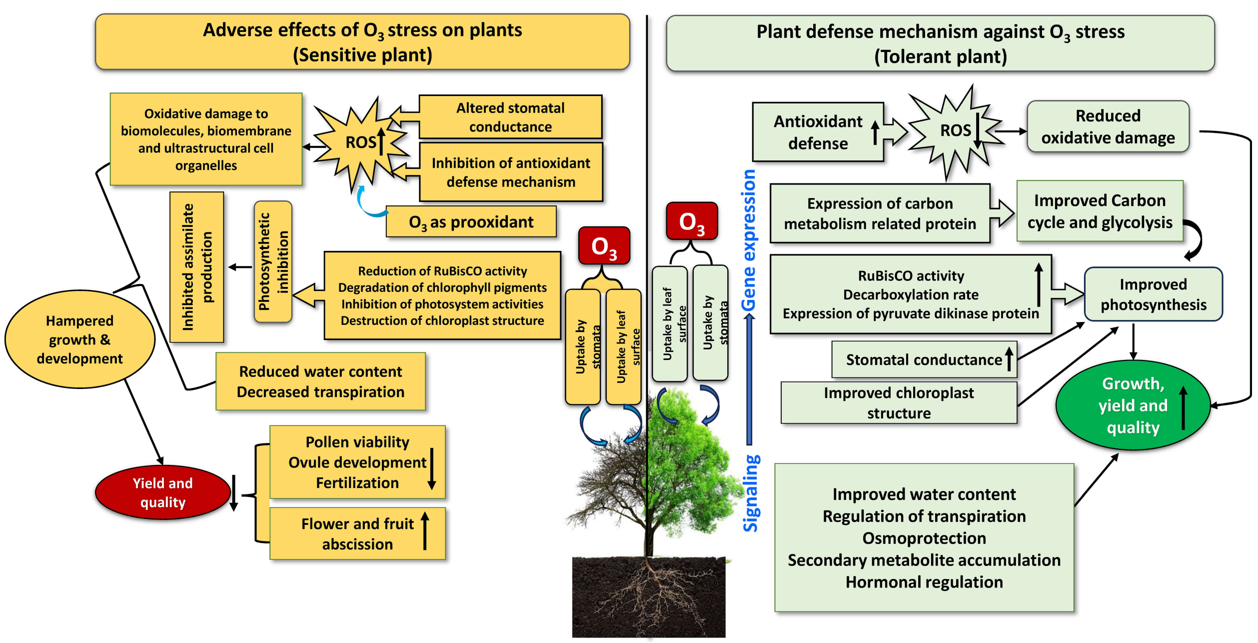

FIGURE 2
A schematic illustration of the response of sensitive and tolerant plants to ozone. The upward arrow inside boxes indicates increase and downward arrow indicates decrease.

TABLE 2 Overview of research on O_3 responses in crop growth of different plant species.

Plant species	O_3 levels	Observed parameters and effects		References
Brassica chinensis (L.)	150 ppb O_3 for 4 h day^{-1}	Crop dry matter	Reduced by 7%	Han et al. (2020)
Oryza sativa (L.)	50 ppb	Chlorophyll content	Reduced by 17-35%	Ramya et al. (2021b)
Pisum sativum (L.)	151.2 ppb	Reduced vegetative growth and increased visible foliar injury		Yendrek et al. (2015)
Triticum aestivum (L.)	Average charcoal filtered O_3 (13.3 ppb) and average non-filtered O_3 (34.6 ppb)	Total above-ground biomass	Reduced by 5%	Pleijel et al. (2018)
T. aestivum (L.) *cvs.* Akbar, Sufi, Bijoy, Shatabdi, BARI gom -26, Gourab, BARI gom-25, Prodip, Sourav and Kanchan	50, 60, 80, 100, 120, 135, 150, and 200 ppb for 14, 11, 8, 6, 5, 4, 3, and 1 days, respectively, for 8 h day^{-1}	Visible leaf injury	Increased in all cultivars	Saitanis et al. (2014)
		Total shoot biomass	Decreased by 25% (Akbar) and 20% (Sufi)	
		Total dry weight	Reduced	
T. aestivum (L.) *cvs.* HUW-37 and K-9107	Elevated O_3 at 40, 60 and 80 days after germination (DAG)	Plant height at 60 DAG	Decreased by 29% (HUW-37) and 21% (K-9107)	Mishra et al. (2013)
		Leaf number at 80 DAG	Reduced by 28.2% (HUW-37)	
		Total biomass at 80 DAG	Decreased by 49.2% (HUW-37) and 43.8% (K-9107)	
T. aestivum (L.) *cvs.* HUW 510 and Sonalika	Ambient + 10 ppb, Ambient + 20 ppb	Vegetative parts	Reduced plant height, root length, leaf number, and leaf area in both cultivars	Sarkar and Agrawal (2010)
Vigna radiata (L.) *cvs.* HUM-1, HUM-2, HUM-6, HUM-23, HUM-24 and HUM-26	Ambient and elevated O_3 (ambient + 10 ppb O_3)	Plant height at 40 DAG	Decreased by 26% (HUM-1) and 10% (HUM-23)	Chaudhary et al. (2013)
		Number of leaves plant^{-1} at 40 DAG	Reduced by 24% (HUM-1), 17% (HUM-2),12% (HUM-6), 9% (HUM-26) and 8% (HUM-24)	
V. unguiculata (L.) *cvs.* Blackeye and Asontem	39 ppb, 24 h mean	Leaf area per plant^{-1}, specific leaf area, whole-plant dry masses, and root–shoot ratio were decreased		Tetteh et al. (2015)

and seedling vigor have been demonstrated in several studies. For example, Gillespie et al. (2015) demonstrated that O_3 adversely affected pollen viability, pollen germination, germ tube growth, pollen-stigma interactions, and fertilization in tomatoes, leading to decreased seed size, weight, and quality. Consequently, O_3-induced lower pollen viability or ovule development leads to reduced fertilization. Moreover, elevated O_3 levels promoted flower and pod abscission. For example, decreased pod production without an effect on flower production was observed in soybeans when plants were exposed to 150 ppb of O_3 inside O_3 chambers. Fruit number, fruit size, seed number, and seed size also decrease at this level of O_3 stress in soybeans (Leisner et al., 2014). Additionally, research suggests that the effects of O_3 exposure vary depending on the stage of plant growth, affecting flowering patterns in various ways and affecting the pollination and reproduction of annual crops and wild species. According to a recent investigation by Duque et al. (2021a), wild mustard (*Sinapis arvensis* L.) plants exposed to 120 ppb O_3 for 6 h day^{-1} at earlier stages had more open flowers than the corresponding controls, whereas plants exposed to the same concentrations of O_3 at later stages tended to have fewer open flowers. Similarly, when *S. arvensis* plants were exposed to 120 ppb O_3 for 6 h day^{-1} during the flower initiation stage, flowering accelerated, increasing the proportion of open flowers in O_3-exposed plants at the start of the flowering phase (Duque et al., 2021b). Furthermore, the O_3-induced reduction in the photosynthesis rate inhibits the accumulation of carbohydrates in pollen in some plant species, conferring adverse effects on pollen germination (Zhang et al., 2017). O_3-induced reductions in the number, size, weight, and quality of grains in cereal crops such as rice and wheat have also been observed in some studies (Banerjee and Roychoudhury, 2019; Schauberger et al., 2019). Although O_3 has been found to have detectable effects on reproductive growth, the precise sites of action and mechanisms underlying these effects remain unknown.

TABLE 3 Ozone-induced responses in crop physiology and metabolism of different plant species.

Plant species	O_3 levels	Observed parameters and effects		References
Capsicum baccatum (L.)	Mean O_3 concentration of 171.6 ppb for 62 days	Secondary metabolite profile, e.g., total phenolic compounds	Increased by 17% in pericarp	Bortolin et al. (2016)
		Total antioxidant potential	Decreased by 87% in seeds	
Glycine max (L.)	Elevated O_3 (ambient + 40 ppb O_3)	Photosynthetic pigment contents at both flowering and seed filling stages	Reduced	Zhang et al. (2014)
		Net photosynthetic rate at both flowering and seed filling stages	Diminished	
		Chlorophyll *a* fluorescence rate at both flowering and seed-filling stages	Reduced	
Hordeum vulgare (L.)	0 ppb and 100 ppb O_3	Accumulation of twenty-five secondary metabolites, including phenylpropanoids, phenolamides and hydroxynitrile glucosides	Altered	Mikkelsen et al. (2015)
Nicotiana tabacum (L.)	Ambient O_3	Epicuticular wax on leaves and stomatal aperture movement	Damaged and reduced	Alves et al. (2016)
Solanum lycopersicum (L.)	0.5 ppb of O_3	PS II activities	Hampered	Thwe et al. (2014)
Triticum aestivum (L.)	50, 60, 80, 100, 120, 135, 150, and 200 ppb for 14, 11, 8, 6, 5, 4, 3, and 1 days, respectively, for 8 h day^{-1}	Total chlorophyll	Reduced	Saitanis et al. (2014)
		Leaf greenness	Decreased	
		Carotenoid content	Reduced	
T. aestivum (L.)	30 and 80 ppb; 4 weeks	Stomatal conductance (g_s)	Reduced	Harmens et al. (2019)
		Light-saturated photosynthesis	Reduced	
		Chlorophyll content index	Decreased	
Vigna unguiculata (L.)	39 ppb, 24 h mean	g_s	Reduced	Tetteh et al. (2015)

5.4 Crop yield and quality

A significant proportion of crop yield losses is caused by tropospheric O_3, which is a transient, volatile, secondary air pollutant and a powerful phytotoxic compound (Ainsworth, 2017). According to the results of several controlled environmental and field studies, current O_3 concentrations in the environment have been found to negatively influence the yield and quality of several crop species worldwide, according to the results of several controlled environment and field studies (Table 4). For example, according to McGrath et al. (2015), yield losses of 10% and 20% were observed in soybean and maize (*Zea mays* L.), respectively, in combination with dry conditions and high seasonal temperatures, and 5% and 10%, respectively, under rainfed conditions at field levels from 1980 to 2011 due to O_3 exposure. Additionally, when two cultivars of tropical maize were exposed to two different doses of O_3 (ambient+15 ppb and ambient +30 ppb), a reduction in the test weight of kernel $plant^{-1}$ of 6% and 10%, respectively, in DHM117, and 4% and 6%, respectively, in HQPM1 was observed (Singh et al., 2014a). Another long-term investigation conducted by Sinha et al. (2015) revealed that yield losses in rice, wheat, and maize were 21–26%, 27–41%, and 3–5%, respectively, under elevated O_3. Additionally, according to the Intergovernmental Panel on Climate Change's Special Report on Emission Scenarios (IPCC SRES) A2 Scenario, O_3-induced worldwide yield losses in 2030 will range from 5 to 26% for wheat and 4 to 9% for maize (Avnery et al., 2011). Moreover, a recent study by Ghosh et al. (2020a) revealed that the grain yield was reduced by 45% in wheat cv. HD 2967 under ambient and elevated (ambient+20 ppb) O_3 stress for 4 hours day^{-1} from 2 weeks after germination to maturity. Baqasi et al. (2018) reported a 49% decline in grain dry mass in wheat after 50 ppb O_3 exposure. Ozone also influences the quality of crops in terms of starch, protein, nutrients, and oil content. For example, it affects grain quality by decreasing starch content and increasing the protein and nutritional contents of crops such as wheat and rice (Broberg et al., 2015; Frei, 2015).

TABLE 4 Overview of recent studies on O_3 responses in crop yield and quality of different plant species.

Plant species	O_3 levels	Observed parameters and effects		References
Brassica napus (L.)	Ambient + 10 ppb of O_3	Grain yield	Reduced by 13% (Sanjukta) and 47% (Vardan)	Tripathi and Agrawal (2012)
Glycine max (L.)	Elevated O_3 (ambient + 40 ppb O_3)	Yield	Decreased by 40%	Zhang et al. (2014)
G. max (L.)	5.78 ppb and 137.7 ppb; 1 week	Seed production, seed protein content	Reduced by 10% and 12%, respectively	Biancari et al. (2021)
Solanum tuberosum (L.)	Ambient + 20 ppb of O_3	Total fresh weight of tuber	Reduced by 48%	Kumari and Agrawal (2014)
Triticum aestivum (L.)	55.2 ppb	Number of ears plant^{-1}	Reduced by 27% (HUW-37) and 20% (K-9107)	Mishra et al. (2013)
		Weight of ears plant^{-1}	Decreased by 31% (K-9107)	
		Number of grains plant^{-1}	Reduced by 21% (HUW-37) and 18% (K-9107)	
		Weight of grains plant^{-1}	Reduced by 12% (K-9107) and 39% (HUW-37)	
T. aestivum (L.)	AOT40-21, 121 ppb of O_3	Total grain weight	Reduced by 11%	Monga et al. (2015)
T. aestivum (L.)	80 ppb; 4 weeks	Grain yield	Decreased by 24%	Harmens et al. (2019)
		1000-grain weight	Reduced by 20%	
Vigna radiata (L.)	Ambient and elevated O_3 (ambient + 10 ppb O_3)	Yield	Reduced by 15% (HUM-1), 14% (HUM-2), 13% (HUM-6), 12% (HUM-24), 10% (HUM-26) and 9% (HUM-1)	Chaudhary et al. (2013)
V. unguiculata (L.)	50 ppb for 5 hr for 88 days after emergence	Number of seeds pod^{-1}, 100-seed weight and yield plant^{-1}	Reduced	Tetteh et al. (2015)
Zea mays (L.)	Ambient+15 ppb and ambient+30 ppb of O_3	Kernel weight	Reduced by 10% (HQPM1) and 13% (DHM117)	Singh et al. (2014a)

6 Developing plant tolerance to O_3

Higher O_3 exposure causes greater yield losses through foliar damage, inhibition of photosynthesis with altered carbon translocation, and faster plant senescence (Osborne et al., 2019). There are opportunities to develop plant tolerance to O_3 which ultimately protects the yield under stressful conditions. Breeding for stress tolerance and variety development can be time consuming and costly. Using physiological gateways such as the photosynthetic pathway, antioxidant defense mechanisms, and hormonal regulation to enhance plant tolerance to O_3 could be a short-term option (Parankusam et al., 2019; Emberson, 2020). It is mandatory to identify the available options, followed by the most suitable option, to increase plant productivity where mitigation actions can be implemented. In this section, we present an overall discussion focusing on various approaches for developing plant tolerance to O_3 and how O_3 sensitivity can be lowered.

6.1 Improving photosynthetic pathways

Leaf health, g_s, photosynthesis, and photosynthetic machinery are hampered by elevated O_3 levels. Improving photosynthesis may be an important approach for attaining higher plant tolerance to O_3 exposure. Ozone-mediated chlorophyll decline causes early senescence, but ethylene diurea (EDU) supplementation delays senescence in maize by increasing the chlorophyll content (Gupta et al., 2020; Poornima et al., 2022; Dhevagi et al., 2023). Increased expression of carbon metabolism-related proteins, which are part of the Calvin cycle and glycolysis, contributed to higher O_3 tolerance by accumulating more starch, which was reflected in better biomass production in EDU-treated maize. In addition, EDU-induced elevation of RuBisCO activity also supports higher photosynthesis in combating O_3 stress. Exogenous EDU increases the C_3 and C_4 photosynthesis rates by increasing the decarboxylation rate and expression of pyruvate phosphate dikinase protein, respectively, leading to higher O_3 tolerance (Alfonso and Brüggemann, 2012). Sensitive wheat cultivars showed higher photosynthetic rates with exogenous EDU application under elevated O_3 stress, which was correlated with EDU-induced higher chlorophyll content (Fatima et al., 2019). Therefore, improved photosynthesis and chlorophyll content may explain the higher biomass and yield, which later resulted in increased O_3 tolerance due to EDU supplementation.

Exogenous catechin (5 mM) supplementation in rice under elevated O_3 conditions reversed O_3-induced damage by enhancing chlorophyll content and its precursor (Mg^{2+} content), g_s, which resulted in higher grain production (Kittipornkul et al., 2020). Catechins can improve photosynthetic processes, thereby improving O_3 tolerance. Recently, calcium acetate application was shown to increase photosystem (PS)-II efficiency and improve the yield performance of rice under O_3 stress (Lakaew et al., 2022). There is a lack of knowledge regarding the mechanisms involved in improving the photosynthetic pathways to regulate plant growth and yield under O_3 stress conditions. Further in-depth research is required to elucidate these mechanisms and the associated pathways.

6.2 Enhancing antioxidant defense

Plant antioxidant defenses must be upregulated to scavenge O_3-induced excess ROS, thereby protecting cellular functions (Gupta et al., 2020). The inhibition of cellular component peroxidation and the maintenance of the subsequent stability of the cell membrane under O_3-induced ROS overgeneration of ROS are distinct features of O_3-tolerant plants, which are made possible by the activation of defense mechanisms, including both enzymatic and non-enzymatic antioxidant components (Gill and Tuteja, 2010; Noctor et al., 2014; Pellegrini et al., 2019; Hasanuzzaman et al., 2020). Similarly, Czarnocka and Karpiński (2018) stated that insufficient responses to plant antioxidants cause oxidative damage and strengthen the defense responses of plants, which are required to develop tolerance to O_3.

Both AsA and GSH are major antioxidants in the AsA-GSH cycle and are involved in regulating oxidative damage by scavenging ROS and maintaining cellular redox balance (Hasanuzzaman et al., 2020). Biogenic AgNPs and EDU-mediated higher antioxidant content (AsA and GSH) and enzymatic activities (SOD, CAT, APX, and GR) in wheat resulted in improved O_3 tolerance, which was correlated with lower H_2O_2 and MDA accumulation (Pellegrini et al., 2019).

Fatima et al. (2018) evaluated the ROS-scavenging capability of O_3-sensitive and tolerant wheat genotypes through their antioxidant responses. Kharchiya 65 (tolerant) displayed a maximum level of AsA, GSH, and flavonoids along with high free radical scavenging activities as well as lower ROS content than genotypes such as HD 2987 (sensitive) and PBW 502 (intermediately sensitive). At high O_3 (ambient+30 ppb), both enzymatic and non-enzymatic antioxidant responses varied among the three cultivars. The highest SOD, peroxidase (POD), GR, and GPX activities were observed in HD 2987, whereas Kharchiya 65 and PBW 502 showed the lowest increases. The maximum APX and the lowest CAT activity was observed in HD 2987 and Kharchiya 65. Sensitive cultivars showed higher enzymatic antioxidant responses when they suffered from O_3-induced elevated ROS levels. However, lowered SOD and POD activities were required in the tolerant cultivars, where ROS levels were lower than those in the sensitive ones. Higher free radical scavenging activities were observed in the tolerant (Kharchiya 65) cultivar than in the sensitive cultivar. Non-enzymatic antioxidant levels, such as those of AsA, GSH, and flavonoids, were also higher in the tolerant cultivar than in the sensitive cultivar, which is probably the most efficient mechanism for combating the elevated O_3. This may be because higher O_3 tolerance is highly associated with the genetic competence to preserve high AsA/DHA (Burkey et al., 2003). In cabbage (cv. Tekila and Primero), proline content was increased by 32.24%, ascorbic acid by 64.75%, CAT activity by 3.58%, and POD activity by 56%, which helped to reduce oxidative stress under O_3 stress (200 ppb) (Ramakrishnan et al., 2023). Therefore, variations in antioxidant responses to counteract O_3-induced oxidative stress are highly dependent on the crop species and the cultivars of the same crop species (Singh et al., 2014b).

Plant researchers are becoming increasingly interested in selecting appropriate techniques to increase plant tolerance by stimulating antioxidant activity. The supplementation of exogenous chemical substances to O_3-exposed plants is one of the most efficient approaches for reducing oxidative stress and cellular damage through the enrichment of antioxidant defense systems (Qiu et al., 2019).

Ethylene diurea is widely used as an anti-ozonant to increase plant tolerance to O_3 phytotoxicity and protect plants from damage (Manning et al., 2011). EDU-induced plant protection under O_3 stress depends on the activation of antioxidant activity (Gupta et al., 2021). Therefore, EDU supplementation in wheat revealed an EDU-mediated active role of apoplastic SOD, CAT, and amino methyltransferase, which facilitated the maintenance of ROS at optimum levels and decreased O_3-induced damage (Gupta et al., 2021).

Foliar spraying of catechin and SA can significantly affect the overexpression of *APX* and *CAT* genes, followed by their higher enzymatic activities, leading to lower lipid peroxidation (MDA), and thus increased tolerance of rice to O_3 (Kittipornkul et al., 2020).

6.3 Phytohormone regulation

Similar to ROS and Ca, phytohormones, such as abscisic acid (ABA), SA, jasmonic acid (JA), and ethylene (ET), are involved in the regulation of stomatal aperture movement upon O_3 exposure, leading to increased plant tolerance, which is mainly related to the cell signaling cascade (Pellegrini et al., 2016). Moreover, O_3-induced stomatal movement is controlled by anion channels, such as slow anion channel 1 and open stomata 1 (Vahisalu et al., 2010). These channels are regulated by ABA (Negi et al., 2008). However, variations in O_3 tolerance have been attributed to g_s and other protective mechanisms involved (Castagna and Ranieri, 2009). How phytohormones are associated with plant tolerance to O_3 exposure needs to be explored to better understand plant responses to stressful conditions. It was reported that the O_3-mediated increases in JA, asmonoyl-l-isoleucine, and ABA reduce leaf damage in Habataki rice (Tsukahara et al., 2015). Stress-induced apoplastic ROS exacerbates SA synthesis, which contributes to *OsORAP1* expression and causes O_3 sensitivity and tolerance (Ueda et al., 2015).

Salicylic acid is essential for maintaining antioxidant defense mechanisms and cellular redox responses in plants upon O_3 exposure (Hasan et al., 2021). Ozone-induced leaf damage accompanying ET biosynthesis has been confirmed by the inhibition of ET biosynthesis in tobacco following O_3 treatment (Bandurska et al., 2009). This suggests that the suppression of ET biosynthesis can increase O_3 tolerance in plants. Abscisic acid controls ET and ABA biosynthesis by limiting ABI1 phosphatase activity and ROS homeostasis to induce O_3 tolerance (Pellegrini et al., 2016). Jasmonic acid is responsible for suppressing ROS-dependent leaf damage under O_3 stress (Hasan et al., 2021). Ozone exposure induces ET-dependent damage, which can be inhibited by JA when *AT2G24850* and *AT5G24770* are induced by JA (Wang et al., 2017). The significant role of SA in increasing the tolerance of rice to O_3 was studied by Kittipornkul et al. (2020), where exogenous 100 μM SA supplementation decreased MDA due to higher activity of CAT, APX upon 100–150 ppb (8 h day^{-1}) O_3. Additionally, plants inhibit O_3 uptake by increasing SA under O_3 exposure as a mechanism of O_3 tolerance (Pheomphun et al., 2019). Therefore, a comprehensive research on exogenous phytohormones is required to understand the mechanisms underlying their protective roles in the development of O_3 tolerance in cultivated crops.

7 Mitigation of O_3 stress in crops

7.1 Improving crop management practices

Because abiotic stress is inevitable, it is crucial to develop strategies to combat stress-induced losses in crop production. Agronomic practices, such as changing the cropping season, air quality management, proper irrigation, and adequate plant protection measures, can be used to manage O_3-induced damage in crops. As O_3 is strongly linked to seasonal and regional changes, shifting the crop growing season by manipulating the sowing time has been suggested by Teixeira et al. (2011). Seasonal variation in crops can influence the physiological responses of plants by altering their gas exchange capacity, PS I function, and stomatal density upon exposure to O_3 stress. The generation of adaptive measures has been recorded through decreased g_s, increased stomatal density, and increased PS I activity when plants are exposed to stress at a later stage in their life cycle (Fusaro et al., 2016). Moreover, early sown crops exhibit higher sensitivity to O_3 owing to their longer life cycles, lengthy post-anthesis stages, higher g_s, and lower threshold levels. In contrast, the comparatively higher enzymatic antioxidant activity of late-grown crops, with increased energy allocation toward growth, facilitates reduced O_3-induced damage in crops (Yadav et al., 2019; Yadav et al., 2021). Air quality management to check for O_3 precursors is beneficial during O_3 exposure. For instance, decreasing methane, an important precursor of O_3 and greenhouse gases, has proven to be beneficial in combating O_3 (Shindell et al., 2012). Furthermore, controlling nitrogen oxide emissions in air is beneficial for reducing O_3 stress in northern China (Lu et al., 2021). By calculating the O_3 depletion potential of the substances (responsible for O_3 depletion), Ravishankara et al. (2009) reported that nitrous oxide (N_2O) is one of the most important greenhouse gases responsible for O_3 layer depletion. Nitrous oxide production can be mitigated using mulches (e.g., rice straw) and by minimizing fertilizer requirements in the soil. It is evident that soil water-filled pore spaces play an important role in N_2O emissions in the field; therefore, altering nitrogenous fertilizer with mulch can contribute to N_2O mitigation (Wu et al., 2018). Intercultural operations, such as mulching, can also reduce O_3-induced losses.

Although irrigation is desirable for improving crop production, it can enhance the susceptibility of crops to O_3 toxicity. In irrigated crops, the widely open leaf pores are subjected to stimulated g_s with elevated uptake of O_3 (Mills et al., 2018). This phenomenon is in agreement with another study (Harmens et al., 2019) that concluded that reduced irrigation could be an effective strategy to mitigate O_3-induced negative impacts partially or completely by delaying adversities on flag leaves at the time of flower initiation or during the gain-filling period of wheat. Therefore, it is imperative to manage irrigation properly to protect crops from the adverse effects of O_3 without causing water stress. For example, alternate wetting and drying irrigation was found to be beneficial for increasing rice productivity with decreasing g_s (Carrijo et al., 2017) and was effective in mitigating the adverse impacts of O_3. In addition, protective measures should be implemented to control plant competition and prevent plant injury in response to O_3 stress. It has been reported that when weed infestation is coupled with O_3, it enhances the susceptibility of crops to increased losses compared with O_3 exposure alone (Li et al., 2016). Ghosh et al. (2020b) reported a higher yield loss in wheat under O_3 stress owing to weed competition and concluded that strong weed management should be introduced to combat O_3 stress-affected production loss.

7.2 Nutrient management

Although nutrient supplementation is important for enriching soil fertility, improper nutrient maintenance can increase crop vulnerability to various stressors, including O_3 (Tiwari and Agrawal, 2018). Zhang et al. (2018) conducted an experiment to assess the O_3 risk management capacity of plant nutrients, where N fertilizer was recorded to elevate the sensitivity of plants to stress; conversely, P improved tolerance by increasing the critical level of O_3 exposure in crops. Biomass loss caused by O_3 also differed between the two nutrients. Nitrogen fertilization caused the maximum g_s, which increased the sensitivity to O_3 and, in turn, resulted in a loss of biomass production, whereas P fertilization decreased g_s. In another study (Tatsumi et al., 2019), it was claimed that a lack of N supplementation in rice caused N deficiency while giving rise to photosynthetic assimilate translocation to the roots in an attempt to increase nutrient uptake, thereby protecting against O_3-induced damage to plant growth. Nutrient supplementation contributes to detoxification of ROS-induced O_3. Both N and P were recorded to encourage the mobilization of integrated participation of antioxidant compounds (carotenoid and AsA) and osmoprotectant (proline) and consequently reduce oxidative stress by keeping minimum $O_2^{\bullet-}$ and H_2O_2 while maintaining membrane integrity (Podda et al., 2019). Moreover, N addition can participate in diversifying stored carbohydrates and photosynthates to synthesize amino acids that help repair damage caused by O_3 (Podda et al., 2019). Although some studies have shown the negative impacts of O_3 and nutrient interactions, improved plant nutrient management can help mitigate O_3 stress to some extent (Gautam and Tiwari, 2020).

7.3 Carbon dioxide (CO_2) fertilization

Plant growth can be stimulated by the subsidiary carbon supply provided by CO_2, which is known as CO_2 fertilization. This phenomenon can help relieve stress by reducing ROS production during the oxidative damage caused by various stressors, including O_3 (AbdElgawad et al., 2016). When CO_2 is used under O_3 concentrations in chickpeas (*Cicer arietinum* L.), a source-sink imbalance changes with the accumulation of photosynthates in leaves and, subsequently, an alteration of phenological characteristics that ultimately accelerate the crop life cycle to early maturity to escape the damage caused by O_3 exposure (Singh et al., 2021). Elevated CO_2 can counteract the damaging effects of O_3 by increasing shoot biomass and pod weight compared to O_3-exposed plants alone. Moreover, CO_2 fertilization can increase protein, starch, and certain mineral nutrients, even under O_3 exposure, thus combatting the negative impacts of imposed stress (Bhatia et al., 2021). A similar compensation tendency was observed in maize (Yadav et al., 2020). Interactive treatment with CO_2 and O_3 resulted in a positive result, as this combination resulted in a higher photosynthesis rate with improved growth attributes and, consequently, an increased yield component compared to the O_3 treatment alone. Carbon dioxide fertilization also results in improved g_s and carbon assimilation (Yadav et al., 2020). When elevated CO_2 levels were coupled with elevated O_3, reduced lipid peroxidation and solute leakage decreased, indicating improved cell membrane integrity. Enhanced antioxidant enzyme activity, which indicates reduced oxidative stress, has also been observed (Kumari et al., 2015). Therefore, CO_2 fertilization can help mitigate O_3-induced damage to some extent by reducing O_3 uptake, increasing carbon assimilation, and reducing oxidative damage, which helps to overcome the detrimental effects on plant growth, physiology (especially photosynthesis), and yield.

7.4 Selecting tolerant crop varieties

The use of tolerant cultivars can be an effective strategy that needs to be expanded by including agricultural practices, particularly in O_3 risk areas (Tiwari and Agrawal, 2018). A noticeable variation in rice genotypes in response to stress was observed by Arshad (2021), in which plant height, dry mass, leaf area, plant damage, and leaf damage were visible between susceptible and tolerant genotypes. Biochemical attributes such as total amino acids, sugar, protein profile, and phenolic content were not affected in varieties tolerant to O_3 stress, indicating tolerance capacity, whereas a trend of reduction was observed in susceptible genotypes. The susceptible varieties showed early visual symptoms through yellow to brown spots, which later turned into necrosis and early leaf senescence; however, this phenomenon was slower in the tolerant varieties (Arshad, 2021). In wheat, the tolerant variety (HD2967) sensed O_3 stressors at an early vegetative stage through increased MDA and initiated ROS scavenging activity, and consequently uplifted better antioxidant defense prior to the reproductive stage to protect against yield losses compared to the sensitive genotype (Sonalika), which was later in sensing (Pandey et al., 2018). Moreover, they added that the tolerant variety has tended to translocate more photosynthetic assimilates to increase biomass and husk weight with better resource partitioning, thus ensuring better protection of the reproductive parts under higher levels of O_3 exposure. These findings agree with those of Dhevagi et al. (2021), who concluded that the tolerant variety of mung beans (*Vigna mungo* L.) had higher AsA under exaggerated O_3 concentrations, which resulted in better morphological, physiological, biochemical performance, and antioxidant defense than the susceptible variety. Bailey et al. (2019) demonstrated the distinctiveness of a soybean-tolerant variety in the exclusion of O_3 uptake through stomata by lowering g_s and the transpiration rate, followed by higher water-use efficiency than the sensitive variety. Furthermore, under O_3 exposure, variations in phosphoenolpyruvate, carboxylase activity, and RuBisCO content and activity controlled the tolerance mechanism of maize hybrids more than the antioxidant defense mechanism of g_s. Stable leaf N content and RuBisCO activity under reduced O_3 exposure indicated better tolerance to late senescence and better yield than sensitive hybrids (Choquette et al., 2020). Therefore, the selection of tolerant varieties under O_3 exposure could be an effective approach for adaptation to O_3.

7.5 Using chemical elicitors

Elicitors with different chemical structures can be used against different stressors through exogenous application to crops or incorporation of transcription factors through breeding (Chakraborty et al., 2019). The use of various chemical elicitors to protect against O_3-induced phytotoxicity has also been demonstrated. For example, chitosan positively influenced wheat growth under O_3 exposure (Picchi et al., 2021). In addition to improving crop yield and quality, chitosan serves as a protectant that enhances the defense metabolism of plants by increasing the concentration of AsA within a short period by activating the APX enzyme to control H_2O_2 and oxidative stress. It also showed limited symptoms on the leaf surface area of chitosan-treated plants compared to O_3-stressed plants. The same trend of increasing antioxidant enzyme levels under stress conditions was reported by Kittipornkul et al. (2020), using catechins in rice. Application of catechin to rice under O_3 exposure activated antioxidant enzymes and helped to maintain chlorophyll, g_s, and Mg contents at the vegetative stage, which consecutively resulted in increased panicle number, filled grain weight, and starch, conferring protection against stress. These chemical elicitors protect plants by forming chemical barriers that detoxify O_3 (Li et al., 2018). It has been shown that trichomes have the capacity to deplete O_3 near the leaf surface, resulting in reduced O_3 uptake through stomata (Li et al., 2018). In addition, stress-induced lipoxygenase (LOX) activity (a consequence of O_3 uptake) were maintained through trichome density, providing plant tolerance to O_3 exposure. Ethylene diurea is widely used to enhance tolerance to O_3 exposure, and its effectiveness has been demonstrated in rice (Ashrafuzzaman et al., 2018), maize (Gupta et al., 2020), groundnuts (*Arachis hypogaea* L.; Chaudhary and Rathore, 2020) and castors (*Ricinus communis* L.;

Rathore and Chaudhary, 2018). As EDU actively participates in the upregulation of crop growth, photosynthesis, and maintenance of improved membrane properties by activating defense metabolism by increasing AsA and flavonoid content, it can protect against O_3 stress (Rathore and Chaudhary, 2018; Chaudhary and Rathore, 2020). Furthermore, in addition to improving plant mechanisms (increased SOD, CAT, and APX activities) to regulate the defense system, EDU enhances protein accumulation in plants, which in turn enhances metabolic functions to mitigate damage under O_3 stress (Gupta et al., 2020). Foliar spraying with calcium acetate and calcium chloride improved tolerance through protectant-induced mechanisms of the antioxidant defense system in O_3-stressed rice plants (Lakaew et al., 2022). Calcium acetate-treated plants tolerated longer periods of O_3 exposure by augmenting NAD kinase and NADPH activities. This calcium acetate-mediated increase in NADPH content was associated with higher AsA and GSH levels, and higher APX and GR activities, resulting in an approximately 29% reduction in MDA generation. This calcium acetate-mediated oxidative stress mitigation contributes to improved plant growth and yield (Lakaew et al., 2022).

8 Conclusion

Over the past few decades, several studies on the effects of O_3 on plants have demonstrated that elevated levels of O_3 hamper overall plant growth and productivity. Ozone can be degraded into ROS in the mesophyll and guard cell walls, which damage the chloroplast ultrastructure and block photosynthetic electron transport after entering directly through the leaves. Leading to stomatal closure and modification of stomatal conductance O_3 hinder CO_2 fixation. Ozone induces leaf chlorosis, necrosis, and abscission. Reduced photosynthesis, altered respiration and transpiration, decreased water uptake, disrupted nutrient homeostasis, and the assimilate translocation caused by O_3 lead to reduced growth. Both chronic and severe O_3 stress can lead to growth reduction, anomalous reproductive development, yield loss, and crop quality deterioration. Approaches for protecting plant physiological pathways such as photosynthesis, antioxidant defense mechanisms, and hormonal regulation have been reported to enhance plant tolerance to elevated O_3. Agronomic approaches, such as adjusting planting dates and cropping systems, nutrient management, CO_2 fertilization, and the use of several chemical stress elicitors have been shown to improve plant performance under elevated O_3. However, it is difficult to conduct research on ambient O_3 because it is difficult to measure the amount of ambient O_3 and the amount of O_3 entering plants. An appropriate method should be developed to understand these issues, and research on meteorological, biochemical, and physiological aspects should be considered. Literature on the effects of O_3 on various aspects of plants is readily available, but there is limited availability of literature on strategies for mitigating O_3-induced stress. Therefore, various agronomic approaches that may mitigate O_3 stress in plants should be determined. Understanding the biochemistry and physiology of O_3-stressed plants is vital for developing O_3-tolerant plants. Therefore, integrated research themes and their implementation are vital for reducing O_3-induced damage and developing O_3-tolerant cultivars.

Author contributions

MH and PV conceived and designed the project; FN, AS, KP, PG, and KN wrote the MS. MH and PV edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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