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Exogenous phytohormones and nutrient management for the build-up of abiotic stress resilience in crops

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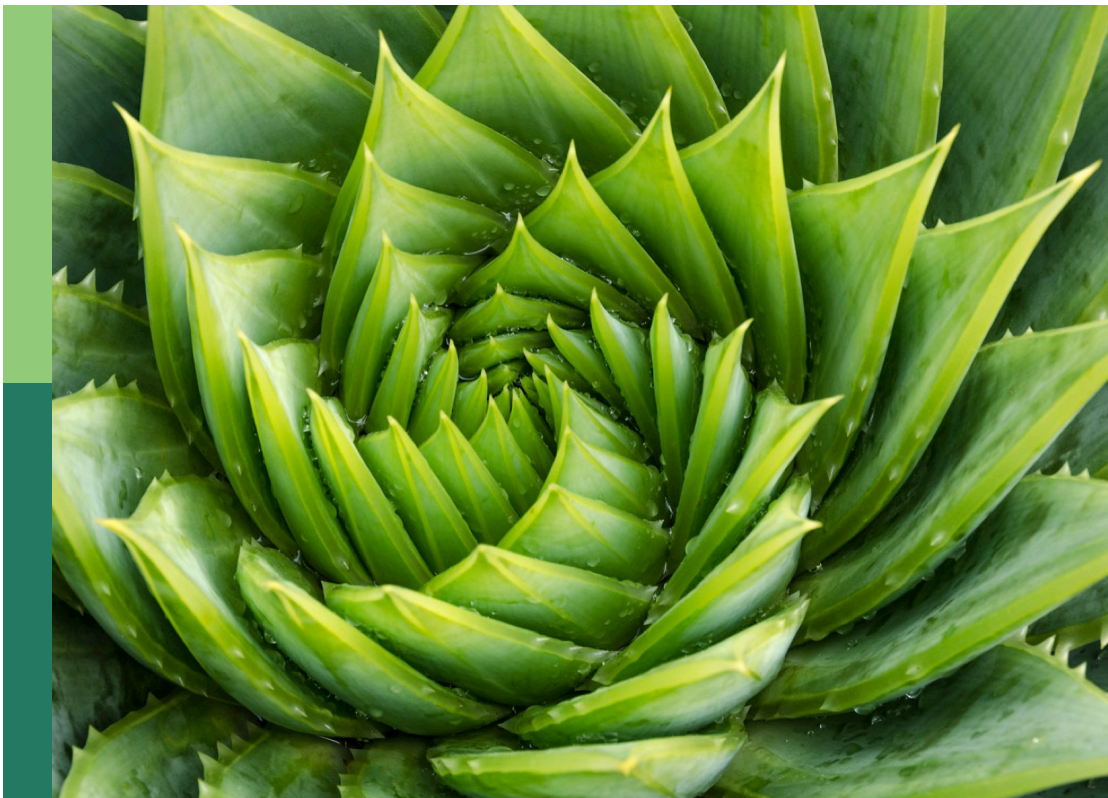

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Exogenous phytohormones and nutrient management for the build-up of abiotic stress resilience in crops

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Abscisic acid alleviates chilling injury in cold-stored peach fruit by regulating ethylene and hydrogen peroxide metabolism

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Peach (*Prunus persica* (L.) Batsch) is susceptible to chilling injury under improper low-temperature storage (2°C–5°C). Previous research has shown that abscisic acid (ABA) alleviates chilling injury in fruits and vegetables, but the potential mechanism is still unclear. To explore its effectiveness and potential mechanism in alleviating chilling injury during cold storage, exogenous ABA was applied to peach fruit by immersion in 100μmolL^{-1} solutions for 10min. In our experiment, ABA alleviated chilling injury by reducing hydrogen peroxide (H_2O_2) content and ethylene production. In addition, ABA inhibited the expression of the ethylene synthesis-related genes *PpACO1* and *PpEIN2*. At the same time, ABA activated the antioxidant enzymatic pathway and the ascorbate-glutathione (AsA-GSH) cycle, the transcript abundance encoding genes related to antioxidant enzyme activities also changed correspondingly. The results suggested that ABA alleviated chilling injury by scavenging excessive H_2O_2 by promoting antioxidant enzymes and the AsA-GSH pathway.

KEYWORDS

***Prunus persica*, chilling injury, ascorbate-glutathione cycle, hydrogen peroxide, ethylene metabolism**

Introduction

Peach [*Prunus persica* (L.) Batsch] is a typical climacteric fruit that ripens and rots quickly at ambient temperature after harvest. Low-temperature storage is a common method of storing and transporting peaches. However, peaches are sensitive to chilling injury during cold storage, especially from 2.2°C to 7.6°C (Lurie and Crisosto, 2005). The symptoms of chilling injury include browning or woolly texture of the flesh, reduced ethylene release, increased decay susceptibility, and abnormal ripening (Lurie and Crisosto, 2005). The chilling injury severely affects commercial value and prompts quality deterioration (Yao et al., 2021; Islam et al., 2022; Zhu et al., 2022). Therefore, investigating proper methods to alleviate chilling injury, is important for maintaining the commercial value of peach fruit.

As a critical phytohormone, ethylene is a key factor that regulates the ripeness and senescence of peach fruit. In addition to being highly correlated with fruit softening, ethylene is also associated with postharvest metabolic disorders and rot (Netlak et al., 2021). Previous research has recently confirmed that immature fruits stored at low temperatures affected ethylene synthesis by altering the expression of ethylene metabolism-related genes (Megías et al., 2016). Candan et al. (2006) have confirmed the widespread involvement of ethylene in regulating cold tolerance in postharvest fruit. 1-methylcyclopropane (1-MCP), a typical inhibitor of ethylene, can effectively alleviate chilling injury symptoms of climacteric fruits such as persimmon (Kou et al., 2020), plums (Menniti et al., 2004), and peach (Liu et al., 2018) by inhibiting the signal transfer of ethylene. In addition, previous research indicated that peach fruit treated with Jasmonic acid can reduce chilling injury symptoms by regulating ethylene metabolism (Zhao et al., 2021a). Ethylene has been proved associated with abnormal ripening and softening during refrigeration storage. Therefore, how to regulate ethylene production may be a very critical factor in alleviating chilling injury.

Postharvest fruit ripening and senescence are considered to be highly linked to oxidative damage. When plants are exposed to adversity stresses, such as low temperature, leading to the imbalance of reactive oxygen species (ROS) metabolism (Babalar et al., 2018; Yang et al., 2021). Excessive accumulation of ROS accumulations eventually leads to oxidative damage (Zhou et al., 2014), such as DNA damage, lipid peroxidation, and finally results in cell death. The prevailing view is that the excessive accumulation of ROS causes the decline of quality in postharvest fruits and vegetables (Meitha et al., 2020). The natural antioxidant defense system present in plants helps them to scavenge ROS, thus reducing oxidative damage. Several enzymatic antioxidant pathways contain superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). SOD can convert O^{2-}·into H_2O_2 (Lounifi et al., 2012). Thereafter, the effect of POD and CAT decomposed H_2O_2 to H_2O. AsA-GSH cycle was another important section of reactive oxygen scavenging system consisting of some typical enzymes like ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione reductase (GR). Ascorbate peroxidase also contributed to the degradation of H_2O_2. Previous study showed that the regulation of the antioxidant system could enhance drought stress tolerance (Hasan et al., 2021), heat tolerance (Tao et al., 2020), toxicity defense (Altaf et al., 2021), and cold tolerance in many plants, such as phalaenopsis seedlings (*Phalaenopsis aphrodite* H. G. Reichenbach; Chen and Ko, 2021), mung bean (*Vigna radiata* L.; Nahar et al., 2015) and cucumber (*Cucumis sativus* L.; Zhang et al., 2019).

Abscisic acid (ABA) as a natural plant hormone, regulates several physiological functions including stomatal closure, leaf abscission, senescence, fruit ripening and so on (Parwez et al., 2022). At present, there are numerous studies related to molecular mechanisms of ABA biosynthesis. Abscisic acid biosynthesis involves many crucial enzymes such as Zeaxanthin epoxidase (ZEP), 9′ cis epoxycarotenoid dioxygenase (NCED) and abscisic aldehyde oxidase (AAO; Zhou et al., 2021). Abscisic acid is widely involved in responding to various adversities and stresses, and it is one of the important regulators of chilling injury mitigation (Ciura and Kruk, 2018). There is the evidence that exogenous application of ABA can effectively alleviate chilling injury of vegetables and fruits. For example, zucchini squash fruit storage under high relative humidity (HRH) conditions alleviates chilling injury by promoting the accumulation of endogenous ABA (Zuo et al., 2021). Abscisic acid can effectively reduce internal browning (IB) in pineapple (Zhang et al., 2015) and maintain the color and quality of postharvest grapes (Cantín et al., 2007). An increasing amount of literature is devoted to explain that improved antioxidant capacity is associated with fruit quality, such as lemon (*Citrus limon* (L.) Burm. f.; Zhang and Zhou, 2019), goji (*Lycium barbarum* L.; Zhang et al., 2021), sweet cherry (*Prunus avium* L.; Zhao et al., 2019), and Rosa sterilis fruit (*R. sterilis*; Dong et al., 2022). In this result, we found that ABA treatment exactly improved the activity of antioxidant enzymes consistent with slight chilling injury symptoms during cold storage. However, resistance to cold stress is not exclusively controlled by a single ABA signal, but often acts in concert with other regulatory factors and the mechanism needs to be deeply explored.

Based on the above background, we assessed the effect of ABA on essential enzymes related to ROS metabolism and analyzed transcript abundance accordingly. Insight to explore the deep mechanism of ABA in alleviating chilling injury and offer an effective strategy to preserve postharvest peach fruit.

Materials and methods

Plant materials and sampling

The peaches (*Prunus persica L. Batsch.*, "Jinqiuhongmi") were picked from an orchard in Weifang, Shandong Province, China, after they reached an eighth mature stage, which means that the peel of green color has faded and turned white, and the flesh is fuller and less fuzz. On the same day, the fruit was delivered to the Chinese Academy of Agricultural Science (CAAS) lab. Each group contained 150 fruits, consisting of three replicates of 50 fruit each. One of the following solutions was used to soak the peaches for 10 min: (1) distilled water (control); (2) 100 μmol L^{-1} abscisic acid (Solarbio Life Sciences, Beijing, China). Then all fruit were stored at 4°C for 5 weeks under 90%–95% relative humidity. Three biological replicates containing three fruit each were used for the analysis of quality and biochemistry at 0, 7, 14, 21, 28, and 35 days following harvest. After the physiological measurement, fruit slices from each of the three replicates were mixed and stored at −80°C for the following experiment.

Evaluation of internal browning index

The evaluation criteria of chilling injury are IB. The fruit was cut along the axial diameter and assessed chilling injury visa visual IB of each fruit. The calculation of the IB index is referenced to Wang et al. (2018).

The severity of the chilling injury was estimated by the scale of the IB region of peach fruit and scored from 0 to 4 as follows:

0, nearly no browning; 1, browning scale was 1%–25%; 2, browning scale was 26%–50%; 3, browning scale was 51%–75%; and 4, browning scale was 76%–100%. The chilling injury index was calculated using the formula: chilling injury index = [(chilling injury score) × (number of fruits with this chilling injury score)]/(4 × total number of fruits in each treatment).

Measurement of ethylene production

Ethylene production was calculated as follows: Select six fruit per treatment from the refrigerator house and divide them into three groups at each sampling time point. Each treatment group was placed in a 1.5 L sealed container for 2 h at room temperature, the ethylene production was measured following the methods described by Zhao et al. (2021a).

Measurement of respiration rate

For each treatment, the respiration rate was recorded following the methods described by Song et al. (2021).

Six peach fruit at each sampling point were composed 2 fruits in each group in three replicates, and they were sealed in 1.5-L boxes for 2 h, using a portable CO_2 infrared analyzer (F950, Felix Instruments) to measure the total amount of CO_2 and calculate the respiration rate based on the total amount of CO_2.

Measurement of H_2O_2 content

Hydrogen peroxide (H_2O_2) content was measured using the H_2O_2 content detection kit (Solarbio, BC3590-50T/48S, Beijing, China), taking 0.1 g of powdered sample and adding the extraction solution. The homogenized sample was then centrifuged at 8,000 *g* for 10 min at 4°C, strictly following the instructions.

Measurement of enzymes activities related to antioxidant enzyme pathway and AsA-GSH pathway

SOD, POD, APX, and GR activities in peach peel tissues were measured using a POD assay kit (Solarbio, BC0090-50T/48S, Beijing, China), a SOD assay kit (Solarbio, BC0175-100T/48S, Beijing, China), an APX assay kit (Solarbio, BC0220-50T/48S, Beijing, China), and a GR assay kit (Solarbio, BC1160-50T/48S, Beijing, China), respectively, based on the guidelines of the manufacturer, taking 0.1 g of powdered sample and adding the extraction solution. The homogenized sample was then centrifuged at 8,000 *g* for 10 min at 4°C, strictly following the instructions.

Total RNA extraction and RNA-seq

Total RNA was isolated according to acetyltrimethylammonium bromide (CTAB) method with little modifications (Zhao et al., 2021a). At the same time, considering that the chilling symptoms of peach fruit occurred 21 days after ABA treatment, fruits were selected after 21 days storage as the time points for RNA-seq. The samples were labeled as CK_21A, CK_21B, CK_21C, ABA_21A, ABA_21B, and ABA_21C. Libraries for RNA sequencing (RNA-Seq) were prepared according to Zhao et al. (2022). RNA-seq was performed on an Illumina HiSeq 2500 sequence platform at ouyi company (Shanghai, China). The adaptor sequences and low-quality sequence reads were first removed from the data sets completely (Bolger et al., 2014), then the paired and clean reads were mapped to the peach genome.[1] Transcript abundance level was expressed by Fragments Per Kilobase Million (FPKM). Differential expression analysis was performed using DESeq2 software. Genes with fold change >2 and false discovery rate (FDR) < 0.05 were regarded as differentially expressed genes (DEGs). The specific calculation is referred to in our previous study (Zhao et al., 2022).

Reverse transcription-quantitative PCR (RT-qPCR) analysis

cDNA synthesis was referred to the previous method with slight modifications for real-time quantitative PCR (Zhao et al., 2021a). cDNA was generated using A SYBR Green Q-PCR Kit (Takara, RR420A, Japan) following the manufacturer's guidelines and qRT-PCR was completed using the Applied Biosystems 7,500 Fast Real-Time PCR System (Thermo Fisher Scientific, United States). According to Livak and Schmittgen (2001), relative gene expression was calculated using the Comparative $2^{-\Delta\Delta CT}$ method. Primer sequences can be found in Supplementary Tables S1, S2.

Statistical analysis

SPSS version 17.0 was used for all statistical analyses, and the figures were prepared using Origin 8.6. Differences between control and treated fruit were assessed with significance at $p < 0.05$.

1 https://phytozome-next.jgi.doe.gov/info/Ppersica_v2_1

Results

Effect of ABA on chilling injury index of peach fruit

To assess whether the alleviation of chilling injury was affected by ABA, we examined the IB region. Examples of IB are shown in Figure 1, the control fruit demonstrated slight chilling injury symptoms after 14 days of storage. On the contrary, IB nearly did not appear in ABA-treated fruit at day 14, but a little appeared at day 21. The chilling injury index in the control fruit was ~4 times and 3 times that of ABA-treated fruit, respectively, at day 21 and day 28, but during the end of storage, the chilling injury index of the two groups was very close. According to this result, we concluded that ABA was effective in reducing chilling injury symptoms during cold storage ($p < 0.05$). However, the effect of ABA treatment beyond 4 weeks of storage was not significant, especially at day 35, peach fruit showed severe IB and lost commodity value absolutely.

Effect of ABA on respiration and ethylene production rate of peach fruit

Levels of ethylene production remained relatively stable between the control and ABA-treated fruit for the first 7 days. After that, ethylene production by both the control and ABA-treated fruit increased rapidly; application of ABA was effective in reducing ethylene production at day 14 (Figure 2A; $p < 0.05$), and there was no difference in later stages of storage. At the end of the storage, ABA treatment nearly keep the same value of ethylene production compared with the CK fruit.

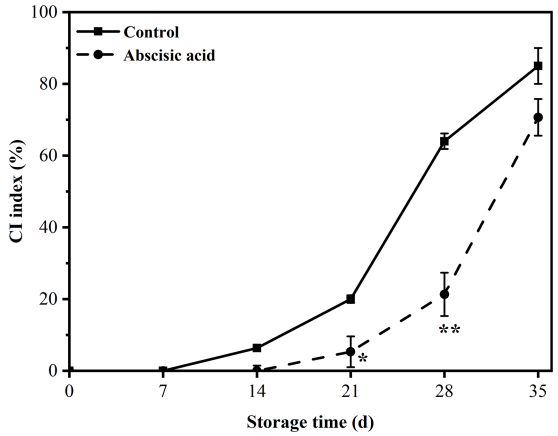

FIGURE 1
Effect of abscisic acid (ABA) treatment on chilling injury index of peach fruit during cold storage at 4°C. Vertical bars represent the standard deviation of the means of triplicate samples (n=3). The symbol (*) indicates significant differences among different treatments at $p < 0.05$. The symbol (**) indicates significant differences among different treatments at $p < 0.01$.

Respiration rate showed a similar trend both in treated fruit and control fruit during the entire storage period. At first, respiration rate declined sharply in the first 7 days of storage and then increased gradually regardless of treatment. However, treatment with ABA significantly ($p < 0.05$) inhabited the respiration rate at day 14 and day 35 (Figure 2B). The significant reduction in ABA-treated fruit reached 30% and 17%, respectively, compared with that in control fruit ($p < 0.05$).

Effect of ABA on H_2O_2 content of peach fruit

As shown in Figure 3, ABA treatment reduced the H_2O_2 content in peach fruit. H_2O_2 content rose sharply at first and reached a peak at day 14, but the content of H_2O_2 in ABA-treated fruit decreased at day 7. In the following storage period, H_2O_2 content in control exhibited a decreasing trend during this period, as for ABA-treated fruit, the H_2O_2 content was at a relatively stable value during the next 4 weeks. It is worth noting that ABA significantly reduced the H_2O_2 content, especially during the first

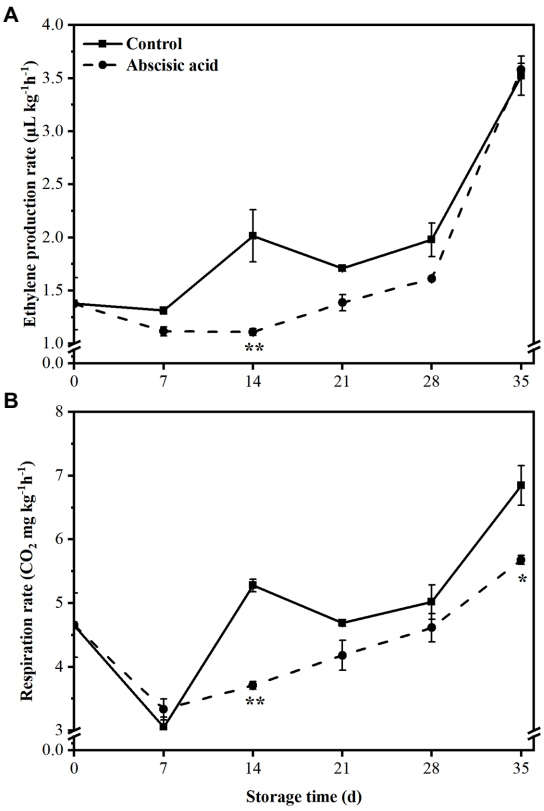

FIGURE 2
Effect of abscisic acid (ABA) treatment on ethylene production **(A)** and respiration rate **(B)** of peach fruit during cold storage at 4°C. Vertical bars represent the standard deviation of the means of triplicate samples (n=3). The symbol (*) indicates significant differences among different treatments at $p < 0.05$. The symbol (**) indicates significant differences among different treatments at $p < 0.01$.

4 weeks of refrigeration period ($p < 0.05$). It is suggested that a significant function of ABA is to reduce the level of H_2O_2 content, thus lessening peroxide damage to cells and maintaining the balance of ROS metabolisms in plants.

Effect of ABA on the expression of genes related to ethylene synthesis and its signaling pathway

We have analyzed the transcript abundance of all genes by RNA-seq (Supplementary Figure 1). After 21 days of low-temperature storage, ABA significantly inhibited the expression of *PpACO1* ($p < 0.05$), which is one of the important members of ethylene synthesis. The results of RT-qPCR (Figure 4B) and RNA-seq were basically the same. In addition, we also examined genes involved in the ethylene signaling pathway (Figure 4B). The data show that gene expression of ethylene receptors *PpEIN2* was significantly inhibited by ABA.

Effect of ABA on the gene expression of the antioxidant enzyme pathway and the AsA-GSH pathway

Plants have a sophisticated metabolic network for ROS. In a bid better to investigate the underlying mechanism of exogenous ABA regulating ROS metabolism in peach fruit, we examined several genes related to antioxidant enzymes including *PpSODs*, *PpPODs*, *PpCATs*, *PpAPXs*, *PpGPXs*, *and PpGRs*. SOD can catalyze the dismutation of O_2^- to H_2O_2. Figure 5B showed that *PpSOD* expression level of the control was relatively lower during the cold storage. In addition, the relative expression of *PpSOD* of ABA treatment was significantly up-regulated ($p < 0.05$), and it was ~2.13 times that of control. CAT and POD can catalyze H_2O_2 and oxidize phenols. *PpPOD* was annotated by five genes (LOC18768241, LOC18769960, LOC18770065, LOC18773443, and LOC18781284). The expression levels for all five genes exhibited an upward trend during the cold storage. As shown in Figure 5B, the expression level of *PpPOD* was markedly increased by ABA treatment at day 21 ($p < 0.05$). *PpCATs* (LOC109949510, LOC18777304, and LOC18776773) expression abundance in ABA-treated fruit both increased and decreased at day 21 (Figure 5B). *PpAPXs* and *PpGPXs* are, respectively, annotated by (LOC18768608, LOC109950245, LOC18769065, LOC18788446, and LOC18772001) and (LOC18788389, LOC109946232, LOC18777404, LOC18776398, and LOC18766597). Moreover, the five *PpAPXs* had different expression patterns. The relative expression of *PpAPX2* of ABA treatment was up-regulated, but there was no difference between control and ABA treatment. In addition, the rest of the genes were down-regulated by ABA treatment (Figure 5B). As for *PpGPXs*, compared with CK, all five genes were induced by ABA at day 21 (Figure 5B).

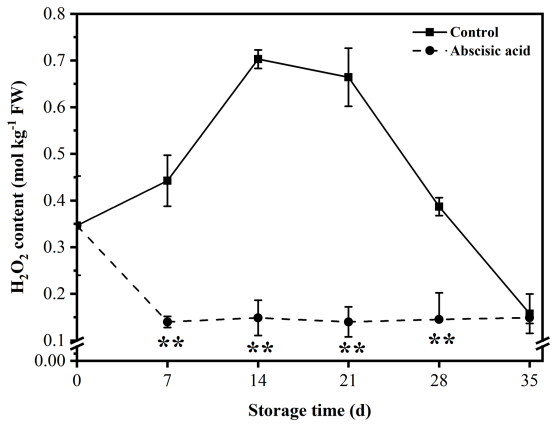

FIGURE 3
Effect of abscisic acid (ABA) treatment on H_2O_2 content of peach fruit during cold storage at 4°C. Vertical bars represent the standard deviation of the means of triplicate samples (n=3). The symbol (**) indicates significant differences among different treatments at $p < 0.01$.

Effect of ABA on the enzyme activities of the antioxidant enzyme pathway and the AsA-GSH pathway

During cold storage, ROS maybe induced by cold stress and the accumulation of ROS eventually cause plant irreversible injury. One of the most important effects of antioxidant enzymes is to scavenge ROS. Therefore, we detected antioxidant enzyme activities including SOD, POD, APX, and GR. As illustrated in Figure 6A, during the cold storage, the SOD activity showed a decreasing trend during the whole period, regardless of treatment. Moreover, ABA treatment alleviated the decline in SOD activity, especially in the last 3 weeks. The SOD activity in ABA treatment was 16% and 23% higher than that of control at day 21 and day 35, respectively ($p < 0.05$).

Unlike the change of SOD activity, Figure 6B showed that APX enzyme activity of control gradually increased and rapidly decreased at day 14, then maintained a stable trend until the end of storage. On the contrary, the APX activity of ABA-treated fruit was higher than that of control, especially in the first 28 days ($p < 0.05$). In addition, we found that APX activity treated by ABA has risen nearly eight times at day 28. Compared with the control, the GR activity in ABA treatment rose sharply first and reached a peak at day 14. At this time, the activity of GR was 2.43 times higher than that of the control ($p < 0.05$). In the later storage period, GR activity decreased sharply and we found treatment with ABA almost had no effect on GR activity in the last 2 weeks. The variation of GR activity in control fruit was quite small and always remained at a low level (Figure 6C). Finally, the POD activity showed a similar trend regardless of treatment. During the

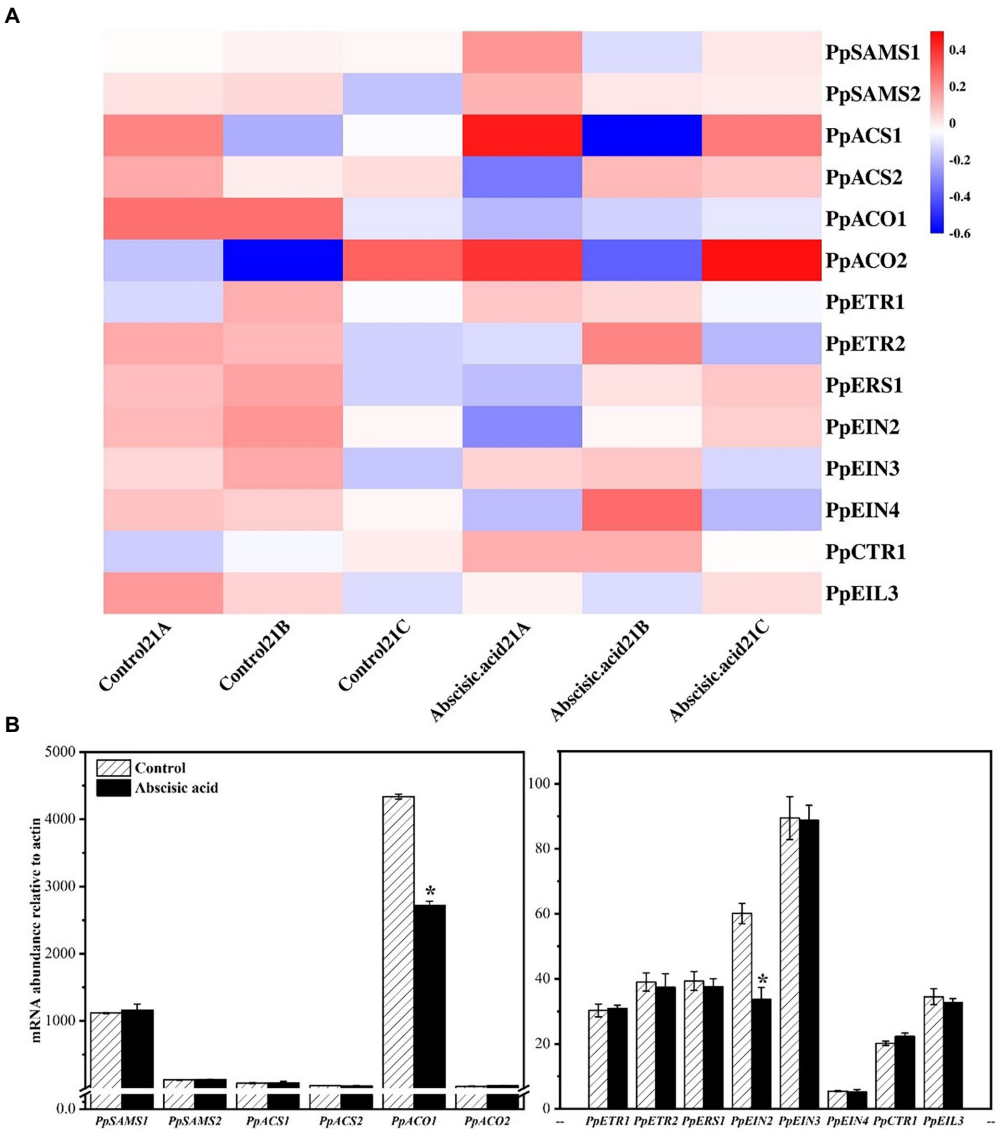

FIGURE 4
Effect of abscisic acid (ABA) treatment on the mRNA abundance of genes related to ethylene biosynthesis and the ethylene signaling pathway in fruit stored at 4°C. **(A)** Heat map showing transcript abundances of differentially expressed genes as determined by RNA-seq. **(B)** Validation of mRNA abundances as determined using RT-qPCR. Data are means of three biological replicates ±SE. The symbol (*) indicates significant differences among different treatments at $p < 0.05$.

first 21 days of storage, the POD activity increased significantly and subsequently fell until the end of storage. Compared with the control, ABA treatment significantly enhanced the activities of POD ($p < 0.05$), except for day 7 and day 28 (Figure 6D).

Discussion

For postharvest fruit and vegetables, storage and preservation at low temperature is a wide and common method to extend the shelf life. Low temperature usually causes the occurrence of chilling injury, resulting in fruit quality deterioration and decline in commodity value seriously. The main limiting factor for applying low temperatures to preserve peach fruit is chilling injury. Therefore, it is necessary to seek effective methods to alleviate chilling injury. Previous research has shown that ABA alleviated chilling injury effectively by maintaining cell membrane stability (Guo et al., 2012; Carvajal et al., 2017; Li et al., 2021; Xiong et al., 2021). Our previous study showed that alleviation of chilling injury symptoms in peach fruit by exogenous ABA was associated with the regulation of sucrose metabolism (Zhao et al., 2022). Moreover, Kim et al. (2016) illustrated that in another example of exogenous ABA application leading to improved chilling tolerance, with oriental melon plants, there was an

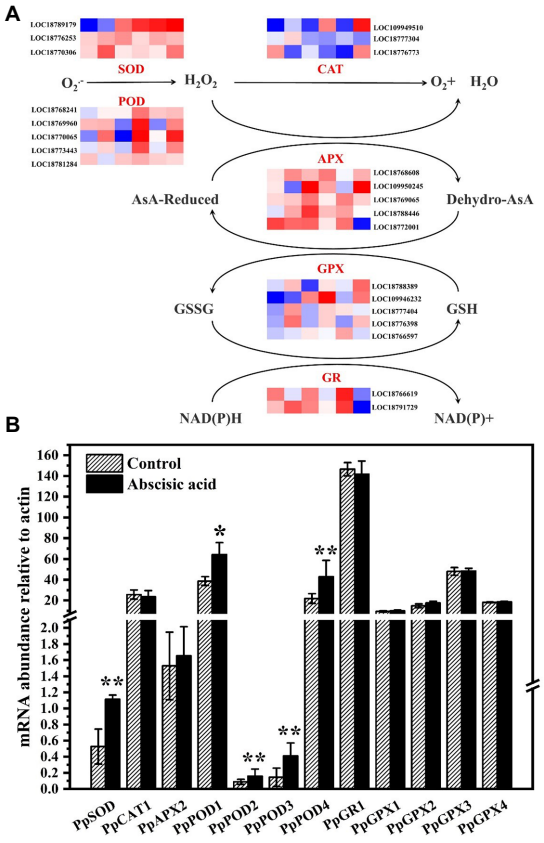

FIGURE 5
(A) Effects of abscisic acid (ABA) treatment on the expression profiles of genes associated with reactive oxygen species metabolic pathways in peach fruit stored at 4°C. The rows in each heat map represent the indicated genes, and the six columns indicate the following storage times (left-to-right): CK_21dA, CK_21dB, CK_21dC, ABA_21dA, ABA_21dB, and ABA_21dC. The colors represent the FPKM values between different samples in the heat map. Each value represents the mean for three replicates. SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; APX, ascorbate peroxidase; GPX, glutathione peroxidase; GR, glutathione reductase, monodehydroascorbate reductase. **(B)** Validation of mRNA abundances of sugar metabolism genes as determined using RT-qPCR. Data are means of three biological replicates±SE. The symbol (*) indicates significant differences among different treatments at $p < 0.05$. The symbol (**) indicates significant differences among different treatments at $p < 0.01$.

associated increase in endogenous gibberellin (GA_4) and salicylic acid (SA). Internal browning is a representative symptom of chilling injury and is associated with damage of cell membranes when peach fruit is exposed to improper low temperature conditions (Duan et al., 2022). The results showed that postharvest application of ABA significantly reduced the IB index of peach fruit during storage at 4°C (Figure 1). Based on the findings both in past and present studies together demonstrate that ABA is a promising approach to enhance cold tolerance in diverse fruits and vegetables after harvest.

Fruit softening, ripening, and rotting is mainly controlled by ethylene. As a plant hormone, ethylene is widely involved in physiological and biochemical reactions in plant cells. For example, endogenous ethylene has been implicated in enhancing the cold tolerance of postharvest fruit in earlier investigations (Wei et al., 2019; Yu et al., 2019). In peach, as a typical climacteric fruit, endogenous ethylene and respiration rates increase dramatically during ripening (Hayama et al., 2006; Wang et al., 2017). Ethylene is also broadly involved in plant response to various stressful conditions (Wei et al., 2019). Candan et al. (2006) found that ethylene can increase chilling injury symptoms of plums. Moreover, Pesis et al. (2002) showed that avocado treated with endogenous ethylene caused chilling injury symptoms such as severe pulp browning. 1-aminocyclopropane-1-carboxylic acid (ACC) can be oxidized by ACC oxidase (ACO) to generate ethylene (Hu et al., 2019). As a crucial rate-limiting enzyme in ethylene synthesis, inhibiting the activity of ACO enzyme can effectively reduce ethylene production. In addition, respiration continues after harvesting and increases with tissue deterioration (Li et al., 2022). Respiratory metabolism provided energy for various metabolic processes in plant cells, but also produced large amounts of ROS (del Río et al., 2002). In this research, we found that exogenous ABA effectively reduced ethylene production and respiration rate by inhibiting the expression of genes related to ethylene synthesis, including *PpACO1* and *PpEIN2* (Figures 2, 4). These findings suggest that ethylene is taken part in regulating chilling injury in peach fruit, and that lower ethylene production may help alleviate chilling injury in ABA-treated fruit. At the same time, discrepancies in ethylene responsiveness are likely due to variances in ethylene production as well as ethylene perception machinery abundance.

The imbalance of ROS production usually leads to severe and irreversible damage in plant cells. The occurrence and development of chilling injury are closely related to the excessive accumulation of ROS (Zhao et al., 2021b; Wang et al., 2022). Antioxidant enzymes are the main approach to scavenge excessive ROS and alleviate cell damage induced by oxidative stress, which included SOD, POD, and CAT. Compared with control fruit, the activity of SOD and POD markedly increased in ABA-treated fruit (Figures 6A,D), and this may account for the lower H_2O_2 content in ABA-treated fruit (Figure 3). AsA-GSH cycle also takes part in this chemical progress (Tian et al., 2013). Some non-enzymatic substances with antioxidant activity respond to different stress conditions by regulating H_2O_2 metabolism (López-Vidal et al., 2016; Piechowiak, 2021). Gao et al. (2016) found that melatonin can effectively reduce the chilling injury index of peaches during cold storage, and this effect may be due to the enhanced activity of antioxidant enzymes thus reducing the accumulation of ROS. Tareen et al. (2012) illustrated that peach fruit treated with SA displayed the highest activity of enzymatic antioxidants, and, in turn, maintained higher quality and showed delayed rotting during storage. Moreover, Cao et al. (2009) showed that loquat fruit treated with methyl jasmonate exhibited lower chilling injury along with higher activities of antioxidant enzymes.

AsA-GSH cycle is also vital for improving the antioxidant capacity of plant cells. Kaya et al. (2021) demonstrated that the

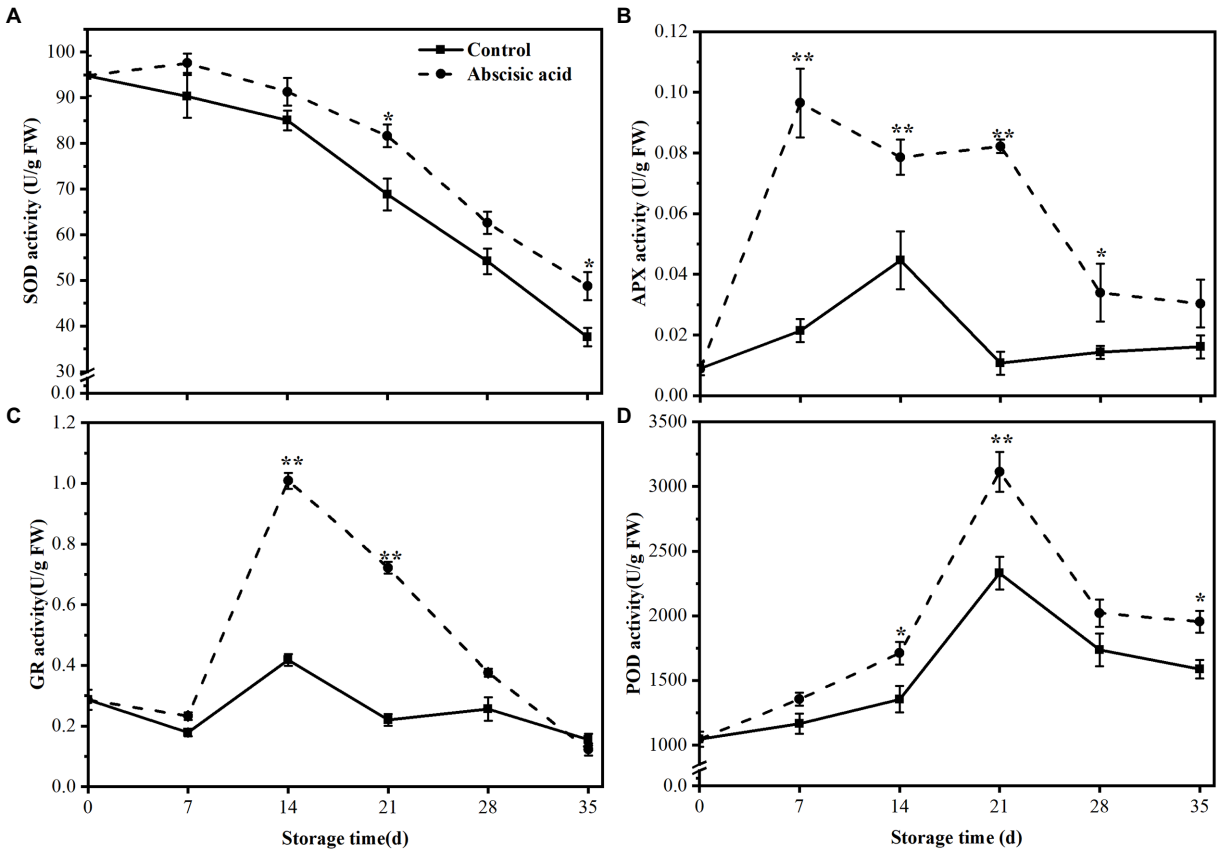

FIGURE 6
Activity of **(A)** Superoxide dismutase (SOD), **(B)** ascorbate peroxidase (APX), **(C)** glutathione (GR), and **(D)** peroxidase (POD) of peach fruit treated with abscisic acid (ABA) during storage at 4°C. The symbol (*) indicates significant differences among different treatments at $p < 0.05$. The symbol (**) indicates significant differences among different treatments at $p < 0.01$.

AsA-GSH cycle enhanced resistance to environmental stress and delayed senescence of postharvest through scavenging excessive ROS. Figure 6B showed that ABA treatment significantly increased the activity of APX. As an important enzyme for scavenging ROS in the AsA-GSH cycle, APX could catalyze H_2O_2 into H_2O. This finding suggested that the lower H_2O_2 content was closely associated with higher APX activity. The presence of both APX and POD allows plants to defend against the toxicity of H_2O_2 (Jannatizadeh, 2019). Abscisic acid treatment also increased the activity of GR The effect of ABA on APX and GR activity suggested that ABA is involved in the regulation of the ASA-GSH cycle. Yao et al. (2021) found exogenous GSH treatment could trigger the AsA-GSH cycle and improve antioxidant capacity, therefore alleviating chilling injury in pepper fruits during cold storage. In our study, peach fruit treated with ABA exhibited higher antioxidant enzyme activities, comprising SOD, POD, APX, and GR (Figure 6). This result was consistent with lower H_2O_2 content in ABA-treated fruit (Figure 3). All the results suggest that antioxidant capacity is a crucial character for maintaining a balance of ROS. In the present study, we detected the impact of ABA on the transcript abundance level of *PpSOD*, *PpPOD*, *PpCAT*, *PpAPX*, and *PpGR* by using RNA-Seq, and found that the transcript abundance of *PpSOD*, *PpPOD* gene were up-regulated after ABA treatment (Figure 5). This indicates that ABA regulates antioxidant enzyme activity probably by regulating the genes of transcript abundance related to the antioxidant enzyme. Our finding confirms that ABA could eliminate oxidative damage and enhances the antioxidant capacity of plant cells.

Conclusion

In conclusion, ABA treatment is beneficial in preventing chilling injury and maintaining the commodity quality of peach fruit. The results revealed that treatment with ABA may improve the chilling tolerance of peach fruit by inhibiting the production of ethylene and promoting the scavenging ability of H_2O_2. This research clarifies the mechanisms of ABA alleviated chilling injury and provides a theoretical method for applying ABA treatment to maintain a better quality of peach fruit after harvest.

Data availability statement

The data presented in the study are deposited in the SRA repository, accession number PRJNA866347.

Author contributions

JT: performed the experiments and analyzed the data, writing and editing. YZ: funding acquisition, conceived the study, and writing–review and editing. SQ: performed the experiments and analyzed the data. QD: methodology, data curation, and investigation. QL: project administration. YD: funding acquisition and validation. 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.2022.987573/full#supplementary-material

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Study on phytotoxicity evaluation and physiological properties of nicosulfuron on sugar beet (*Beta vulgaris* L.)

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Nicosulfuron is an herbicide widely used in corn fields. In northeast China, sugar beet is often planted adjacent to corn, resulting in frequent phytotoxicity of nicosulfuron drift in sugar beet fields. This study was conducted by spraying nicosulfuron to assess the phytotoxicity and clarify the mechanism of nicosulfuron toxicity on sugar beet. The results showed that nicosulfuron impaired growth and development by reducing photosynthetic capacity and disrupting antioxidant systems at a lethal dose of 81.83 g a.i. ha^{-1}. Nicosulfuron damaged the function of photosynthetic system II (PSII), lowered photosynthetic pigment content, and inhibited photosynthetic efficiency. Compared with the control, the electron transfer of PSII was blocked. The ability of PSII reaction centers to capture and utilize light energy was reduced, resulting in a weakened photosynthetic capacity. The maximum net photosynthetic rate (Amax), light saturation point (LSP), and apparent quantum yield (AQY) decreased gradually as the nicosulfuron dose increased, whereas the light compensation point (LCP) and dark respiration (Rd) increased. Nicosulfuron led to reactive oxygen species (ROS) accumulation in sugar beet leaf, a significant rise in malondialdehyde (MDA) content, electrolytic leakage (EL), and considerable oxidative damage to the antioxidant system. This study is beneficial for elucidating the effects of nicosulfuron toxicity on sugar beet, in terms of phytotoxicity, photosynthetic physiology, and antioxidative defense system.

KEYWORDS

herbicide, phytotoxicity, lethal dose, chlorophyll fluorescence, oxidative defense

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1 Introduction

Weeds are an important limiting factor in agricultural production (Ghosh et al., 2020; Skalicky et al., 2020). Herbicide application is a common means of weed control in agricultural production, but improper use can easily cause phytotoxicity. In recent years, herbicide has attracted widespread attention. Studies have shown that crops such as rice (Bellaloui et al., 2006), soybean (Brown et al., 2009), peanut (Koger et al., 2010), sorghum (Steppig et al., 2017), maize (Egan et al., 2017), wheat (Wiersma and Durgan, 2018) and melon (Xu et al., 2018) have been infested with herbicide toxicity, affecting growth and crop yield.

Nicosulfuron is widely used in corn fields because of its fast-acting, strong persistence and high safety. It is an Acetolactate Synthase (ALS) inhibitor herbicide that inhibits ALS enzyme activity in sensitive plants, thereby inhibiting the formation of branched-chain multiple amino acids (Wright et al., 2017). As a result, plants affected by nicosulfuron will eventually stop growing, or even die. Field weed resistance and the relentless pursuit of crop yield have resulted in an increase in the use of nicosulfuron in agricultural production year after year. Herbicide phytotoxicity not only occurs in crops but also causes phytotoxicity to neighboring crops due to herbicide drift of droplets formed during herbicide spraying (Meloni and Bolzón, 2021).

Sugar beet (*Beta vulgaris* L.), a widespread sugar crop in temperate climates, meets about 20% of the global sugar demand (Song et al., 2022). At the same time, sugar beet is a susceptible crop to herbicides. It is often damaged by herbicide drift from adjacent field crops (Li et al., 2021). Corn and sugar beet are frequently cultivated adjacent in northeast China. Nicosulfuron toxicity is a common phenomenon in local production areas and is an important cause of sugar beet yield decline (Wang et al., 2009; Ellis and Miller, 2010). Since corn is a monocotyledonous plant, nicosulfuron is commonly used in corn fields to control dicotyledonous weeds. Because of this, nicosulfuron drift is more harmful to sugar beet that grows next to corn fields (Li et al., 2017).

Under herbicide stress, plants usually produce large amounts of reactive oxygen species (ROS), leading to oxidative stress in plants. The surge of ROS activates the plant's antioxidant system, which allows the plant to scavenge excess ROS (Jervekani et al., 2018; Li et al., 2022b). At the same time, plants are also able to respond to herbicide stress by regulating hormonal activity and promoting or inhibiting the formation of key metabolites. Therefore, it is common to mitigate herbicide toxicity in crops by application of plant hormones (Li et al., 2022a).

The effects of Herbicide toxicity stress on crop growth parameters, photosynthetic properties, and antioxidant systems have received extensive attention, including wheat (Yadav et al., 2019; Feng et al., 2021), maize (Wang et al., 2018; Wang et al., 2021a; Sun et al., 2022) and black bean (Meloni and Bolzón, 2021). However, fewer studies have been reported on sugar beet toxic symptoms and photosynthetic physiology under herbicide toxicity. In particular, the response of sugar beet under nicosulfuron stress in terms of physiology, photosynthetic system and the antioxidant system is not clear. Consequently, a pot experiment was conducted to explore the phytotoxic effects of nicosulfuron on sugar beet, to provide a reference for assessing herbicide phytotoxicity and addressing nicosulfuron drift damage on sugar beet.

2 Materials and methods

2.1 Experimental material

Sugar beet variety KWS1176 was provided by Seed Co., Ltd. (Germany). Qingdao Hansen Bioscience Co., Ltd. supplied the 24% nicosulfuron oil suspension. The soil type is black soil with the following initial properties, pH: 6.64; bulk density: 1.26 g cm^{-3}; organic matter content: 21.23 g kg^{-1}; alkali-hydrolyzable N: 122.64 mg kg^{-1}; available P: 46.30 mg kg^{-1}; available K: 330.92 mg kg^{-1}.

2.2 Experimental design

The experiment was carried out in a greenhouse at Heilongjiang University, China. The test soil was filled with 0.073 g kg^{-1} of urea, 0.078 g kg^{-1} of phosphate diamine, and 0.095 g kg^{-1} of potassium sulfate in polyethylene plastic pots (300 g per pot) and poured with 45 mL of distilled water. Each pot was sown with 3 sugar beet seeds, and covered with 100 g of soil. The seedlings were cultivated in a greenhouse under natural light with a light intensity of 138 mol m^{-2} s^{-1}, 14 h of light per day, 25°C/20°C (day/night), and 50–60% relative humidity. One plant was left in each pot after one week of cultivation.

The recommended dose of nicosulfuron in the corn field was 60 g a.i. ha^{-1}. Considering the herbicide over-application in agricultural production, the nicosulfuron doses of the five treatment groups were designated 1/100, 1/10, 1/3, 1, and 2 times the recommended dose in the field, noted as N0.6, N6, N20, N60, N120. Water was sprayed as a control group (CK) and each treatment was replicated six-time. The sugar beet seedlings were sprayed with various concentrations of nicosulfuron solution once the second pair of sugar beet leaves were utterly extended. Control treatments were sprayed with distilled water.

2.3 Measurement of phytotoxicity index and physiological properties

Phytotoxicity index, growth indexes, photosynthetic parameters, and fluorescence parameters were measured within 20 days after being treated with nicosulfuron. On 20

DAT (days after treatment), samples of the second pair of true leaves of sugar beet were taken and stored at −20°C to determine physiological indicators.

2.3.1 Determination of growth parameters

SPAD values of the second pair of true leaves of sugar beet were measured using SPAD chlorophyll meter (Minolta SPAD-502Plus, Tokyo, Japan). The plant height, leaf length and leaf width of the second pair of true leaves in the natural state of the sugar beet were measured using a straightedge. To collect the samples, the beets were removed from the pots, cleaned of root soil, and placed flat on a glass plate. The plants extended naturally and the length of the underground part of the plants was recorded as root length with a straightedge. The root thickness was measured with vernier calipers. Leaf area was calculated from the leaf area index (Hoffmann and Blomberg, 2004). The above- and below-ground parts were split with scissors and the fresh weight of the plants was determined separately. Beets were killed in an oven at 120°C for 2 h, dried at 80°C to a constant weight, and weighed for dry weight after natural cooling.

2.3.2 Calculation of phytotoxicity index and dose-fresh weight response curve

The phytotoxicity index was calculated based on the phytotoxicity grade (Dai et al., 2017) (Table 1).

To get the dose-fresh weight response curve, a three-parameter log-logistic model in R Studio was utilized to perform regression analysis on the dose-fresh weight response data (Stevan et al., 2007). The effective herbicide dosage that resulted in a 50% growth reduction (GR_{50}) was determined.

2.3.3 Determination of leaf photosynthetic parameters

The photosynthetic pigment content was determined using the ethanol method (Arnon, 1949). The net photosynthetic rate (P_n), stomatal conductance (G_s), transpiration rate (T_r), and intercellular CO_2 concentration (C_i) of the second pair of true leaves of sugar bee were determined with a portable photosynthesis instrument, TARGAS-1 (Deligiosa et al., 2019). The investigations were run during 9:00-11:00 AM under the photosynthetically active radiation (PAR) level of 250 μmol m^{-2} s^{-1}. The PAR levels of 1500, 1200, 800, 600, 400, 300, 200, 100, and 0 μmol m^{-2} s^{-1} were measured to get the P_n-light curve, G_s-light curve, T_r-light curve and C_i-light curve. The non-rectangular hyperbola model was utilized to calculate photosynthetic parameters, including the maximum net photosynthetic rate (Amax), light compensation point (LCP), light saturation point (LSP), apparent quantum yield (AQY), and dark respiration (Rd) (Ye et al., 2014).

TABLE 1 Classification standard of phytotoxicity grades.

Phytotoxicity grade	Description of phytotoxicity symptoms
0	Control treatment
1	Seedlings' height and leaf color slightly different from the control
2	Seedlings were slightly deformed, lower in height than the control
3	Seedlings were shorter, with thicker stalks, slightly thicker leaves, and yellow color
4	Seedlings stopped growth. Seedlings were deformed and stiff or the whole leaf was yellow and dead.
5	Seedlings death

2.3.4 Determination of chl a fluorescence parameters

The chl a fluorescence transient (OJIP transient) of the second fully expanded sugar beet leaf under different treatments was determined using Pocket PEA continuous excitation fluorimeter (Handy, UK). The initial fluorescence (F_O) was set as O (50 μs), K (300 μs), J (2 ms) and I (30 ms) are the intermediates (F_K, F_J and F_I, respectively) and P (1000 ms) as the maximum fluorescence (F_m). The original (without normalization) chl a fluorescence intensity (F_t) curves were plotted. The original OJIP transients were double normalized between the two fluorescence extreme O (F_O) and P (F_m) phases and the variable fluorescence between OP expressed as V_{O-P} was determined. The difference in transients (ΔV_{O-P}) concerning a reference was calculated. Further, the chl a fluorescence transients were double normalized between F_O and F_J expressed as V_{O-J} and the difference between transients expressed as ΔV_{O-J} was determined.

Maximal Photochemical Efficiency of PSII (F_V/F_m), performance index on absorption basis (PI_{abs}), electron transport flux per reaction center (RC) (ET_O/RC), dissipated energy flux per RC (DI_O/RC), absorption flux per RC (ABS/RC), dissipated energy flux per CS (DI_O/CS_M), electron transport flux per CS (ET_O/CS_M), section absorption flux per CS (ABS/CS_M) were measured based on the above fluorescence parameters as reported by Strasser et al. (1995).

2.3.5 Determination of physiological indicators

Physiological indicators were determined using the second pair of true leaves of sugar beet from the stored samples. The content of superoxide anion (O_2^-) was measured as reported by Zhang et al. (2007). The hydrogen peroxide (H_2O_2) content was measured as reported by Wang et al. (2021b). The malondialdehyde (MDA) content was measured by the thiobarbituric acid reaction (Dhindsa and Matowe, 1981). Electrolytic leakage (EL) was measured by a multi-parameter water quality analyzer (DZS-706-A) according to Belkhadi et al. (2010).

The activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) were determined according to the approach of NBT reduction

(Giannopolites and Ries, 1977), guaiacol method (Chance and Maehly, 1955), UV absorption method (Khorram et al., 2016), and the way of Jiang and Zhang (2001), respectively.

2.4 Data analysis

The data were analyzed by one-way ANOVA and Duncan's method, and differences across groups were assessed. All data were expressed as 'Means ± SD'. IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA) were applied for data analysis. Origin 2018 (OriginLab, Northampton, 210 MA, USA) was employed to draw graphs.

3 Results

3.1 Effects of nicosulfuron on the growth parameters of sugar beet

The symptoms of phytotoxicity appeared on 4 DAT. On 20 DAT, sugar beet stopped growth when the dose of nicosulfuron reached 20 g a.i. ha^{-1}. The plants were deformed, and yellow spots on leaves were obvious. Sugar beet seedlings were wilted and deformed at a recommended dosage of 60 g a.i.ha^{-1}. The plant mortality rate was 60%, with the growing point as the starting point and extending upward to the petiole blackened. All plants died at 120 g a.i. ha^{-1} (Figure 1A). As the dose of nicosulfuron increased, the area of sugar beet leaves was enlarged and damage was visible (Figures 1B, C). The phytotoxicity index showed a remarkable difference between treatment groups and CK at 6 g a.i. ha^{-1} and above ($p < 0.05$) (Figure 1D).

The dose-fresh weight response regression equation for nicosulfuron was calculated as $y = 64.13 \times \exp(-x/65.99) + 31.63$ (y represents the percentage of fresh weight in each treatment to the fresh weight in the control group and x represents the dose of nicosulfuron). The lethal dose GR_{50} value was 81.83 g a.i. ha^{-1}, which was higher than the recommended field dose (60 g a.i. ha^{-1}) by 36.38% (Figure 1E).

The biomass of shoot and root were reduced with expanding doses of nicosulfuron. The shoot biomass were more affected than the root. There was a remarkable difference in shoot DW compared with CK when the dose reached 6 g a.i. ha^{-1}, with a 45% reduction ($p < 0.05$). At this dose, the dry weight of the shoot did not change significantly, which was only 7.69% lower than the control (Table 2).

All plant growth parameters were significantly reduced with increasing dose, such as plant height, leaf area, and *SPAD* value. The plant height, leaf area, and *SPAD* value were significantly different from the control at 20 DAT as the dose reached 0.6 g a.i. ha^{-1} ($p < 0.05$) (Figures 2A, B, C). There was a remarkable inhibition in shoot water content, leaf length, leaf width, and root length at 6 g a.i. ha^{-1} compared to the control, 10.45%, 10.63%, 10.76% and 18.24% ($p < 0.05$) (Figures 2D, E, F).

3.2 Effects of nicosulfuron on the photosynthetic parameters of sugar beet leaf

The content of photosynthetic pigment was reduced with increasing doses of nicosulfuron. When the dose of nicosulfuron reached 6 g ai ha^{-1}, the content of chlorophyll a, b, and carotenoids were decreased by 31.43%, 29.29% and 31.36%, compared to CK, respectively ($p < 0.05$). When the dose reached the highest dose in this study (120 g a.i. ha^{-1}), the content of carotenoids and total chlorophyll decreased by 75.75% and 58.48% (Figure 3).

The P_n of sugar beet leaf showed a linear enhancement trend with light intensity as PAR increased when the PAR was under 400 μmol m^{-2} s^{-1}. After that, the increase in P_n slowed under each treatment as PAR continued to grow. Under different doses of nicosulfuron treatment, the changing pattern of the P_n-light curve began to differ as the PAR was over 400 μmol m^{-2} s^{-1}. The highest P_n-light curve changes were observed in CK treatment and the lowest in N120 treatment. As the PAR reached 1200 μmol m^{-2} s^{-1}, P_n gradually saturated (Figure 4A). Both G_s and T_r showed an upward trend with increased PAR and nicosulfuron dose while C_i declined (Figures 4B, C, D).

The inhibitory effects of nicosulfuron on P_n, G_s, T_r and C_i increased with increased doses of nicosulfuron. There were remarkable differences in P_n, G_s, T_r, and C_i at 60 g a.i. ha^{-1} as compared with CK ($p < 0.05$) (Figures 4E, G, H). Only the difference in G_s reached significance at the lowest dose of nicosulfuron (0.6 g a.i. ha^{-1}) in the study, with a reduction of 22.39% as compared with CK ($p < 0.05$) (Figure 4F).

The Amax, LSP and AQY gradually decreased with increasing nicosulfuron dose as compared with CK. Specifically, the differences in Amax, LSP and AQY reached significance when the dose reached 0.6 g a.i. ha^{-1} were 12.45%, 8.05%, and 15.79% lower than the control ($p < 0.05$). The LCP and Rd increased with increasing nicosulfuron dose. The difference between Rd and the control was significant when the dose was over 0.6 g a.i. ha^{-1}, and the Rd increased by 35.14% at 0.6 g a.i. ha^{-1} ($p < 0.05$) (Table 3).

3.3 Effects of nicosulfuron on the chl a fluorescence parameters of sugar beet leaf

On the OJIP transient, fluorescence intensity at the O point showed an upward trend as the doses of nicosulfuron treatment increased. In contrast, it showed the opposite at the P point. (Figure 5A). The effects of nicosulfuron on F_m and F_v were more

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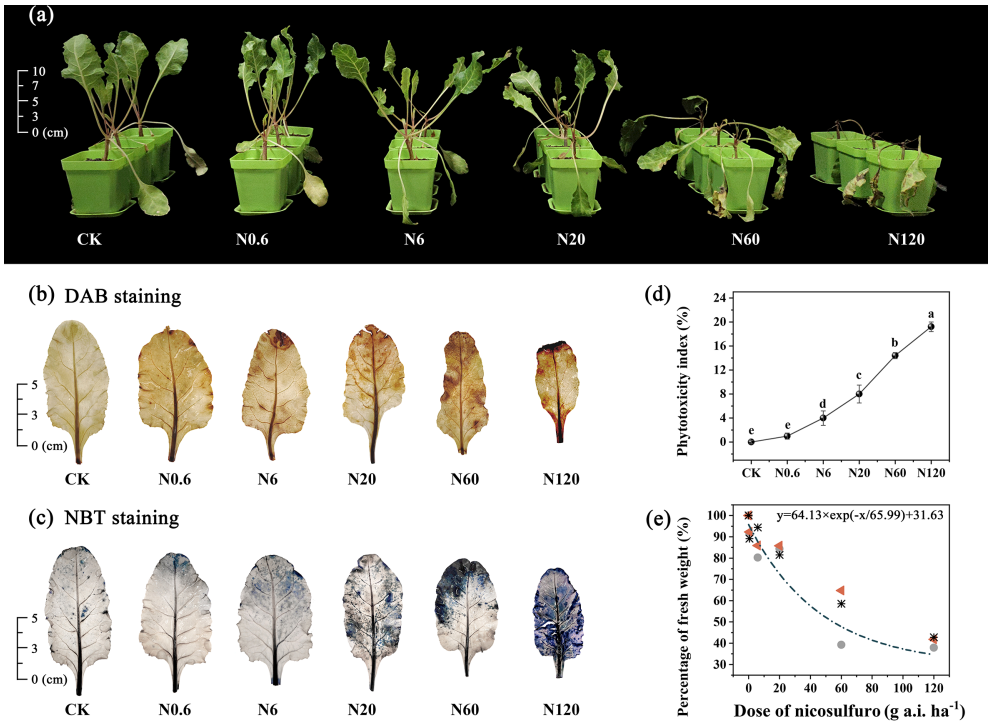

FIGURE 1
Effects of nicosulfuron on visible symptoms of phytotoxicity on sugar beet. The growth of sugar beet **(A)**, DAB staining **(B)**, NBT staining **(C)**, phytotoxicity index **(D)**, and dose-fresh weight response curve **(E)** in sugar beet on 20 DAT with different doses of nicosulfuron. Triangles, circles and asterisks represent different repetitions. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, p < 0.05).

pronounced compared to F_O. At 6 g a.i. ha^{-1}, F_m showed remarkable differences at 8 DAT (Figures 5B, C, D). The variations of F_v/F_m and PI_{abs} were decreased with increasing doses. On 20 DAT, F_v/F_m and PI_{abs} significantly decreased by 18.75% and 53.86% at 60 g a.i. ha^{-1} ($p < 0.05$) (Figures 5E, F).

The V_J and V_K of sugar beet leaf significantly lowered under nicosulfuron toxicity. The V_K was more remarkably affected than V_J (Figures 6A, B). At 20 DAT, V_J and V_K increased significantly by 84.20% and 96.33% at 60 g a.i. ha^{-1} contrasted with CK ($p < 0.05$) (Figures 6C, D). The ABS/CS_M and ET_O/CS_M of sugar beet leaf declined with the increase of nicosulfuron dose while DI_O/CS_M increased significantly. The DI_O/RC and ABS/RC increased, while ET_O/RC reduced (Figure 6E). The trends of each light energy absorption and distribution parameter on 20 DAT were consistent with those of the 4 DAT, but the changes were significantly greater than those of the 4 DAT (Figure 6F).

TABLE 2 Effects of nicosulfuron on biomass of sugar beet.

Treatment	Shoot		Root		Root-shoot ratio
	FW (g $plant^{-1}$)	DW (g $plant^{-1}$)	FW (g $plant^{-1}$)	DW (g $plant^{-1}$)	
CK	4.31 ± 0.42a	0.78 ± 0.02a	0.25 ± 0.02a	0.15 ± 0.03a	0.09 ± 0.01a
N0.6	3.91 ± 0.33a	0.77 ± 0.04a	0.23 ± 0.02a	0.13 ± 0.01a	0.07 ± 0.01ab
N6	3.76 ± 0.64a	0.72 ± 0.04ab	0.22 ± 0.06ab	0.10 ± 0.03a	0.05 ± 0.02bc
N20	3.63 ± 0.28a	0.69 ± 0.03b	0.19 ± 0.01ab	0.09 ± 0.06a	0.05 ± 0.01bc
N60	2.33 ± 0.59b	0.57 ± 0.01c	0.16 ± 0.03b	0.11 ± 0.03a	0.04 ± 0.01c
N120	1.87 ± 0.25b	0.60 ± 0.04c	0.07 ± 0.01c	0.11 ± 0.02a	0.03 ± 0.01c

FW, fresh weight; DW, dry weight. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, p < 0.05).

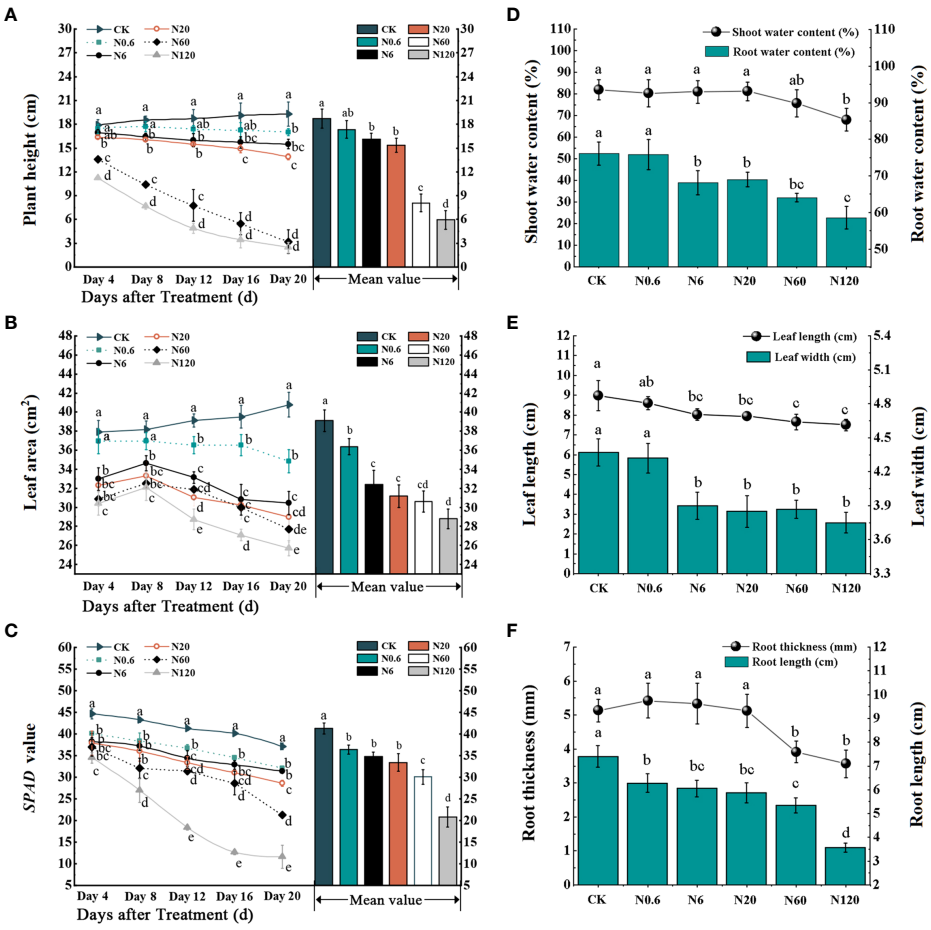

FIGURE 2
Effects of nicosulfuron on the growth parameters of sugar beet. Plant height **(A)**, leaf area **(B)**, *SPAD* value **(C)**, shoot water content, root water content **(D)**, leaf width, leaf length **(E)**, root width and root length **(F)** in sugar beet with different doses of nicosulfuron. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, $p < 0.05$).

3.4 Effects of nicosulfuron on the physiological indicators of sugar beet leaf

An increased dose of nicosulfuron enhanced the generation rate ofO_2^-, the contents of H_2O_2, MDA and EL in sugar beet leaf. When the dose reached 0.6 g a.i. ha^{-1}, the generation rate of O_2^- and H_2O_2 contents increased significantly by 84.73% and 65.96% ($p < 0.05$) (Figures 7A, B). The differences in MDA content and EL reached significant amounts at 6 g a.i. ha^{-1}, increasing by 183.15% and 102.46% ($p < 0.05$) (Figures 7C, D).

The SOD, POD and CAT activities were enhanced first and afterward lowered, reaching a peak at 6 g a.i. ha^{-1} as the dose of nicosulfuron increased, while the APX activity decreased gradually. The difference in SOD and CAT activities was significantly contrasted with CK lowered by 5.74% and 7.44% at 20 g a.i. ha^{-1} ($p < 0.05$) (Figures 7E, G). The POD activity was in an upward trend at 0.6 g a.i. ha^{-1}, which significantly increased 77.78% compared to the control ($p < 0.05$). And it was on the decline at 60 g a.i. ha^{-1}, with a remarkable reduction of 25.93% contrasted with the control ($p < 0.05$) (Figures 7F). APX activity was significantly lowered by 23.29% contrasted with CK at 6 g a.i. ha^{-1} ($p < 0.05$) (Figures 7H).

4 Discussion

4.1 Nicosulfuron phytotoxicity repressed the growth of sugar beet seedlings

Growth parameters are the most visible indicator of the degree of crop phytotoxicity in a stress condition. The most commonly used method for describing herbicide phytotoxicity is a simple and subjective visual estimation of the observed crop injury (Weber et al., 2017). Nicosulfuron can be harmful to

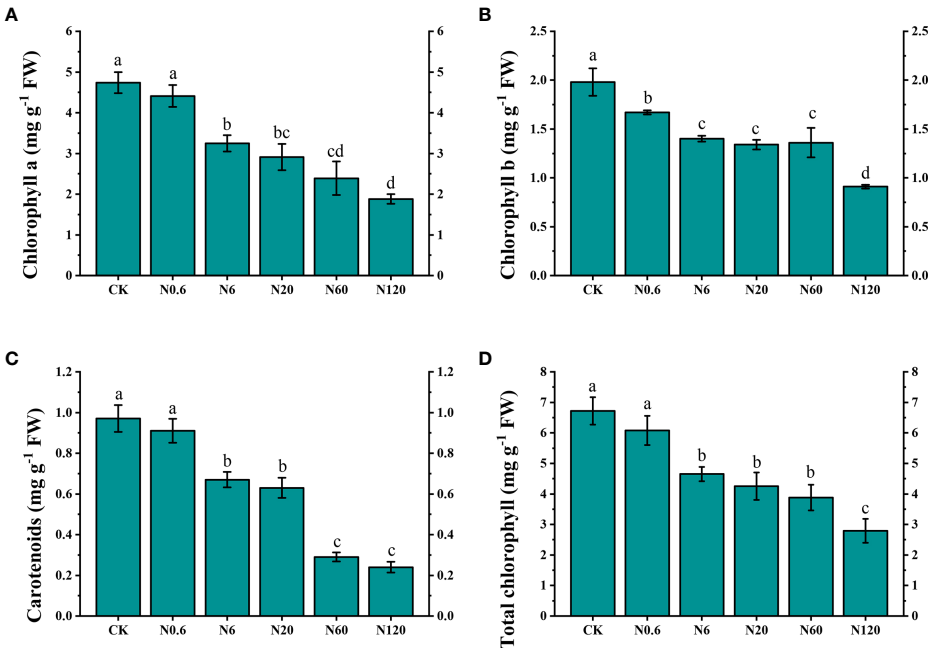

FIGURE 3
Effects of nicosulfuron on the photosynthetic pigment of sugar beet leaf. Chlorophyll a **(A)**, chlorophyll b **(B)**, carotenoids **(C)** and total chlorophyll **(D)** in sugar beet on 20 DAT with different doses of nicosulfuron. FW: fresh weight. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, $p < 0.05$).

plants, leading to the inhibition of plant growth indicators (Wang et al., 2021b). Under the influence of nicosulfuron at the recommended dose of nicosulfuron in the field (60 g a.i. ha^{-1}), plants showed the symptoms of phytotoxicity on 4 DAT. On 20 DAT, plants were deformed with blackened growing points and yellow leaves. Biomass was significantly suppressed, and the mortality rate was 60%. It was noteworthy that the symptoms were more pronounced on new leaves than on old leaves. This might be because nicosulfuron was a systemic herbicide that stems, leaves, and roots can taken up. The new leaves were young and metabolically active, so they were more susceptible to damage by such herbicides (Rey Caballero et al., 2016).

The phytotoxicity degree of nicosulfuron to plants depends on the dose. The range of variation in GR_{50} for different plants ranges from 0.95 to 169.93 g a.i. ha^{-1} (Xu et al., 2018), which indicates that the resistance to nicosulfuron varies widely among different plants. The reason is that the resistance of nicosulfuron depends mainly on the plant's genetic material. There are differences in resistance even among different varieties of the same plant (Choe and Williams, 2020). In this experiment, the GR_{50} was 81.83 g a.i. ha^{-1} of sugar beet, which was close to the response of cocklebur (*X. strumarium* L.) to nicosulfuron (Božić et al., 2015). The GR_{50} of nicosulfuron on sugar beet increased by 36.38% of the recommended field dose of nicosulfuron (60 g a.i. ha^{-1}). In comparison, the total drift of nicosulfuron was generally less than 25% of the total applied dose in agricultural production (Wang et al., 2009). So, the toxic effects of nicosulfuron drift on sugar beet were usually not deadly, even considering the over-application of nicosulfuron in agriculture.

4.2 Nicosulfuron inhibited photosynthetic performance in sugar beet

In this study, the contents of chlorophyll and carotenoid were significantly reduced by nicosulfuron toxicity. This might be due to the over-production of reactive oxygen species (ROS) inhibiting the photochemical activity of chloroplasts and blocking the formation of photosynthetic pigment. Another reason could be the degradation of chlorophyll due to cell damage caused by the accumulation of ROS. ALS inhibitors induced a reduction in P_n after being treated in plants according to numerous studies (Orcaray et al., 2010), which were consistent with the findings of this study. The reduction of P_n in sugar beet leaf by nicosulfuron toxicity was similar to the change in photosynthetic pigment content, which indicated that the decrease in photosynthetic pigment is one of the essential reasons for the decrease in P_n. The stomata of plant leaf control gas exchange which can further affect photosynthetic capacity by limiting water loss and controlling CO_2 uptake, affecting T_r and C_i (Hetherington and Woodward, 2003; Geiger et al., 2009). In

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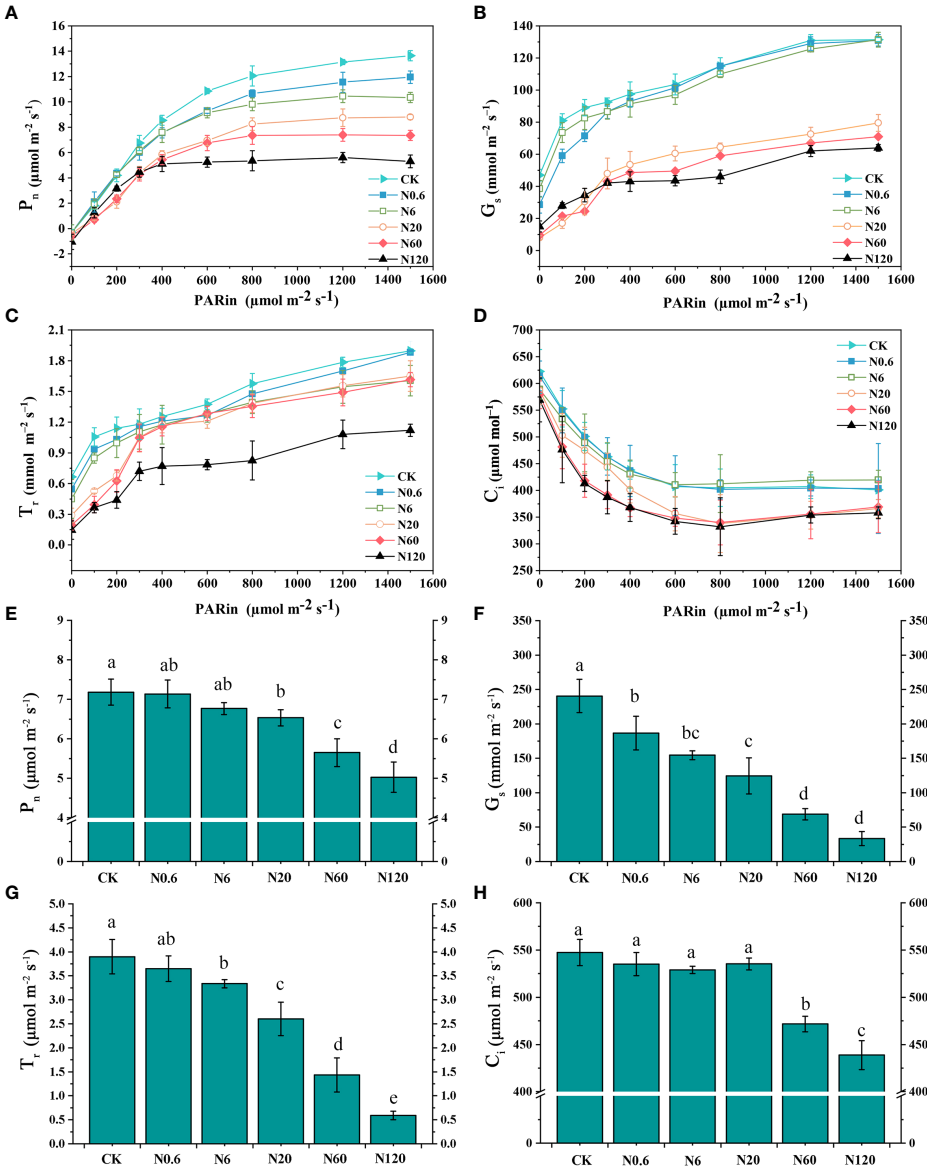

FIGURE 4
Effects of nicosulfuron on the gas exchange parameters of sugar beet leaf. P_n-light curve **(A)**, G_s-light curve **(B)**, T_r-light curve **(C)**, C_i-light curve **(D)**, Net photosynthetic rate (P_n) **(E)**, stomatal conductance (G_s) **(F)**, transpiration rate (T_r) **(G)** and intercellular CO_2 concentration (C_i) **(H)** in sugar beet on 20 DAT with different doses of nicosulfuron. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, $p < 0.05$).

this study, G_s gradually decreased with increasing doses, which led to a decrease in T_r. Notably, C_i also showed a decreasing trend, suggesting that photosynthesis capacity might be limited by stomatal and non-stomatal factors (Singh et al., 2013).

Photosynthetic light-response curves can determine the extent to which the photosynthetic efficiency of plants is affected by environmental change. It is shown that nicosulfuron significantly suppressed the Amax, LSP and AQY, while the LCP, was increased. This indicated that the photosynthetic efficiency of sugar beet was significantly reduced in response to light environment change. This might be due to the fact that phytotoxicity reduced the pigment-protein complexes that absorb and convert light energy in sugar beet, resulting in a reduced ability of sugar beet to utilize both strong and weak light (He et al., 2018). The Rd of sugar beet leaf was significantly higher under nicosulfuron poisoning conditions, probably due to the inhibition of assimilate transport in sugar beet. Increased Rd consumed the excess assimilation accumulated in the leaves and

TABLE 3 Effects of nicosulfuron on the P_n-PAR curve parameters of sugar beet leaf.

Treatments	Amax (μmol m^{-2} s^{-1})	LCP (μmol m^{-2} s^{-1})	LSP (μmol m^{-2} s^{-1})	AQY	Rd (μmol m^{-2} s^{-1})
CK	13.57 ± 0.01a	12.60 ± 1.42a	1433.71 ± 76.26a	0.038 ± 0.001a	0.37 ± 0.03a
N0.6	11.88 ± 0.07b	15.94 ± 1.61a	1318.94 ± 12.43b	0.032 ± 0.002b	0.50 ± 0.05b
N6	10.58 ± 0.01c	20.87 ± 1.56a	1166.65 ± 9.81c	0.032 ± 0.001b	0.62 ± 0.09bc
N20	9.03 ± 0.02d	32.40 ± 3.66b	1205.10 ± 9.78c	0.030 ± 0.001c	0.65 ± 0.06bc
N60	7.79 ± 0.01e	34.56 ± 1.76bc	1082.99 ± 2.64d	0.022 ± 0.002d	0.72 ± 0.14cd
N120	5.69 ± 0.03f	42.88 ± 5.42c	915.21 ± 3.74e	0.020 ± 0.003d	1.17 ± 0.04d

Amax, maximum net photosynthetic rate; LSP, light saturation point; AQY, apparent quantum yield; Rd, dark respiration. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, $p < 0.05$).

slowed the inhibition of photosynthesis (Pavithra et al., 2020). This indicated that sugar beet adapted to the toxicity mainly by promoting respiration.

4.3 Nicosulfuron inhibited PSII activity and photosynthetic energy

By blocking the electron transport chain in chloroplasts, ALS inhibitors can damage the structure and function of the photosynthetic system II. F_v/F_m and PI_{abs} are considered the most common indicators to characterize PSII reaction center activity (Yi et al., 2016). The most sensitive fluorescence parameter to different stress treatments was the PI_{abs}. It is used to quantify the overall photosynthetic performance of the sample. The reduction of F_v/F_m and PI_{abs} in this study demonstrated that nicosulfuron remarkably repressed the PSII reaction center activity of sugar beet leaf. The restraint extent was connected with nicosulfuron dose, similar to the investigation by Zhang et al. (2018). Compared with CK, both V_K and V_J increased to different degrees with increasing doses of nicosulfuron. The enhancement in V_J demonstrated the electron transfer process from Q_A to Q_B is blocked, which leads to a large accumulation ofQ_A^-, a typical inhibition of the PSII receptor side.

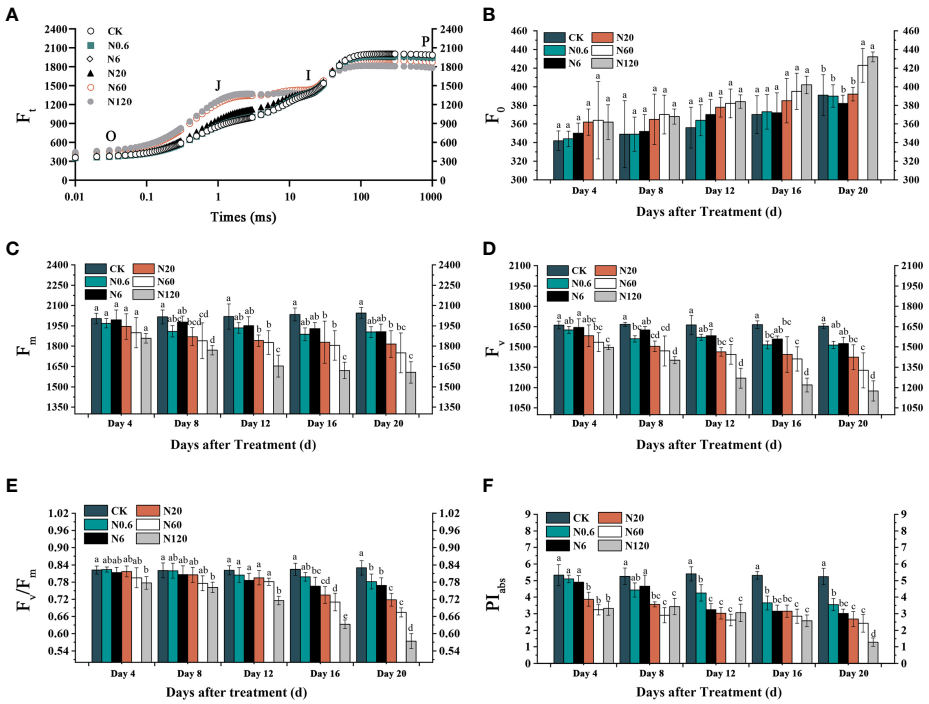

FIGURE 5
Effects of nicosulfuron on the PSII activity of sugar beet leaf. OJIP transient on 20 DAT **(A)**, F_O **(B)**, F_m **(C)**, F_v **(D)**, F_v/F_m **(E)** and PI_{abs} **(F)** in sugar beet with different doses of nicosulfuron. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, $p < 0.05$).

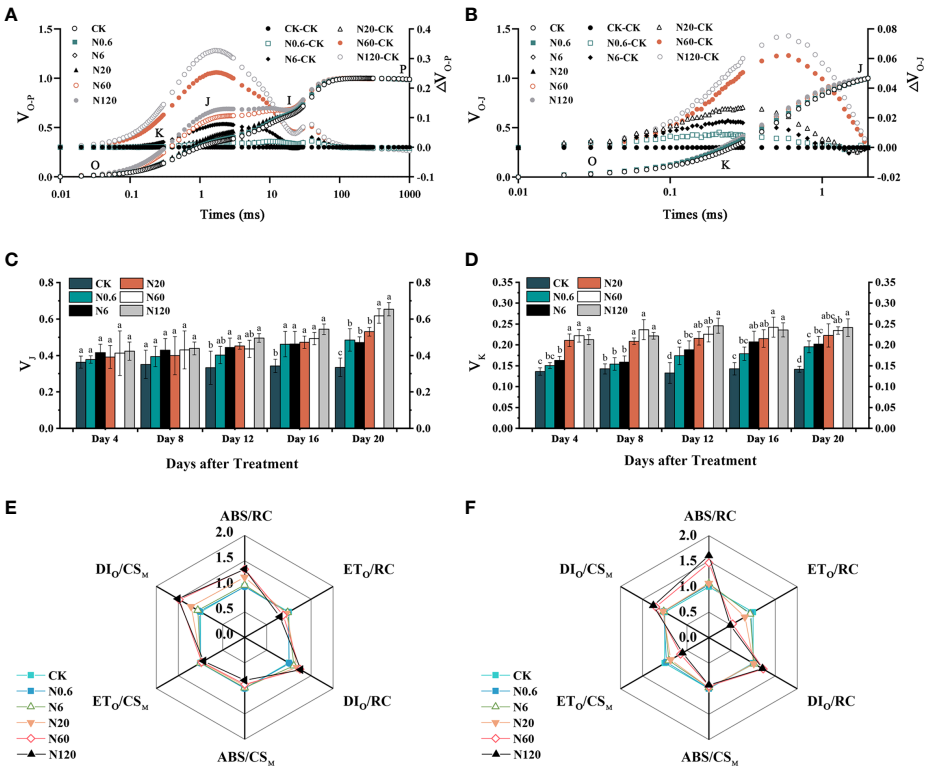

FIGURE 6
Effects of nicosulfuron on the photosynthetic energy of sugar beet leaf. V_{O-P}, ΔV_{O-P} curves on 20 DAT **(A)**, V_{O-J}, ΔV_{O-J} curves on 20 DAT **(B)**, V_J **(C)**, V_K **(D)**, energy distribution parameters on 4 DAT **(E)** and 20 DAT **(F)** in sugar beet leaf after treatment with different doses of nicosulfuron. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, $p < 0.05$).

The increase in V_K indicated that the PSII electron donor side of the oxygen-evolving complex OEC was destroyed (Strasser, 1997). This was consistent with the response of mulberry (Liu et al., 2018) and alfalfa (Guo et al., 2020) under herbicide stress. The most significant change in each characteristic point of the OJIP transient was the elevation of the J point. From this, we conclude that the PSII receptor side of sugar beet plants is more sensitive to nicosulfuron toxicity.

Under phytotoxic stress, plants often improve adaptation by adjusting energy distribution (Arthaud et al., 2021). With the increase of nicosulfuron dose, the variation of ABS/CS$_M$ was decreased. This indicated that nicosulfuron caused inactivation of partial reaction centers in sugar beet leaf on the one hand and also damaged antenna pigment-protein which then resulted in a decrease in the amount of captured light energy and the reduction of ET$_O$/CS$_M$. The experiment showed a reduction in DI$_O$/CS$_M$ with nicosulfuron dose increased, indicating a decrease in the amount of active reaction centers and the rate of excess excitation energy consumption in the leaf. In this experiment, as the dose of nicosulfuron increased, the ABS/RC enhanced and the DI$_O$/RC boosted, implying that the dissipation of the remaining active reaction centers expanded. This might be due to the increased burden on the remaining active reaction centers, compelling them to be more efficient in better dissipating the energy in the electron transfer chain.

4.4 Nicosulfuron increased ROS accumulation

In adverse circumstances, the content of ROS increases, and cell membranes are disrupted inside the plant. ROS causes oxidative damage to the photosynthetic apparatus in chloroplasts, resulting in the photoinhibition of PSII (Ajithkumar and Panneerselvam, 2014; Alzandi and Naguib, 2020). The study showed that under nicosulfuron stress, the generation rate of O_2^- and H_2O_2 contents of sugar beet were considerably enhanced, along with MDA content and EL, which indicated significant oxidative damage to sugar beet. This might be due to the accelerated generation rate of O_2^-in plants under adversity and the reduced ability of plants to utilize photosynthetic excitation energy. Excess electrons in the excited state synthesized electron transport chains to scavenge freeO_2^-. ROS triggered membrane lipid peroxidation and

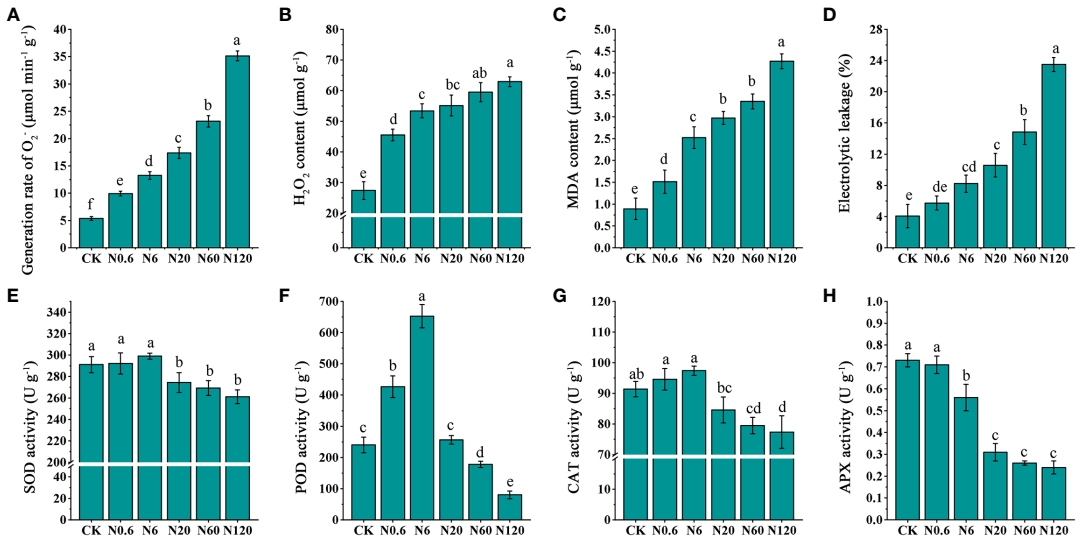

FIGURE 7
Effects of nicosulfuron the on physiological indicators of sugar beet leaf. The generation rate of O_2^- **(A)**, H_2O_2 content **(B)**, malondialdehyde (MDA) content **(C)**, electrolytic leakage (EL) **(D)**, SOD activity **(E)**, POD activity **(F)**, CAT activity **(G)**, and APX activity **(H)** in sugar beet on 20 DAT with different doses of nicosulfuron. Data with the different letters indicate significant differences between different doses of nicosulfuron drift (n = 6, $p < 0.05$).

produced MDA, which altered the structure and function of cell membranes and disrupted membrane stability (Lanza and Dos Reis, 2021), thus leading to a significant increase in EL.

It was found that toxic treatment increased the activities of SOD and CAT in leaf to stable ROS content within a certain toxic concentration range (Wu et al., 2020). SOD, POD, and CAT activities enhanced afterward and decreased with increasing doses of nicosulfuron at 6 g a.i. ha^{-1}, while APX activity gradually decreased. This may be because when ROS in plants exceeded the capacity of antioxidant enzymes, the antioxidant enzyme system cannot scavenged ROS in time. Excess ROS might decreased antioxidant enzyme activity, making plant cells more susceptible to oxidative damage (Li et al., 2011; Meloni and Martínez, 2021). In addition, plants can respond to toxic damage by regulating hormone levels to affect key enzyme activities in plants. Studies have shown that topical application of salicylic acid to valerian can reduce the toxic effects of bentazon herbicides by enhancing oxidative defense mechanisms and altering POD, CAT and APX enzyme activities (Khatooni et al., 2022).

5 Conclusion

Nicosulfuron led to the disruption in the function of PSII in sugar beet leaf. Photosynthetic parameters were altered, resulting in lower photosynthetic efficiency and significant photoinhibition. The ROS content, MDA content and EL of sugar beet leaf were enhanced significantly. The oxidative defense system of sugar beet was disrupted, and SOD, POD, CAT, and APX enzyme activities were inactivated considerably. The GR_{50} of nicosulfuron toxicity on sugar beet was 81.83 g a.i. ha^{-1}. This study showed how nicosulfuron affected sugar beet. It also showed that the toxicity of nicosulfuron on sugar beet is a cause for concern and that the risk of herbicide in agricultural ecosystems should be taken into account.

Data availability statement

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

Author contributions

LW: investigation, validation, formal analysis, and writing-Original draft preparation. MR: validation and writing-reviewing. BS: conceptualization, resources, supervision, and writing-reviewing. XS: writing-reviewing. WH: validation. XB: conceptualization, resources, supervision, and writing-reviewing. XZ: writing-reviewing. 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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Exogenous naphthaleneacetic acid alleviated alkalinity-induced morpho-physio-biochemical damages in *Cyperus esculentus* L. var. sativus Boeck

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Cyperus esculentus L. var. sativus Boeck (commonly called Chufa) is a perennial species that produces nutritious underground tubers and contributes to the diet and health of human worldwide. However, it is salt-sensitive and its adaptation to salinity stress remains an enigma. Naphthaleneacetic acid (NAA) plays a vital role in regulating plant salt stress tolerance. Thus, we aimed to investigate the impact of NAA (150 mg/L) application on growth and physio-biochemical response mechanisms of Chufa plants to different levels of salinity stress (0-, 90-, and 180 mM of alkaline stress ([1:1 ratio of Na_2CO_3 and $NaHCO_3$]). In response to increasing stress levels, shoot-root growth decreased, whereas malondialdehyde (MDA), hydrogen peroxide (H_2O_2), osmolytes (soluble protein, proline, and soluble sugars), and activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) significantly increased. Alkalinity led to significant increase in Na^+ and Cl^-, but decrease in Mg^{2+} concentration in both roots and leaves; however, K^+ decreased significantly in leaves under both stresses. Additionally, $NO_3^-$and. levels, nitrate reductase (NR) activities, and glutamate synthase (GOGAT) decreased significantly. However, glutamine synthetase (GS) increased non-significantly at 90 mM but declined at 180 mM. Foliar NAA application reduced Na^+ and Cl^-, MDA, and H_2O_2 but increased photosynthetic pigments, K^+ and Mg^{2+}, osmolytes, nitrogen (N) metabolism, and upregulating the enzymatic antioxidant system to reduce oxidative stress under alkaline conditions. Hence,

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our findings manifest that NAA application is an effective strategy that can be utilized to enhance tolerance of chufa plants to alkaline stress. Future studies should explore whether NAA can positively alter the nutrient composition of chufa tubers at deeper molecular levels, which might offer solutions to nutritious problems in developing countries.

KEYWORDS

salinity, alkalinity, physiology, growth regulators, nitrogen metabolism, antioxidants

Introduction

Salinization and alkalization of soils have become major socio-economic issues that have negative impacts on crop growth and productivity (Li et al., 2014; Hassani et al., 2021). In general, soil alkalinity occurs simultaneously with soil salinity. It is estimated that more than 25% of the earth's surface is covered by alkaline soils, affecting more than 434 million hectares of land (FAO, http://www.fao.org/soils-portal/soil-management). Alkalinity is caused by carbonates and bicarbonates, which are common constituents of irrigation water. They adversely affect physiological homeostasis (Munns and Tester, 2008; Guo et al., 2019) by altering the ionic balance within plant cells (Li et al., 2017). Alkalinity, for example, causes the high pH in the rhizosphere, which reduces the availability and uptake of important nutrients such as K^+, Ca^{2+}, Mg^{2+}, NO_3^-, and $H_2PO_4^-$, by causing their precipitation (Yang et al., 2007; Ullah et al., 2019). Plants can respond to alkaline salt stress by modifying certain metabolic processes, such as ion transport and accumulation, photosynthesis, osmotic solute accumulation, and hormone synthesis, and nitrogen metabolism (Shi and Wang, 2005; Yang et al., 2009; Wang et al., 2012; Ullah et al., 2019). Alkalinity stress also causes an increase in antioxidant processes, which is one of the mechanisms for the scavenging of free radicals under stress (Mir et al., 2018). Nitrogen (N) metabolism is of great importance to plants, as it provides proteins and nucleic acids that control many of their cellular functions. Plants absorb nitrogen from the soil primarily as nitrates (NO_3^-) and ammonia (NH_4^+) (Luo et al., 2013). Next, NO_3^- is reduced into nitrite (NO_2^-) and then into NH_4^+ through the actions of nitrate reductase (NR) and nitrite reductase (NiR) enzymes, respectively. In addition, NH_4^+ is assimilated into amino acids through glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle or through glutamate dehydrogenase (GDH) (Shi et al., 2009; Ullah et al., 2019). N is an essential constituent of amino acids, proteins, amides, and polyamines and secondary metabolites and hence interferes in several physio-biochemical processes. Its regulations therefore, contribute to salinity stress tolerance in plants (Zaki, 2016; Arghavani et al., 2017). However, salinity stress and N metabolism interact in a complex manner affecting nearly almost every physiological process in plants (Läuchli and Lüttge, 2002). However, previous studies have demonstrated that alkaline stress has a far greater impact on nitrogen metabolism than saline stress. Several studies have shown that alkaline stress impacts nitrate assimilation and/or uptake, resulting in lower concentrations of nitrate in Suaeda glauca (Yang et al., 2008), barley (Yang et al., 2009), blackseed grass (Yang et al., 2010), and soybeans (Ullah et al., 2019). Consequently, regulating N metabolism might be as important for alkaline-tolerance as for salt-tolerance.

Normal growth conditions produce relatively low levels of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion radical (O_2^-). However, saline-alklaine stress conditions disrupt this homeostasis, and causes excessive ROS production, leading to intracellular oxidative stress. In response plants activates enzymatic [(superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD)] and non-enzymatic antioxidant defence mechanisms to eliminate the excess ROS and protect plants cells from oxidative damage (Guo et al., 2019; Sarker and Oba, 2020).

Moreover, several studies have demonstrated that alkaline stress has a more severe effect on plants than saline stress (Yang et al., 2009; Wang et al., 2012; Ullah et al., 2019). For instance, saline stress generally causes ionic damage and osmotic stress in plants (Kiełkowska et al., 2019), whereas alkaline stress leads high pH injuries along with the above-mentioned damages (Yang et al., 2009; Mao et al., 2019). The studies comparing the effects of alkaline stress on *Lathyrus quinquenervius* and *Glycine soja* revealed that the former is more damaging than the latter (Zhang & Mu, 2009; Ullah et al., 2019). In the case of alkalinity, for example, there was a greater reduction in the growth, photosynthesis, ionic regulation, carbon and nitrogen metabolism of soybean (Ullah et al., 2019). Likewise, alkaline stress inhibited germination, root system activities, photosynthesis, organic acid imbalance, reactive oxygen species (ROS), and malondialdehyde (MDA) concentrations, resulting in impaired growth (Zhang and Mu, 2009). However,

there have been few studies examining the effects of alkaline stress in plants and the mechanisms by which plants adapt to alkaline stress, as compared to saline stress (Ahmad et al., 2014; Hu et al., 2015). It is therefore imperative to unravel the physio-biochemical mechanisms by which crop species, respond to alkaline stress in order to meet the growing population's food needs in the face of climatic changes and diminishing fresh water resources.

Phytohormones, also known as plant growth regulators, are molecules derived from the plant biosynthetic pathway that can mediate the growth and development of plants both under normal and stressed conditions. Several phytohormones, including auxins (IAA), gibberellins (GA), cytokinins (CKs), brassinosteroids (BRs), and ethylene (ETHY), regulate plant growth and development in a coordinated fashion. These phytohormones influence a variety of physiological and biochemical processes (Fahad et al., 2015). They enhance abiotic stress tolerance and productivity of economic crop species (Iqbal et al., 2012; Fahad et al., 2015). Foliar applications of growth regulators are recommended as considerably quick and timely approach to achieve tolerance in plants grown in salinity-alkalinity-affected soils (Mir et al., 2018; Nigam et al., 2022). Naphthalene acetic acid (NAA) is a synthetic plant hormone, similar to naturally occurring indole acetic acid (IAA), in the auxin family. It stimulates cell division, elongation, membrane permeability, leaf chlorophyll content, photosynthesis, mRNA synthesis, water uptake and other physiological processes (Basuchaudhuri, 2016; Ullah and Sajjad, 2017). Plant growth regulators, whether natural or synthetic, affect endogenous hormonal patterns in the plant, either by supplementing sub-optimal levels or by interfering with their synthesis, translocation, or inactivation (Basuchaudhuri, 2016). Foliar application of plant growth regulators, including NAA, have been reported to increase, plant photosynthesis, ions regulation and anti-oxidant defense mechanism and hence protect plant tissues from salinity-induced damages (Ullah and Sajjad, 2017; Muhammed et al., 2022). However, our understanding of the role of exogenous NAA application in alleviating the alkalinity-induced physio-biochemical responses of plants is sparse, and needs further investigation.

The cultivated yellow nutsedge (*Cyperus esculentus* L. var. sativus Boeck), commonly known as chufa, is a perennial crop plant (Cyperaceae family). It produces underground almond-like tubers that are remarkably sweet and nutritious, having several benefits to human health (Pascual et al., 2000; Sánchez-Zapata et al., 2012). Its tubers are also used in preparing a non-alcoholic, milk-like drink, known as horchata, which has been the subject of recent studies (Sebastia et al., 2010; Sánchez-Zapata et al., 2012). Additionally, this crop has aphrodisiac, carminative, diuretic, stimulant, emmenagogue, and tonic properties and is commonly used to treat excessive stomach gassiness, indigestion, diarrhea, and dysentery (Adejuyitan, 2011). The tubers of chufa contain many nutrients and bioactive compounds, making them an extremely important cash crop for humans and animals (Adejuyitan, 2011; Sánchez-Zapata et al., 2012; Maduka and Ire, 2018). Despite of being neglected, it has become an increasingly important crop due to the health benefits and nutritional value they provide. Chufa plant has many uses in the food industry, for instance, its flour is now commonly used to thicken bread, cakes, or prepared into alcoholic and nonalcoholic beverages. In addition to providing dietary diversification to alleviate micronutrient deficiency, especially amongst the poor and children, chufa can contribute to the agricultural gross domestic product (GDP) both locally and internationally (Asare et al., 2020). Given the rising human population, this neglected and underutilized species can contribute to food security and poverty reduction. This species prefers moist, sandy soils and are tolerant of drought and flooding as well as temperature fluctuations in the soil, but not tolerant of salinity (Halverson, W. L. 2003). A variety of aspects related to cultivating chufa have been thoroughly examined, including, cultivar selection and plant characterization, crop management techniques, irrigation, nutrition and fertilization (Pascual-Seva et al., 2018). However, to the best of our knowledge, investigation of salinity-alkalinity responsive mechanism is entirely missing in the case of salt sensitive Chufa plant species. Therefore, examining its growth and physio-biochemical changes in response to soil alkalinity is imperative.

This study aimed to investigate, how alkalinity impact growth Chufa plant in terms of physiological and biochemical changes. We hypothesized that, alkalinity stress (high pH) would decrease the growth of chufa plants by disrupting their metabolism; however, exogenous NAA application might enhance their growth by alleviating the negative effects associated with alkalinity stress. To test our hypothesis, we have performed morphological and physio-biochemical investigations to gain deeper insights into the salinity tolerance mechanism of chufa plants by evaluating various parameters, including, (i) Shoot and root growth and biomass, (ii) photosynthetic chlorophyll pigments, (iii) salt ions accumulation, (iv) Lipid peroxidation and reactive oxygen species, (v) enzymatic antioxidant mechanism (vi) osmolytes accumulation and (vii) nitrate (NO_3^-) reduction and ammonium (NH_4^+) assimilation in response to increasing levels of simulated alkalinity stress conditions and foliar NAA application.

Materials and methods

Study area and growth conditions

We conducted this study at the Cele National Station of Observation and Research for Desert-Grassland Ecosystem (37° C00′ 56′′ N, 80° C43′ 81′′ E), Chinese Academy of Sciences. It is located at the southern fringe of a hyper-arid saline desert

known as the Taklamakan. The mean yearly temperatures, precipitation, and evaporative potential are 11.9°C, 35 mm, and 2600 nm, respectively. We obtained the tubers of *Cyperus esculentus* L. var. sativus Boeck (Fengchan No1) chufa plant from Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi, China. In August 2022, four same-sized healthy tubers were sown in each 1.5 L plastic pot (Height, 12.0 cm; top diameter, 15.01 cm; and basal diameter 11.02) with a 2 cm hole at the bottom (ca. 18%), filled with 1 kg of homogenized soil (aeolian loamy sand with organic C, 2.99 g kg^{-1}; total N, 0.23 g kg^{-1}; total P, 0.60 g kg^{-1} and total K, 23.11 g kg^{-1}, pH 8.43, and EC 177.7 µs.cm^{-1}) The pots were arranged in a complete randomized block design (RCBD) in August 2022. During the first three weeks, water was supplied every three days to each pot using a weight method to field capacity (18% w/w). In the study area, groundwater has a salinity of approximately 40-50 mM Na^+ and is ordinarily used for local irrigation.

Treatments and experimental design

At the fifth leaf stage (four weeks after sowing), we selected 60 pots (2 seedlings/pot) with uniform seedlings and divided them into six groups for the alkaline stress (1:1 ratio of Na_2CO_3 and $NaHCO_3$) and NAA application. Three groups for alkaline stress treatments were treated with (i) 0 mM (controlled condition), (ii) 90 mM, and (iii) 180 mM alkaline stress. The other three groups were subjected to the same three levels of alkaline stress but applied with NAA application (150 mg/L) using a sprinkler four times (20, 30, 35, and 40 days after sowing). In a preliminary growth experiment, NAA concentrations ranging from 50 to 150 mg were studied (Table S1). We selected the optimal concentration of NAA based on improved growth of chufa seedlings under 90 mM alkalinity stress. Each treatment was replicated three times. Finally, we harvested the 50-days old plants, and were immediately frozen in liquid nitrogen and stored (-80°C) for the determination of physiological indexes.

Measurement of plant growth and biomass

After harvesting, the shoot height and root length were measured using a measuring tape. Next fresh and dry weights, and shoot and root dry weights of the seedlings were measured using an electric balance. For dry weight determination, the plants were oven-dried at 105° C for 30 min and then dried at 75° C until constant weight (Shao et al., 2015).

Measurement of photosynthetic pigments

The chlorophyll (0.1–0.3 g fresh leaves) was extracted from the leaves using ethanol (95 percent, vol/vol) and measured at 665 nm and 649 nm following a standard method (Lichtenthaler and Buschmann, 2001) calculated the chlorophyll contents using the following equations (mg g^{-1} FW).

$$\text{Chl a} = 13.98\ A665 - 6.88\ A649 \quad (1)$$

$$\text{Chl b} = 24.96\ A649 - 7.32\ A665 \quad (2)$$

$$\text{Chl a/b} = \text{Chl a} / \text{Chl b} \quad (3)$$

$$\text{Chl} = \text{Chl a} + \text{Chl b} \quad (4)$$

Determination of mineral elements

Dry leaf and root samples oven dried at 105° C for 30 min and then grinded into fine powder using an electric mortar. Following this, the finely powdered samples (0.05 g) were transferred into a centrifuge tube with deionized water (4 ml) and placed in a boiling water bath for 40 min. After centrifuging the tubes for 10 min at 4000 rpm, the supernatant was collected. The concentrations of sodium (Na^+), potassium (K^+), and (magnesium) (Mg^{2+}) ions were determined using an atomic absorption spectrophotometer (Super 990F, Beijing Purkinje General Instrument Co. Ltd. Beijing, China) (Mostofa et al., 2015). Furthermore, Cl^- ion concentration was determined using ion chromatography (DX-300 ion chromatographic system, AS4A-SC chromatographic column, CDM-II electrical conductivity detector, mobile phase: $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM; Dionex, Sunnyvale, CA, USA) (Ullah et al., 2019).

Determination of H_2O_2 and cell membrane injury

Hydrogen peroxide (H_2O_2) concentrations were determined by a standard procedure (Patterson et al., 1984). The 0.2 g freshly leaf samples were homogenized in 5 ml of trichloroacetic acid (TCA) (0.1%) in an ice bath, transferred to tubes, and centrifuged at 5000 × *g* for 10 min (4°C). Next, we centrifuged the supernatant comprising 0.1 ml of titanium reagent (50 µL of 20% titanium tetrachloride) and 0.2 ml of ammonia, centrifuged at 10,000 × *g* for 10 min. Following five washes with acetone, the precipitate was centrifuged at 10,000 × *g* for 10 min, after which 3 ml of 1 M H_2SO_4 was added.

Malondialdehyde (MDA) concentration was assessed based on the thiobarbituric acid (TBA) test for the evaluation of lipid peroxidation (Heath and Packer, 1965). Fresh leaf samples (0.5 g) were homogenized in 1ml of 5% TCA and centrifuged for 10 min at 5,000 × *g* (4°C). Using a separate tube, 4 ml of the supernatant was added to 2 ml of 20% TCA, and the mixture was heated at 100°C for 15 min before centrifugation at 5,000 × *g* for

10 min. A spectrophotometer was used to measure absorbance at 450, 532, and 600 nm, and the concentration of MDA was calculated according to the following equation:

$$\text{MDA (mol g}^{-1}\text{FW)} = = 6.45\,(\text{A532 - A600}) - 0.56\text{A450}$$

Antioxidant enzyme activities

Fresh leaves samples were ground and homogenized in a chilled mortar with 0.1 M phosphate buffer (pH 7.3) and 0.5 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 8000 × *g* (10 min at 4°C). The activity of SOD was assayed by measuring the reduction rate of nitroblue tetrazolium (NBT) at 560 nm (Giannopolitis and Ries, 1977). One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of NBT reduction at 560 nm. Moreover, with minor modifications, the POD activity was determined according to standard methods (Wang et al., 2018), with minor changes. A reaction mixture was prepared by mixing 2 ml of buffer substrate (8 mM guaiacol and 100 mM Na_3PO_4 pH 6.4), 24 mM H_2O_2 in 0.5 ml of enzyme extract). At 460 nm, absorbance values were measured twice at 1-minute intervals.

We calculated enzyme activity by increasing the absorbance of the reaction system by 0.01 up to a maximum of 1U per min, which was then converted into U/g·min^{-1}. CAT activity was determined by monitoring the disappearance of H_2O_2 (Sabra et al., 2012). Initially, 50 ml of enzyme extract was poured into 1.5 ml of reaction mixture containing 50 mM K-phosphate buffer (pH 7.0) and 15 mM H_2O_2. One unit of CAT corresponds to one mole of H_2O_2 degradation per minute measured at 240 nm for 1 min. The absorbance was recorded at 240 nm for 1 min. One unit of CAT corresponds to one mole of H_2O_2 degradation per min.

Determination of N-metabolizing enzymes

The nitrate reductase activity was determined by homogenizing 0.2 g using 2 ml of 25 mM phosphate buffer saline (PBS, pH 8.7), which contained 10 mM cysteine and 1 mM EDTA, and then centrifuging for 20 min at 30,000 × *g*. the resulting supernatant was tested for NR activity using a diazo-coupling method employing Griess reagent (Sánchez et al., 2011).

The GS activity was determined by homogenizing them in 2 ml of 50 mM TrisHCl buffer (pH 7.8; containing 15% glycerol, 0.1% TritonX-100, 1 mM of EDTA, and 14 mM of 2-mercaptoethanol) and centrifuging them twice at 4°C for 10 min. After complexing with acidified ferric chloride, the supernatant was used to determine GS by forming glutamine hydroxamate using a 540 nm fluorescence measurement (Liu et al., 2014).

Determination of soluble sugar, proline and soluble protein

We ground dried leaves samples into a fine powder using a ball mill. Next, the powdered samples were added to a centrifuge tube containing 2 ml of 80% ethanol. Following incubation at 80° C in a shaking water bath (30 min), the mixture was centrifuged at 4,000 g for 5 min. Further two extractions were performed using 80% ethanol on the pellets. We retained, combined and stored the supernatant at 20°C for soluble sugar determination, following a standard method (Yemm and Willis, 1954). Proline extraction (0.2 g samples) was conducted using two ml of 10% acetic acid and five ml of 3% salicylic acid, respectively. The mixture was centrifuged at 12,000 × *g* for 10 min. The resulting supernatants were analyzed using a standard method (Liu et al., 2014). For soluble protein determination, extracts were made from 0.3 g of frozen fresh leaf samples in sodium phosphate buffer (50 mmol, pH 7.8), and centrifuged at 4000 × *g* for 10 min (4°C). The concentration of soluble proteins using bovine serum albumin as the standard (Bradford, 1976).

Statistical analysis

Measurements were replicated three times and sorted using Microsoft Excel 2019. SPSS version 16.0 (Chicago, IL, United States) was used to perform descriptive statistics and one-way analysis of variance (ANOVA). Duncan's multiple range tests were used to compare means at a significance level of 0.05. GraphPad Prism 8 was used to create the figure graphics. For interpretation purposes, Pearson correlation analyses were conducted using OriginPro 2019 software (Origin Lab Carporation Northampton, MA, USA) regarding the growth parameters, chlorophyll pigment concentrations in the leaf samples, N metabolism, osmolytes accumulation, mineral nutrition, and reactive oxygen species production rate, as well as antioxidant enzymatic activities.

Results

NAA-induced alkaline stress alleviation in chufa growth parameters

Both levels of alkaline stress produced a substantial reduction in growth parameters of chufa plant (Figures 1A-G). The SL, SFW, SDW, RL, RFW, and RDW experienced the 18.8, 28.2, 32.2, 13.0, 22.6, and 22.8% reduction in 90 mM alkaline stress and 30.9, 47.8, 43.8, 25.7, 44.8, and 45.4% inhibition after 180 mM alkaline stress, respectively (Fig 1). While, the exogenous application of NAA improved the SL, SFW, SDW, RL, RFW, and RDW 10, 22, 11.0, 15.4, 35.4, and 14% relative to

untreated plants, 5.8, 18.0, 23.5, 6.2, 8.8, and 21.5% as compared to 90 mM alkaline stress, and 5.0, 12.0, 3.6, 5.3, 12.6, and 13.0% after 90 mM alkaline stress, respectively. The root-shoot ratio exhibited the inverse trend of other growth indices since it did not display any substantial alterations after either treatment relative to control plants (Figure 1).

Ions regulation in chufa plants under alkaline stress followed by NAA supplementation

In both the studied organs (leaves and roots), the plants showed abrupt modifications in the nutritional profile under alkaline stress followed by NAA application (Fig 2A-J). Particularly, in leaves of chufa plant, the concentration of Na^+ and Cl^- were greatly elevated under both alkaline stress levels. For instance, a 3.4-fold enhancement was noted in Na^+ concentration, and 1.5- and 1.9-fold up-regulation in Cl concentration was experienced by the chufa leaves when exposed to 90 mM and 180 mM alkaline stress, respectively (Figures 2A, B). Nevertheless, the NAA exogenous application declines the concentrations of Na^+ by 15.6, 6.5, and 27.0% under 0 mM, 90 mM, and 180 mM alkaline stress, respectively. Interestingly, the highest reduction (23.7%) for Cl- concentration was seen when the plants were grown under 180 mM alkaline stress (Figures 2A, B).

Contrarily, the K^+, $Mg^{2+,}$ and K^+/Na^+ concentrations were observed to be declined in the alkaline stress-affected plants except in the case of K^+ under higher alkaline levels (Figures 2C-E). In contrast, the 90 mM alkaline stress-induced 7.5% reduction in K^+ concentration of chufa. leaves between Mg^{2+} and K^+/Na^+, the most pronounced inhibition (55.3 and 70%) was detected in K^+/Na^+, when the plants were cultivated in 90 mM and 180 mM alkaline stress, respectively. Nonetheless, the NAA exogenous application induced the improvement of 15.0, 15.0, and 3.6% in K^+, 7.6, 3.0, and 7.3% in Mg^{2+}, and 37.0, 22.6, and 41.5% in K^+/Na^+ in the 0 mM, 90 mM, and 180 mM alkaline stress-treated plants, respectively (Figures 2C-E).

All the nutrients exhibited similar trends in the case of roots (Figures 2F-J). The 90 mM and 180 mM levels of alkaline stress produced the upregulation of Na^+ by 2.0- and 2.5-fold, and Cl^- by 1.4- and 1.8-fold, respectively Figures 2F, G). Further, the NAA supplementation enhanced the Na^+ and Cl^- concentrations in all the treatments except Cl^- concentration at 0 mM alkaline stress. However, the alkaline stress (90 mM and 180 mM) profoundly reduced the K^+ concentration by 28.7 and 26.6%, the Mg^{2+} concentration by 19.4 and 26.6%, and the K^+/Na^+ concentration by 64.7 and 70.6%, respectively (Figures 2H-J). Contrarily, the NAA application significantly improved the K^+, Mg^{2+}, and K^+/Na^+ concentrations except for Mg^{2+}, where the plants were not treated with alkaline stress (Figures 2H-J).

Modifications in chlorophyll pigments under alkaline stress followed by NAA application

The chufa plants showed lower chlorophyll contents after alkaline stress compared to untreated plants (Figures 3A-C). This inhibition was also seen in the case of NAA application. Briefly, the chl-*a* and chl–*b* showed a 9.6 and 12% reduction after 90 mM alkaline stress, while 180 mM alkaline stress-induced 19.5 and 16% decline in Chl-*a* and –*b* contents, respectively. However, a significant improvement was shown by the chufa plants when the NAA was applied under both stress levels (Figures 3A, B). The Chl a/b presented an interesting trend as it was not remarkably modified after both stress levels. In addition, the plants displayed only significant change when treated with NAA supplementation under no alkaline stress (Figure 3C).

Reduction of MDA and H_2O_2 levels by NAA application under alkaline stress

The higher accumulation of MDA and H_2O_2 indicates oxidative damage in plants (Figures 4A, B). The same was the case in our experiment since the alkaline stress-subjected plants showed abrupt elevation in MDA and H_2O_2 levels. Interestingly, after 90 mM alkaline stress, the chufa. plants did not show substantial up-regulation in MDA levels. However, a 30% increase was recorded in the 180 mM alkaline stress-treated plants. Nevertheless, the NAA-treated plants displayed a 20, 10, and 15.4% decrease in MDA levels after 0 mM, 90 mM, and 180 mM alkaline stress, respectively (Figures 4A, B).

In contrast to MDA, the H_2O_2 levels were remarkably up-regulated (53.3 and 76%) by both respective alkaline concentrations. Nonetheless, the NAA application resulted in a 13, 13.5, and 21% decline in H_2O_2 levels when applied under 0 mM, 90 mM, and 180 mM alkaline stress conditions, respectively (Figures 6A, B).

Enhancement in antioxidant enzymes under alkaline stress followed by NAA supplementation

The plants activate their antioxidant machinery to deal with higher production of MDA and H_2O_2, as evident in SOD, POD, and CAT activities (Figures 4C-E). More specifically, a 1.3-, 5.2-, and 2.2-fold increment was detected in SOD, POD, and CAT activities when the chufa. plants were subjected to 90 mM alkaline stress, respectively. Moreover, the 180 mM alkaline stress induced the elevation of 1.5-, 2.8-, and 2-fold in SOD, POD, and CAT activities,

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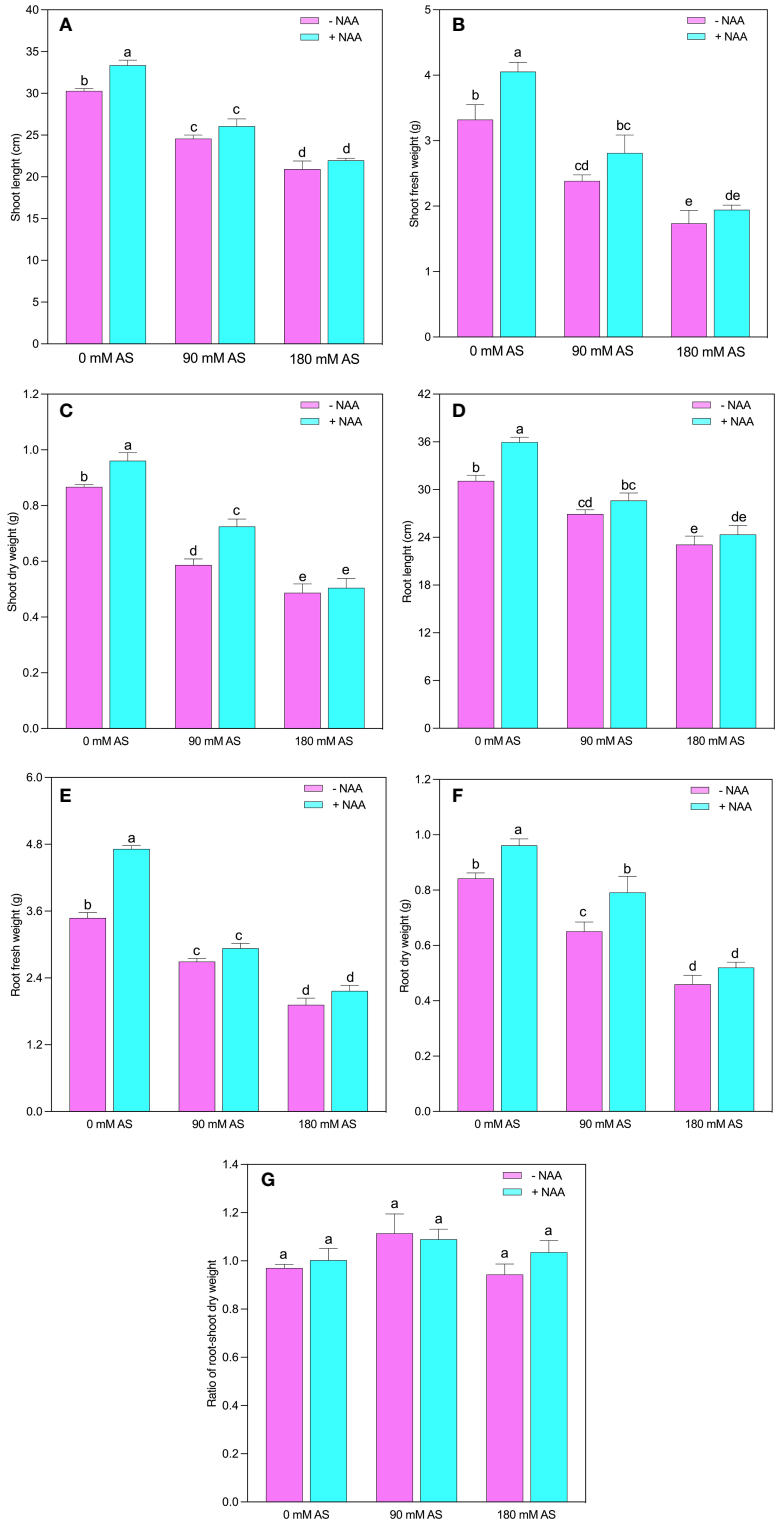

FIGURE 1
Changes in **(A)** shoot length, **(B)** shoot fresh weight and **(C)** shoot dry weight, **(D)** root length, **(E)** root fresh weight **(F)** root dry weight and, **(G)** root-shoot ratio of chufa plants under alkaline stress (AS) and exogenous naphthaleneacetic acid (NAA) application. Bars represents SD of mean, n=3. Different letters indicate significantly different values at $P<0.05$ (Duncan's method).

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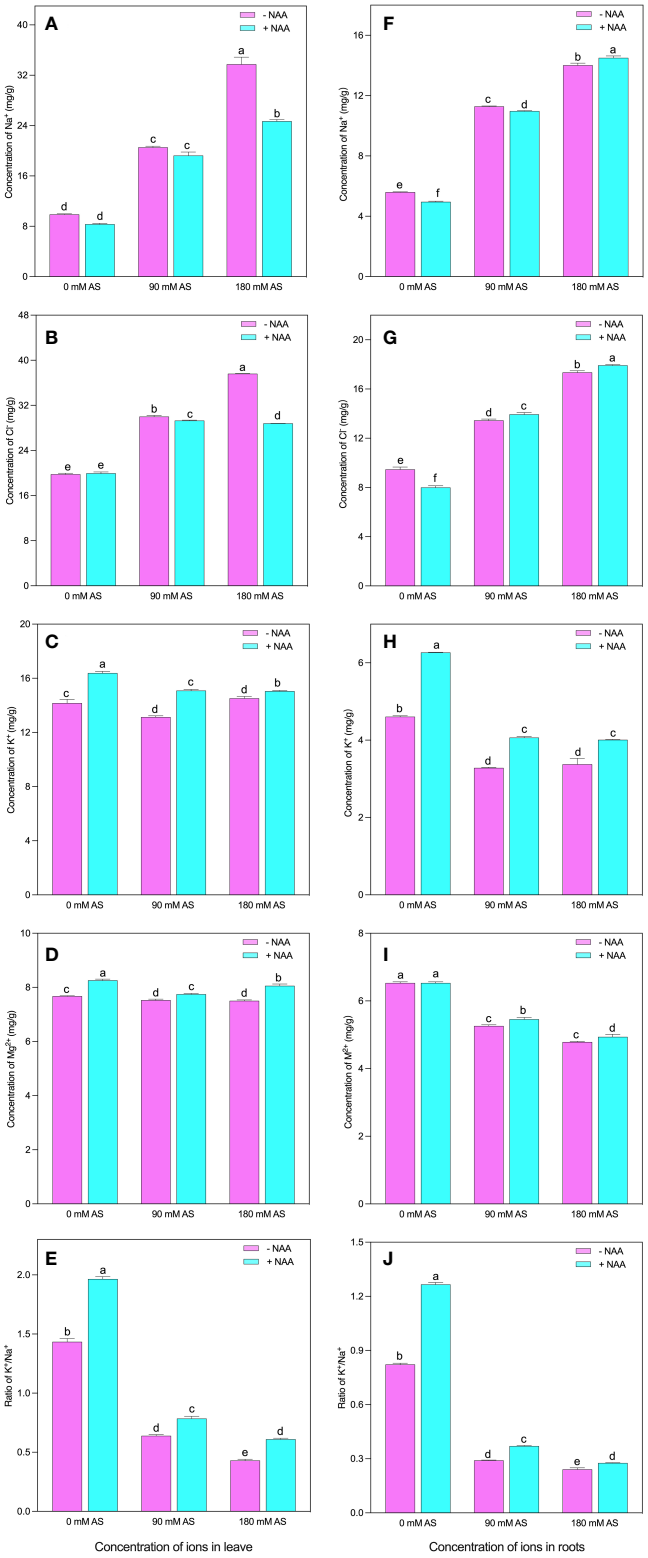

FIGURE 2
Changes in ions concentration of leaf **(A)** Na^+, **(B)** Cl^- **(C)** K^+ **(D)** Mg^{++} and **(E)** $K^{+/}Na^+$ ratio and root **(F)** Na^+, **(G)** Cl^- **(H)** K^+ **(I)** Mg^{++} and **(J)** $K^{+/}Na^+$ ratio of chufa plants under alkaline stress (AS) and exogenous naphthaleneacetic acid (NAA)application. Bars represents SD of mean, n=3. Different letters indicate significantly different values at $P<0.05$ (Duncan's method).

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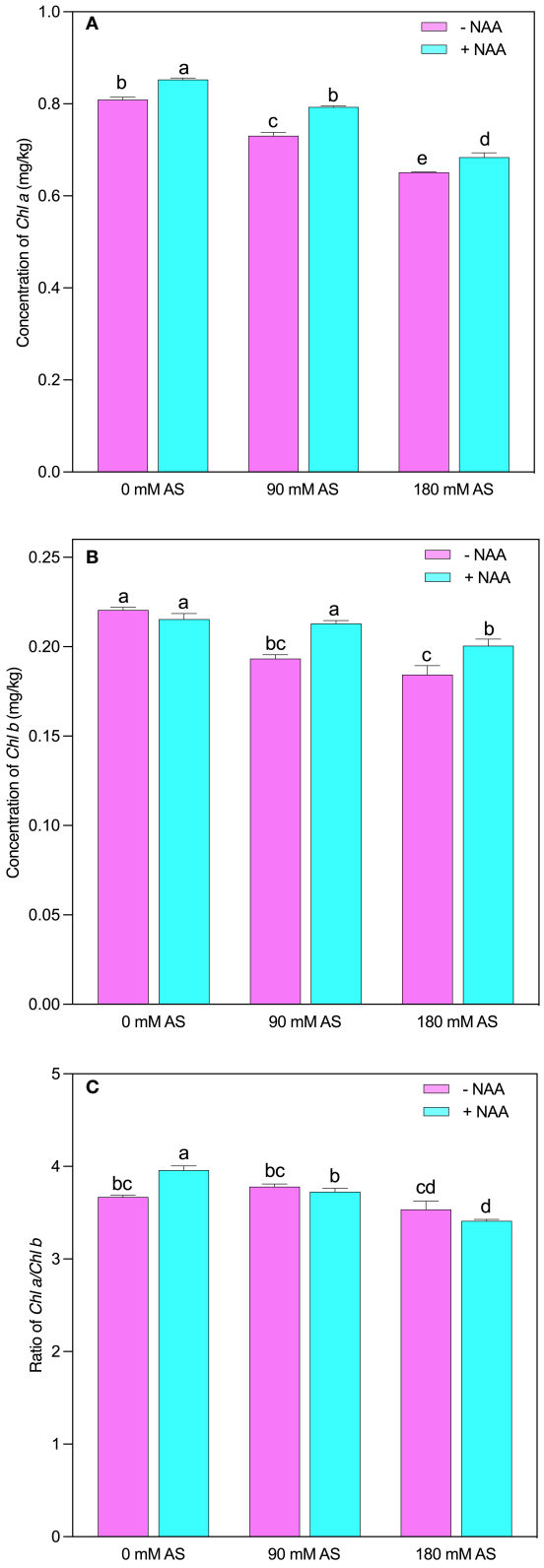

FIGURE 3
Changes in concentrations of **(A)** chlorophyll *a* **(B)** chlorophyll *b* and **(C)** ratio of chlorophyll *a*/*b* in chufa plants under alkaline stress (AS) and exogenous naphthaleneacetic acid (NAA) application. Bars represents SD of mean, n=3. Different letters indicate significantly different values at $P<0.05$ (Duncan's method).

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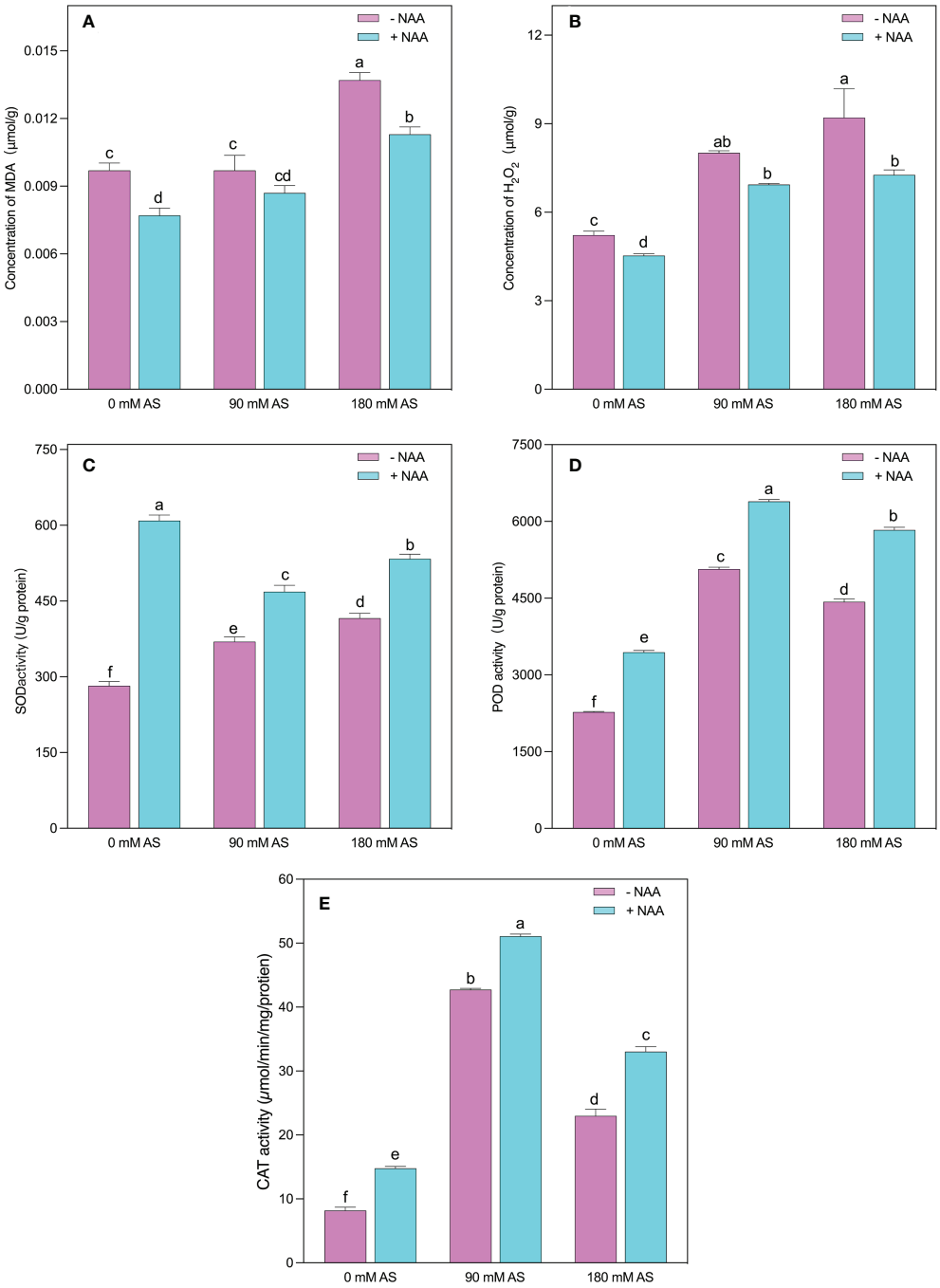

FIGURE 4
Changes in concentrations of **(A)** MDA **(B)** H_2O_2 and enzymatic activity of **(C)** SOD **(D)** POD, and **(E)** CAT in chufa plants under alkaline stress (AS) and exogenous naphthaleneacetic acid (NAA)application. Bars represents SD of mean, n=3. Different letters indicate significantly different values at $P<0.05$ (Duncan's method).

respectively. The NAA application significantly improved the SOD activities by 2.1-, 1.2-, and 1.3-fold, POD activities by 1.8-, 1.2-, and 1.4-fold, and CAT activities by 1.5-, 1.3-, 1.3-fold under 0 mM, 90 mM and 180 mM alkaline stress, respectively (Figures 4C-E).

Alterations in N-metabolism under alkaline stress followed by NAA addition

The NO_3^- concentration was not significantly influenced by the alkaline stress. Nonetheless, NH_4^+ and NH_4^+/NO_3^- reduced

substantially after alkaline stress. The 90 mM and 180 mM alkaline stress caused 47.5 and 52.5% reduction in NH_4^+ concentration, while, NH_4^+/NO_3^- exhibited 43.3 and 40.5% inhibition, respectively (Figures 5A–C). Interestingly, no significant improvement was seen in NO_3^- concentration under 0 mM alkaline stress; however, the 7.5 and 11.5% elevation was exhibited under respective alkaline levels. In addition, NAA application produced 5, 15 and 25% improvement in NH_4^+ and 5, 6 and 12% in NH_4^+/NO_3^- under 0 mM, 90 mM and 180 mM alkaline stress, respectively. Moreover NO_3^- reduction and NH_4^+ assimilation also decreased under alkalinity stress (Figures 5D–F).

The NR and GOGAT activities were inhibited by both alkaline levels. A 53.7 and 78% decline were observed in NR activities when the plants were exposed to 90 mM and 180 mM alkaline stress. This reduction was 66 and 48% for GOGAT activities in the respective alkaline levels. Additionally, the chufa. plants displayed 1.1-, 1.7- and 2-fold up-regulation in NR and 1.6-, 2.4- and 1.6-fold in GOGAT activities, under NAA foliar application, relative to untreated 0 mM, 90 mM and 180 mM alkaline stress, respectively (Figures 5D, E).

Although the alkaline stress could not induce any significant change in GS activities, the NAA application improved its activity by 1.3-, 12- and 1.2-fold under 0 mM, 90 mM, and 180 mM alkaline stress, respectively (Figure 5F).

Changes in the osmoprotectants of chufa under alkaline stress followed by NAA application

Likewise, in other parameters, the studied osmolytes were also influenced by the alkaline stress followed by NAA application (Figures 6A-C). The respective alkaline levels significantly reduced sugar contents (48.5 and 61%). Interestingly, the NAA could not improve the sugar contents at 0 mM and 180 mM stress levels, while a 19.4% up-regulation was recorded in sugar contents under 90 mM alkaline stress (Figure 6A). However, in the case of proline, this elevation (45%) was only detected after a 180 mM alkaline level (Figure 6B). The significant elevation in soluble protein (20.4 and 33.6%) and proline (21.3 and 40%) was observed after respective alkaline levels, respectively. The only significant up-regulation (27%) in soluble protein was found in the case of a 90 mM alkaline stress level (Figure 6C).

Relationship between the investigated parameters

According to Pearson's correlation analysis, all the studied growth indices positively correlated with Chl a, Chl b, Chla/b, NR, GS, GOGAT, NH_4^+ and NO_3^-, NH_4^+/NO_3^-, L-K^+/Na^+, R-K^+, R-Mg^{2+}, and R L-K^+/Na^+. While their negative correlation with MDA, H_2O_2, CAT, POD, Pro, SP, L-Na^+, L-Cl^-, R-Na^+, and R-Cl^- indicates alkaline-induced oxidative stress persistence in chufa plant. (Figure 7).

Discussion

Soil alkalinity (high pH) is an important abiotic stress, causing osmotic stress, ionic injuries, high pH-induced damages, and nutritional deficiencies, which result in acute physiological changes, thereby significantly affecting plant growth and productivity. Phytohormones have multiple functions in improving plant physiology both in normal growth conditions and in stress conditions (Fahad et al., 2015). The present study examined the effects of exogenously applied NAA on growth and physio-biochemical characteristics of chufa plants grown under different levels of simulated alkalinity stress.

We observed that alkaline stress significantly affected the growth and metabolism of chufa plants. For instance, the root and shoot length, root and shoot fresh weight, and dry weight were reduced significantly with increasing alkaline stress levels. The harmful effects of alkaline salt stress on growth parameters of chufa could be attributed to rise in pH, decrease in cell division and elongation, metabolic disruptions, nutrients deficiencies, and ionic imbalances which can be seen in Figure 7 in the current experiment (Yang et al, 2009; Zhang and Mu, 2009; Abd-Alla et al., 2014; Mohsenian and Roosta, 2015; Li et al., 2019; Ullah et al., 2019). However, root-shoot ratio (RSR) non-significantly increased compared to controlled conditions. Increased RSA may be an adaptive response that provides a greater capacity for water and nutrient absorption (Lv et al., 2013). However, exogenous NAA application improved growth parameters of alkaline-stressed chufa plants, demonstrating that NAA's ameliorative action in reducing alkaline stress damages. NAA, similar to naturally occurring IAA stimulates cell division, and cell elongation leading to increased growth. Consequently, the results of the current study and previously published reports indicate that NAA provides protection against a wide variety of environmental stresses (Zhou et al., 2012; Abou El-ghit, 2015; Ullah & Sajjad, 2017; Muhammed et al., 2022).

The pigment chlorophyll is essential for photosynthesis. Plant growth is most commonly reduced under alkalinity conditions due to a decrease in photosynthetic capacity (Ullah et al., 2019). In the present study, we observed that alkaline stress significantly decreased chlorophyll concentrations (Chl a and Chl b) which might be the result of (*a*) Mg^{2+} precipitation that degrades green pigments, (*b*) increased oxidative stress causing damage to chloroplasts, and (*c*) increased chlorophyllase activity, which is responsible for chlorophyll destruction (Kariola et al., 2005; Abdel Latef and Chaoxing, 2011;

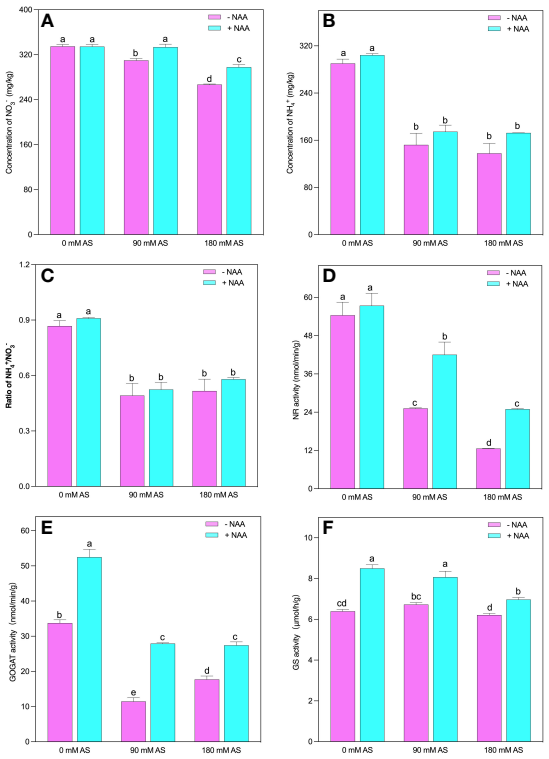

FIGURE 5
Changes in concentrations of **(A)** NO_3^- and **(B)** NH_4^+ **(C)** NH_4^+/NO_3^- ratio, and enzymatic activity of **(D)** NR **(E)** GS, **(F)** GOGAT in chufa plants under alkaline stress (AS) and exogenous naphthaleneacetic acid (NAA) application. Bars represents SD of mean, n=3. Different letters indicate significantly different values at $P<0.05$ (Duncan's method).

Fu et al., 2018). Compared to unsprayed chufa plants, NAA increased chlorophyll concentrations under controlled and alkaline stress conditions. Several studies have shown that NAA reduces the damage caused to chlorophyll pigments in leaves by saline-alkaline stress, which is in agreement with our findings (Burondkar et al., 2009; Abou El-ghit, 2015; Ullah and Sajjad, 2017; Muhammed et al., 2022). Importantly, NAA-induced restoration of chlorophyll pigments in the alkaline-stressed chufa plants exhibited an osmoprotective and membrane-protective role of NAA for chufa plants subjected to alkalinity. Our findings suggest that, the increase in chlorophyll pigments caused by NAA could be the result of its ability to promote pigment synthesis and retard pigment degradation by increasing antioxidant capacity or by stimulating the synthesis of stabilizing substances.

In the current study, an increase in alkaline stress caused an increase in Na^+ and Cl^- concentration while a decrease in Mg^{2+} and K^+ concentration, low K^+/Na^+ ratio in both roots and leaves as shown by the Figure 7. There is evidence that alkalinity causes high pH in the rhizosphere which reduces the availability of ions of nutrient elements in the soil, such as K^+, Mg^{2+}, Ca^{2+}, and $H_2PO_4^-$ by causing their precipitation (Yang et al., 2007). The alkalinity-induced reduction in K^+ and Mg^{2+} concentration is likely due to stress-driven repression of K^+ and Mg^{2+} absorption. Further, the reduced K^+ and K^+/Na^+ ratio could also be attributed to the competition between K^+ and Na^+ ions for binding sites necessary for cellular functions (Azooz et al., 2015; Ullah et al., 2019). Toxic accumulations of salt ions compromise plant development, metabolism and growth. We suggest that, the poor growth of chufa plants as a consequence of alkaline stress might be the result of osmotic, ionic, and pH-induced cellular membrane damage, associated with higher levels of Na^+ and Cl^- ions and a reduction in beneficial K^+ and Mg^{2+} ions (Zhu, 2001; Chartzoulakis 2005; Ullah et al., 2019; Noor et al., 2022). During transpiration, the fast-moving xylem carries Na^+ and Cl^- to the shoots (Tester and Davenport, 2003). Consequently, we suggest that chufa leaves accumulated more Na^+ and Cl^- ions than roots, making them more susceptible to salt ions. The results of our study agree with those of previous studies, which reported significantly lower levels of Na^+ and K^+ in roots compared to shoots (Wang et al., 2012; Menezes et al., 2017). Our work suggests that chufa plants use these excess accumulations of Na^+, Cl^-, K^+, and Mg^{2+} to survive under water deficit conditions. For example, these excess ions might act as osmolytes for reducing leaf water to enhance water absorption and improve their photosynthesis and other metabolic processes.

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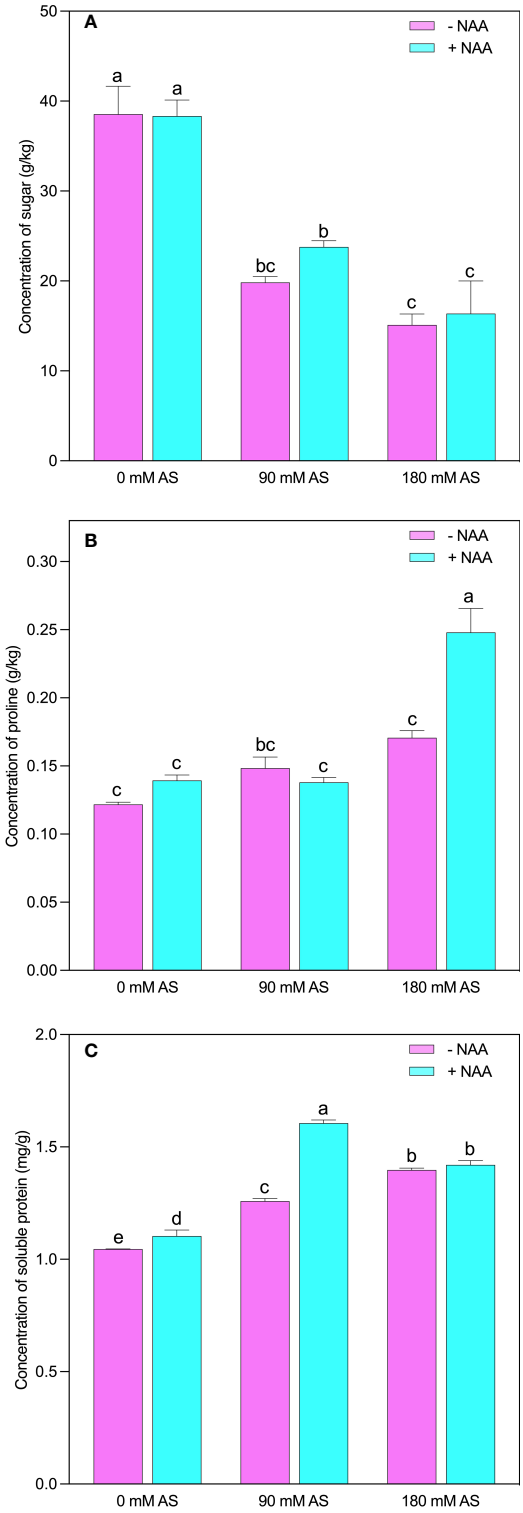

FIGURE 6
Changes in concentrations of **(A)** sugar, **(B)** proline and **(C)** soluble protein in chufa plants under alkaline stress (AS) and exogenous naphthaleneacetic acid (NAA) application. Bars represents SD of mean, n=3. Different letters indicate significantly different values at $P<0.05$ (Duncan's method).

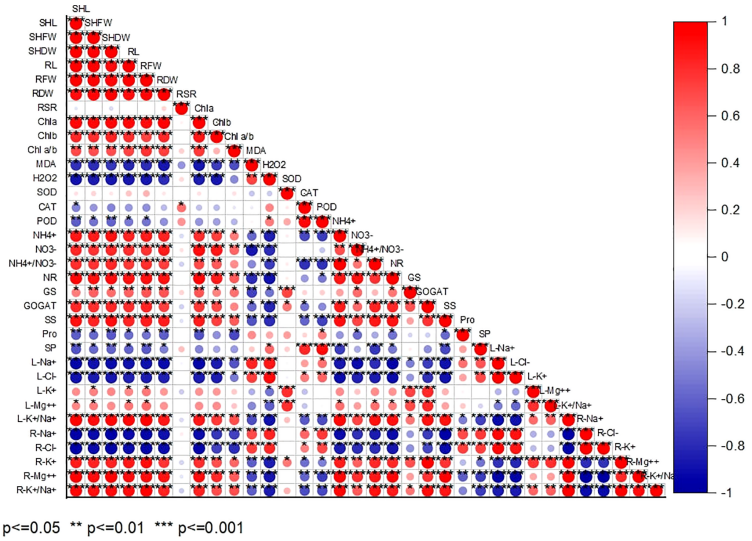

FIGURE 7
Pearson correlation analysis between study parameters in chufa plants under alkaline stress (AS) and exogenous naphthaleneacetic acid (NAA) application. SHL (Shoot lenght), SHFW (Shoot fresh shoot), SHDW (Shoot DW), RL (Root length), RFW (RFW), RDW (Root dry weight), Root-shoot ratio, Chla (Chlorophyll a), Chlb (Chlorophyll b), Chlab (Chlorophyll a/Chlorophyll b ratio), MDA (Malondialdehyde), H_2O_2, (Hydrogen peroxide), SOD (Superoxide dismutase), CAT (Catalase), POD (peroxidase), NR (Nitrate reductase), GS (Glutamine synthetase), GOGAT (Glutamine oxoglutarate aminotransferase), SS (soluble sugar), Pro (Proline), SP (Soluble protein), NO_3^- (nitrate), NH_4^+ (ammonium), L-Na^+ (Leaf N), L-Cl^- (Leaf Cl^-), L-K^+ (Leaf K^+) L-Mg^{++} (Leaf Mg^{++}), L-K^+/Na^+ ratio (Leaf K^+/Na^+ ratio), R-Na^+ (Root N^+), R-Cl^- (Root Cl^-), R-K^+ (Root K^+) R-Mg^{++} (Root Mg^{++}), R-K^+/Na^+ ratio (Root K^+/Na^+ ratio).

In contrast, NAA-applied chufa plants significantly decreased Na^+ and Cl^- concentrations while improving Mg^{2+}, K^+, and K^+/Na^+ ratios in both roots and leaves. Previous studies reported that IAA and NAA reduced the Na^+ accumulation and enhanced K^+, Ca^{+2}, and P contents in plants grown under salinity conditions (Kaya et al., 2010; Olaiya and Anyanwu, 2013; Muhammed et al., 2022). Therefore, we propose that NAA application ameliorated the adversity of alkalinity on *C. esculentus* by declining the toxic accumulations of salt ions and increasing beneficial mineral nutrients (i.e., K^+, Mg^{2+}) to optimize its cellular metabolism and growth. Considering the high K^+/Na^+ ratio, it appears that exogenous NAA application has a balancing effect on K^+ and Na^+ uptake under alkaline stress, suggesting that NAA may be used as an alleviating agent in alkaline soil grown crops.

Alkalinity stress often causes plants to experience oxidative stress damages and membrane lipid peroxidation (Gong et al., 2013; Guo et al., 2019). In our study, alkaline stress increased concentration of H_2O_2, resulted in higher MDA accumulation, which strongly indicates ROS bursts and potential oxidative damage of chufa plant cells, indicating that there is a positive correlation between MDA, H_2O_2 and other oxidative stress biomarkers as indicated in Figure 7 (Ahmad et al., 2014; Guo et al., 2019). However, alkalinity-stressed chufa plants significantly upregulated the activity of SOD, POD and CAT. There is evidence that plants upregulate several antioxidant enzymes (SOD, POD and CAT) which contribute significantly to the metabolism of excessive ROS under alkalinity stress (Zhang and Mu, 2009; Gong et al., 2013; Fu et al., 2017; Guo et al., 2019), which corroborates our findings. In contrast, NAA application significantly inhibited the accumulation of H_2O_2 and MDA, but increased activity of antioxidant enzymes including SOD, CAT and POD in alkalinity-stressed chufa plants compared with control plants and alkalinity-treated plants alone, suggesting that NAA activated adaptive mechanisms against oxidative damages in stressed plants. These findings demonstrate that NAA-application increased antioxidant activity to protect chufa plants against oxidative damage associated with alkalinity, as evidenced by the observed decrease in MDA concentration, which are in line with previous studies (Olaiya & Anyanwu, 2013; Ullah & Sajjad, 2017; Khedr et al., 2022; Muhammed et al., 2022). We suggest that chufa plant is able to significantly increase the activity of antioxidant enzymes in order to resist alkaline stress. However, this, diverting energy and protein into anti-oxidant mechanisms come at a significant cost in terms of growth.

In addition to oxidative stress, alkaline stress also causes osmotic stress in sensitive plants. In response, plants accumulate compatible solutes, such as glycine betaine, proline, and soluble sugars, to regulate osmotic pressure and enhance stress tolerance (Yang et al., 2007; Wang et al., 2012; Guo et al., 2017). In our study, chufa plants were significantly enriched in soluble protein

and proline under alkaline stress, and NAA application further increased their concentration. Hence, it seems that the accumulation of soluble protein and proline as osmolytes played a critical role in the physiological response of chufa plants to alkaline stress. In addition, exogenous NAA application to alkalinity-stressed chufa plants might have further facilitated the stabilization of membranes, enzymes, and proteins, and elimination of excess ROS for protecting the photosynthetic machinery (Verdoy et al., 2006; Ahmad et al., 2016; Muhammed et al., 2022). Furthermore, soluble sugar levels decreased as alkalinity stress increased; however, NAA application increased the concentration of soluble sugar compared to unsprayed alkalinity-stressed chufa plants. There have been reports that exogenous application of NAA and IAA increases the proline and soluble sugar under salinity stress for various plant species, which is consistent with our observation (Ullah & Sajjad, 2017; Mir et al., 2020). Soluble sugars and proline accumulate during stress, contributing to the maintenance of metabolism, for example, by alleviating ROS-induced oxidative stress damages (Sami et al., 2016). Moreover, in alkalinity-subjected chufa plants, high levels of soluble protein may serve as a form of nitrogen storage that can be reclaimed when stress is relieved (Abdel Latef, 2010; Abdel Latef and Chaoxing, 2014; Zhang et al., 2014). The increase in soluble protein was accompanied by a significant decline in growth of plants subjected to alkalinity stress. Therefore, our results suggest that chufa plants utilize most of the synthesized proteins for osmoregulation rather than growth in response to alkaline stress.

Plants typically absorb nitrogen (N) derived from inorganic sources, such as NO_3^- and NH_4^+ NH_4^+, through their roots which are then distributed for cellular functions. We observed that both NO_3^- and NH_4^+ levels decreased with increasing alkalinity stress gradients as presented in Figure 7. A plethora of studies demonstrated that alkalinity inhibits NO_3^- and NH_4^+ uptake, resulting in low concentrations (Yang et al., 2008; Yang et al., 2009; Yang et al., 2010; Ullah et al., 2019). Several transport systems utilize by roots of plants to absorb NO_3^- and NH_4^+ For instance, H^+/NO_3^- symport mediates NO_3^- uptake through a transmembrane proton gradient (Crawford and Glass, 1998). The alkalinity-induced decrease in NO_3^- uptake by roots might be the result of high pH injuries, resulting in large reduction of OsNR1 expression in roots of rice seedlings (Wang et al., 2012). Further, the AMT protein regulates the absorption of NH_4^+ (Crawford and Glass, 1998; Wang et al., 2012). Further, it has been reported that alkalinity (pH, 9.11) reduces NH_4^+ for root uptake in the surrounding rhizosphere by changing it to NH_3 , or decreases NO_3^- concentrations and OsNR1expression, resulting in a reduction in NH_4^+ synthesis (Wang et al, 2012). Hence, we suggest that alkaline salt stress might hamper the activity of NRT and AMT, resulting in a decreased uptake of NO_3^- and NH_4^+. There is a possibility that this phenomenon may affect virtually all processes of plant metabolism (Guo et al., 2017).

The NO_3^- is further reduces into nitrite (NO_2^-) and then to NH_4^+ by the actions of nitrate reductase (NR) and nitrite reductase respectively. Further incorporation of NH_4^+ into organic molecules is carried out by glutamine synthetase and glutamate synthase cycle (GS/GOGAT) or alternatively *via* glutamate dehydrogenase (GDH) (Shi et al., 2009). The present study indicated significant decreases in NR and GOGAT activities due to increased alkaline stress, while there was no significant effect on GS activity. Alkalinity stress has been reported to interfere in NO_3^- reduction and NH_4^+ assimilation by affecting the activities of NR, NiR, GS/GOGAT and GDH enzymes in plants (Song et al., 2006; Yang et al., 2007; Wang et al., 2012; Zhang et al., 2013; Guo et al., 2017; Ullah et al., 2019), which corroborates our findings. The decrease in in NO_3^- and NH_4^+ assimilation hampers N metabolism and amino acid synthesis, leading to reduced plant growth and dry weight (Queiroz et al., 2012; Ullah et al., 2019). The observed decline in NR activity could be attributed to decreased carbon fixation, decreased NO_3^- uptake by roots, or its low translocation in the xylem, which consequently decreases NO_3^- availability to plants (Kumar and Joshi, 2008; Gloser et al., 2020). In addition, there may be a negative effect of alkalinity stress on the enzyme protein synthesis and/or activity that contributes to the decrease in NR activity. Further, the decrease in GOGAT activity at both stress levels and GS at high stress level, could be attributed to an increase in protein oxidation (Balestrasse et al., 2006). Due to the fact that alkalinity stress is known to stimulate the generation of ROS which may contribute to the destruction of these enzyme proteins by oxidative stress (Zhang and Mu, 2009). In contrast, NAA-applied chufa plants increased the concentration of NO_3^- and NH_4^+ as well as up-regulated NR, GS and GOGAT enzymes in leaves of chufa plants. Additionally, Sharma and Dubey (2005) demonstrated that NR is highly sensitive to H_2O_2. Therefore, we suggest that, NAA application indirectly increased NR enzymes by reducing the accumulation of excessive H_2O_2 under alkalinity stress condition. In addition, NAA-induced increase in GS and GOGAT activities in in leaves of chufa plants subjected to alkalinity stress, indicating its role in proper incorporation of NH_4^+ into glutamate pool and further amino acid biosynthesis.

In plants, nitrogen is an integral component of nucleic acid, amino acids, proteins, and chlorophyll pigments and secondary metabolites. Hence, it plays a significant role in numerous physio-biochemical mechanisms. In fact, the regulation of N metabolism contributes to stress tolerance through various mechanisms (Rais et al., 2013; Arghavani et al., 2017). We therefore propose that NAA application facilitated the N assimilation of alkalinity stressed chufa plants, by increasing the concentration of NO_3^- and NH_4^+ as well as their metabolizing enzymes, thus improving their tolerance to alkalinity stress. Further, we noticed that NAA application increased N assimilation along with enzymatic antioxidants in alkalinity-stressed chufa plants. Consequently, we propose that alkalinity stressed chufa plants need amelioration of N assimilation for maximizing protein synthesis needed to divert to anti-oxidant mechanism for protecting the cells from alkalinity induced

oxidative stress damage. Consequently, we propose that chufa NAA-application to alkalinity stressed chufa plants ameliorated their N assimilation, enabling them to synthesize the maximum amount of protein necessary to enhance their anti-oxidant mechanism against alkalinity-induced oxidative stress. The increased N assimilation, protein concentration, and increased enzymatic anti-oxidant mechanisms, and reduced H_2O_2 and MDA concentration in NAA-applied alkalinity stressed chufa plants could explain this conclusion.

Conclusion

We used exogenous NAA application to check whether it can alleviate the adverse effects of alkalinity on the growth and physio-biochemical features of chufa plants. We found that exogenous application of NAA promoted the growth and metabolism of chufa by reducing the damage of alkalinity stress. For instance, exogenous NAA resulted in (i) reduction of toxic salt ions (Na^+ and Cl^-), (ii) protection of photosynthetic apparatus (iii), maintenance of beneficial mineral ions (K^+ and Mg^{2+}), (iv) reduction of ROS and lipid peroxidation by upregulating enzymatic antioxidant defense mechanism (v) improvement of nitrogen assimilation, and (vi) stimulation of osmolytes. In addition, chufa leaves accumulated a larger quantity of Na^+, Cl^-, K^+, and Mg^{2+} ions than roots, suggesting that leaves are capable of increasing their cellular osmolality, thereby facilitating the upward flux of water from the soil to leaves; an important strategy for adaptation to hyper-arid conditions. Our results show that NAA acts as a powerful plant growth regulator capable of modulating chufa growth and physiological responses. We suggest that application of exogenous NAA may therefore be a an effective strategy for reducing alkalinity-induced negative effects on chufa and other salt-sensitive vital economic crop species in the hyperarid Taklamakan desert, where salty groundwater is the sole source of agricultural irrigation water.

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

Conceptualization: AU, FZ. Data curation: AU. Formal analysis: AU, MA, and JN. Funding acquisition; FZ. Investigation: AU, AT. Methodology: AU. Project administration; FZ. Resources: FZ. Software: AU, AT, JN, MAA, KS, AR, and ZZ. Supervision: FZ. Validation: FZ, AU, AT, and ZZ. Visualization: JN, KS, AR, MN, and MA. Writing - original draft: AU. Writing - review and editing: AU, FZ, AT, ZZ, JN, MN. 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.2022.1018787/full#supplementary-material

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Exogenous 6-benzylaminopurine enhances waterlogging and shading tolerance after anthesis by improving grain starch accumulation and grain filling

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Due to the frequent occurrence of extreme weather events, the area of wheat affected by continuous cloudy rainfall is increasing, with waterlogging becoming a major limiting factor of wheat yield. To alleviate the effect, spraying exogenous plant growth regulators is often used. In this study, two wheat cultivars, waterlogging-tolerant Yangmai 18 and waterlogging-sensitive Sumai 188, were selected for waterlogging and shading (WS) after anthesis for 7, 11, and 15 days respectively. Three concentrations of 6-benzylaminoadenine (6-BA) solution (15, 25, and 35 mg·L^{-1}) were sprayed after WS treatment and water was sprayed as the control. Then, the effect of spraying 6-BA on photosynthetic characteristics, starch content, grain filling characteristics, and yield was explored under artificially stimulated continuous cloudy rainfall during anthesis. Compared with the control, the application of 6-BA caused a significant increase in grain plumpness throughout grain filling, as well as increases in the net photosynthetic rate (P_n), stomatal conductance (G_s), and transpiration rate (T_r), and a significant decrease in the intercellular CO_2 concentration (C_i) of the flag leaves, all of which enhanced the photosynthetic capacity. The content of total starch, amylose, and amylopectin in the grains also increased significantly compared with the control. After WS for 15 days, the starch content increased by 3.81%–11.41% compared with the control. Spraying 6-BA also prolonged grain filling, increased the average grain filling rate, and significantly increased the 1000-grain weight and yield. The thousand-grain weight increased by 5.06%–43.28%, and wheat yield increased by 8.93%–64.27% after spraying 25 mg·L^{-1} of the 6-BA solution. These findings suggest that the application of 6-BA after WS stress could significantly improve the photosynthetic performance, which is propitious to the accumulation and transport of photosynthetic products after

anthesis. Besides, spraying 6-BA can also increase the duration and rate of grain filling and starch accumulation content and improve grain weight, thereby alleviating the adverse effects of WS on wheat yield. Overall, spraying 25 mg·L^{-1} of the 6-BA solution had an optimal effect. These findings provide a theoretical basis for the exploration of cultivation techniques and measures aimed at alleviating damage caused by continuous rainfall during wheat anthesis.

KEYWORDS

wheat (*Triticum aestivum* L.), waterlogging and shading, 6-benzylaminoadenine, photosynthetic performance, starch accumulation, grain filling, yield

1 Introduction

In the recent years, continuous or heavy rainfall caused by climatic factors, coupled with poor irrigation and drainage facilities as well as a low-lying terrain, has had widespread effects on global wheat production, with waterlogging becoming a major limiting factor of wheat yield (Boru et al., 2001; Singh and Setter, 2015). This is particularly apparent in the wheat-growing regions of the middle and lower reaches of the Yangtze River, China, where rice–wheat rotation has resulted in heavy soil with saturated water content (Shao et al., 2013). Moreover, much of the high rainfall in this region occurs in spring, a critical wheat growth period in terms of booting and anthesis. Studies have shown that the frequency of waterlogging in wheat-growing areas along the Yangtze River was as high as 60% during the 50-year period from 1961 to 2010, with detrimental effects on wheat yield (Wu et al., 2016; Wu H. et al., 2018). At the same time, the weak light effect caused by continuous precipitation has an additional effect during later growth stages, greatly reducing grain dry matter accumulation, which also affects yield (Mu et al., 2010). However, waterlogging stress and shading stress on the damage mechanism of wheat are different (Geigenberger, 2003; Gao et al., 2017). At present, there are many studies on the effects of single-factor stress such as waterlogging or shading, while there are few studies on the effect of compound stress on wheat yield. Under natural conditions, waterlogging is generally caused by continuous rainy weather, which leads to insufficient light. Therefore, the study of the combined stress of WS can better simulate the natural disaster situation of wheat. New cultivation techniques and measures aimed at effectively alleviating the damage caused by the combined stress of WS are therefore essential.

About 70% of wheat grain yield is associated with the accumulation of photosynthetic products after anthesis (Ziaei and Sepaskhah, 2003). Under waterlogging stress, the nitrogen content of wheat plants is significantly reduced, leaves become chlorotic, senescence is accelerated, and plants lose their ability to capture light and carboxylate (Ziaei and Sepaskhah, 2003; Wu W. et al., 2018). This has a direct effect on the rate of photosynthesis, dramatically slowing growth and development. Meanwhile, studies have also shown that while mild shading (88% light transmittance) delays the senescence of wheat leaves, increasing the P_n, canopy apparent photosynthetic rate, 1000-grain weight and yield, and moderate (67% light transmittance) and heavy shading (35% light transmittance) has significant negative effects on all of the above traits (Xu et al., 2015). The later stages of wheat growth and development center on the formation of grains, the main component of which is starch, which accounts for about 65% of the dry weight. Starch in the wheat endosperm is composed of amylose and amylopectin, with differences in the composition having an important impact on flour quality (Golay et al., 1991; Holm and Björck, 1992). Waterlogging during heading and anthesis causes damage to endosperm cell structure, resulting in the formation of irregular starch granules (Zhou Q. et al., 2018a), in addition, post-anthesis waterlogging affects the content of starch components by altering the activity and expression of enzymes related to starch synthesis (Zhou Q. et al., 2018b). Similarly, shading treatment in the wheat filling stage also causes a decrease in the starch content of wheat grains, significantly reducing overall quality (Andrade et al., 2018). In addition, shading also causes a reduction in dry matter accumulation before anthesis and wheat yield (Demotes-Mainard and Jeuffroy, 2004; Mu et al., 2010).

The exogenous application of plant growth regulators is often used to alleviate the impact of water or shading stress on crops. For example, studies have shown that the application of abscisic acid (ABA) before waterlogging can improve the antioxidant and photosynthetic capacity of waterlogged crops (Kim et al., 2018). Application of methyl jasmonate increased grain yield and biomass and water use efficiency under water stress during the vegetative stage (Ghasem et al., 2022). It was also shown that exogenous application of salicylic acid (SA) inhibited the uptake of Na and stimulated the uptake of N, P, and K in wheat under water stress, decreasing stomatal conductivity and increasing the grain number per spike and 1000-grain weight (Hafez and Farig, 2019). 6-Benzylaminoadenine (6-BA) promotes cell division and the

transport and accumulation of photosynthetic products, enhancing stress resistance in plants. Moreover, in comparisons of five hormones (auxin, cytokinin, abscisic acid, gibberellin, and brassino steroid), 6-BA was found to have the greatest effect on duckweed biomass and, along with ABA, was most effective in enhancing biomass and starch accumulation (Liu et al., 2019). A previous study also revealed that after waterlogging during anthesis, foliar application of 6-BA significantly heightened the P_n of wheat leaves and the aboveground biomass and grain yield (Wang X. et al., 2020a). Evidence also suggests that spraying exogenous 6-BA was able to postpone leaf senescence and improve the chlorophyll content and photosynthetic capacity, thereby effectively lessening the harmful effects of flooding in summer maize (Ren et al., 2016). Furthermore, spraying the 6-BA solution before shading during anthesis was also found to delay the senescence of wheat flag leaves under shading treatment, as well as increase dry matter accumulation and reduce yield losses (Li et al., 2019).

At present, research into the mitigating effects of 6-BA application tends to focus on single stress events such as waterlogging or high temperatures, with the research content centering on photosynthetic characteristics and yield. However, few reports have examined the mitigating effects of exogenous 6-BA application on photosynthetic product transport, grain starch, and its starch component content in wheat under the combined stress of WS. This study aimed at ascertaining the effects of spraying several different concentrations of the 6-BA solution on photosynthetic characteristics, photosynthetic product transport, grain starch accumulation, grain filling characteristics, and yield under shading and waterlogging stress after anthesis. Furthermore, the physiological reasons for reducing the damage of WS to wheat yield after spraying the 6-BA solution were analyzed by studying the process of the photosynthetic performance and dry matter accumulation of wheat. This research could provide the theoretical foundation for effective cultivation techniques and measures to alleviate the damage of continuous rainfall after the anthesis of wheat.

2 Materials and methods

2.1 Experimental design

The experiment was carried out from November 2020 to June 2022 at Anhui Agricultural University Wanzhong Experimental Base, Guohe Town, Lujiang County, Hefei City (117°01′E, 30°57′N). The test site has a subtropical humid monsoon climate, with an average annual temperature of 15°C–16°C, average annual precipitation of 900–1000 mm, an annual sunshine duration of about 2000 h, and an average annual frost-free period of 228 days. The mean daily temperature and monthly cumulative rainfall from 2020 to 2022 during the wheat growing season is shown in Figure 1. The waterlogging-tolerant cultivar Yangmai 18 (bred by the Academy of Agricultural Sciences of Lixiahe District, Jiangsu Province) and the waterlogging-sensitive cultivar Sumai 188 (bred by Jiangsu Fengqing Seed Industry Technology Co., Ltd.) were used as test materials. Each box contained 150 kg of paddy soil taken from the 0–20 cm tillage layer. Before sowing in 2020 and 2021, the soil contains 22.3 and 15.8 g·kg^{-1} of organic matter, 1.4 and 0.8 g·kg^{-1} of total nitrogen, 106.5 and 104.4 mg·kg^{-1} of alkali-hydrolyzable nitrogen, 105.0 and 91.0 mg·kg^{-1}

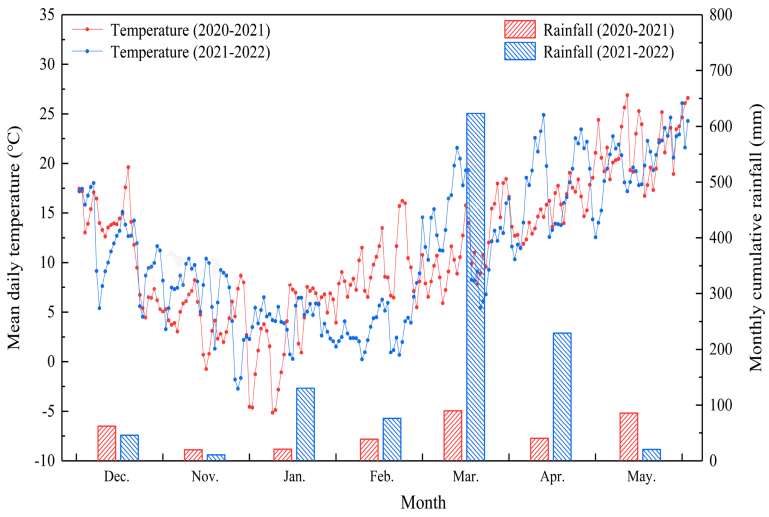

FIGURE 1
The mean daily temperature and monthly cumulative rainfall during wheat growing seasons (2020–2022).

of available potassium, and 21.2 and 22.7 mg·kg^{-1} of available phosphorus, respectively. Plants were grown in boxes measuring 70 cm in length, 50 cm wide, and 43 cm high, with a volume of 120 L. Each box contained 150 kg of soil. Round drainage holes with a diameter of 2 cm were arranged around the sides of each box approximately 8 cm from the bottom, with eight along each long side and six on each end. During WS treatment, the drainage holes were plugged with rubber stoppers, while at all other times, they were kept open to ensure sufficient water permeability. The wheat plants were sown on 9 November 2020 and 2 November 2021, five rows per box at a row spacing of 15 cm. Before sowing, each box was supplied with 105 g organic fertilizer, 2.6 g phosphate fertilizer (P_2O_5), 5.2 g potassium fertilizer (K_2SO_4), and 15.98 g nitrogen fertilizer (46% urea), followed by 6.85 g nitrogen fertilizer as topdressing during the jointing stage. All other field management ways were the same as high-yield cultivation measures in the field.

WS were carried out during anthesis at durations of 7, 11, and 15 days. Shading and waterlogging treatment were carried out simultaneously, with shade nets used to create a shading rate of approximately 45%. The light intensity after shading is shown in Figure 2. Yangmai 18 underwent WS treatment at 19:00 on 10 April 2021 and 4 April 2022, while Sumai 188 began treatment at 19:00 on 12 April 2021 and 4 April 2022. The shade net was fixed to an open arched structure, the top of which was 1.8 m above the ground to allow sufficient ventilation. During waterlogging, the drainage holes were plugged to allow approximately 2 cm of water to accumulate on the soil surface. Rainproof measures were also taken during WS to mitigate the effect of external rainfall events. At 9:00 a.m. the day following WS treatment, plants were sprayed with one of three concentrations of the 6-BA solution (15, 25, and 35 mg·L^{-1}). As a control, the same amount of distilled water was used. The spraying amount was 500 ml for each box, so as to ensure that each leaf was evenly covered with the reagent. Each treatment set consisted of 12 boxes.

2.2 Acquisition of samples

Single stems that bloomed on the same day and had similar-sized wheat ears were marked in each treatment to indicate anthesis. Following WS treatment, and starting from 10 days after anthesis, 20–25 of the marked wheat ears were then randomly selected from each treatment every 5 days (see Table 1 for sampling times). The grains were then collected and the remaining kernels were removed after heating at 105°C for 15 min. These samples were kiln-dried to a steady weight at 80°C for the analysis of starch and each starch component, and for grain dry matter accumulation.

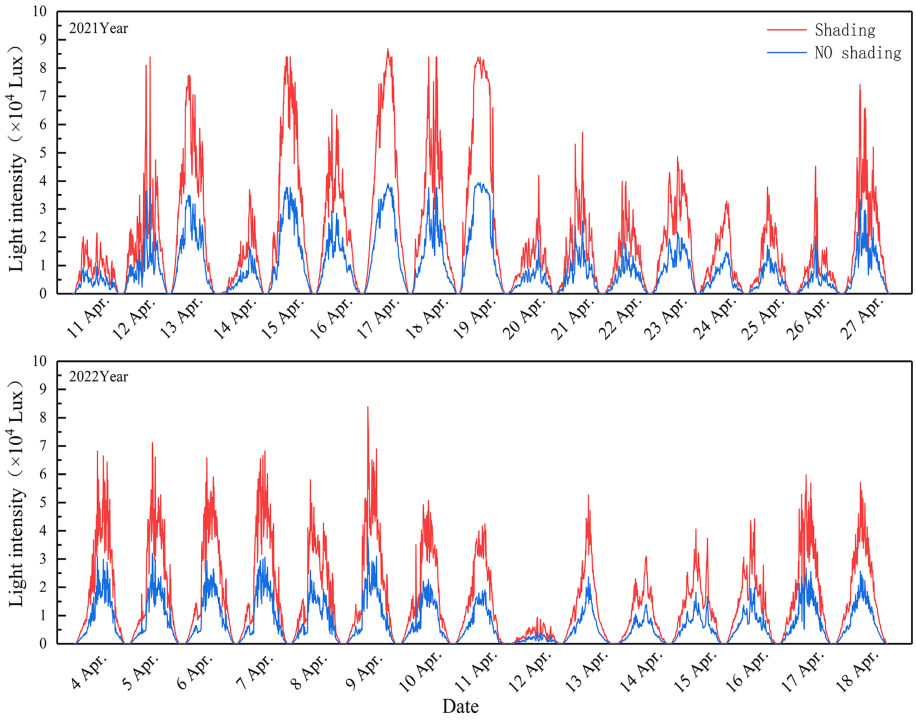

FIGURE 2
Changes of light intensity under shading treatment.

TABLE 1 Sampling time for different treatments under waterlogging and shading after anthesis.

Treatment	Sample time					
WS_7	10 DAA	15 DAA	20 DAA	25 DAA	30 DAA	35 DAA
WS_{11}	—	15 DAA	20 DAA	25 DAA	30 DAA	35 DAA
WS_{15}	—	—	20 DAA	25 DAA	30 DAA	35 DAA

WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. DAA represents days after anthesis. — represents not sampling.

2.3 Measurements

2.3.1 Morphology of grains and starch granules

Grain morphology was observed in samples obtained as described above and in Table 1, every 5 days from 10 days after anthesis. One to two seeds from the bottom of the intermediate spikelet were selected for observations using a SZX16 stereo microscope (OLYMPUS, Japan).

For observations of starch grain morphology, one to two wheat ears were randomly selected from the marked wheat plant after reaching maturity. One to two grains at the bottom of the middle spikelet were then selected and cut along the middle with a blade to provide cross-sections for observations of the endosperm. The samples were fixed on a copper column with double-sided carbon glue then plated with gold using an ion sputtering device then the ultrastructure of the starch granules was observed using an S-4800 scanning electron microscope (HITACHI, Japan).

2.3.2 Photosynthetic parameters of flag leaves

The LI-6400XT portable photosynthetic system (LI-COR Co., USA), with an open gas circuit, was used to set the rate of photosynthetically active radiation (PAR) to 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. In each treatment, three flag leaves of wheat with the same growth and development process were measured from 9:00 a.m. to 11:00 a.m. When the net photosynthetic rate of wheat tended to be stable, the photosynthetic parameters at this time were recorded. Each flag leaf was measured three times then the average value was determined. Measurements were performed every 5 days from 10 to 25 days after anthesis.

2.3.3 Isotope $\delta^{13}C$ analysis

After spraying with the 6-BA solution the day after WS, 10 flag leaves were randomly selected from the marked single stem of wheat from each treatment; then the flag leaves were then sealed with transparent PVC plastic bags, injected with 5 ml of $^{13}CO_2$ gas into the plastic bags using a medical syringe, and assimilated for 60 min under natural light conditions at 10:00–11:00 a.m. on the same day. Three flag leaves were then removed, fixed at 105°C for 15 min, and kiln-dried at 80°C. After reaching maturation, three unsampled wheat ears were selected, the grains removed, fixed, and dried. They were then ground to a powder using an A11 basic analytical mill (IKA, Germany) and passed through a 100-mesh screen. The Sumai 188 after 11 days under WS was selected for analysis of $\delta^{13}C$ using flag leaves and grains were treated with 25 $mg \cdot L^{-1}$ of the 6-BA solution. To do so, a Sercon Integra 2 Elemental Analysis-Stable Isotope Ratio Mass Spectrometer (EA-IRMS) was used with the $^{13}CO_2$-labeled flag leaves and grains (SerCon, Britain).

$\delta^{13}C_{TOC}$ values were obtained using the PDB international standard as a reference, as follows:

$$\delta^{13}C_{TOC}(‰) = \left[\frac{R(^{13}C/^{12}C_{sample})}{R(^{13}C/^{12}C_{VPDB})}\right] - 1 \times 1000 \quad (1)$$

where R ($^{13}C/^{12}C_{VPDB}$) is the carbon isotope abundance ratio of the international standard Vienna Pee Dee Belemnite (VPDB). The analytical precision of the $\delta^{13}C_{TOC}$ values was set at ± 0.2‰.

2.3.4 Grain starch and its component content

The total starch content of the grains was determined by anthrone colorimetry (Jia and Zhang, 2008). After drying, the wheat kernels were ground and then passed through a 100-mesh sieve. Next, 8 ml of 80% ethanol was added to about 0.1 g (accurate to 1mg) of the sample, heated in a water bath at 80°C for 30 min, cooled, and then centrifuged at 5000×*g* for 15 min. The supernatant was then discarded, 2 ml of distilled water was added to the precipitate, shaken, and then boiled for 20 min before cooling and adding 2 ml of 9.2 $mol \cdot L^{-1}$ HCl_4. The samples were then shaken for a further 10 min before adding 6 ml of distilled water and centrifuging at 5000×*g* for 15 min. The resulting supernatant was then poured into a 50 ml volumetric flask. The process was repeated three times, giving a total sample volume of 50 ml, respectively. Next, 0.1 ml of the extract solution was added to 4 ml of 0.2% anthrone, boiled for 15 min, cooled, and observed using a spectrophotometer at a wavelength of 620 nm (Agilent Cary 300, USA). The starch standard curve was then used to calculate the starch content of each sample.

The amylose content was determined using a coupled spectrophotometer (Zhang et al., 2009). After drying, the wheat grains were ground into a powder, passed through a 100-mesh sieve, and then degreased with ether. Next, 10 ml of 0.5 $mol \cdot L^{-1}$ KOH solution was added to about 0.1 g (accurate to 1 mg) of defatted sample, boiled for 10 min, then diluted to 50 ml with distilled water. Any foam was removed with ethanol then the sample was left to stand for 20 min. Next, 30 ml of distilled water was added to 2.5 ml of solution; the pH was adjusted to 3.5

using 0.1 mol·L^{-1} HCl, and 0.5 ml iodine reagent was added (diluted 2.0 g potassium iodide and 0.2 g iodine with distilled water to 100 ml). A blank solution without an iodine reagent was diluted to 50 ml then left to stand for 20 min for use as a control. Colorimetry was then performed using a spectrophotometer at wavelengths of 630 and 460 nm (Agilent Cary 300, USA). The amylose content was obtained using the amylose standard curve then the amylopectin content was determined as follows:

Amylopectin content (%) = total starch content (%) −amylose content (%).

2.3.5 Grain filling measurements

Every 5 days from 10 days after anthesis, 15–20 wheat ears showing consistent growth and development were sampled. The grains were removed then fixed at 105°C for 15 min, dried at 80°C to a constant weight, weighted, and then converted into the 1000-grain weight. The Logistic equation $Y = K/[1+\exp(A+Bt)]$ (Darroch and Baker, 1990) was used to fit the variation in 1000-grain weight with the number of days after anthesis, where Y is the 1000-grain weight of the observed grain (g), t is the number of days (d) from anthesis until observation, A and B are determined parameters, and K is the fitted maximum 1000-grain weight (g). Least-squares estimates of K, A, and B were determined by nonlinear regression. The first and second derivatives of this Logistic equation are obtained, and the following parameters were then determined:

Effective days of grain filling (D):

$$D = \frac{\ln(1/9) - \mathbf{A}}{\mathbf{B}} \quad (2)$$

Duration of the gradual grain filling period (D_1):

$$D_1 = \frac{\mathbf{A} - 1.317}{\mathbf{B}} \quad (3)$$

Duration of the rapid grain filling period (D_2):

$$D_2 = \frac{\mathbf{A} - 1.317}{\mathbf{B}} - \frac{\mathbf{A} + 1.317}{\mathbf{B}} \quad (4)$$

Duration of the slow grain filling period (D_3):

$$D_3 = D - D_1 - D_2 \quad (5)$$

Average grain-filling rate (V_{mean}):

$$V_{\mathbf{mean}} = \mathbf{K}/D \quad (6)$$

Maximum grain-filling rate (V_{max}):

$$V_{\mathbf{max}} = -\mathbf{KB}/4 \quad (7)$$

Time point of the maximum grain-filling rate (T_{max}):

$$T_{\mathbf{max}} = -\mathbf{A}/\mathbf{B} \quad (8)$$

2.3.6 Yield and contributing factors

After reaching maturity, three boxes of unsampled wheat were selected from each treatment for the analysis of spikes, kernel numbers per spike, thousand-grain weight, and grain yield.

2.4 Data processing and analysis

Data were analyzed using the SPSS 22.0 software to fit the logistic curve with related parameters of starch, each starch component, and grain dry matter data. Duncan's method was used to perform multiple comparisons of the measured data. Origin 2017 was used for graphing and Photoshop was used for image annotation and processing.

3 Results

3.1 Effects of spraying 6-BA after WS on the morphology of grain and starch granules

3.1.1 Grain morphology

The degree of grain filling gradually deteriorated with increasing WS; however, the effect was alleviated after the application of 6-BA. Under each duration of WS, grain plumpness increased after the application of 6-BA, and the difference in grain fullness was obvious between the treatment and control groups. The grain ripening process was faster in the waterlogging-sensitive variety Sumai 188 (Figure 3B) than the waterlogging-tolerant Yangmai 18 (Figure 3A), while the degree of overall grain fullness was greater in Yangmai 18 than Sumai 188.

3.1.2 Morphology of starch granules

The ultrastructure of the wheat grains at maturity was observed in Sumai 188 using a scanning electron microscope (Figure 4). As shown, A- and B-type starch granules in the grain were closely arranged and filled the endosperm cells, with granular proteins in between. Meanwhile, with increasing WS, the cross-sectional area of the grains decreased; moreover, these grains became shrunken and deformed. In terms of starch granule morphology, the longer the duration of WS, the smaller the individual volume of A-type starch granules and the greater the number of deformed B-type granules. After WS for 11 days, some irregular B-type starch granules were observed, while after 15 days, most B-type granules formed irregular polygons. Compared with the control, the volume of A-type starch granules in the grains increased significantly after the application of 6-BA, and deformation of the B-type granules decreased.

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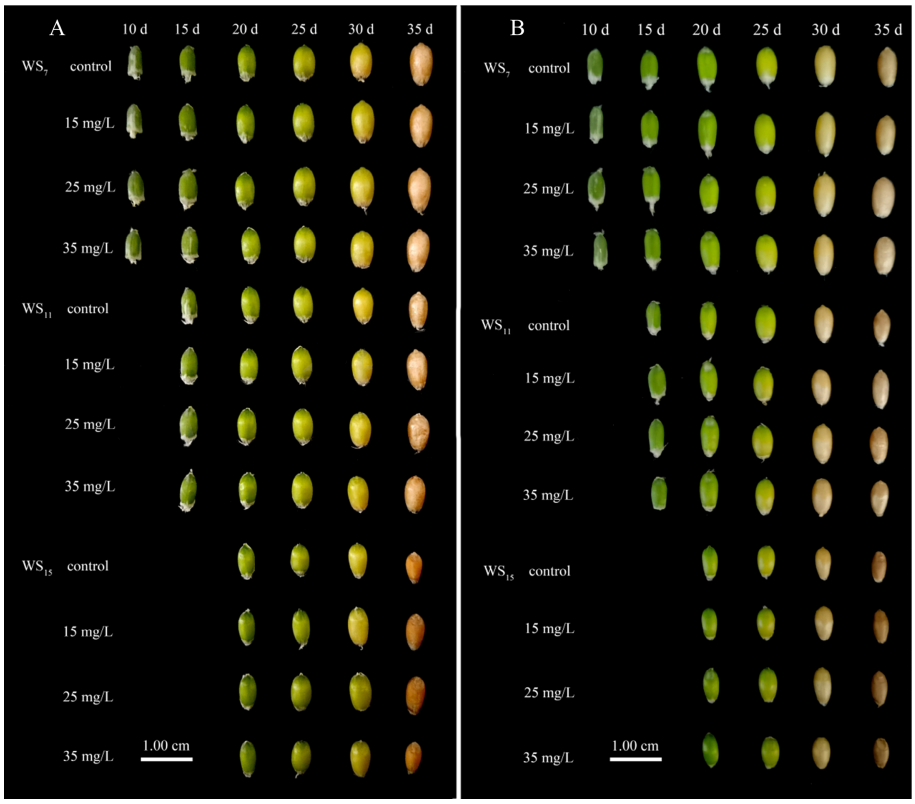

FIGURE 3
The effect of exogenous 6-BA on grain morphology of wheat under waterlogging and shading. The waterlogging-tolerant (Yangmai 18, 2021 **A**) and the waterlogging-sensitive (Sumai 188, 2021 **B**) were waterlogging and shading during anthesis. WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. Control is the treatment of spraying the same amount of distilled water; 15 mg·L^{-1}, 25 mg·L^{-1}, and 35 mg·L^{-1} are the concentrations of 6-BA sprayed.

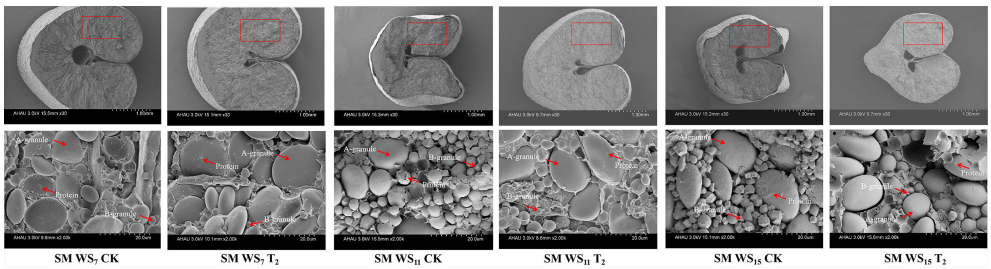

FIGURE 4
The effects of exogenous 6-BA on final starch grain morphology under waterlogging and shading. SM, Sumai 188, 2021. WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. CK refers to the control sprayed with distilled water; T_2: the concentration of 6-BA sprayed is 25 mg·L^{-1}. The red rectangles in the graphs indicate the range of areas to be observed.

3.2 Effects of spraying 6-BA after WS on photosynthetic parameters of the flag leaves

As shown in Figure 5A, the P_n of the flag leaves continued to decrease with growth after anthesis, and this decrease increased with increased WS. The P_n of the flag leaves increased significantly after the application of the 6-BA solution ($p< 0.05$), and was highest at a concentration of 25 mg·L^{-1} of 6-BA, with significant differences compared with the remaining two treatments ($p< 0.05$). Taking Sumai 188 after 20 days after anthesis as an example, the P_n of the flag leaves increased by 19.74%, 34.13%, and 25.97% compared with the control under WS treatment for 7 days after spraying 15, 25, and 35 mg·L^{-1} of the 6-BA solution, respectively. Meanwhile, after 11 days under WS, increases of 28.57%, 37.66%, and 34.38% were observed, while after 15 days, increases of 22.80%, 50.83%, and 36.60% were noted, respectively.

As shown in Figures 5B, C the changes in G_s and T_r in the control flag leaves after anthesis were consistent with those of P_n, with a continual decrease with growth, and WS. In contrast, compared with the control, both G_s and T_r increased significantly after the application of 6-BA ($p< 0.05$). Meanwhile, as shown in Figure 5D, the C_i of the control flag leaves after anthesis increased gradually with growth and WS. In comparison, the application of 6-BA caused a significant decrease in the C_i of the flag leaves ($p< 0.05$).

3.3 Effect of spraying 6-BA after WS on the $\delta^{13}C$ of the flag leaves and mature grains

As shown in Figure 6, the $\delta^{13}C$ of the flag leaves increased significantly by 8.67% compared with the control after spraying 25 mg·L^{-1} of the 6-BA solution for 24 h, while that of the mature grains increased significantly by 10.77% ($p< 0.05$). The transport rate of $\delta^{13}C$ from the flag leaves to the grains was 92.02% in the control group, while that spraying 25 mg·L^{-1} of the 6-BA solution was 94.18%. Overall, after application of each concentration of 6-BA, the transport of assimilates from the flag leaves to the grains was significantly improved.

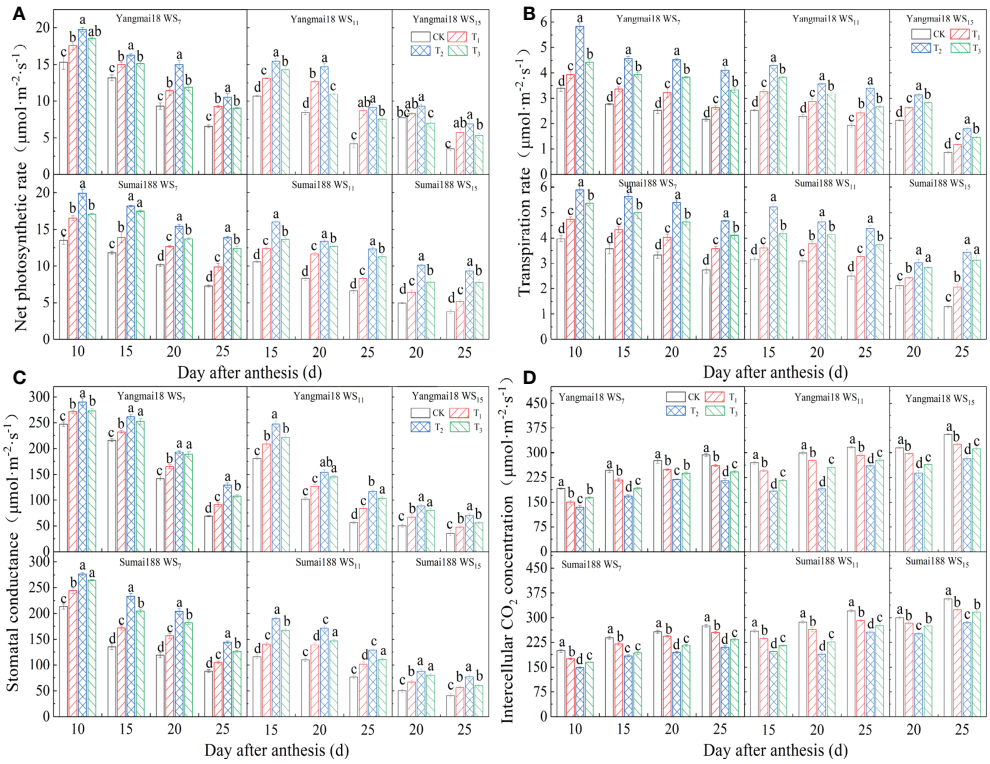

FIGURE 5
The effect of exogenous 6-BA on Pn **(A)**, Tr **(B)**, Gs **(C)**, Ci **(D)** of wheat under waterlogging and shading in 2021. WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. CK refers to the control sprayed with distilled water. T_1, T_2, and T_3 refer to the concentrations of 6-BA sprayed (15, 25, and 35 mg·L^{-1}). Each value is expressed as mean ± SE (n = 3). Different lowercase letters embedded at the top of the histogram express significant differences between treatments ($p< 0.05$).

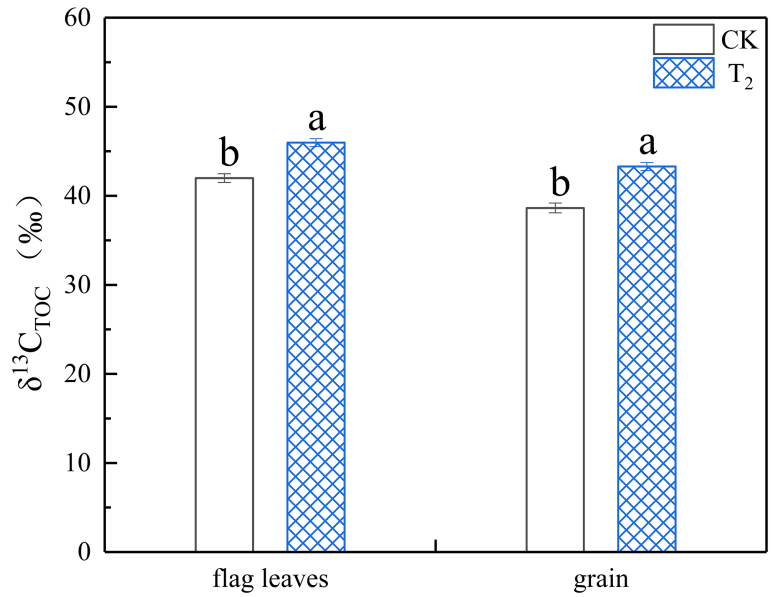

FIGURE 6
The effect of exogenous 6-BA on flag leaves and grain $\delta^{13}C$ of wheat under waterlogging and shading treatment of 11 days in 2021. CK refers to the control sprayed with distilled water. T_2: the concentration of 6-BA sprayed is 25 mg·L^{-1}. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. Each value is expressed as mean ± SE (n = 3). Different lowercase letters embedded at the top of the histogram express significant differences among treatments (p< 0.05).

3.4 Effect of spraying 6-BA after WS on the content of grain starch and its component

3.4.1 Starch content

As shown in Figure 7, the growth of the grain starch showed a rapid increase from 15 to 25 days after anthesis followed by gradual stabilization from 25 to 35 days. The longer the duration of WS, the lower the final accumulation of starch; a significant increase was observed following the application of 6-BA during the entire grain-filling process compared with the control (Figures 7A, B, p< 0.05). Moreover, starch accumulation was significantly higher after spraying 25 mg·L^{-1} of the 6-BA solution compared with the remaining two concentrations. Take the 2021 year results as an example, after WS for 15 days, the final accumulation of starch in the Sumai 188 grains increased by 3.85%, 10.24%, and 5.28% compared with the control after spraying 15, 25, and 35 mg·L^{-1} of the 6-BA solution, respectively.

3.4.2 Amylose and amylopectin content

As shown in Tables 2, 3, the amylose and amylopectin content gradually increased with wheat grain development; however, the longer the duration of WS, the lower the final accumulation. The different cultivars, treatment durations of WS, and spraying concentrations of 6-BA significantly affected the final amylose and amylopectin contents from wheat grains. Under the same treatment duration of WS, except at 10 days after anthesis, the content of amylose and amylopectin was improved remarkably following the application of 6-BA in contrast with the control treatment during the grouting period (p< 0.05), suggesting that 6-BA improved the accumulation of amylose and amylopectin in the wheat grains. As an example, after WS for 15 days, the final amylose content of the Sumai 188 grains increased by 3.44%, 6.60%, and 3.15% compared with the control after spraying 15, 25, and 35 mg·L^{-1} of the 6-BA solution, while the final accumulation of amylopectin increased by 3.95%, 11.15%, and 5.82%, respectively.

3.5 Effect of spraying 6-BA after WS on grain filling

A logistic equation served to fit the grain-filling, with the coefficient factor of each equation reaching a notable level (Table 4). The maximum theoretical 1000-kernel weight was higher after the application of 6-BA compared with the control, and the number of effective days of grain filling (D) was prolonged in both cultivars (except in Yangmai 18 after WS for 15 days and Sumai 188 after WS for 11 days in 2021, and after WS for 7 days during in 2022). Moreover, under the same treatment duration of WS, the average grain filling rate (V_{mean}) increased significantly after spraying the 6-BA solution, and the

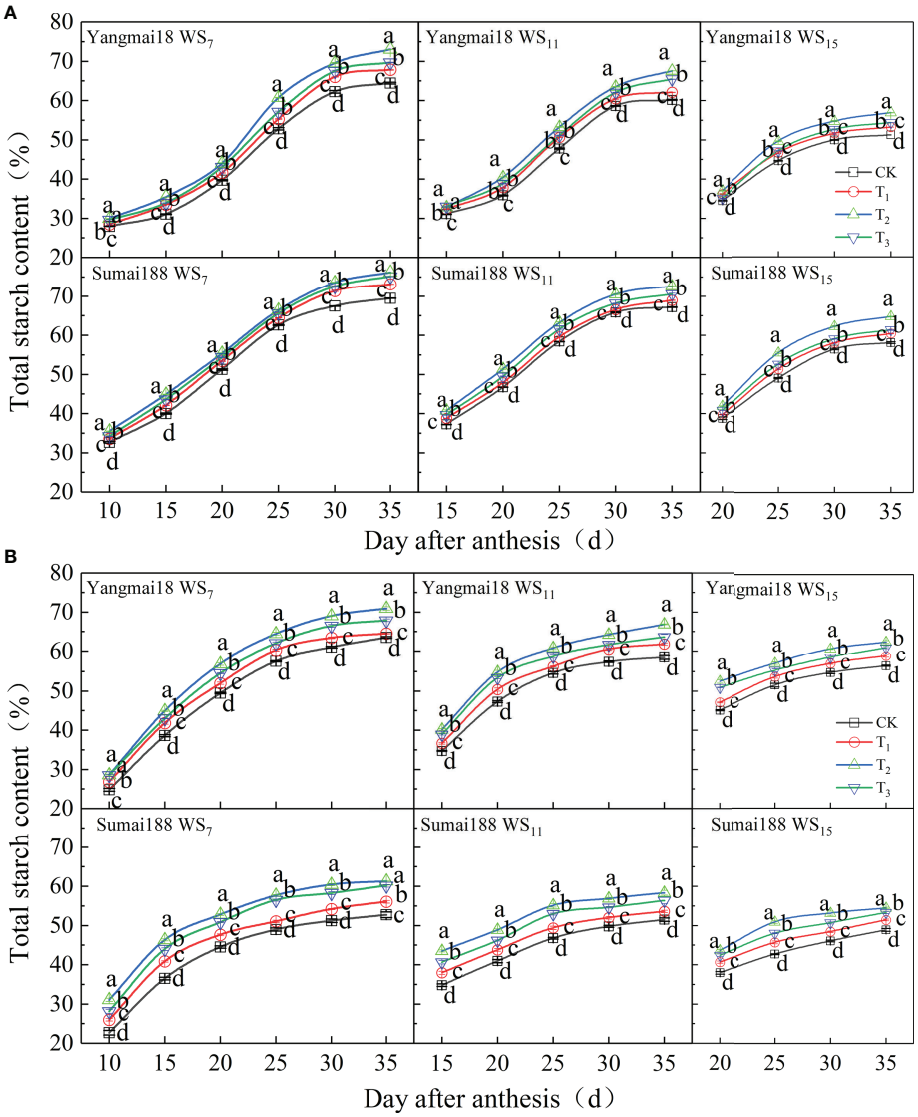

FIGURE 7
The effect of exogenous 6-BA on total starch accumulation of wheat under waterlogging and shading in 2021 **(A)** and 2022 **(B)**. WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. CK refers to the control sprayed with distilled water. T_1, T_2, and T_3 refer to the concentration of 6-BA sprayed (15, 25, and 35 mg·L^{-1}). Each value is expressed as mean ± SE (n = 3). Different lowercase letters embedded in the linear graph express significant differences among treatments (p< 0.05).

longer the treatment duration of WS, the lower the average grain filling rate. Compared with the control, spraying the 6-BA solution increased the duration of gradual grain filling in Yangmai 18 following 7 days of WS in 2021, while the rapid and slow grain filling periods increased following 11 and 15 days of WS in 2021 and 2022. Of the three concentrations of 6-BA, spraying 25 mg·L^{-1} of the 6-BA solution had the greatest effect. Take the 2021 results as an example; in Yangmai 18, spraying 25 mg·L^{-1} of the 6-BA solution following 7, 11, and 15 days of WS resulted in an increase in V_{mean} by 6.27%, 5.59%, and 21.20% compared with the control, while in Sumai 188, increases of 7.84%, 5.08%, and 15.11% were observed, respectively.

3.6 Effect of spraying 6-BA after WS on yield and contributing factors

As shown in Table 5, the longer the duration of WS, the lower the number of grains per spike, the thousand-grain weight, and the yield. The different cultivars, treatment durations of WS, and

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TABLE 2 The effect of exogenous 6-BA on amylose accumulation of wheat under waterlogging and shading in 2021.

Cultivar	Treatment		Amylose content (%)					
			10DAA	15DAA	20DAA	25DAA	30DAA	35DAA
Yangmai 18	WS_7	CK	1.14a ± 0.06	2.46c ± 0.06	4.03c ± 0.04	7.76d ± 0.06	11.93c ± 0.14	13.50c ± 0.09
		T_1	1.20a ± 0.05	2.83b ± 0.09	4.30c ± 0.05	8.30c ± 0.03	12.94b ± 0.11	14.00bc ± 0.08
		T_2	1.22a ± 0.12	3.25a ± 0.06	5.16a ± 0.17	9.34a ± 0.05	14.21a ± 0.35	15.47a ± 0.33
		T_3	1.17a ± 0.05	3.01ab ± 0.08	4.67b ± 0.07	8.56b ± 0.10	12.96b ± 0.30	14.30b ± 0.07
	WS_{11}	CK	—	2.13c ± 0.08	3.50d ± 0.11	6.46d ± 0.12	11.12c ± 0.07	12.11d ± 0.07
		T_1	—	2.47b ± 0.04	4.02c ± 0.08	7.30c ± 0.17	11.93b ± 0.06	12.76c ± 0.09
		T_2	—	2.83a ± 0.06	4.85a ± 0.03	8.47a ± 0.11	13.07a ± 0.07	14.43a ± 0.11
		T_3	—	2.64b ± 0.04	4.36b ± 0.09	7.91b ± 0.10	12.16b ± 0.09	13.60b ± 0.02
	WS_{15}	CK	—	—	3.01d ± 0.07	6.06d ± 0.09	10.61d ± 0.06	11.45c ± 0.08
		T_1	—	—	3.65c ± 0.08	7.07c ± 0.10	11.23c ± 0.10	11.99b ± 0.10
		T_2	—	—	4.57a ± 0.11	8.02a ± 0.10	12.28a ± 0.04	13.11a ± 0.09
		T_3	—	—	3.92b ± 0.06	7.50b ± 0.05	11.69b ± 0.07	12.21b ± 0.09
Sumai 188	WS_7	CK	1.22a ± 0.09	2.78c ± 0.10	6.31d ± 0.07	10.95d ± 0.08	13.49d ± 0.06	13.98c ± 0.09
		T_1	1.15a ± 0.03	3.21b ± 0.04	6.61c ± 0.05	11.34c ± 0.04	14.11c ± 0.06	14.84b ± 0.06
		T_2	1.29a ± 0.05	3.80a ± 0.04	7.37a ± 0.05	12.24a ± 0.14	15.09a ± 0.04	15.49a ± 0.08
		T_3	1.20a ± 0.03	3.41b ± 0.06	6.93b ± 0.04	11.64b ± 0.07	14.51b ± 0.09	14.95b ± 0.06
	WS_{11}	CK	—	2.61d ± 0.06	5.89d ± 0.07	10.33d ± 0.05	13.22d ± 0.03	13.52d ± 0.09
		T_1	—	2.96c ± 0.04	6.25c ± 0.03	11.30b ± 0.06	13.42c ± 0.03	13.88c ± 0.02
		T_2	—	3.40a ± 0.07	6.83a ± 0.04	11.85a ± 0.04	14.47a ± 0.05	14.77a ± 0.02
		T_3	—	3.14b ± 0.01	6.57b ± 0.04	10.87c ± 0.05	13.95b ± 0.04	14.25b ± 0.07
	WS_{15}	CK	—	—	5.58d ± 0.05	8.26c ± 0.12	11.55c ± 0.07	12.06d ± 0.03
		T_1	—	—	5.82c ± 0.03	8.69b ± 0.08	12.03b ± 0.08	12.49c ± 0.07
		T_2	—	—	6.79a ± 0.02	9.49a ± 0.11	12.45a ± 0.05	13.31a ± 0.03
		T_3	—	—	6.36b ± 0.03	9.19a ± 0.06	12.07b ± 0.02	12.83b ± 0.08

Significant level	10DAA	15DAA	20DAA	25DAA	30DAA	35DAA
Cultivar (C)	ns	**	**	**	**	**
Treatment duration (D)	—	**	**	**	**	**
Concentrations of 6-BA (T)	ns	**	**	**	**	**
C×D	—	**	**	**	**	**
C×T	ns	**	ns	**	*	**
D×T	—	**	ns	**	**	**
C×D×T	—	ns	ns	**	*	**

WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. CK refers to the control sprayed with distilled water, T_1, T_2, T_3 refer to 6-BA sprayed at a concentration of 15, 25, and 35 mg·L^{-1}, respectively. DAA represents days after anthesis. — represents not sampling. Each value is expressed as mean ± SE (n = 3). Different letters labeled after the value among the treatments with different concentrations of 6-BA refer to significant differences at $p< 0.05$. ** represents significant differences at the 1% level. * represents significant differences at the 5% level. ns represents no significant difference.

spraying concentrations of 6-BA significantly affected the thousand-grain weight and yield from wheat. Moreover, under the same treatment duration of WS, compared with the control, the application of 6-BA after WS caused a remarkable improvement in the thousand-grain weight and yield in both varieties ($p< 0.05$); however, there were little difference in the spike numbers and kernel numbers per spike. Of the three 6-BA concentrations, spraying 25 mg·L^{-1} of the 6-BA solution had the greatest effect on the thousand-grain weight and yield. Take the results of the wheat growing season from 2020 to 2021 as an example; in Yangmai18, spraying 25 mg·L^{-1} of the 6-BA solution following 7, 11, and 15 days of WS caused an increase in the thousand-grain weight by 5.06%, 7.97%, and 30.52%, and an increase in yield of 9.43%, 18.23%, and 28.15% compared with the control, respectively. Meanwhile, in Sumai 188, the thousand-grain weight increased by 11.21%, 7.23%, and 17.73%, while yield increased by 15.64%, 8.93%, and 24.89%, respectively. Taken together, these findings suggest that the application of 6-BA had the greatest influence on yield after 15 days of WS.

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TABLE 3 The effect of exogenous 6-BA on amylopectin accumulation of wheat under waterlogging and shading in 2021.

Cultivar	Treatment		Amylopectin content (%)					
			10DAA	15DAA	20DAA	25DAA	30DAA	35DAA
Yangma 18	WS_7	CK	13.07a ± 0.33	28.39b ± 0.99	35.59c ± 0.67	45.22d ± 0.27	50.29c ± 0.48	50.92c ± 0.23
		T_1	13.25a ± 0.48	30.43ab ± 0.27	37.40b ± 0.45	47.18c ± 0.12	52.42b ± 0.77	53.85b ± 0.15
		T_2	13.96a ± 0.34	32.26a ± 0.72	40.58a ± 0.22	51.62a ± 0.05	55.99a ± 0.49	57.87a ± 0.65
		T_3	13.88a ± 0.36	31.04a ± 0.71	38.53b ± 0.35	48.73b ± 0.30	54.51a ± 0.35	55.04b ± 0.28
	WS_{11}	CK	—	22.41c ± 0.38	32.58c ± 0.42	42.76d ± 0.29	32.58c ± 0.42	48.84d ± 0.42
		T_1	—	24.35b ± 0.27	34.31bc ± 0.60	44.88c ± 0.40	34.31bc ± 0.60	50.77c ± 0.59
		T_2	—	26.09a ± 0.16	37.50a ± 0.37	48.65a ± 0.50	37.50a ± 0.37	55.08a ± 0.33
		T_3	—	24.46b ± 0.59	35.02b ± 0.72	46.37b ± 0.41	35.02b ± 0.72	53.28b ± 0.14
	WS_{15}	CK	—	—	28.18c ± 0.52	38.82d ± 0.40	42.71d ± 0.33	44.91c ± 0.24
		T_1	—	—	31.05b ± 0.61	40.19c ± 0.37	46.67c ± 0.40	47.92b ± 0.40
		T_2	—	—	33.48a ± 0.09	44.96a ± 0.38	51.25a ± 0.56	51.39a ± 0.51
		T_3	—	—	31.20b ± 0.08	42.15b ± 0.45	48.64b ± 0.56	49.02b ± 0.46
Sumai 188	WS_7	CK	14.37c ± 0.21	27.84d ± 0.35	42.59c ± 0.50	50.23d ± 0.34	53.52c ± 0.47	53.33d ± 0.35
		T_1	15.18bc ± 0.20	29.81c ± 0.29	44.07bc ± 0.83	52.66c ± 0.47	56.78b ± 0.60	56.79c ± 0.39
		T_2	16.14a ± 0.36	33.44a ± 0.29	46.37a ± 0.52	55.12a ± 0.14	58.47a ± 0.15	60.55a ± 0.13
		T_3	15.61ab ± 0.24	32.08b ± 0.18	44.94ab ± 0.47	54.04b ± 0.25	57.52ab ± 0.38	59.33b ± 0.39
	WS_{11}	CK	—	26.70c ± 0.31	39.73d ± 0.23	46.90b ± 0.34	51.15c ± 0.24	50.15d ± 0.23
		T_1	—	28.73b ± 0.41	41.49c ± 0.37	47.65b ± 0.15	52.22bc ± 0.48	54.15c ± 0.28
		T_2	—	30.53a ± 0.26	44.38a ± 0.35	52.00a ± 0.33	56.48a ± 0.42	59.00a ± 0.17
		T_3	—	28.91b ± 0.18	42.76b ± 0.28	50.04a ± 0.52	53.32b ± 0.23	55.33b ± 0.22
	WS_{15}	CK	—	—	33.01d ± 0.37	40.76c ± 0.36	45.25d ± 0.36	43.87d ± 0.20
		T_1	—	—	34.04c ± 0.18	42.66b ± 0.36	46.94c ± 0.33	48.30c ± 0.25
		T_2	—	—	37.34a ± 0.29	45.98a ± 0.15	51.43a ± 0.44	51.82a ± 0.35
		T_3	—	—	35.82b ± 0.27	43.34b ± 0.23	49.15b ± 0.29	49.71b ± 0.40

Significant level	10DAA	15DAA	20DAA	25DAA	30DAA	35DAA
Cultivar (C)	**	**	**	**	**	**
Treatment duration (D)	—	**	**	**	**	**
Concentrations of 6-BA (T)	**	**	**	**	**	**
C×D	—	**	**	**	**	**
C×T	*	**	**	**	ns	**
D×T	—	**	**	**	**	**
C×D×T	—	**	**	**	**	**

WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. CK refers to the control sprayed with distilled water, T_1, T_2, and T_3 refer to 6-BA sprayed at a concentration of 15, 25, and 35 mg·L^{-1}, respectively. DAA represents days after anthesis. — represents not sampling. Each value is expressed as mean ± SE (n = 3). Different letters labeled after the value among the treatments with different concentrations of 6-BA refer to significant differences at $p< 0.05$. ** represents significant differences at the 1% level. * represents significant differences at the 5% level. ns represents no significant difference.

4 Discussion

4.1 Effects of spraying 6-BA after WS on photosynthetic parameters and photosynthetic product transport

Studies have shown that improvements in the photosynthetic area of functional leaves, the photosynthetic duration, and the photosynthetic capacity can improve the grain yield of crops (Parry et al., 2011). Studies have shown that waterlogging accelerates the leaf senescence in wheat, especially under soil compaction, thereby significantly reducing leaf SPAD values as well as P_n, G_s, and T_r, which affects the overall photosynthetic capacity (Wu W. et al., 2018b; Wu X. et al., 2018a). Similarly, insufficient light is also known to limit photosynthesis in wheat (Shimoda and Sugikawa, 2020). This study also found that the P_n of

TABLE 4 The effect of exogenous 6-BA on the grout characteristic of wheat grain under waterlogging and shading.

Year	Cultivar	Treatment		Model	R^2	D (d)	D_1 (d)	D_2 (d)	D_3 (d)	V_{mean} ($g \cdot d^{-1}$)	V_{max} ($g \cdot d^{-1}$)	T_{max} (d)
2021	Yangmai 18	WS_7	CK	Y=40.8284/(1+exp(4.0300-0.209079t))	0.9959	29.7841	12.9760	12.5981	4.2100	1.3708	2.1341	19.2750
			T_1	Y=42.1515/(1+exp(3.8009-0.198696t))	0.9970	30.1874	12.5010	13.2564	4.4300	1.3963	2.0938	19.1292
			T_2	Y=43.7910/(1+exp(4.2770-0.215386t))	0.9936	30.0587	13.7428	12.2292	4.0867	1.4568	2.3580	19.8574
			T_3	Y=43.0673/(1+exp(4.2196-0.209505t))	0.9940	30.6285	13.8546	12.5725	4.2014	1.4061	2.2557	20.1408
		WS_{11}	CK	Y=39.6341/(1+exp(4.5285-0.214369t))	0.9951	31.3745	14.9812	12.2872	4.1061	1.2633	2.1241	21.1248
			T_1	Y=40.5208/(1+exp(4.3343-0.207992t))	0.9979	31.4028	14.5068	12.6639	4.2320	1.2904	2.1070	20.8388
			T_2	Y=43.2746/(1+exp(3.7295-0.179306t))	0.9971	33.0723	13.4622	14.6983	4.9118	1.3085	1.9388	20.8114
			T_3	Y=41.2151/(1+exp(4.0977-0.198904t))	0.9961	31.6481	13.9801	13.2426	4.4254	1.3023	2.0495	20.6014
		WS_{15}	CK	Y=24.6955/(1+exp(6.1919-0.324780t))	0.9997	25.8302	15.0099	8.1101	2.7102	0.9561	2.0052	19.0649
			T_1	Y=27.3199/(1+exp(6.4432-0.335064t))	0.9995	25.7874	15.2992	7.8612	2.6270	1.0594	2.2885	19.2298
			T_2	Y=30.4738/(1+exp(6.1855-0.318768t))	0.9999	26.2973	15.2729	8.2631	2.7613	1.1588	2.4285	19.4044
			T_3	Y=27.8256/(1+exp(4.8744-0.258264t))	0.9994	27.3814	13.7743	10.1989	3.4082	1.0162	1.7966	18.8737
	Sumai 188	WS_7	CK	Y=37.0025/(1+exp(4.4644-0.244162t))	0.9951	27.2836	12.8906	10.7879	3.6051	1.3562	2.2587	18.2846
			T_1	Y=38.5689/(1+exp(4.2625-0.233686t))	0.9935	27.6428	12.6045	11.2715	3.7667	1.3953	2.2533	18.2403
			T_2	Y=41.0969/(1+exp(4.2318-0.230217t))	0.9936	27.9259	12.6611	11.4414	3.8235	1.4716	2.3653	18.3818
			T_3	Y=38.9439/(1+exp(4.2545-0.234300t))	0.9952	27.5362	12.5373	11.2420	3.7568	1.4143	2.2811	18.1583
		WS_{11}	CK	Y=34.9371/(1+exp(4.9973-0.262400t))	0.9935	27.4182	14.0255	10.0381	3.3545	1.2742	2.2919	19.0446
			T_1	Y=35.7117/(1+exp(5.2951-0.284353t))	0.9902	26.3487	13.9900	9.2631	3.0955	1.3554	2.5387	18.6216
			T_2	Y=36.2078/(1+exp(4.9271-0.264143t))	0.9914	26.9715	13.6672	9.9719	3.3324	1.3424	2.3910	18.6532
			T_3	Y=36.3340/(1+exp(4.8815-0.260203t))	0.9931	27.2046	13.6989	10.1229	3.3828	1.3356	2.3636	18.7604
		WS_{15}	CK	Y=25.2967/(1+exp(3.6929-0.199288t))	0.9974	29.5558	11.9219	13.2171	4.4168	0.8559	1.2603	18.5305
			T_1	Y=29.2296/(1+exp(3.5198-0.181609t))	0.9999	31.4799	12.1294	14.5037	4.8468	0.9285	1.3271	19.3812
			T_2	Y=29.8813/(1+exp(3.8062-0.202551t))	0.9995	29.6391	12.2893	13.0041	4.3457	1.0082	1.5131	18.7913
			T_3	Y=29.3759/(1+exp(3.9273-0.205097t))	0.9995	29.8616	12.7271	12.8427	4.2917	0.9837	1.5062	19.1485
2022	Yangmai 18	WS_7	CK	Y=46.5062/(1+exp(3.6992-0.191187t))	0.9988	30.8411	12.4601	13.7771	4.6040	1.5079	2.2228	19.3486
			T_1	Y=51.0984/(1+exp(3.5655-0.181740t))	0.9985	31.7086	12.3721	14.4932	4.8433	1.6115	2.3217	19.6187

(Continued)

TABLE 4 Continued

Year	Cultivar	Treatment	Model	R^2	D (d)	D_1 (d)	D_2 (d)	D_3 (d)	V_{mean} ($g \cdot d^{-1}$)	V_{max} ($g \cdot d^{-1}$)	T_{max} (d)
		T_2	Y=50.8789/(1+exp(3.6187-0.196395t))	0.9987	29.6134	11.7197	13.4117	4.4819	1.7181	2.4981	18.4256
		T_3	Y=51.1861/(1+exp(3.5978-0.189887t))	0.9992	30.5183	12.0114	13.8714	4.6355	1.6772	2.4299	18.9471
	WS_{11}	CK	Y=31.5899/(1+exp(4.6557-0.276685t))	0.9973	24.7680	12.0668	9.5199	3.1813	1.2754	2.1851	16.8267
		T_1	Y=35.8646/(1+exp(4.0393-0.231770t))	0.9912	26.9082	11.7457	11.3647	3.7978	1.3328	2.0781	17.4281
		T_2	Y=38.8952/(1+exp(3.8009-0.222926t))	0.9920	26.9063	11.1423	11.8156	3.9485	1.4456	2.1677	17.0501
		T_3	Y=36.6372/(1+exp(3.9614-0.235234t))	0.9911	26.1808	11.2416	11.1974	3.7419	1.3994	2.1546	16.8403
	WS_{15}	CK	Y=23.9258/(1+exp(3.5457-0.238614t))	0.9999	24.0678	9.3402	11.0387	3.6889	0.9941	1.4273	14.8596
		T_1	Y=26.3812/(1+exp(1.8507-0.153667t))	0.9985	26.3422	3.4731	17.1410	5.7281	1.0015	1.0135	12.0436
		T_2	Y=35.5361/(1+exp(2.3404-0.155732t))	0.9999	29.1374	6.5715	16.9137	5.6522	1.2196	1.3835	15.0284
		T_3	Y=32.4776/(1+exp(1.9379-0.137511t))	0.9999	30.0712	4.5153	19.1548	6.4011	1.0800	1.1165	14.0927
Sumai 188	WS_7	CK	Y=46.9604/(1+exp(3.7339-0.189572t))	0.9981	31.2869	12.7492	13.8945	4.6432	1.5010	2.2256	19.6965
		T_1	Y=47.6664/(1+exp(3.5938-0.189730t))	0.9983	30.5225	12.0002	13.8829	4.6394	1.5617	2.2609	18.9417
		T_2	Y=48.3420/(1+exp(3.7755-0.210788t))	0.9978	28.3352	11.6634	12.4960	4.1759	1.7061	2.5475	17.9114
		T_3	Y=47.4846/(1+exp(3.6954-0.207033t))	0.9962	28.4622	11.4880	12.7226	4.2516	1.6683	2.4577	17.8493
	WS_{11}	CK	Y=30.2138/(1+exp(4.0763-0.237924t))	0.9947	26.3678	11.5974	11.0708	3.6996	1.1459	1.7971	17.1328
		T_1	Y=32.5974/(1+exp(3.7223-0.219752t))	0.9930	26.9373	10.9455	11.9862	4.0055	1.2101	1.7908	16.9386
		T_2	Y=35.1059/(1+exp(3.5807-0.218164t))	0.9954	26.2929	10.3012	11.9862	4.0055	1.3352	1.9286	16.2943
		T_3	Y=34.5883/(1+exp(3.5672-0.211169t))	0.9965	27.2977	10.6559	12.4734	4.1683	1.2671	1.8260	16.8926
	WS_{15}	CK	Y=21.7192/(1+exp(2.9027-0.164092t))	0.9994	31.0797	9.6635	16.0520	5.3642	0.6988	0.8910	17.6895
		T_1	Y=26.4782/(1+exp(2.6624-0.134967t))	0.9992	36.0060	9.9684	19.5159	6.5218	0.7354	0.8934	19.7263
		T_2	Y=29.4422/(1+exp(2.4965-0.134233t))	0.9997	34.9670	8.7870	19.6226	6.5574	0.8420	0.9880	18.5983
		T_3	Y=27.9205/(1+exp(2.3131-0.126460t))	0.9999	35.6660	7.8768	20.8287	6.9605	0.7828	0.8827	18.2912

WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. CK refers to the control sprayed with distilled water, T_1, T_2, and T_3 refer to 6-BA sprayed at a concentration of 15, 25, and 35 $mg \cdot L^{-1}$, respectively. Y is the 1000-grain weight of the observed grain. t is the number of days from anthesis until observation. D is the effective days of grouting. D_1, D_2, and D_3 are the duration of grain filling increasing stage, rapid increasing stage, and slow increasing stage respectively. V_{mean} is the average grouting rate, V_{max} is the maximum grouting rate, T_{max} is the occurrence time of the maximum grouting rate. Each value is expressed as mean ± SE (n = 3).

wheat flag leaves decreased rapidly with the increase of WS treatment duration, especially after 15 days of WS treatment. However, the self-regulation effect of wheat can only resist the damage caused by WS stress in a short time (Liu et al., 2007), thus taking flag leaf photosynthesis at a low level under the 15 days of WS. Meanwhile, studies have also shown that spraying the 6-BA solution before waterlogging can increase the photosynthetic rate of the flag leaves, slowing down the rate of plant senescence, and

TABLE 5 The effect of exogenous 6-BA on wheat yield and its components under waterlogging and shading.

Cultivar	Treatment		2020–2021				2021–2022			
			Spikes(10^4 per ha)	Kernel numbersper spike	Thousand grainweight (g)	Grain yield ($kg{\cdot}ha^{-1}$)	Spikes(10^4 per ha)	Kernel numbersper spike	Thousand grainweight (g)	Grain yield ($kg{\cdot}ha^{-1}$)
Yangmai 18	WS_7	CK	440.22a ± 15.28	39.67a ± 1.20	40.48c ± 0.44	7053.76c ± 55.74	483.33a ± 14.53	42.00a ± 1.53	44.00c ± 0.21	8935.57b ± 467.73
		T_1	440.22a ± 5.78	41.33a ± 0.88	40.78bc ± 0.33	7415.81b ± 26.06	486.67a ± 6.67	42.00a ± 1.53	48.07b ± 0.07	9828.73ab ± 426.56
		T_2	450.23a ± 5.78	40.33a ± 0.88	42.53a ± 0.16	7719.05a ± 58.21	506.67a ± 8.82	40.67a ± 0.88	49.57a ± 0.38	10206.91a ± 160.61
		T_3	443.56a ± 3.34	40.67a ± 0.67	41.72ab ± 0.06	7523.59ab ± 95.60	483.33a ± 14.53	42.33a ± 0.33	49.27a ± 0.24	10074.29ab ± 194.10
	WS_{11}	CK	416.88a ± 6.67	39.33a ± 0.88	38.53c ± 0.40	6313.01c ± 26.72	493.33a ± 13.33	38.67a ± 1.20	31.87c ± 0.19	6070.35c ± 108.35
		T_1	433.55a ± 8.82	39.00a ± 0.58	39.27bc ± 0.34	6634.99b ± 44.12	500.00a ± 10.00	39.33a ± 0.88	36.13b ± 0.26	7102.46b ± 143.81
		T_2	460.23b ± 5.78	39.00a ± 0.58	41.60a ± 0.10	7463.93a ± 12.66	506.67a ± 8.82	40.33a ± 1.20	38.93a ± 0.24	7954.47a ± 254.27
		T_3	433.55a ± 8.82	40.33a ± 0.88	40.00b ± 0.18	6989.16ab ± 78.70	500.00a ± 5.77	40.33a ± 1.20	37.03b ± 0.46	7466.85ab ± 236.78
	WS_{15}	CK	450.23a ± 10.01	37.33a ± 0.88	25.03c ± 0.88	4198.41d ± 29.21	496.67a ± 18.56	36.00b ± 0.58	23.73d ± 0.15	4238.78c ± 103.67
		T_1	470.24a ± 5.77	36.33a ± 0.88	28.33b ± 0.92	4832.51b ± 48.82	570.00a ± 15.28	38.00ab ± 0.58	25.57c ± 0.03	5538.66b ± 186.98
		T_2	466.90a ± 12.02	35.33a ± 0.88	32.67a ± 0.65	5380.31a ± 33.70	533.33a ± 37.12	38.33a ± 0.67	34.00a ± 0.31	6962.96a ± 571.19
		T_3	453.57a ± 6.67	36.33a ± 0.33	28.42b ± 0.31	4681.22c ± 33.75	526.67a ± 39.30	37.00ab ± 0.58	30.73b ± 0.07	5990.28ab ± 469.63
Sumai 188	WS_7	CK	423.55a ± 8.82	36.33a ± 0.67	36.75c ± 0.21	5651.24c ± 47.26	496.67a ± 6.67	38.00a ± 0.58	43.93c ± 0.37	8287.86c ± 35.83
		T_1	440.22a ± 5.78	36.33a ± 0.33	38.45b ± 0.18	6148.49b ± 39.01	496.67a ± 6.67	39.33a ± 0.88	45.50b ± 0.17	8884.36b ± 128.31
		T_2	440.22a ± 5.78	36.33a ± 0.33	40.87a ± 0.25	6534.98a ± 52.86	503.33a ± 8.82	39.67a ± 0.33	46.80a ± 0.57	9339.07a ± 26.03
		T_3	440.22a ± 5.78	36.00a ± 0.58	38.38b ± 0.32	6080.64b ± 66.04	490.00a ± 5.77	39.67a ± 0.67	46.10ab ± 0.17	8958.93b ± 149.49
	WS_{11}	CK	426.88a ± 3.34	36.00a ± 0.58	35.28c ± 0.10	5421.71c ± 82.75	503.33a ± 3.33	40.33a ± 1.20	30.20c ± 0.15	6132.50c ± 212.73
		T_1	433.56a ± 8.82	36.67a ± 0.88	36.30b ± 0.10	5764.90ab ± 19.75	496.67a ± 6.67	39.67a ± 0.88	32.63b ± 0.27	6424.44bc ± 24.21
		T_2	430.22a ± 11.55	36.33a ± 0.88	37.83a ± 0.15	5905.69a ± 37.28	496.67a ± 8.82	41.00a ± 0.58	35.00a ± 0.46	7125.16a ± 144.63
		T_3	433.55a ± 6.67	36.00a ± 0.58	36.43b ± 0.13	5683.77b ± 35.63	493.33a ± 12.02	39.00a ± 1.00	34.37a ± 0.22	6604.31b ± 66.58
	WS_{15}	CK	420.21b ± 5.78	37.00b ± 0.58	24.53d ± 0.25	3812.93c ± 46.25	490.00a ± 5.77	32.67b ± 1.20	20.57d ± 0.35	3291.25c ± 128.13
		T_1	426.88a ± 3.34	38.67a ± 0.33	27.62c ± 0.21	4558.34b ± 60.82	500.00a ± 5.77	35.33a ± 0.33	23.43c ± 0.12	4140.52b ± 82.31
		T_2	430.21a ± 5.78	38.33ab ± 0.33	28.88a ± 0.07	4762.15a ± 23.67	496.67a ± 12.02	37.00a ± 0.58	26.47a ± 0.34	4859.88a ± 69.60
		T_3	433.55a ± 8.82	38.00ab ± 0.58	28.20b ± 0.09	4643.08ab ± 29.39	493.33a ± 6.67	36.00a ± 0.58	24.90b ± 0.06	4423.73b ± 122.92
Significance level			Spikes (10^4 per ha)	Kernel numbers per spike	Thousand grain weight (g)	Grain yield ($kg{\cdot}ha^{-1}$)	Spikes (10^4 per ha)	Kernel numbers per spike	Thousand grain weight (g)	Grain yield ($kg{\cdot}ha^{-1}$)
Cultivar (C)			**	**	**	**	ns	**	**	**
Treatment duration (D)			*	**	**	**	*	**	**	**
Concentrations of 6-BA (T)			**	ns	**	**	ns	*	**	**
C×D			**	**	**	**	*	**	**	**
C×T			ns	ns	ns	**	ns	ns	**	*
D×T			ns	ns	**	**	ns	ns	**	ns
C×D×T			ns	ns	**	**	ns	ns	**	ns

WS_7, WS_{11}, and WS_{15} respectively represent treatments under waterlogging and shading for 7, 11, and 15 days after anthesis. Shade nets with a shading rate of about 45% were used for shading, and waterlogging preserves about 2 cm of moisture on the surface of the soil. CK refers to the control sprayed with distilled water; T_1, T_2, and T_3 refer to 6-BA sprayed at a concentration of 15, 25, and 35 $mg{\cdot}L^{-1}$, respectively. Each value is expressed as mean ± SE (n = 3). Different letters labeled after the value among the treatments with different concentrations of 6-BA refer to significant differences at $p< 0.05$. ** represents significant differences at the 1% level. * represents significant differences at the 5% level. ns represents no significant difference.

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reducing yield losses (Wang X. et al., 2020a). Notably, maintaining the photosynthetic rate of flag leaves after anthesis was found to effectively increase biomass and yield (Luo et al., 2006; Chen et al., 2010). In this study, spraying the 6-BA solution after WS caused a significant increase in the P_n, G_s, and T_r of the flag leaves, and a reduction in the C_i. These findings suggest that the stomatal limitations of photosynthesis under WS was relieved by 6-BA, elevating the photosynthesis of wheat flag leaf. In line with this, maintaining a longer photosynthetically active period during grain filling, which allows the transfer of more assimilates into the grains, was previously found to improve resistance to abiotic stress after anthesis (Chen et al., 2010). Therefore, in this study, spraying 6-BA prolonged the photosynthetic activity period of wheat leaves after anthesis, especially under the treatment of WS for 7 days; and spraying 6-BA prolonged the duration of grain filling; the photosynthetic rate of flag leaves of wheat decreased slowly, which increased the amount of photosynthetic products produced by leaves into grains. Moreover, spraying 6-BA can alleviate the effects of waterlogging on the chloroplast and mitochondrial structure, thereby increasing the chlorophyll content and improving the photosynthetic performance of maize after waterlogging (Ren et al., 2017). These results confirmed that spraying 6-BA could improve the photosynthetic capacity of leaves.

After the wheat leaves absorbed $^{13}CO_2$, we found that the ability of flag leaves to assimilate CO_2 improved after spraying the 6-BA solution, and the accumulation of photosynthetic products in grains also increased. 6-BA improved the ability of the flag leaves to assimilate CO_2, thereby increasing the transportation rate of assimilates. This may be because exogenous 6-BA reduces the damage to the photosynthetic system, improving the assimilation efficiency of CO_2, which allows the generation of more carbohydrates, thus meeting the needs of plant growth (Ding et al., 2013). Therefore, the results of this study showed that spraying 6-BA could not only improve the photosynthetic capacity of flag leaves but also alleviate the transport process of carbohydrates generated by flag leaves to grains, and this process was also related to the transport of photosynthates in stem and stem sheath. It was found that 7 days after spraying the 6-BA solution, the contents of soluble sugar and starch in different organs of maize (leaves, sheaths, stems and young corn cobs) increased, and 6-BA could significantly improve the translocation rate of photosynthetic products from maize leaves to maize grains (Yang et al., 2019).

4.2 Effects of spraying 6-BA after WS on starch granules and starch content

Wheat grain starch exists as starch granules that differ in shape, size, composition, and properties (Shewry, 2010; Vamadevan, 2013; Guo et al., 2019; Yan et al., 2021). Studies have shown that WS reduces the number and volume of A-type starch granules (Li et al., 2020), with deformation of the granules under stress (Wang and Copeland, 2012). However, 6-BA can restore the adverse effects of ethephon on the number and size of starch granules and increase the storage capacity of grains (Liu et al., 2018). In this study, spraying 6-BA after WS could increase the volume of A-type starch granules and reduce the deformation of B-type starch granules, which indicated that spraying 6-BA can alleviate the formation and development of starch granules.

The enrichment process of wheat grains under WS is largely related to the accumulation of starch in the endosperm (Tomlinson and Denyer, 2003). The content and ratio of amylose to amylopectin has an effect on starch structure, gelatinization, and thermal properties (Wang H. et al., 2020b). 6-BA and ABA are the most potent phytohormones in terms of increased biomass and starch accumulation (Liu et al., 2019). In this study, in contrast with the control, the application of 6-BA significantly increased the starch content and starch accumulation rate in grains, and alleviated the effect of WS stress on starch accumulation. The enhanced photosynthetic capacity of wheat after spraying 6-BA increased the photosynthate produced by flag leaves, which improved the amount of carbohydrates transported to grains and resulted in the increase of starch accumulation. Moreover, the application of 6-BA was found to increase the content of starch in various organs, promoting the transport and subsequent accumulation of carbohydrates in the grains (Yang et al., 2019). The activity of starch-related enzymes is able to regulate the starch synthesis rate, thereby affecting carbohydrate synthesis (Ran et al., 2020; Wang et al., 2021); however, spraying the 6-BA solution can improve the activity of enzymes related to starch synthesis, which causes an increase in starch accumulation (Hsu et al., 2014; Luo et al., 2015; Wang et al., 2022).

4.3 Effects of spraying 6-BA after WS on grain filling and yield

The rate and duration of grain filling are the decisive factors of the final grain weight of wheat kernels (Duguid and Br Lé-Babel, 1994; Gelang et al., 2000). Previous studies revealed a significant increase in wheat grain weight and yield with increasing waterlogging (Arata et al., 2019; Ding et al., 2020), while shading treatment caused a significant drop in wheat grain yield (Yang et al., 2020). Meanwhile, studies have also shown that spraying the 6-BA solution before shading can delay the senescence process of flag leaves under shading treatment, improving dry matter accumulation and, ultimately, grain yield (Li et al., 2019). In this study, the application of 6-BA after WS stress could prolong the photosynthetic activity period of wheat leaves after anthesis, which is propitious to the accumulation and transport of photosynthetic products, and

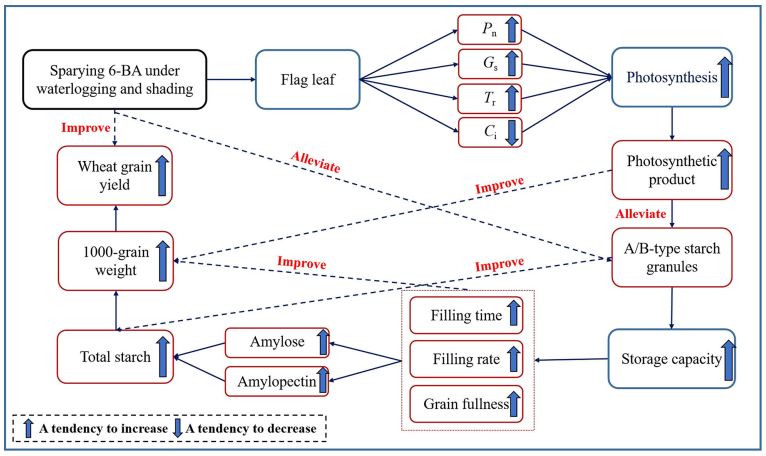

FIGURE 8
Analysis of the ways of spraying 6-BA to improve wheat yield under waterlogging and shading after anthesis.

thus increase grain weight and grain yield (Figure 8). These findings are related to the fact that exogenous 6-BA can delay leaf senescence, increase the chlorophyll content, and improve photosynthetic performance, all of which effectively enhance the grain-filling characteristics and photosynthesis of crops under waterlogging stress, ultimately having a significant effect on yield (Ren et al., 2016).

The grain filling process is directly affected by the storage capacity, and the number of starch granule can affect the grain-filling, and finally affect the dry matter accumulation in the grain, thereby affecting the yield (Tetlow and Emes, 2017; Zheng et al., 2017; Xie et al., 2018). In this study, spraying 6-BA can alleviate the adverse influence of WS on the formation and development of wheat starch granules, to further expand the storage capacity of wheat grains after WS and increase the duration and rate of grain filling and the fullness degree of grains. However, the duration and rate of grain filling were positively correlated with the grain weight (Dias and Lidon, 2009), so 6-BA application eventually improved wheat grain weight and yield (Figure 8).

5 Conclusions

Exogenous spraying of the 6-BA solution could improve photosynthesis in the flag leaves and alleviate the negative influence on starch granules, increase the content of starch and its components, and improve the duration and rate of grain filling, thus resulting in improvement of grain weight. In addition, under the conditions of this study, spraying 25 mg·L^{-1} of the 6-BA solution had an optimal effect with three different spray concentrations.

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

WZ and ZH designed the experiment. WZ and BW initiated statistical analysis and drafted the manuscript. BW, AZ, and QZ performed the experiments and determined related data. YL and LL contributed to the experiments proceeding and data interpretation. SM and YF assisted in polishing 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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WRKY transcription factors (TFs): Molecular switches to regulate drought, temperature, and salinity stresses in plants

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The WRKY transcription factor (TF) belongs to one of the major plant protein superfamilies. The WRKY TF gene family plays an important role in the regulation of transcriptional reprogramming associated with plant stress responses. Change in the expression patterns of WRKY genes or the modifications in their action; participate in the elaboration of numerous signaling pathways and regulatory networks. WRKY proteins contribute to plant growth, for example, gamete formation, seed germination, post-germination growth, stem elongation, root hair growth, leaf senescence, flowering time, and plant height. Moreover, they play a key role in many types of environmental signals, including drought, temperature, salinity, cold, and biotic stresses. This review summarizes the current progress made in unraveling the functions of numerous WRKY TFs under drought, salinity, temperature, and cold stresses as well as their role in plant growth and development.

KEYWORDS

WRKY TFs, drought-stress, salinity-stress, temperature-stress, cold-stress, plant development and growth, plants/crops

Introduction

The WRKY family is a group of transcription factors (TFs) that are widely distributed in plants and play important roles in plant growth and development, and biotic and abiotic stress management. The increased exposure in plants to various stresses, such as extreme temperatures, drought, and salinity is a global threat to key crops which significantly affect plant/crop growth and productivity. Many TF genes help plants withstand to adverse conditions and remain potential genomic candidates for widespread use in crop breeding. WRKY TFs represent important molecular switches that evaluate plant development processes and are involved in regulating responses to various stresses. Under stress conditions, plants can initiate a variety of changes at the molecular, cellular, and physiological levels, including stomatal closure, reduced photosynthesis, higher osmolality accumulation, and induction of many stress response genes (Shinozaki and Yamaguchi-Shinozaki, 2007; Masclaux-Daubresse et al., 2010; Kapoor et al., 2020). Genetic engineering is considered an alternative to increasing stress tolerance and has made significant contributions to changing the agronomic properties of crops. Many genes encoding functional proteins, TFs, and proteins involved in signal transduction pathways have been identified as genes responding to abiotic stresses (Turan et al., 2012; Rashid et al., 2020; Cohen et al., 2021). Many TF families, such as WRKY, AP2 (APETLA2)/ERF (ethylene responsive factor), and NAC (NAM, ATAF1/3, and CUC1/2), are unique to plants and have important and specific functions (Jiang et al., 2017).

TABLE 1 Number of WRKY TFs genes in plants.

S. No	Name of plant	Number of WRKY TF gene
1	*A. thaliana*	74
2	*B. distachyon*	81
3	*C. sinensis*	51
4	*C. clementina*	48
5	*D. carota*	38
6	*G. max*	179
7	*J. curcas*	58
8	*M. esculenta*	117
9	*M. domestica*	123
10	*M. notabilis*	54
11	*O. sativa Indica*	116
12	*O. sativa japonica*	137
13	*P. vulgaris*	88
14	*P. trichocarpa*	119
15	*S. lycopersicum*	79
16	*S. tuberosum*	82
17	*V. vinifera*	98
18	*Z. mays*	180

Structural features and homology of the WRKY TFs

WRKY protein have the unaltered sequence *WRKYGQK* (hence called WRKY) and a 60 amino acid DNA binding domain comprising a zinc finger-like domain (CX7CX23HXC or CX4-5CX22-23HXH) (Rushton et al., 1996; Finatto et al., 2018). WRKY TFs are classified into different groups; several WRKY proteins are placed in group I, containing two WRKY domains. WRKY proteins comprising one WRKY domain and a Cys2-His2 zinc finger motif are placed in group II. Furthermore, based on additional structural motifs maintained outside the WRKY domain, group II is subdivided into five subgroups (group IIa, group IIb, group IIc, group IId, and group IIe). Group III proteins represent WRKY domains with different zinc finger motifs (Cys2-His/Cys Cys-His2) (Eulgem et al., 2000; Finatto et al., 2018). The genomes of various plants have sequenced—presenting important knowledge about WRKY TFs and revealed that the WRKY TF family consists of a large number of genes (Zhang et al., 2011b; Xiong et al., 2013; Ayadi et al., 2016; Li et al., 2016a; Mohanta et al., 2016; Liu et al., 2017; Finatto et al., 2018) (Table 1). Plant-specific WRKY TFs, a major family of TFs, are a class of DNA-binding proteins found primarily in plants that have a variety of roles in plant processes, including growth, development, and stress signaling through autonomic and cross-regulation with TF and various other genes (Bakshi and Oelmüller, 2014). The first member of WRKY SPF1 superfamily was isolated from the sweet potato (*Ipomoea batatas*) (Ishiguro and Nakamura, 1994). In general, WRKY TF is expected to function as a key regulatory protein through precise binding to the W-box (TTGAC (C/T)) that regulates gene expression (Chi et al., 2013).

The coding sequence (CDS) of each WRKY gene was obtained from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) to build the phylogenetic tree using MEGA X and 1000 BS. It was shown in (Figure 1) that each homolog of WRKY genes showed the closest similarity, such as *AtWRKY53* with *TcWRKY53*, *AtWRKY46* with *BrWRKY46*, and *AtWRKY70*, *BrWRKY70*, *MfWRKY70* with *TaWRKY70*. As it was mentioned before that, *AtWRKY53* expression was induced by drought stress (Jiang et al., 2012). In contrast, *TcWRKY53* was induced by cold stress (Wei et al., 2008), illustrating that these two WRKY genes have a different role under different abiotic stress and species as well. It was also assumed that each WRKY gene might also contribute to multiple abiotic stresses.

Drought stress-related WRKY TFs

The expression of WRKY TF is induced when plants are exposed to various stresses or defense signals, including salicylic

acid (SA) or other molecules. In addition to the fact that WRKY TF expression is rapid, transient, and tissue-specific, WRKY proteins also play diverse functions in plant defenses against different stresses including drought, plant growth, development, metabolism, trichome and embryonic morphogenesis, senescence, biosynthesis and regulation of hormonal signals (Wei et al., 2017) (Figure 2). The WRKY TFs present important roles in response and adaptation to drought stress (Table 2). Overexpression of *AtWRKY57* increased drought tolerance in *A. thaliana*. It has been studied that the Arabidopsis *WRKY57* transcription factor may confer drought tolerance to transgenic rice *O. sativa* plants. The overexpression of *AtWRKY57* in rice improved drought, salinity, and polyethylene gylcol (PEG) tolerance, indicating a possible role of *AtWRKY57* in crop development (Jiang et al., 2016). The *MaWRKY80* was up-regulated under drought stress conditions and was identified as a TF capable of binding to the W-box in *A. thaliana*. *MaWRKY80* overexpression exhibits improved phenotypic morphology, improved survival, lower water loss rate, and lower malondialdehyde (MDA) levels than WT (wild-type) under drought stress. Under drought stress, the transgenic *MaWRKY80*-leaves of *A. thaliana* showed lower reactive oxygen species (ROS) than WT. The *MaWRKY80* also promoted leaf stomata motility and water retention by regulating 9-cis-epoxycarotenoid dioxygenase (NCED) transcript and abscisic acid (ABA) biosynthesis in *A. thaliana* (Liu et al., 2020).

The sorghum WRKY TF, *SbWRKY30* primarily expressed in leaves and roots was induced *via* drought stress. In *A. thaliana* and rice, heterologous expression of *SbWRKY30* confers drought tolerance *via* disturbing root architecture. In addition, *SbWRKY30* induced *SbRD19* (a homologous gene of the drought stress response gene *RD19* in *A. thaliana*) expression in sorghum and the overexpression of *SbRD19* increased drought tolerance in Arabidopsis compared to WT plants. This suggests that *SbWRKY30* functions as a positive regulator in response to drought stress (Yang et al., 2020). Suppression of *GhWRKY21* has been shown to improve drought tolerance in cotton, although *GhWRK21* exhibits a negative role in drought response in cotton (Wang et al., 2021b). Overexpression of the *MuWRKY3* TF gene in peanuts (*A. hypogaea L.*) showed increased tolerance to drought stress and exhibited reduced and delayed wilting symptoms in transgenic plants than WT under drought stress imposition. This indicated that *MuWRKY3* (nuclear-localized) TFs controlled the expression of stress response genes and the actions of ROS scavenging enzymes, thereby led to increased drought tolerance in peanuts (Kiranmai et al., 2018). The expression analysis of *GhWRK25* revealed that *GhWRK25* gene is induced by biotic stress and several defense-related signaling molecules (Liu et al., 2016). Overexpression of *GhWRKY25* in *N. benthamiana* reduced plant tolerance to drought stress and increased tolerance to salt stress (Liu et al., 2016). The *GmWRKY12*, clustered in WRKYII, is 714 bp in length and encodes 237 amino acids. The *GmWRKY12* is expressed in various tissues, not only under normal conditions in soybean, but also strongly expressed under drought and salt treatments (Shi et al., 2018).

The *GhWRKY68* overexpression in *N. benthamiana*, a novel group of WRKY group IIC genes, responds to drought and salt stresses by regulating ABA signaling and modulating cellular ROS (Chi et al., 2013; Jia et al., 2019). The gene *BdWRKY36*

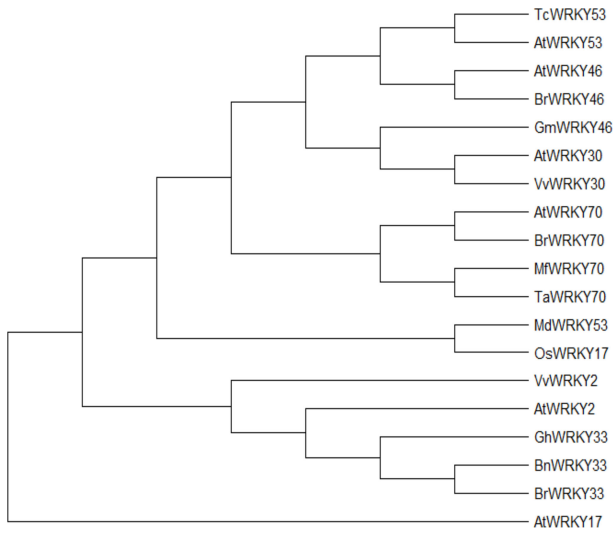

FIGURE 1
Neighbor-joining phylogeny of WRKY-related protein. MEGA X reconstructed the neighbor-joining phylogeny with 1000 bootstrap replicates and used maximum composite likelihood.

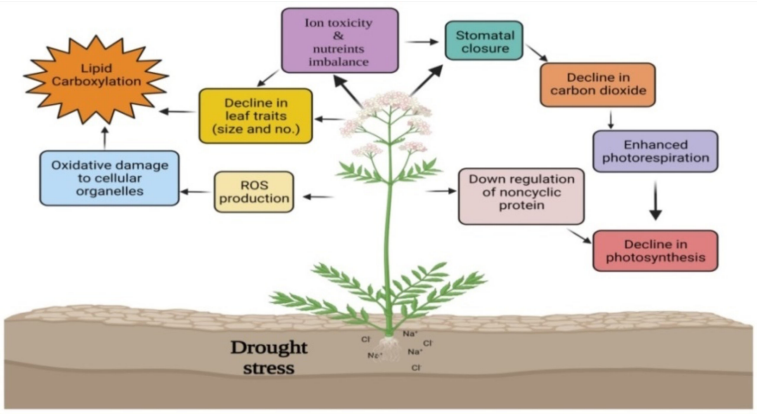

FIGURE 2
Effect of drought stress and the role of WRKY TFs in mitigating drought stress. Drought stress causes ROS production, oxidative damage, ion toxicity, and nutrient imbalance impairing plant growth and development. WRKY TFs regulate the expression of stress response genes and ROS scavenging enzymes. Overexpression of various WRKY TFs reduces ion loss and ROS accumulation, induces leaf stomatal mobility, decreases water loss rate thereby promote water retention, which overall improves phenotypic morphology and plant survival.

TABLE 2 Drought Stress-related WRKY TFs in plants.

S. No.	Gene	Species	Tolerance to stress	Reference
1	*AtWRKY53*	*A. thaliana*	drought	(Jiang et al., 2012)
2	*SlWRKY81*	*S. lycopersicum*	drought	(Ahammed et al., 2020)
3	*GhWRKY33*	*G. hirsutum L.*	drought	(Wang et al., 2019)
4	*SlWRKY72*	*S. lycopersicum*	drought	(Karkute et al., 2018)
5	*MuWRKY3*	*M. uniflorum lam.verdc.*	drought	(Kiranmai et al., 2018)
6	*TaWRKY2*	*T. aestivum L.*	drought	(Gao et al., 2018)
7	*TaWRKY1/33*	*T. aestivum L.*	drought	(He et al., 2016)
8	*ZmWRKY40*	*Z. mays*	drought	(Wang et al., 2018b)
9	*SbWRKY30*	*S. bicolor*	drought	(Yang et al., 2020)
10	*AtWRKY30*	*A. thaliana*	drought	(El-Esawi et al., 2019)
11	*VlWRKY48*	*CV.kyoho*	drought	(Zhao et al., 2018a)
12	*XsWRKY20*	*X. sorbifolium*	drought	(Xiong et al., 2020)
13	*GhWRKY41*	*G. hirsutum L.*	drought	(Chu et al., 2015)
14	*ZmWRKY106*	*Z. mays*	drought	(Wang et al., 2018a)
15	*VfWRKY1/2*	*V. faba L.*	drought	(Abid et al., 2017)
16	*AhWRKY*	*A. hypogaea L.*	drought	(Zhao et al., 2020b)
17	*VvWRKY13*	*V. vinifera L.*	drought	(Hou et al., 2020)
18	*BdWRKY36*	*B. distachyon*	drought	(Sun et al., 2015)
19	*GhWRKY27a*	*G. hirsutum*	drought	(Yan et al., 2015)
20	*MbWRKY1*	*M. baccata L.*	drought	(Han et al., 2018a)
21	*SpWRKY1*	*Phy. infestans*	drought	(Li et al., 2015)
22	*EjWRKY17*	*E. japonica*	drought	(Wang et al., 2021a)
23	*PheWRKY86*	*Phy. edulis*	drought	(Wu et al., 2022a)
24	*BoWRKY1o*	*B. oleracea* var. *A. DC*	drought	(Guo et al., 2021)
25	*TaWRKY46*	*T. aestivum L.*	drought	(Yu and Zhang, 2021)
26	*OsWRKY5*	*O. sativa*	drought	(Lim et al., 2021)
27	*CsWRKY26*	*C. sinensis*	drought	(Chen et al., 2021)

belongs to the WRKY IIe group, designated from *B. distachyon*, the *BdWRKY36* localization in the nucleus is identified *via* the transient expression in onion epidermal cell. The C-terminal region of *BdWRKY36* was found to be transcriptionally active by transactivation assays in transgenic tobacco lines under drought stress. Overexpression of *BdWRKY36* resulted in less ion loss (IL) and ROS accumulation in tobacco lines. Whereas, under drought stress in *BdWRKY36*-overexpressing tobacco lines, the expression levels of ROS scavenging and stress response genes were up-regulated. Overall, *BdWRKY36* was found to act as a positive regulator of drought stress response through regulation of ROS homeostasis and regulation of transcription of stress-related genes (Sun et al., 2015; Li et al., 2020b). The *OsWRKY11* activates the drought-responsive gene transcription, namely *RAB21*, *via* binding directly to the promoter site, and the protein levels of *OsWRKY11* controlled by the system known as ubiquitin-proteasome (Lee et al., 2018; Liu et al., 2020). It was studied that *GmWRKY54* improved stomatal closure to reduce water loss, thus confirming drought tolerance in soybean through improved gene ontology (GO), co-expression network analysis, and physiological parameters. In transgenic soybean plants, expression of *GmWRKY54* confers drought tolerance by the constitutive promoter (*pCm*) and drought-induced promoter (*RD29a*). In soybean, the *GmWRKY54* activates genes (*PYL8, SRK2A, CIPK11*, and *CPK3*) by directly binding to the promoter region, and it has revealed that *GmWRKY54* played its function *via* ABA and Ca^{2+} signaling pathways. In transgenic Arabidopsis, *GmWRKY54* could also improve drought stress tolerance (He et al., 2016; Wei et al., 2019). The *GhWRKY59* plays an important role in regulating cotton's response to drought. Studies have identified that key WRKY TFs are activated and phosphorylated by the MAP kinase cascade, which exhibited *GhMAP3K15*, *GhMKK4*, *GhMPK6*, *GhWRKY59*, and *GhDREB2*, as regulatory modules involved in regulating the response of cotton to drought (Li et al., 2017a).

Wheat (*Triticum aestivum*) is the main crop worldwide; its production in various areas is affected by drought. Therefore, improving the drought tolerance of wheat *via* breeding cultivars is an essential step for food security. It has been examined that *TaWRKY2* isolated from *T. aestivum* enhanced drought tolerance and increased grain productivity in wheat (Niu et al., 2012; Gao et al., 2018; El-Esawi et al., 2019). The WRKY30 TF, *AtWRKY30*, cloned from *A. thaliana*, overexpressed in wheat, which exhibited lower levels of hydrogen peroxide, electrolyte leakage, and malondialdehyde in transgenic plants compared to WT. Moreover, in transgenic wheat plants, some enzyme encoding stress-responsive genes (*WRKY19*, *TIP2, ERF5a*, *DREB1*, *DREB3*, and *AQP7*), were induced, which indicates *AtWRKY30* to be a possible candidate gene to improve stress-tolerance in wheat (El-Esawi et al., 2019). A WRKY TF, *GhWRKY33*, established in cotton, localizes to the cell nucleus and can bind to (W-box) cis-acting elements of target promoters. Moreover, *GhWRKY33* overexpression in Arabidopsis acts as negative regulator that mediates drought stress responses and contributes to ABA signaling (Wang et al., 2019; Khuman et al., 2020; Shaheen et al., 2020). It has been reported that the grape gene *WRKY48* is upregulated due to drought stress, fungal infection, and response to exogenous addition of plant hormones. In *A. thaliana*, over-expressed *V1WRKY48* form (cv. Kyoho), regulates a variety of drought stress responses and exhibits resistance to powdery mildew infection (Han et al., 2018c; Zhao et al., 2018a). The maize WRKY gene promoter region contains C-repeats, dehydration response element (DRE), cold response element (LTR), microbial biomass-C (MBC), and TCA elements that act on drought stress, flocculation, and SA. In transgenic Arabidopsis, the overexpression of *ZmWRKY106* (from the maize member WRKY group II) acted as a positive factor, which improved the drought and heat tolerance (Wang et al., 2018a; Hou et al., 2020). It has been recognized that the WRKY TF gene *ZmWRKY40*, is located in the core of mesophyll protoplasts and the promoter region of *ZmWRKY40* and has numerous transcriptional regulatory elements. A candidate gene, *ZmWRKY40*, improved drought tolerance in transgenic *A. thaliana* through regulation of stress-related genes under drought stress in transgenic lines where ROS levels decreased by enhancing the activity of two enzymes, peroxide dismutase (POD) and catalase (CAT) (Wang et al., 2018b; Leng and Zhao, 2020). The WRKY genes, *TaWRKY1* and *TaWRKY33* (group III and II) have reported to be localized in nucleus in wheat mesophyll protoplasts. In the promoter regions of these genes, several abiotic cis-acting elements were detected. Due to high temperature and ABA, *TaWRKY1* gene was up-regulated and down-regulated *via* low temperature. In addition, the *TaWRKY33* gene shows the higher response to ABA, jasmonic acid methyl ester, and to high and low temperatures. In Arabidopsis transgenic lines, *TaWRKY33* exhibited less water loss than the *TaWRKY1* gene, and the overexpressed *TaWRKY1* and *TaWRKY33* genes were associated in activation of various downstream stress-related genes, and higher germination rates under various stress conditions (He et al., 2016).

Temperature stress-related WRKY TFs

Most plants grow in specific environments and repeatedly experience changes in external conditions. As a result, plants have evolved many complex mechanisms to resist various stresses. WRKY TFs are key proteins that respond to environmental stimuli by regulating gene expression (Xu et al., 2018; He et al., 2019). WRKY TFs are major plant-specific TFs that regulate numerous downstream stress response genes and play important roles in plant biotic and abiotic stress responses. Abiotic stressors, such as drought, heat, salinity, and cold are the

main reasons why plants are undermining productivity around the world (Surendran et al., 2017). At the molecular level, WRKY-TFs are one of the most important families of plant-specific regulatory proteins in the plant kingdom, and are known to contribute to biotic and abiotic stress responses (Sarris et al., 2015; Joshi et al., 2016).

The high and low temperatures cause widespread agricultural damage, reducing crop yields and plant quality. To Protect plant cells from damage caused by extreme temperature changes essential for increasing agricultural production (Ohama et al., 2017). Due to global change, extremely high temperatures are getting a lot of attention and there is evidence that heat stress is responsible for biochemical changes in plants (Li et al., 2020b). Extremely high temperatures have become a major factor affecting plant growth, crop yield, fruit quality, flowering, plant biochemistry, morphology, and physiology (Goraya et al., 2017; Li et al., 2018). WRKY TF plays an important role in plant responses to heat stress. Most studies have shown that WRKY TF responds positively to plant tolerance to high temperatures. For example, in *A. thaliana* high-temperature treatment induces the expression of *AtWRKY25* and *AtWRKY26*, and inhibits *AtWRKY33*, whereas overexpression of *AtWRKY25/26* increases tolerance to heat stress in *A. thaliana* (Li et al., 2011). In peppers, *CaWRKY40* promotes stress resistance at high temperatures and the overexpression of *CaWRKY40* in tobacco reduces susceptibility to heat treatment, whereas loss of *CaWRKY40* reduces this tolerance (Liu et al., 2021). Inhibition of *AtWRKY41* expression in *A. thaliana* leads to reduced seed dormancy and suppression of high temperature (Chen et al., 2012; Ding et al., 2014). The overexpression of *TaWRKY33* in wheat enhances the high-temperature tolerance (El-Esawi et al., 2019). It has been studied that WRKY-TFs increase ROS production in the cell because of high-temperature stress in plants results in an excessive accumulation of ROS produced oxidative stress. Recent studies have shown that WRKY-TF is induced through ROS and contributes to the ROS elimination transformation pathway.

Oxidative stress is a severe stress caused by a variety of stresses, and ROS-mediated signaling is regulated by a delicate balance between production and clearance (Salvucci et al., 2001; Alvarez-Venegas et al., 2007). There are four types of reactive oxygen species in plants: oxygen, hydrogen peroxide, hydroxyl radicals, and superoxide anions. Several WRKY TFs (*WRKY6*, *WRKY30*, *WRKY22*, *WRKY8*, *WRKY53*, *WRKY48*, *WRKY39*, and *WRKY75*) are activated in *A. thaliana* in response to hydrogen peroxide treatment (Davletova et al., 2005; Jiang et al., 2017). It has been investigated that treatment of H_2O_2 activated higher expression of (*WRKY6*, *WRKY8*, *WRKY22*, *WRKY30*, *WRKY39*, *WRKY48*, *WRKY53*, and *WRKY75*) that could respond to a higher temperature in *A. thaliana* (Chen et al., 2010). *OsWRKY42* has been shown to play an important role as a negative regulator of oxidative stress, and overexpression of *OsWRKY42* in rice results in higher ROS accumulation (Han et al., 2014). Overexpression of *TaWRKY10* in wheat showed reduced malonaldehyde (MDA) accumulation, and low MDA was associated with a low rate of lipid peroxidation. This showed that the transgenic seedlings exhibited high resistance to oxidative stress due to increased expression of *TaWRKY10*, which resists reduced heat damage. The *AtWRKY28* was found to regulate the expression of downstream-associated genes through ROS in *A. thaliana* when exposed to oxidative stress (Niu et al., 2012; Babitha et al., 2013). The *ClWRKY20* belongs to group III of the WRKY family, and intracellular localization of *ClWRKY20* was found in the nucleus. The expression level of *ClWRKY20* was increased due to salinity, drought, and phytohormones (ABA, ET, and SA) treatment. *ClWRKY20* overexpression in transgenic Arabidopsis increased sensitivity to ABA at low temperatures, salinity, and during seed germination (Zhu et al., 2022). This study showed that WRKY-TF enhances plant tolerance to high temperature through transcriptional regulation (Table 3).

Cold stress-related WRKY TFs

Cold stress (cold below 20°C and freezing below 0°C) adversely affects plant growth and development and greatly limits agricultural productivity. Plants adapt tolerance to cold stress, chilling and freezing by various physiological, protective, and molecular response systems. It has been studied *via* analyzing regulatory mechanism in plants, many genes have been identified that respond to cold stress at the transcriptional level (Ahmadizadeh and Heidari, 2014; Ritonga et al., 2021). Many WRKY TFs known to have important role in cold stress tolerance in various species (Table 4). Recent studies have shown that transgenic lines of Arabidopsis overexpressing *CsWRKY46* and cucumber WRKY show higher seedling viability when frozen at 4° C. In addition, the study identified transgenic *A. thaliana* in which overexpression of *GmWRKY21* (soybean WRKY) showed increased resistance to cold stress (Zhou et al., 2008; Zhang et al., 2016). Another study showed that *CsWRKY46* (belonging to the group II WRKY family) was localized in the nucleus, as determined by transient expression analysis. After freezing treatment, Arabidopsis lines, overexpressing *CsWRKY46*, *WRK46-OE1*, and *WRK46-OE5* had a higher survival rate than the WT. *CsWRKY46* confers cold tolerance to transgenic plants and modulates cold signaling pathways in an ABA-dependent manner. Whereas, overexpression of *OsWRKY76* was found to enhance cold stress tolerance at 4°C (Zhang et al., 2016). Overexpression lines compared to WT exhibited better surveillance under -20°C after 80 minutes and until 72 hours. The over-expressing plant lines had

TABLE 3 Temperature stress-related WRKY TFs.

S. No.	Gene	Species	Tolerance to stress	References
1	*AtWRKY30*	*A. thaliana*	temperature	(El-Esawi et al., 2019)
2	*AtWRKY46*	*A. thaliana*	temperature	(Suzuki et al., 2005)
3	*OsWRKY77*	*O. Sativa*	temperature	(Lan et al., 2013)
4	*CaWRKY27*	*C. annuum*	temperature	(Dang et al., 2018)
5	*CaWRKY40*	*C. annuum*	temperature	(Dang et al., 2013)
6	*AtWRKY41*	*A. thaliana*	temperature	(Ding et al., 2014)
7	*TaWRKY70*	*T. aestivum*	temperature	(Wang et al., 2017)
8	*AtWRKY54*	*A. thaliana*	temperature	(Li et al., 2020b)
9	*PtWRKY13*	*P. tomentosa*	temperature	(Ren et al., 2019)
10	*PtWRKY50*	*P. tomentosa*	temperature	(Ren et al., 2019)
11	*ZmWRKY106*	*Z. mays*	temperature	(Wang et al., 2018a)
12	*AtWRKY39*	*A. thaliana*	temperature	(Li et al., 2010b)
13	*AtWRKY72*	*A. thaliana*	temperature	(Cheng et al., 2021)
14	*AtWRKY7*	*A. thaliana*	temperature	(Park et al., 2005)
15	*AtWRKY8*	*A. thaliana*	temperature	(Han et al., 2015)
16	*AtWRKY15*	*A. thaliana*	temperature	(Han et al., 2015)
17	*AtWRKY26*	*A. thaliana*	temperature	(Fu and Yu, 2010)
18	*AtWRKY33*	*A. thaliana*	temperature	(Fu and Yu, 2010)
19	*TaWRKY1*	*T. aestivum*	temperature	(Ren et al., 2019)
20	*NtWRKY6*	*N. tabacum*	temperature	(Macková et al., 2013)
21	*HaWRKY6*	*H. annuus*	temperature	(Giacomelli et al., 2012)
22	*ClWRKY20*	*C. lanatus*	temperature	(Zhu et al., 2022)

lower ion content leakage related to WT plants. From that, it could be assumed that overexpression lines could possess higher membrane stability (Yokotani et al., 2013).

Salinity stress-related WRKY TFs

Soil salinity is one of the major abiotic stresses that affect the productivity of crops. Because the ionic and osmotic stresses of high salt concentrations in the soil affect the growth and development of plants. Salt stress is highly common in arid regions because of excessive evaporation leading to the accumulation of inorganic salts, which affects plant metabolism. With the success of traditional breeding approaches to improve stress-tolerant traits, transformation methods appear to be particularly beneficial for breeding stress-tolerant crops. In this regard, TFs play an important role as mediators in genetic engineering due to their unique

TABLE 4 Cold stress-related WRKY TFs.

S. No.	Gene	Plant species	Factors	Responses	Reference
1	*AtWRKY34*	*A. thaliana*	Cold	Play a role as a negative regulator in cold stress	(Zou et al., 2010)
2	*VvWRKY24*	*V. vinifera*	Cold	Up-regulate regulation of hypothermia	(Wang et al., 2014b)
3	*OsWRKY76*	*O. sativa*	Cold	Tolerance to cold	(Yokotani et al., 2013)
4	*BcWRKY46*	*B. campestris*	Cold and Salt	Drought and salt tolerance	(Wang et al., 2012)
5	*VbWRKY32*	*V. bonariensis*	Cold	Tolerance to cold stress	(Wang et al., 2020)
6	*GmWRKY21*	*G. max*	Cold, Drought,	Tolerance to cold stress	(Zhou et al., 2008)
7	*VpWRKY2*	*V. pseudoreticulata*	Cold, ABA, and Salt	Tolerance to cold and salt stress	(Li et al., 2010a)
8	*TcWRKY53*	*T. caerulescens*	Cold, NaCl, and PEG	Play a role as a negative regulator in osmatic stress	(Wei et al., 2008)
9	*JrWRKY2*	*J. regia*	Cold and Drought	Cold and drought tolerance	(Yang et al., 2017)
10	*JrWRKY7*	*J. regia*	Cold and Drought	Tolerance to cold and drought stress	(Yang et al., 2017)
11	*LchiWRKY33*	*L. chinense* (*Lchi*)	Cold	Tolerance to cold stress	(Wu et al., 2022b)

roles in the regulation and modification of various stress-sensitive genes (Chaudhry et al., 2021;Hussain et al., 2021).

WRKY TFs also present a key role in salt stress response and tolerance (Table 5). Recent studies have shown that overexpression of *AtWRKY46* enhances root development during salt stress in Arabidopsis through modulation of ABA signaling. In addition, overexpression of *GhWRKY34* (*G. hirsutum*) enhances the plant's ability to selectively absorb Na^+ as well as K^+ and maintain low Na^+/K^+ levels, thereby increasing resistance to salt stress in the leaves and roots of transgenic Arabidopsis plants (Dai et al., 2016). Overexpression of *GmWRKY54* (*WRKY soybean*) in transgenic Arabidopsis plants shows salt tolerance, it has indicated that WT plants showed 25% survival while over-expressing lines showed 70% survival under 180 mM NaCl treatment (Zhou et al., 2008). Another study found that N. benthamiana *GmWRKY17* (cotton WRKY) improved salinity stress tolerance as measured by physiological analyzes of germination rate, root growth, survival, and leaf water loss (Yan et al., 2014). A new WRKY gene was isolated from *M. xiaojinensis*, namely *MxWRKY55*, and it is localized in the nucleus. The expression level of *MxWRKY55* in *M. xiaojinensis* seedlings was affected by salinity, low Fe, and high Fe stresses, and *MxWRKY55* also increased salinity and iron tolerance when introduced into *A. thaliana*. Overexpression of *MxWRKY55* in *A. thaliana* showed high levels of chlorophyll and proline, as well as increased activity of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Similarly, *MxWRKY55* in *A. thaliana* resulted in lower levels of malondialdehyde (MAD), particularly in response to salt stress. In addition, overexpression of *MxWRKY55* in transgenic *A. thaliana* showed greater root length, mass, chlorophyll, and iron content compared to WT (Han et al., 2020). Based on these properties, it has been demonstrated that *MxWRKY55* can play a positive role in the process of salt resistance, resistance to high Fe, and low Fe content. Another study showed that the growth and development of *M. xiaojinesis* (semi-dwarf apple in China) was affected by the salinity and Fe. The novel WRKY *MxWRKY53/64* gene isolated from *M. xiaojinesis* is a nuclear-localized protein and its expression level is strongly influenced by salt as well as Fe, when *MxWRKY53/64* was introduced into transgenic *A. thaliana*, resistance to salinity and iron stress was significantly increased (Han et al., 2021a; Han et al., 2021b). Moreover, the over-expression of wheat WRKY TF, the *TaWRKY93* in *A. thaliana* showed high salt tolerance, low temperature, and osmotic stress tolerance (Qin et al., 2015).

WRKY TFs as key regulators in plant growth and development

The WRKY TF is one of the largest TF families in plants, which in addition to stress response and defense regulation significantly contributes to plant growth and development. Various WRKY

TABLE 5 Salinity stress-related WRKY TFs.

S. No.	Gene	Plant	Tolerance to stress	Reference
1	*FcWRKY70*	*F. crassifolia*	Salt	(Wang et al., 2007)
2	*GmWRKY17*	*G. max*	Salt	(Yan et al., 2014)
3	*ZmWRKY17*	*Z. mays*	Salt	(Cai et al., 2017)
4	*SbWRKY30*	*S. bicolor*	Salt	(Yang et al., 2020)
5	*GbWRKY1*	*G. barbadense*	Salt	(Luo et al., 2020)
6	*IbWRKY47*	*I. batatas*	Salt	(Qin et al., 2020)
7	*PgWRKY33/62*	*P. glaucum*	Salt	(Chanwala et al., 2020)
8	*SbWRKY50*	*S. bicolor*	Salt	(Song et al., 2020b)
9	*VpWRKY1*	*V. pseudoreticulata*	Salt	(Li et al., 2010a)
10	*VpWRKY2*	*V. pseudoreticulata*	Salt	(Li et al., 2010a)
11	*MbWRKY5*	*M. baccata*	Salt	(Han et al., 2018b)
12	*CmWRKY*	*C. pepo*	Salt	(Bankaji et al., 2019)
13	*PbWRKY40*	*P. betulaefolia*	Salt	(Lin et al., 2022)
14	*ClWRKY20*	*C. Lanatus*	Salt	(Zhu et al., 2022)
15	*MxWRKY53*	*M. xiaojinensis*	Salt	(Han et al., 2021b)
16	*MxWRKY64*	*M. xiaojinensis*	Salt	(Han et al., 2021a)
17	*AhWRKY75*	*A. hypogaea L.*	Salt	(Zhu et al., 2021)
18	*MfWRKY70*	*M. Flabellifolia*	Salt	(Xiang et al., 2021)

genes have been reported in different plant species that promote growth and development (Zhang et al., 2017) (Table 6). The *AtWRKY28* gene, *AtWRKY2*, and *AtWRKY34* which are involved in macrospore fate, pollen tube extension, pollen production, seed germination, and early growth after germination. *AtWRKY2* (a knockout mutant exhibiting high sensitivity to ABA) plays an important role in seed germination (Jiang and Yu, 2009). The overexpression of *VvWRKY30* in

TABLE 6 Role of WRKY TFs in plant growth and development.

S. No.	Name	Plant	Function	References
1	*VvWRKY30*	*V. vinifera*	Increasing salt stress resistance by ROS and accumulation of osmoticum.	(Zhu et al., 2019)
2	*GmWRKY12*	*G. max*	Drought and salinity tolerance	(Shi et al., 2018; Zhang et al., 2020)
3	*MdWRKY40*	*M. domestica*	Important regulators of wound-induced anthocyanin biosynthesis	(An et al., 2019)
4	*TaWRKY51*	*T. aestivum L.*	Promotes lateral root formation due to negative regulation of ethylene biosynthesis	(Hu et al., 2018)
5	*GhWRKY59*	*G. hirsutum*	Drought responses	(Li et al., 2017a)
6	*HbWRKY82*	*H. brasiliensis*	Abiotic resistance and leaf aging	(Kang et al., 2021)
7	*MfWRKY70*	*M. Flabellifolia*	Drought and salinity tolerance	(Xiang et al., 2021)
8	*HmoWRKY40*	*H. monacanthus*	Betalain biosynthesis	(Zhang et al., 2021b)
9	*MxWRKY64*	*M. xiaojinensis*	It plays an important role in response to Fe and salt stress	(Han et al., 2021a)
10	*AhWRKY75*	*A. hypogaea L*	Conferred salt tolerance in transgenic peanut lines	(Zhu et al., 2021)
11	*BoWRKY10*	*B. oleracea* var.*acephala DC*	Regulation of drought stress tolerance	(Guo et al., 2021)
12	*AtWRKY28*	*A. thaliana*	Oocyte development	(Zhao et al., 2018b)
13	*AtWRKY2*	*A. thaliana*	Seed germination, growth after germination	(Jiang and Yu, 2009)
14	*AtWRKY10*	*A. thaliana*	The size of the seed	(Luo et al., 2005)
15	*AtWRKY34*	*A. thaliana*	Seed germination, growth after germination	(Guan et al., 2014)
16	*AtWRKY41*	*A. thaliana*	The dormancy of seed	(Ding et al., 2014)
17	*AtWRKY44*	*A. thaliana*	In the proanthocy seed coat of tannins	(Gonzalez et al., 2016)
18	*OsWRKY78*	*O. sativa*	The development of seed and stem elongation	(Zhang et al., 2011a)
19	*OsWRKY24*	*O. sativa*	Increased lamina inclination and grain size through cell elongation.	(Jang and Li, 2018)
20	*GhWRKY42*	*G. hirsutum*	Premature leaf senescence and stem development	(Gu et al., 2018)
21	*AtWRKY23*	*A. thaliana*	Root growth and biosynthesis of flavanols	(Grunewald et al., 2012)
22	*GhWRKY91*	*G. hirsutum*	Leaf senescence and stress response	(Gu et al., 2019b)
23	*OsWRKY93*	*O. sativa*	Leaf senescence and in response to fungi attack	(Li et al., 2021)
24	*BrWRKY6*	*B. rapa ssp.pekinensis*	Leaf senescence	(Fan et al., 2018)
25	*GhWRKY27*	*G. hirsutum*	Leaf senescence	(Gu et al., 2019a)
26	*PyMYB114*	*Red-Skinned pears*	Regulate anthocyanin biosynthesis and transport	(Li et al., 2020a)
27	*WRKY6*	*A. thaliana*	Improve FA accumulation and seed yield	(Song et al., 2020a)
28	*TaWRKY40-D*	*T. aestivum L.*	Association to the promotion of leaf senescence with jasmonic acid and abscisic acid	(Zhao et al., 2020a)
29	*WRKY46/6*	*A. thaliana*	PBZ/SA-mediated leaf senescence	(Zhang et al., 2021a)
30	*WRKY45*	*A. thaliana*	Positive regulator of age-triggered leaf senescence	(Chen et al., 2017)
31	*VvWRKY2*	*V. venifera*	Vigor, yield, and tuber quality	(Chiab, 2021)
32	*AtWRKY26*	*A. thaliana*	Leaf senescence	(Li et al., 2017b)
33	*WRKY12/13*	*A. thaliana*	Regulate flowering time	(Li et al., 2016b)
34	*WRKY42*	*A. thaliana*	Root hair growth and development	(Moison et al., 2021)
35	*OsWRKY11*	*O. sativa*	Flowering time and plant height	(Cai et al., 2014)
36	*AtWRKY45*	*A. thaliana*	Play a key role in Phosphate uptake	(Wang et al., 2014a)
37	*AtWRKY42*	*A. thaliana*	Play a great role in phosphate uptake	(Su et al., 2015)
38	AtWRKY71	*A. thaliana*	Flowering time	(Yu et al., 2016)
39	*MxWRKY55*	*M. xiaojinensis*	Tolerance to salt, low-iron and high-iron stress	(Han et al., 2020)

Arabidopsis increased resistance to salt stress at various growth stages by regulating ROS clearance and osmotic accumulation (Zhu et al., 2019). In soybean, *GmWRKY12* induced a positive role in ABA, salt, and drought stresses (Shi et al., 2018).

There are several WRKY genes involved in plant root development. The *TaWRKY51*, an important WRKY TF that increases lateral root formation through the regulation of ethylene biosynthesis in wheat (Hu et al., 2018). The study also reported that *TaWRKY51* regulates lateral root formation *via* the ethylene and auxin signaling pathways (Hu et al., 2018). *AtWRKY23* expression induced by the auxin response factor7 (ARF7) and auxin response factor 19 (ARF19) (serve as part of the auxin feedback loop), help to regulate the growth of plant roots and the synthesis of flavonoids (Grunewald et al., 2012). Both *AtWRKY75* and *AtWRKY44* are involved in root hair development. *AtWRKY44* is also a downstream gene (*TTG1* and *GLABROUS1*) expressed in root hairs that act jiontly with *GLABRA2* to regulate root hair growth in plants (Johnson et al., 2002). Studies have shown that the number and length of root hairs are increased in *AtWRKY75* (Knockout mutant) compared to the WT, suggesting that *AtWRKY75* is a negative regulator of root hair development (Devaiah et al., 2007).

A novel WRKY TF, designated *HbWRKY82*, was identified based on stress-related WRKY in rubber trees, encoded by nuclear proteins and present an important function as a transcriptional activator. Exogenous ethrel and ABA stimulation induce *HbWRK82* transcriptional activity, which play important roles as transcriptional regulators in ethrel and in response to ABA-mediated leaf senescence and abiotic stress (Kang et al., 2021). The *WRKY70* is involved in biological stress as a positive regulator and has a negative role in abiotic stress signaling in Arabidopsis and several other plant species. The localization of *MfWRKY70* in the nucleus was confirmed by examining *MfWRK70* from *M. flabellifolia* in *Arabidopsis* model plants. The *MfWRKY70* is reported to have an essential role in drought, osmotic pressure, and salinity tolerance by promoting root growth and water retention. Under stress conditions, *MfWRKY70* enhanced the antioxidant enzyme system, maintaining ROS homeostasis and stability of membrane lipids (Xiang et al., 2021).

A novel WRKY TF, the *HmoWRKY40* was identified from the transcriptomic data of pitaya (*H. monacanthus*), and the *HmoWRKY40* transcriptionally activates *HmoCYP76AD*, which regulates ptiaya betalain biosynthesis (Zhang et al., 2021b). Fe and high salinity affect the growth and development of *M. xiaojiensis*, a semi-dwarf apple in China. The newly isolated WRKY gene from *M. xiaojinesis*, namely *MxWRKY64* (localization in the nucleus)was introduced into *A. thaliana*, which showed increased resistance to Fe and salts, and overexpression of *MxWRKY64* in transgenic *A. thaliana* under Fe stress resulted in higher levels of mass, root length, chlorophyll, and Fe content compared to WT (Han et al., 2021a). A novel WRKY-TF gene *AhWRKY75* (WRKYIIC) identified from M34 (salt-tolerant mutant) confers salt tolerance to transgenic peanut strains by increasing the efficiency of ROS removal system and photosynthesis during stress treatment (Zhu et al., 2021). In flowering plants, female gonadal megasporoblasts (MMCs) start as single cells in each ovule, and Arabidopsis cytochrome P450 (KLU) functions through the SWR1 chromatin remodeling complex to promote *WRKY28* expression in oocyte primordial (Zhao et al., 2018b). The studies have suggested that WRKY genes play a key role in seed germination and post-germination growth. The Arabidopsis *WRKY2* TF is involved in seed germination and post-emergence stunting (Jiang and Yu, 2009), plant (male) gamete formation with complex and dynamic changes in gene expression. Studies have shown that WRKY2 and its close homolog WRKY34 (pollen-specific) TFs participated in male gametogenesis in *A. thaliana* (Guan et al., 2014).

Interaction of WRKY genes with some stress-related genes to improve plant abiotic stress tolerance in plants was shown in Figure 3. The interaction network with STRING (https://

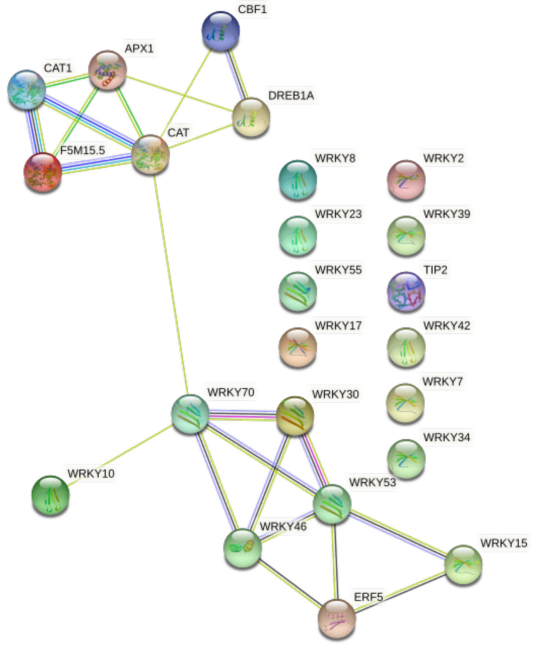

FIGURE 3
The interaction network analysis of some WRKY genes identified in *Arabidopsis thaliana* using STRING: Line colors are associated with interaction types. Around the green line gene, pink lines are experimentally determined, black lines mean co-expression, dark blue lines mean gene co-occurrence, and blue lines mean protein homology.

string-db.org/cgi/) was recognized. The result showed that several WRKY genes correlate with abiotic stress-related genes; for instance, the above mentioned *AtWRKY30* cloned TFs from Arabidopsis; its over-expression in wheat showed improved stress tolerance. Moreover, in transgenic wheat, antioxidant genes such as APX1, CAT, CAT1, F5M15.5, ERF5, CBF1, and DREBIA play key roles as stress-responsive genes (El-Esawi et al., 2019). It was speculated that correlated genes might have a positive or negative correlation in response to abiotic stress.

Conclusion and future prospects

Plants are considered as sessile organisms that cannot avoid adverse abiotic stresses as well as other major environmental stresses and have developed complex signaling networks composed of multiple pathways. One of the largest TF families, WRKY-TFs act as molecular switches that regulates the expression of stress-sensitive genes. Stress-induced WRKY-TF expression is regulated by a complex transcriptional regulatory network that allows plants to maintain the proper balance between growth and stress response. This review discusses the recent studies of WRKY-TF. Many studies have shown that WRKY-TFs play important roles in abiotic stress tolerance (Figure 4). Nowadays the sequencing of plant genomes has increased largely; especially in economically important crops and whole-genome identification of the WRKY gene (with respect to functional plant genes) facilitate screening. Previous studies have demonstrated that the WRKY gene primarily depends on its functional assumptions and transcriptome. In addition, genetic confirmations joined to the latest technologies are increasing to confirm the novel role of the WRKY genes, expression of WRKY-TF or downstream genes regulated by self-regulation of WRKY-TF, which helps to simplify the regulatory network of responses to abiotic stresses. Future studies should explore noncoding RNAs and epigenetic modifications involved in the regulation of WRKY-TFs. Based on current studies the role of WRKY-TFs in regulating plant responses related to abiotic stresses, particularly drought, salinity, and temperature stress, are not sufficiently detailed, particularly at the transcriptional level. Finally, the use of WRKY-TF screening for plant stress tolerance in context to increase climate change significantly improves crop yield and crop quality.

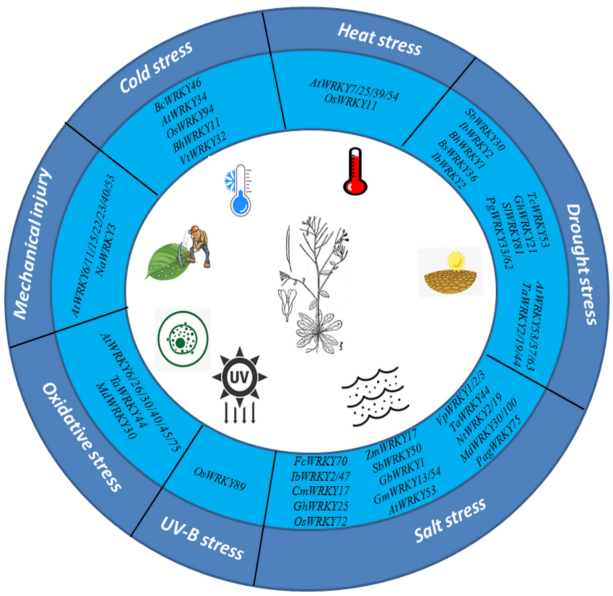

FIGURE 4
WRKY-TFs response to various abiotic stresses.

Author contributions

MK, and AH planned and designed this review manuscript. MK, AH, and HM wrote this review paper. FR, QA, MC, QM, MA, WZ, RMA, and RB helped to improve the manuscript writing. FL and HM contributed to the critically revising of the manuscript. All the authors have reviewed, edited, and approved the manuscript before submission.

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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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Identification of GOLDEN2-like transcription factor genes in soybeans and their role in regulating plant development and metal ion stresses

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The Golden 2-Like (G2-like or GLK) transcription factors are essential for plant growth, development, and many stress responses as well as heavy metal stress. However, G2-like regulatory genes have not been studied in soybean. This study identified the genes for 130 G2-Like candidates' in the genome of *Glycine max* (soybean). These GLK genes were located on all 20 chromosomes, and several of them were segmentally duplicated. Most GLK family proteins are highly conserved in Arabidopsis and soybean and were classified into five major groups based on phylogenetic analysis. These *GmGLK* gene promoters share cis-acting elements involved in plant responses to abscisic acid, methyl jasmonate, auxin signaling, low temperature, and biotic and abiotic stresses. RNA-seq expression data revealed that the GLK genes were classified into 12 major groups and differentially expressed in different tissues or organs. The co-expression network complex revealed that the *GmGLK* genes encode proteins involved in the interaction of genes related to chlorophyll biosynthesis, circadian rhythms, and flowering regulation. Real-time quantitative PCR analysis confirmed the expression profiles of eight GLK genes in response to cadmium (Cd) and copper (Cu) stress, with some GLK genes significantly induced by both Cd and Cu stress treatments, implying a functional role in defense responsiveness. Thus, we present a comprehensive perspective of the GLK genes in soybean and emphasize their important role in crop development and metal ion stresses.

KEYWORDS

soybean, Golden2-like TF, phylogenetic classification, duplication, gene expression, metal ion stress

Introduction

Soybean is a significant oil and food crop with a rich protein content and sources of food and cooking oil worldwide (Kim et al., 2017). However, soybean crops are frequently exposed to a variety of environmental stresses, which restrict crop yields. Accumulation of heavy metals in soil and water can be attributed to several anthropogenic activities such as industrialization and modern farming practices, including extensive exploitation of land resources (Salazar et al., 2012; Zhang et al., 2019). Heavy metal buildup in plant tissues may hinder the plant's significant enzymatic activity, causing a variety of negative effects on germinability, seedling growth, and photosynthetic activity (Sharma and Dietz, 2006). Heavy metals are taken up by the roots of plants and moved to the shoots. This has a negative effect on root and shoot cells as well as organelles such as chloroplasts and mitochondria, which limits energy production and enforces peroxidation (Garg and Singla, 2011; Yan et al., 2020).

Plants have evolved complex defense systems to counteract the effects of environmental factors (Bohnert et al., 2006). Transcription factors (TFs) are important regulators of developmental processes and stress responses, playing a crucial role in signal transduction and gene expression regulation (Davidson et al., 1983; Suo et al., 2003; Ramsay and Glover, 2005; Shin et al., 2016; Zaikina et al., 2019). These TFs are altered during stress, which affects their intracellular allocation, consistency, activity, connections with several other proteins, and eventually the expression of target genes. MYB-like TFs are among the most significant TF families engaged in the plant transcriptional control network and are linked to many stressors, including metal ion stress (Hu et al., 2017; Jalmi et al., 2018; Li X. et al., 2021; Wa Lwalaba et al., 2021). Currently, the completion of whole genome sequencing has allowed us to comprehensively analyze and classify the TFs; however, several of them remain unknown.

Golden2-Like (G2-Like or GLK) transcription factors occur widely in plants. They belong to the GARP superfamily in the Myb class of transcription factors. They play important roles in chlorophyll biosynthesis, leaf senescence, and stress responses, including heavy metal stress (Riechmann et al., 2000; Fitter et al., 2002; Van De Mortel et al., 2008; Kakizaki et al., 2009; Waters et al., 2009; Kobayashi et al., 2013; Garapati et al., 2015; Leister and Kleine, 2016; Nagatoshi et al., 2016; Ahmad et al., 2019). The first GLK transcription factor was reported in *Zea mays* (Hall et al., 1998). A typical GLK protein contains two conserved domains: a Myb-DNA binding domain (DBD) and a GCT box (Rossini et al., 2001). In Arabidopsis, *AtGLK1* and *AtGLK2* have been shown to regulate chloroplast development (Fitter et al., 2002; Yasumura et al., 2005; Waters et al., 2008). Furthermore, *AtGLK2* is important for anthocyanin production. In Arabidopsis seedlings, the accumulation of anthocyanins is limited due to loss of function of *AtGLK2*, and its overexpression increased the accumulation of anthocyanins (Liu et al., 2021a). In barley, the G2-like ALM1 mutant reduces seed weight (Taketa et al., 2021). The overexpression of two *ZmGLK* genes, particularly that of the *ZmGLK2* gene governed by the maize UB promoter has recently shown to increase rice yield by 30-40% (Li et al., 2020). The overexpression of *AtGLK1* gene under control of its silique promoter (PAt1G56100) increased 11% seed weight in Arabidopsis. Moreover, overexpressing *AtGLK1* with its leaf promoter increased leaf photosynthesis and 25% of seed yield (Zhu et al., 2018). Additionally, it has been demonstrated that GLK proteins act with ANAC92 to exhibit leaf senescence (Rauf et al., 2013). Arabidopsis atglk1/atglk2 double mutants exhibited leaf senescence in addition to plant yellowing, whereas plants overexpressing *AtGLK1* or *AtGLK2* exhibited the opposite phenotype (Waters et al., 2009). In atglk1/atglk2 double mutants, overexpression of *AtGLK1* or *AtGLK2* can complement their progeria phenotypes (Waters et al., 2008). GLK genes are also involved in plant senescence and are regulated by signaling pathways such as light, ABA, and brassinolide (BR). Light is required for chloroplast development but is involved in plant senescence by inducing the expression of AtGLKs genes (Fitter et al., 2002). In addition, *AtGLK1* modulates the expression of disease-resistance genes and has varied effects on diverse pathogens (Chen et al., 2016). *AtGLK1* increases resistance to *Fusarium graminearum* in Arabidopsis and promotes cucumber mosaic virus tolerance (Savitch et al., 2007; Schreiber et al., 2011; Han et al., 2016). *AhGLK1b* can enhance tolerance to fungal and bacterial infections and other environmental stresses (Liu et al., 2021b). In rice, *OsGLK1* is involved in disease resistance (Chen et al., 2016). Loss of function of *SlGLK29* may affect cold tolerance in tomato plants (Junfang et al., 2017). Similarly, *GhGLK1* in cotton has been associated with the response to cold and drought stress (Liu et al., 2021b). Several GLK gene functions have been studied extensively. Nevertheless, those associated with abiotic stress have received little attention, and only a few published scientific studies are available. Previously, GLK family genes were discovered and studied in the genomes of several plant species, including Arabidopsis (Alam et al., 2022), cotton (Zhao et al., 2021), maize (Liu et al., 2016), tomato (Wang et al., 2022), and tobacco (Qin et al., 2021). However, there have been no report on the GLK gene family in the soybean genome.

This study identified and classified soybean GLK gene-containing proteins based on phylogenetic tree analysis. Furthermore, the *GmGLK* genes were analyzed and annotated. The cis-regulatory elements of the *GmGLK* promoter region were examined. Interestingly, several of these GLK genes have been shown to be expressed in various soybean tissues. Furthermore, the expression analysis of eight GLK genes in response to cadmium and copper treatments were confirmed using real-time quantitative PCR (qRT-PCR). Our research will not only expand genetic research on GLK genes in soybean while also give a valuable perspective and new insights for researchers to investigate *GmGLK* functions in the future.

Materials and methods

Identification and analysis of soybeans GLK members

The entire set of previously reported genomic and proteomic data on Arabidopsis GLK genes was downloaded from the Arabidopsis database TAIR (http://www.arabidopsis.org) and used as query sequences for BLASTp searches against Phytozome V13 plant databases. To confirm the existence of GLK-associated motifs, all GLK sequences were screened using Hidden-Markov-Model (HMM) profiles and online tool such as SMART (http://smart.embl-hei-delberg.de), Pfam (http://pfam.sanger.ac.uk), and Interpro (http://www.ebi.ac.uk/interpro/). The ProtParam tool was searched to examine the isoelectric point (pI) and molecular weight (Wt) (Protparam, 2017). Furthermore, the PlantRegMap tool was explored to analyze the gene ontology (GO) of the soybean GLK genes.

Phylogenetic tree analysis

Phylogenetic analysis of Arabidopsis and soybean GLK proteins was generated using MEGAX software with the maximum likelihood (ML) algorithm (Kumar et al., 2018) and Jones-Taylor-Thornton (JTT). Bootstrap with 1000 replications was used to evaluate the group support (Jones et al., 1992).

Structure of GmGLK genes, conserved motifs, and phylogenetic analysis

TBtools was used to depict the exon-intron arrangement of the GLK-encoding genes (Chen et al., 2020), the MEME tool for search conserved motifs in GLKs (Bailey and Elkan, 1994), and Tomtom was used to predict the TFs that were most likely to bind to these predicted binding sites (Gupta et al., 2007).

Analysis of GmGLK promoters, expression pattern, and co-expression analysis

Phytozome V13 plant databases (https://phytozome-next.jgi.doe.gov/) were searched to retrieve the promoter sequences (2-kb upstream genomic region) of the *GmGLK* genes. The obtained upstream regions were submitted to the online PlantCARE web tools for important cis-elements of the GLK genes (Lescot et al., 2002). The soybean GLK gene expression data files (RNA-seq expression data) were obtained and examined from the Phytozome V12.1 database. The *GmGLK* protein list was obtained from the "CoExSearch" tool and submitted to the STRING web tool to predict putative interacting proteins (Szklarczyk et al., 2018). An interaction network was generated through the Cytoscape tool.

Physical location of GmGLKs on the chromosome and Ka/Ks analysis

The chromosomal location of soybean GLK genes was determined using genome annotation files. The allocation and segmental duplication of soybean GLK genes on chromosomes were mapped and depicted using MapMan tools. The duplicated *GmGLK* genes were shown *via* numerous color lines. Clustal Omega was used for sequence alignments (Sievers and Higgins, 2014), and the ratios of synonymous (Ks), non-synonymous (Ka), and evolutionary pressures (Ka/Ks) among the *GmGLK* pairs were estimated *via* the PAL2NAL and PAML package (Goldman and Yang, 1994; Yang et al., 1994; Suyama et al., 2006).

Plant materials and qRT-PCR analysis

Good quality Williams 82 cultivar Soybean seeds were apparent clean with 1% sodium hypochlorite for five min with gentle shaking before being washed with ddH_2O. Then the clean seeds were sown in a mixture of soil and sand-filled pots (1:1) and cultivated in a control chamber with 16/8- h of the light–dark cycle at 22°C and 65-70% humidity. To examine the expected role of *GmGLK* genes in response to metal stress treatment, seven days of soybean seedlings were exposed to an excess amount of Cd (50 μM of $CdCl_2$) and Cu (50 μM of $CuSO_4{\cdot}5H_2O$) for 1–6 hour treatments. Total RNA was isolated from the frozen roots of each soybean (0.3 cm) utilizing a plant-specific RNA extraction kit according to the company's guidelines (OMEGA, China). For each treatment, three biological replicates were prepared to reduce the error rate. The RNA quality was evaluated using electrophoresis and the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). To generate first-strand cDNA from 1 ug of total RNA from each sample, the cDNA Synthesis Kit (Takara, China) was used. Before use, the reverse transcription products were diluted 20 times and stored at 20°C. The online tool Primer3Plus was employed to construct GLK genes specific primers in soybean. Soybean actin primers were used as a control (Table S6). Quantitative RT-PCR was performed using a CFX-Bio-Rad RT System. The $2-\Delta\Delta Ct$ method was used to evaluate the data. The relative expression levels were normalized to those of the housekeeping genes.

Results

Identification of GmGLK genes containing protein subsection

To explore the *GmGLK* genes containing proteins in soybean, we accomplished a BLASTp search in the Phytozome v13 databases utilizing previously available *AtGLK* proteins from Arabidopsis (Alam et al., 2022). The non-redundant soybean GLK proteins were screened through SMART and Pfam online tools for the existence of a Myb-like domain. In total, 130 GLK genes were detected in the soybean genome, and were named *GmGLK1* to *GmGLK130* according to the location on the chromosome. Detailed information about *GmGLK* genes, including gene ID, genomic location, length of gene/protein, isoelectric point (pI), and molecular weight (Wt) is listed in Table S1. Furthermore, each of the identified *GmGLK* proteins was screened for the existence of any other domain in addition to the GLK domain (s). Six additional domains were identified, allowing the organization of 130 *GmGLK* proteins into seven groups (Table S2). Group I contained 60 *GmGLK* proteins (46.15%). None of these members had additional domains apart from the GLK domain. Group II comprised of 35 *GmGLK* members (26.92%) with an additional Myb-CC_LHEQLE domain. Group III included 27 GLK members (20.77%) with an additional REC domain, Group IV included two *GmGLK* proteins (1.54%) with an additional coiled-coil domain, and the remaining groups contained one member each with one or more additional domains (Table S2). GO analysis demonstrated that all soybean GLK proteins had DNA-binding activity, were primarily found in the nucleus, and were involved in a variety of biological activities in the cell (Table S3).

Evolutionary relationship of GLK genes containing proteins

A phylogenetic tree was generated based on Arabidopsis and soybean GLK genes encoding proteins through available MEGAX software with the option of the maximum likehood (ML) technique (Figure 1). Based on these results, all GLK members were clustered into five major groups (Groups A-E), with Arabidopsis and soybean orthologous or homologous proteins clustered together (Figure 1). Group A contained 54 (15 *AtGLK*, 39 *GmGLK*), group B contained 43 (12 *AtGLK*, 31 *GmGLK*), group C contained 22 (7 *AtGLK*, 15 *GmGLK*), group

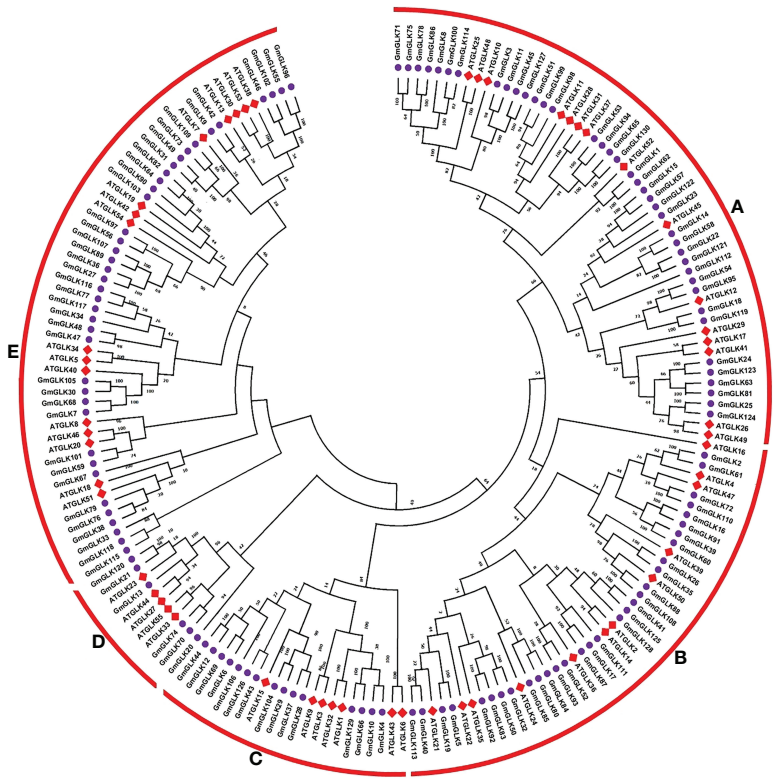

FIGURE 1
Phylogenetic tree and classification of *Glycine max* and *A. thaliana* GLK proteins. The ML-hood with Jones Taylor Thornton (JTT) model tree was constructed using MEGAX. The bootstrap support from 1000 replications is transformed to each node. The GLK proteins were clustered into five major groups **(A–E)**.

D contained 11 (5 *AtGLK*, 6 *GmGLK*), and group E contained 55 (16 *AtGLK*, 39 *GmGLK*) proteins (Figure 1).

Analysis of soybean GLK gene structure and conserved motif composition

The generated phylogenetic tree was analyzed for evolutionary relationships between GLK members using 130 *GmGLK* protein sequences (Figure 2). The numbers of exons and introns from one to eleven in soybean GLK genes. The gene structures were generally well-preserved within the variant groups of the phylogenetic tree classification. However, divergence in exon and intron numbers and lengths was observed between each group.

To study the structural variations of the soybean GLK proteins, we observed the conserved motifs organization of each GLK protein from soybean according to phylogenetic classification (Figure 1). As shown in Figure 2 and Figure S1, 15 conserved motifs were identified using MEME software with a range of 15 - 50 amino acids, and Motifs 1-3 were distributed across all GLK members (which contain the MADS, MYB, and LOBAS2 signature motifs). In addition, the remaining motifs were distributed among the different groups of the phylogenetic tree classification and could conceivably be used to distinguish between subfamilies. Group A includes motifs 6, 7, and 15 (which mostly contain Ab13vp1, bZIP, and G2-like signature motifs). Group B includes motif 14 (which contains the AP2EREBP signature motif). Group C includes motifs 9 and 13, whereas some members have motif 11 (which contains the CCAATHAP3, HB, and Trihelix signature motifs). Group D contains only two members with an additional motif 11 (which contains the Trihelix signature motif), and group E includes motifs 4, 5, 10, 11, and 12 (which contain the WRKY, BBRBPC, ARID, Trihelix, and GRF signature motifs) (Figure S1).

Physical location of *GmGLKs* on the chromosome and Ka, Ks analysis

The chromosomal location of each soybean GLK member was retrieved from the Phytozome v13 database (Table S1) and mapped onto specific soybean chromosomes (Figure 3). The 130 *GmGLK* genes were positioned on 20 chromosomes, including seven *GmGLK* genes on Chr-1, ten on Chr-2, eight on Chr-3, three on Chr-4, six on Chr-5, four on Chr-6, eight on Chr-7, five on Chr-8, eleven on Chr-9, five on Chr-10, six on Chr-11, seven on Chr-12, six on Chr-13, five on Chr-14, eight on Chr-15, two on Chr-16, seven on Chr-17, five on Chr-18, eleven on Chr-19, and six on Chr-20 (Figure 3). Our mapping analysis revealed that 95 out of 130 (73.07%) *GmGLK* pairs of genes were involved in segmental duplications, and one pair in tandem duplication (Figure 3). Gene duplication is a major contributor to the gene family expansion throughout the genome's expansion (Cannon et al., 2004). The ancient tetraploid soybean has experienced two round of genome duplications (Zhao et al., 2017). Most soybean genes are paralogous, meaning that they exist in many numbers. To examine the evolutionary history of *GmGLK* duplicated members, the Ka, Ks, and Ka/Ks ratios between members of the paralogous pairs were analyzed (Table S4). The Ka/Ks ratios ranged from 0.0313–0.7109, with an average of 0.3375 indicating that *GmGLK* genes have undergone strong purifying selection during evolution. The probable divergence times among the segmentally duplicated *GmGLK* gene pairs ranged from 4.598–94.844 million years (my) with an average of 36.195 my (Table S4), indicating that these gene pairs in soybean plant underwent duplication events at approximately 11–35 and 110–170 million years ago (mya).

Promoter analysis of soybean GLK genes

Cis-acting elements found in the promoter region can assist in determining the activity of candidate genes. The 2-kb segment upstream of the soybean GLK genomic sequences from Phytozome v13 and was submitted to the PlantCARE database. The entire 130 *GmGLK* gene promoter regions with 4466 potential cis-acting elements were discovered. They were divided into four major groups: light-responsive (21), phytohormone responsive (10), plant developmental responses (8), and stress-responsive (8) (Table S5). Three light-responsive elements (Box 4-motif, G box-motif, and TCT-motif), three plant hormone-related elements (ABRE, CGTCA-motif, and TGACG-motif) involving ABA, methyl jasmonate, and auxin signaling), three developmental responses (CAT-box motif, O_2-site motif, AT-rich element), and other stress-responses (ARE-motif, MYB-motif, and MYC-motif) were detected at high ratios in the soybean GLK genes promoter regions (Figure 4).

Expression profiles of GmGLK members across different tissues

Soybean GLK gene expression patterns were obtained by examining their RNA-seq expression files from the Phytozome V12.1 database. The retrieved expression values (FPKM) were log2-transformed, and a clustering heat map illustrating the expression profiles of GLK genes in different tissues or organs was constructed (Figure 5)—revealing 125 *GmGLK* genes were clustered into 12 main groups (Figure 3). Group I contained 11 *GmGLK* genes, which are mostly expressed in four tissues or organs (seed, root, root hair, and nodules). Group II included two *GmGLK* genes that are specifically and highly expressed in root hair tissue. Group III included nine genes, that are highly expressed in the root, followed by root hair and nodules. Group IV included 14 genes that are specifically and highly expressed in

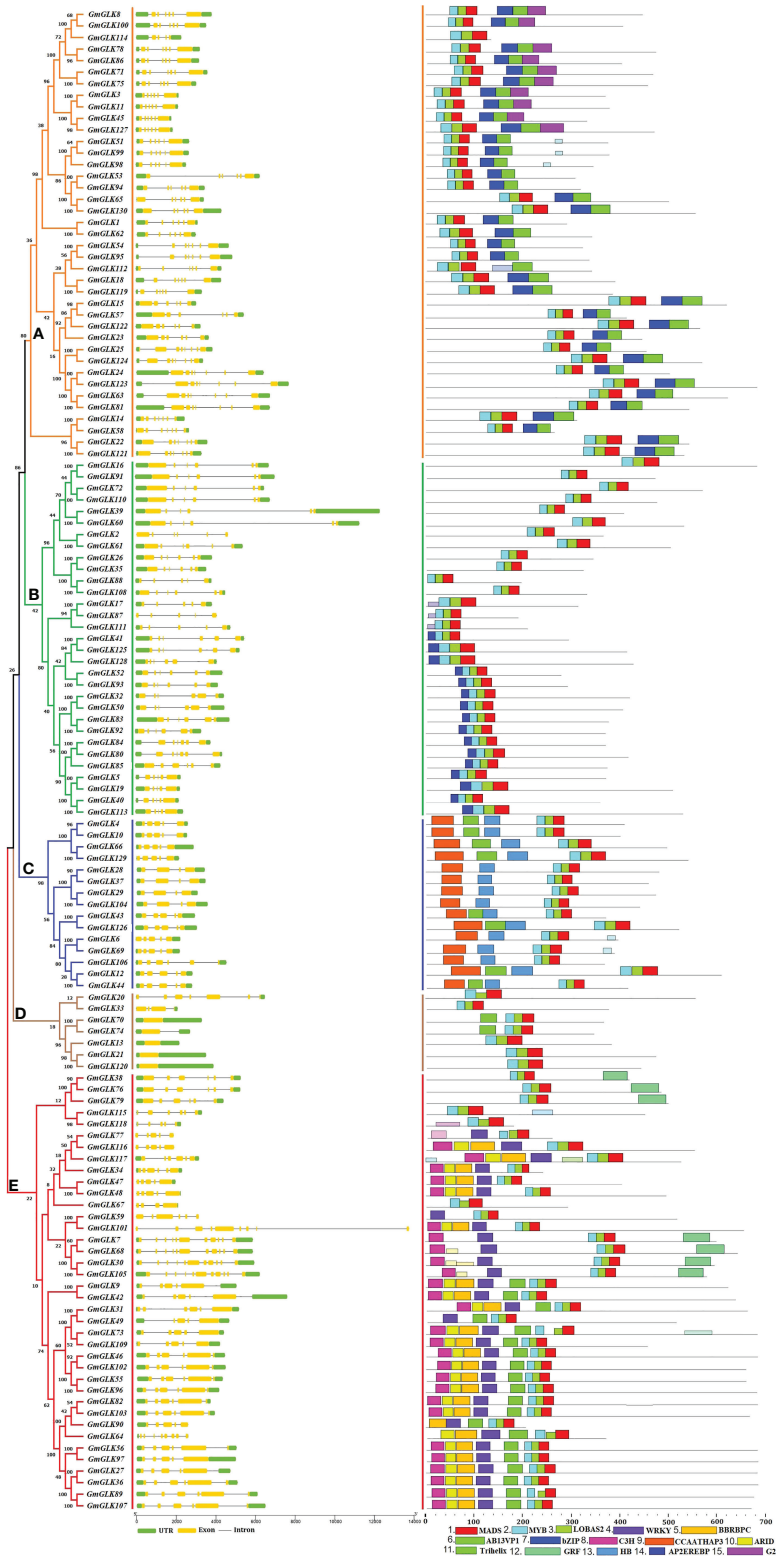

FIGURE 2
Schematic representation of the structural organization and motif composition of the soybean GLK genes. The ML approach evolutionary tree is presented on the left, which was clustered into five major groups **(A–E)**, preceded by the GLK gene structure, including exons and introns, which are represented by yellow color boxes and black lines, respectively. The preserved motifs are shown in different colors. Non-conserved regions are shown in black lines.

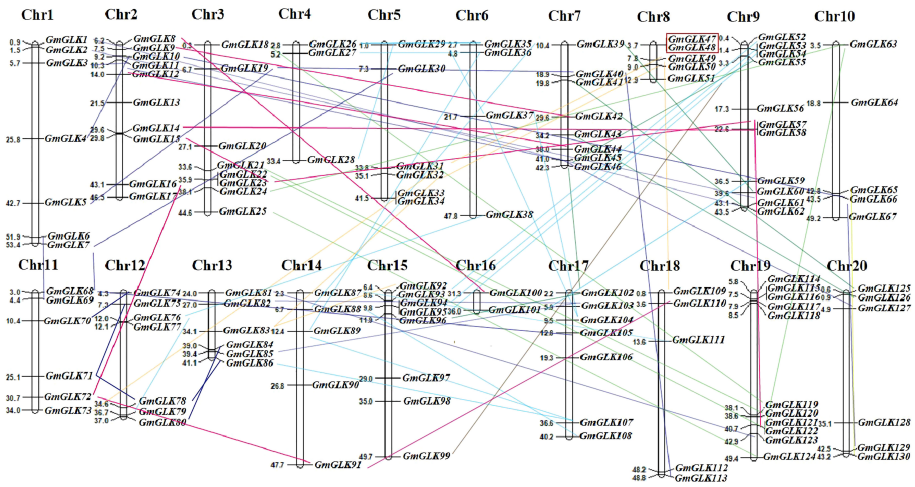

FIGURE 3
The distribution of GmGLK members on 20 soybean chromosomes.The chromosome number (Chr1-Chr20) and the name and physical position (Mb) of GLK members are represented on each chromosome. The segmentally duplicated members are connected with the variant lines, whereas the tandem duplicated members are shown in red color box.

nodules. Group V contained 11 genes. The majority of these genes are highly expressed in the leaves, whereas five are expressed in the roots. Group VI contained 14 genes that are highly expressed in flowers and leaves. Group VII included seven genes that are highly expressed in flowers. Group VIII comprised 18 genes, most of which are highly expressed in pods, followed by seeds and flowers. Group IX included eight genes; that are highly expressed in shoot apical meristem (SAM), followed by seeds and leaves. Group X included 11genes The majority of these genes are highly expressed in seeds, followed by pods and SAMs. Group XI includes 9 genes, which are highly expressed in stem, followed by nodules Group XII includes 11 genes explicitly enriched in SAM.

Expression of soybean GLK genes in response to cadmium and copper stresses

Heavy metal pollution is regarded as one of the most important societal concerns (Mustafa and Komatsu, 2016). When heavy metal concentrations in soil exceed a certain threshold, plant photosynthesis is inhibited, and nutrient uptake is inadequate, harshly restricting plant production (Cai, 2011; Wang et al., 2020a). Cd has the highest mobility and toxicity compared with that of other heavy metals and is hence one of the most harmful metal contaminants in plants and animals. Cd^{+} affects plant cellular activities, decreases root

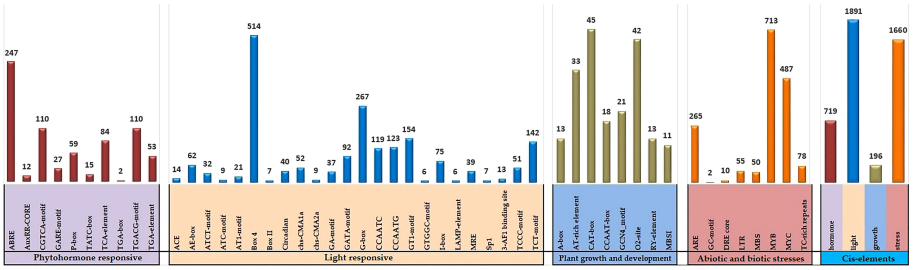

FIGURE 4
The number of cis-acting elements analyzed in the promoter region of *GmGLK* genes. A separate colored histogram shows the ratio of detected cis-regulatory components of each group.

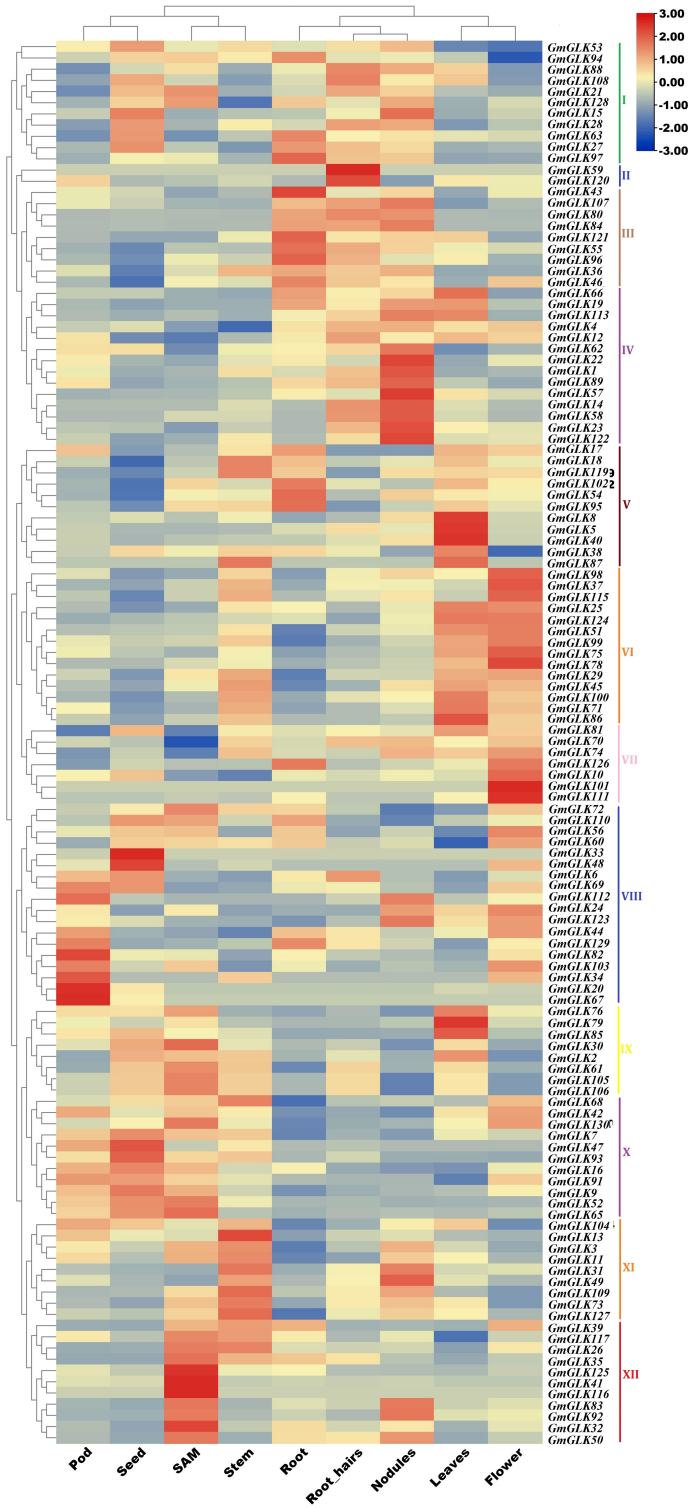

FIGURE 5
The expression pattern of soybean GLK members in various tissues/organs. The Phytozome database V12.1 was utilized to retrieve the expression data of *GmGLK* genes across numerous tissues. The relative bar level is on the top, and the types of tissue or organ are named on the bottom. The names of the *GmGLK* genes are present on the right side of the heatmap.

development, impairs regulatory processes, induces oxidative stress, inhibits nutrient acquisition, damages membranes, and may promote cell death under highly toxic conditions (Song et al., 2017; Chang et al., 2019). Based on our previously study in Arabidopsis, we selected eight orthologous genes in soybean to further understand their expression under Cd and Cu stress in soybean crops (Alam et al., 2022).

The relative expression of the eight *GmGLK* members in soybean seedlings exposed to Cd treatment for 1-6 hours was examined using qRT-PCR (Figure 6). The relative expression of six *GmGLK* genes was improved by exposure to Cd stress, including five GLK genes, namely *GmGLK1*, *GmGLK5*, *GmGLK13*, *GmGLK67*, and *GmGLK129*, which were 1–12-fold upregulated after exposure of Cd stress for 6 h. In addition, three *GmGLK* members, *GmGLK74*, *GmGLK105*, and *GmGLK106*, were downregulated after 6 h of Cd exposure. However, most *GmGLK* members were downregulated after 1 and 3 h of Cd exposure and upregulated after prolonged exposure to Cd (Figure 6).

Cu ion concentration significantly influences several metabolic pathways implicated in plant development. Both the excess and deficiency of Cu can seriously affect metabolic activities *in vivo*, such as limiting plant growth (Adrees et al., 2015). In addition, the expression of eight *GmGLK* members was examined using qRT-PCR in soybean seedlings exposed to Cu treatment for 1-6 hours (Figure 6). Four *GmGLK* genes—namely *GmGLK1*, *GmGLK5*, *GmGLK67*, and *GmGLK129* were 1 to 14 fold upregulated on exposure to Cu stress,. In contrast, three *GmGLK* genes, including *GmGLK74*, *GmGLK105*, and *GmGLK106* were downregulated following a 6-h exposure to Cu (Figure 7).

Co-expression network analysis of GmGLKs

Different proteins interact with one another to generate a protein interaction network, that contributes to the regulation of signal transmission and gene expression. To further investigate the role of GLK proteins in soybean, we examined the protein-protein interaction network of 130 soybean GLK proteins (Figure 8). The results showed that *GmGLK* plays pivotal roles in this network and involved in chlorophyll biosynthesis, circadian rhythms, and flowering regulation. A strong interaction was detected between photosynthesis-related genes and soybean GLK genes, especially *GmGLK38*, *GmGLK76*, and *GmGLK79*—suggesting that these genes may play an essential role in chloroplast development (Nakamura et al., 2009; Kobayashi et al., 2013; Shi et al., 2017). In addition, nine *GmGLK* genes were associated with circadian rhythms, and flowering-related genes—including *GmGLK13*, *GmGLK70* (GmLUXc), *GmGLK74* (GmLUXb), and *GmGLK120*—had strong interaction with *GmELF3a*, *GmELF3b*, *GmELF4b*, and *GmELF4b* genes—also related to the circadian clock and flowering (Nusinow et al., 2011; Preuss et al., 2012; Liew et al., 2017; Uehara et al., 2019).

Discussion

The G2-Like or GLK transcription factor belongs to the Myb transcription factors of the GARP superfamily. It is present in numerous plant species, including Arabidopsis, cotton, maize, and tobacco, and is involved in several stress responses (Liu et al., 2016; Qin et al., 2021; Zhao et al., 2021; Alam et al., 2022). However, to date, no study has reported on these proteins in soybean species. In the current study, we identified 130 GLK genes in soybean. All GLK protein members in soybean contain a Myb-like domain and various additional domains, including Myb CC LHEQLE, REC, transmembrane, coiled-coil, Hox, and DUF4281 domains. The presence of multiple domains in a protein signifies their evolution of the protein to perform numerous functions.

Analysis of evolutionary relationships shows that the *GmGLK* genes can be classified into five major groups with subgroups. The presence of a substantial bootstrap value on the inner tree branches suggests the presence of homologous proteins with the same activities from a common ancestor. Gene duplications are crucial for neofunctionalization and functional divergence (Birchler and Yang, 2022). Duplications, whether segmental or tandem, are major forces driving gene family expansion (Cannon et al., 2004). In this study, 95 out of 130 (73.07%) pairs of *GmGLK* members were involved in segmental duplications, and one pair in tandem duplication. Previous research has shown that the soybean genome has experienced two rounds of segmental duplication across its evolutionary history, with approximately 75 percent of the genes existing in many copies (Schmutz et al., 2010). The segmentally duplicated pairs diverged between 4.598 and 94.844 my, with an average of 36.195 my (Table S4). The results revealed that soybean has undergone two rounds of gene duplication events of about 11–35 and 110–170 mya, and the number of chromosomes in the soybean plant increased from 10 to 20 (Xu et al., 2011). The Ka/Ks ratio of all *GmGLK* duplicated members was reported to range from 0.0313 and 0.7109, and an average of 0.3375, implying the influence of selective pressure on the evolution of these pair of genes because a gene pairs with Ka/Ks less than one could specify purifying selection acting on the diverse protein-encoding genes throughout evolution (Tien et al., 2015).

The phylogenetic tree was generated from 55 *AtGLK* and 130 *GmGLK* genes, which were further distributed into five main groups (A, B, C, D, and E). As indicated in Figure 1, all phosphate starvation response (PHR1) genes were clustered into group A, which is the key regulator of the phosphate deprivation response (PDR) in Arabidopsis and rice (Bari et al., 2006; Zhou et al., 2008; Sun et al., 2016). KANADI

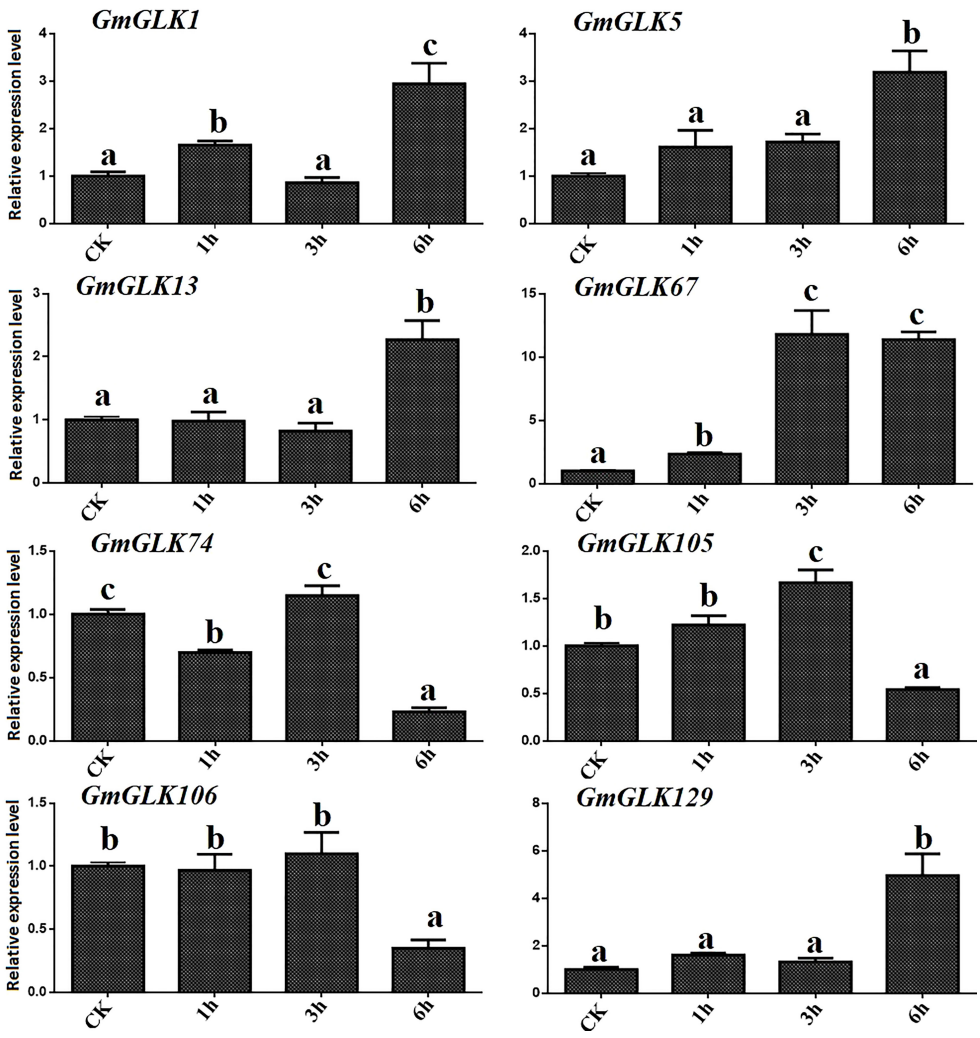

FIGURE 6
qRT-PCR analysis of the relative expression of GLK members in soybean under Cd stress. The time in hours is represented through the x-axis and relative expression level through y-axis. Tukey's HSD tests were utilized to measure the differences among effects on various time frames for Cd exposure. The alphabetical letters indicate significant difference ($p<0.05$).

(KAN)-like genes were found in group B, and they play important roles in organ positioning, cell type patterning, and organ morphogenesis of the SAM (Caggiano et al., 2017; Ram et al., 2020). Members of NIGT1/HRS1/HHO-like genes were clustered in group C. NIGT1 transcription factors have been reported to coordinate N and P responses in Arabidopsis (Kiba et al., 2018; Maeda et al., 2018; Ueda et al., 2020; Wang et al., 2020b; Ludewig et al., 2021). Eleven GLK members were clustered into subgroup D, including AtLUX (*AtGLK33*), AtMYBC1 (*AtGLK23*), and AtBOA (*AtGLK55*), which play important role in circadian oscillation were clustered into subgroup D (Bhutia et al., 2020; Alam et al., 2022). However, 55 GLK members were clustered into subgroup E, including *AtGLK1* and *AtGLK2*, involved in chloroplast formation (Fitter et al., 2002; Yasumura et al., 2005; Nagatoshi et al., 2016), and *AtGLK2*, which also plays a significant role in anthocyanin biosynthesis (Liu et al., 2021a).

The gene architectures were well conserved within a distinct class of phylogenetic categorization, and 15 conserved motifs were found in soybean GLK proteins; a comparable motifs numbers and organization were also reported in Arabidopsis and cotton (Zhao et al., 2021; Alam et al., 2022). The upstream region of the *AtGLK* genes contains essential cis-acting elements associated with phytohormones (ABRE, CGTCA, and TGACG), light response (G-box, Box 4, and TCT), developmental like (AT-rich motif, CAT-box, CCAAT-box), and stress-responsive (ARE, MYB, and MYC), which indicates that underlying hormones and environmental signals can regulate the *GmGLK*

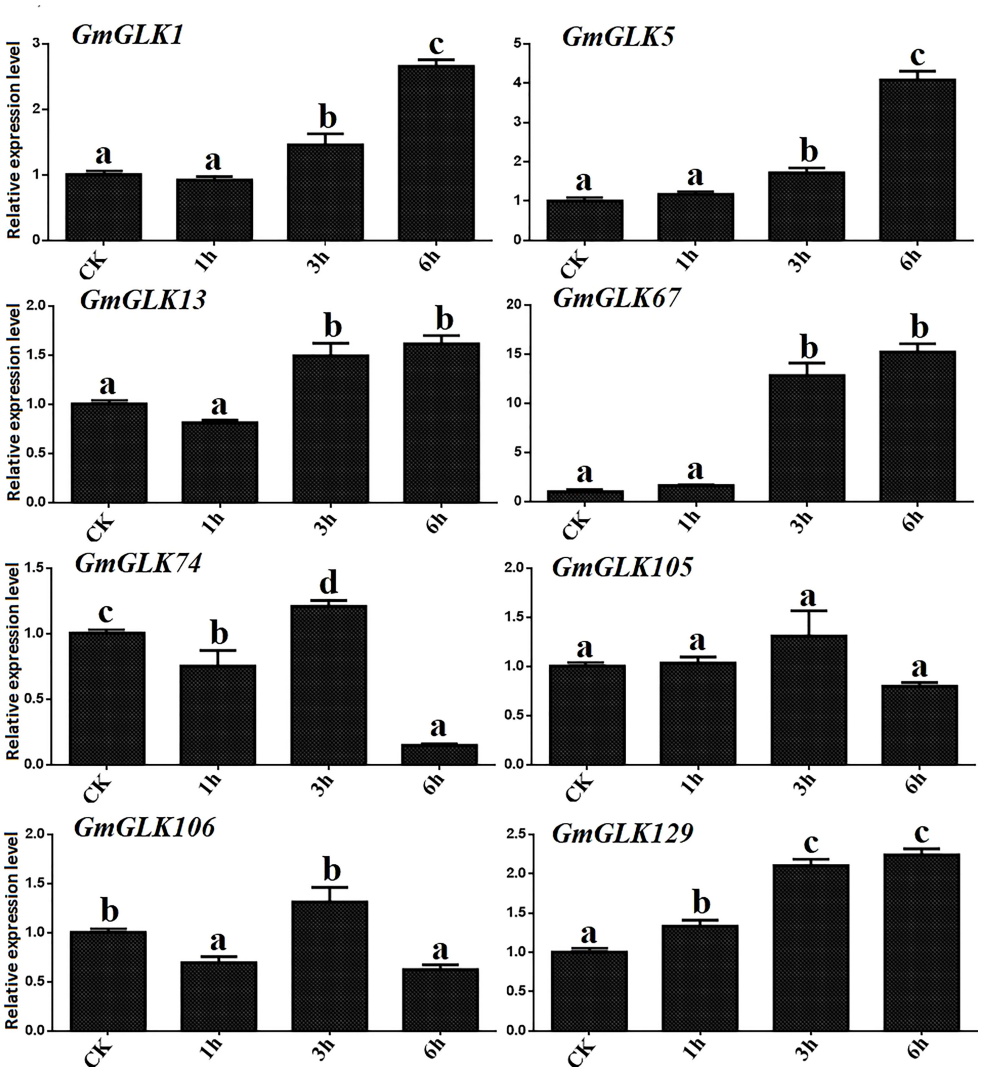

FIGURE 7
qRT-PCR analysis of the relative expression of GLK members in soybean under Cu stress. The time in hours is represented through the x-axis, and the relative expression level through y-axis. Tukey's HSD tests were utilized to measure the differences among effects on various time frames for Cu exposure. The alphabetical letters indicate significant difference ($p<0.05$).

gene expression, similar results were reported by other studies in Arabidopsis (Alam et al., 2022), cotton (Zhao et al., 2021) and tobacco (Qin et al., 2021).

Numerous studies have indicated that the *GmGLK* genes are TF that play important roles in plant development (Hall et al., 1998; Fitter et al., 2002; Yasumura et al., 2005). The expression analysis revealed that 125 *GmGLK* genes were classified into 12 groups and were mainly expressed in one or several of the tested tissues or organs, demonstrating the functional diversity of soybean crops. Several members of the *GmGLK* family that exhibit tissue-specific expression patterns may provide excellent targets for further research into their roles and possible use in plant genetic improvement. For example, *GmGLK38*, *GmGLK76*, and *GmGLK79* are explicitly expressed in leaves, while their orthologous genes in Arabidopsis play important roles in leaf development chloroplast formation, and anthocyanin biosynthesis (Fitter et al., 2002; Yasumura et al., 2005).

Protein-protein interaction analysis revealed that *GmGLK* genes encoding proteins play important role in chlorophyll biosynthesis, circadian rhythms, and regulation of flowering (Figure 8). In the current study, various *GmGLK* genes interacted with important chlorophyll biosynthesis genes, including three genes such as *GmGLK38*, *GmGLK76*, and *GmGLK79*, which were very strongly connected, as previously reported that these chlorophyll biosynthesis genes play a

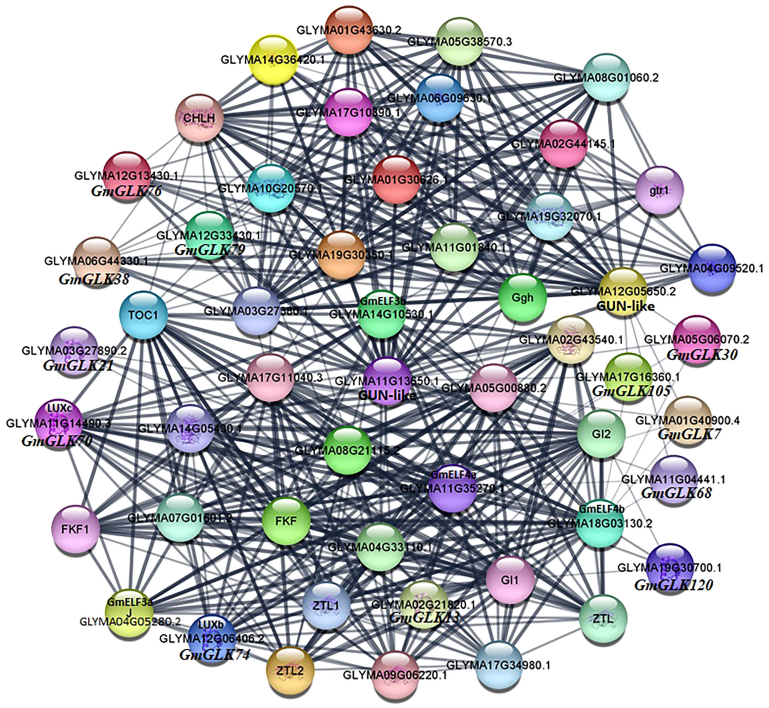

FIGURE 8
Co-expression networks of *GmGLK* proteins in soybean. The nodes indicate distinct proteins, whereas the edges indicate the interaction across proteins. The width of the edge representing the strength of the interaction.

significant role in chloroplast development in different species (Nakamura et al., 2009; Kobayashi et al., 2013; Sakuraba et al., 2017; Shi et al., 2017; Li M. et al., 2021). Furthermore, four *GmGLKs*, such as *GmGLK13*, *GmGLK70* (*GmLUXc*), *GmGLK74* (*GmLUXb*), and *GmGLK120* encode proteins that strongly interacted with the circadian clock and flowering-related proteins. However, previous studies also supported these connections that the *GmLUXb* and *GmLUXc* form protein complexes with *GmELF3a* and *GmELF4b* and share many different phenotypes, including a circadian oscillator, hypocotyl growth, and flowering regulation in various plants species (Hicks et al., 1996; Liu et al., 2001; Hazen et al., 2005; Onai and Ishiura, 2005; Nozue et al., 2007; Thines and Harmon, 2010; Nusinow et al., 2011).

Excessive concentration of metal ions is hazardous to plant cells. Cu and Cd are two of the most hazardous metal ions. The effects of Cd toxicity on plant biological processes include altered root development, disrupted of regulatory systems, oxidative stress, hampered nutrient uptake, membrane destruction, and, cell death in extreme cases (Song et al., 2017; Chang et al., 2019). The Cu ions level significantly affects several metabolic processes linked to plant development. Both the excess and shortage of Cu can affect vital metabolic processes *in vivo*, comprising regulating plant growth and development (Adrees et al., 2015). Previously, it was reported that Myb-like TF has a significant role in heavy metal toxicity. In Arabidopsis, a loss of function MYB72 mutant showed enhanced metal sensitivity (Van De Mortel et al., 2008). In addition, mutations in OsMYB45 cause a Cd hypersensitive phenotype (Hu et al., 2017). Many MYB-like genes, including MYB4, MYB28, MYB43, MYB48, MYB72, and MYB124, have previously been found to be highly expressed in Arabidopsis under Cd and other metal ion stresses (Van De Mortel et al., 2008). In current study, six *GmGLK* genes under Cd stress and four *GmGLK* genes under Cu stress were highly expressed, including *GmGLK1* (orthologous to Arabidopsis *AtGLK52*), *GmGLK5* (orthologous to Arabidopsis *AtGLK21*), and *GmGLK67* (orthologous to Arabidopsis *AtGLK51*) showed similar expression in both species under Cd and Cu stress treatments (Alam et al., 2022). The differential gene expression and activity of GLKs in Cd and Cu stress provides evidence that GLKs have a potential role during metal ion stress.

Conclusions

In this study, we discovered 130 *GmGLK* genes encoding TFs in soybean, which were unevenly distributed across 20

chromosomes. The *GmGLK* members were further evenly divided into the five major groups and were separated into clades A, B, C, D, and E of a phylogenetic tree. The gene structure and conserved motifs of *GmGLK* members from the same group or clade share common features, suggesting that they have similar biological activities. According to gene duplication analyses, segmental duplications play a significant role in the expansion of the *GmGLK* family and generation of novel *GmGLK* genes. Data from cis-regulatory element analyses, transcriptomic expression analysis and qRT-PCR experiments in response to cadmium and copper stress treatments revealed that *GmGLK* genes might be involved in soybean expansion and abiotic stress responses. Co-expression analysis identified many crucial *GmGLK* genes strongly connected with chlorophyll biosynthesis, circadian rhythms, and flowering regulatory networks and has provided valuable information for further functional characterization of each *GmGLK* gene across the legume species. In conclusion, our findings have laid the foundation for further functional studies of *GmGLK* genes, which may increase the present knowledge on soybean genetic improvement in response to Cd and Cu stress.

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

Conceptualization, IA, and LG; methodology, IA, HM, HZ, and QY; software, IA; experiments IA; formal analysis, IA; validation, IA, and LG; investigation, IA; data curation, IA; resources, LG; writing—original draft preparation, IA; writing—review and editing, IA, HM, HZ, QY, and LG; visualization, LG; supervision, LG; project administration, LG; funding acquisition, LG. The manuscript was read and approved for publication by all authors.

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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.

The reviewer AA declared a shared affiliation with the authors to the handling editor at the time of the review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1052659/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Tomtom analysis of transcription factors for 15 conserved motifs.

SUPPLEMENTARY TABLE 1
The list and detailed information of soybean GLK members.

SUPPLEMENTARY TABLE 2
Distribution of soybean GLK members classification based on their additional domain(s).

SUPPLEMENTARY TABLE 3
GO analysis of soybean GLK members.

SUPPLEMENTARY TABLE 4
The Ka, Ks, and Ka/Ks analysis and divergence event of duplicated GLK gene pairs in soybean.

SUPPLEMENTARY TABLE 5
Cis-acting element analysis of soybean GLK member promoter region.

SUPPLEMENTARY TABLE 6
Sequences of Soybean *GLK* gene primers for qRT-PCR.

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Effect of intermittent shade on nitrogen dynamics assessed by ^{15}N trace isotopes, enzymatic activity and yield of *Brassica napus* L.

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Modern era of agriculture is concerned with the environmental influence on crop growth and development. Shading is one of the crucial factors affecting crop growth considerably, which has been neglected over the years. Therefore, a two-year field experiment was aimed to investigate the effects of shading at flowering (S1) and pod development (S2) stages on nitrogen (N) dynamics, carbohydrates and yield of rapeseed. Two rapeseed genotypes (Chuannong and Zhongyouza) were selected to evaluate the effects of shading on ^{15}N trace isotopes, enzymatic activities, dry matter, nitrogen and carbohydrate distribution and their relationship with yield. The results demonstrated that both shading treatments disturbed the nitrogen accumulation and transportation at the maturity stage. It was found that shading induced the downregulation of the N mobilizing enzymes (NR, NiR, GS, and GOGAT) in leaves and pods at both developmental stages. Shading at both growth stages resulted in reduced dry matter of both varieties but only S2 exhibited the decline in pod shell and seeds dry weight in both years. Besides this, carbohydrates distribution toward economic organs was declined by S2 treatment and its substantial impact was also experienced in seed weight and seeds number per pod which ultimately decreased the yield in both genotypes. We also revealed that yield is positively correlated with dry matter, nitrogen content and carbohydrates transportation. In contrast to Chuannong, the Zhongyouza genotype performed relatively better under shade stress. Overall, it was noticed that shading at pod developmental stage considerable affected the transportation of N and carbohydrates which led to reduced

rapeseed yield as compared to shading at flowering stage. Our study provides basic theoretical support for the management techniques of rapeseed grown under low light regions and revealed the critical growth stage which can be negatively impacted by low light.

KEYWORDS

nitrogen, ^{15}N isotopes, carbohydrates, yield, shade, rapeseed

1 Introduction

In the modern era, agriculture is concerned with the environmental impact on crop yield and nutritional quality. Rapeseed (*Brassica napus* L.) is one of the most frequently consumed oilseeds crop worldwide, with double the oil yield per hectare as soybean. After rice, maize, and wheat, rapeseed is China's fourth most farmed crop (Hu et al., 2022). The Yangtze River Basin is the main rapeseed-producing region, where farmers adopt an intensive cropping system to get better yields (Li et al., 2018). Furthermore, the demand for rapeseed oil as a sustainable energy source has risen significantly (Ahmad et al., 2011). Light is possibly the most geographically and temporally variable of all the environmental conditions that affect plant performance (Nascimento et al., 2015). Light signals photomorphogenesis and supplies energy to develop plant assimilatory power (Kumar et al., 2016). Global climate change has reduced daylight hours and solar radiation during the last 50 years (Ren, 2005). Clouds and greater plant populations can restrict light availability, especially in later growth phases. Under the influence of meteorological and environmental factors, the tallest crops are frequently susceptible to low light stress or self-shading (Gao et al., 2018). The impact of shade stress depends on the cultivar, growth stage, shading intensity, and shading duration. Shade stress damages the plant's morphology and ultrastructure, limiting chlorophyll synthesis and lowering the canopy's photosynthetic capability (Li et al., 2010; Mu et al., 2010; Bellasio and Griffiths, 2014). As a result, shading stress lowers photosynthate production and grain yield (Clay et al., 2009; Chikov et al., 2016; Ren et al., 2016). Light plays a vital role in plants' photosynthate accumulation and nutrient intake and distribution (Clay et al., 2009; Cui et al., 2013). Absorption, assimilation, and transport of nitrogen (N) directly impact growth and development (Bu et al., 2014; Jia et al., 2014; Ihtisham et al., 2018). Nitrate is the most prevalent form of nitrogen available to plants due to the quick nitrification of the regularly used reduced forms of nitrogen. After being absorbed by the plant, nitrate needs to be converted to an ammoniacal form in order to be incorporated into amino acids for protein synthesis. The first enzyme that carries out the rate-limiting step in converting nitrate to ammonia in the nitrate assimilatory pathway is nitrate reductase, which is substrate-inducible (Eilrich and Hageman, 1973). Inorganic nitrogen can only be absorbed and utilized when transformed into organic nitrogen, with glutamate and glutamine being the major assimilation metabolites generated from ammonia. Glutamine synthetase (GS)/glutamate synthase (GOGAT) was discovered to catalyze ammonia assimilation (Lea and Miflin, 1974) and it was determined to be the principal mechanism for ammonia assimilation in higher plants (Hirel et al., 2001; Miflin and Habash, 2002; Glevarec et al., 2004; Martin et al., 2006). The transamination that transfers amino groups from glutamate to other amino acids perform crucial functions in nitrogen metabolism (Lea et al., 1992). The accumulation and partition of photosynthate determine the grain yield (Sun et al., 2017; Zhai et al., 2017). Before anthesis, a large number of carbohydrates and nitrogenous chemicals accumulate, which are then reallocated to the grain (Yang et al., 2001; Xu et al., 2006). The content of grain N depends on the rate of nitrogen accumulation and proportion of translocation from distinct organs of crop (Chen et al., 2015a). Additionally, the ratio of nitrogen translocated from the vegetative organs to the grain is influenced by climatic factors, management techniques, soil nutrients, and water availability, which are crucial for crop yield (Dordas and Sioulas, 2009). Yangtze river basin is the part of southern region of China. As a result of the significantly decreased light intensity in southern China, where plants face low light stress during different growth stages of different crops (Setién et al., 2013; Gao et al., 2017b). Thus, it is critical to explore the accumulation and remobilization of dry matter (DM), N and carbohydrates under shading stress at different growth stages of rapeseed. Although many studies have examined the changes in N distribution in response to various growth conditions such as temperature, precipitation, and nitrogen-deposition conditions (Villar-Salvador et al., 2015), but very few have focused on the effects of shade stress on N assimilation and distribution at different growth stages of crops especially the rapeseed. So, we hypothesized that low light stress at pod development stage significantly altered the N dynamic, carbohydrates transportation and ultimately causes the yield reduction. Artificial shade environments were used to simulate the field shade conditions to investigate the plant dry matter and nitrogen accumulation processes. The accumulation and translocation of nitrogen were investigated using the ^{15}N stable

isotope tracer under shading at various growth stages of rapeseed. The specific objectives of this study were to quantify the effects of various shading periods on rapeseed dry matter and N accumulation and to identify the critical growth stage that has the most significant impact on N dynamics and yield in rapeseed plants.

2 Materials and methods

2.1 Experimental location

A two-year field experiment was carried out at Huihe village, Chengdu plain, Sichuan province (102°54-104°53 E, 30°05-31°26 N) from 2020-22. It is a subtropical region with an average temperature of 16.1°C, annual total precipitation of 1780 mm, and a total sunshine duration of 1050 h (Sichuan Province Agro-meteorological Center, China). The basic soil fertility of soil includes organic matter (20.3 g/kg), total nitrogen (1.3 g/kg), available phosphorus (0.015 mg/kg), available potassium (0.118 mg/kg), and pH (6.7) in the topsoil layer (0-20cm). The monthly annual temperature and rainfall of the rapeseed growing season are demonstrated in Figure 1.

2.2 Experimental materials and layout

The two rapeseed genotypes (Chuannong and Zhongyouza) were involved in the two-year field trial. These rapeseed genotypes are abundantly cultivated in Sichuan province, especially in the higher reaches of the Yangtze river basin. The experiment employed a two-factor split-plot design. Three shading treatments were established at various growth stages of rapeseed; S0 = control (ambient light), S1 = shade from GS5 to GS6 and S2 = shade from GS7 to GS8 (Figure 2). The plants were enclosed by a layer of black polyethylene nets, which blocked approximately 35% of solar radiation. Two cultivars were assigned to the main plot and subplots received shading treatment. All treatments were carried out three times, yielding 24 plots with a 12 m^2 plot size. The prior harvested crop was rice, and the soil fertility was medium. The field was rotated before sowing, and a rope was manually pulled on-line while maintaining a row-row distance of 33 cm and a plant-plant gap of 20 cm. One seedling was left in each hole after emergence, and the baseline planting density was 150,000 plants/ha. Phosphorus and potassium fertilizers were applied at a rate of 90 kg/ha as base fertilizer. Nitrogen fertilizer was used at a 90 kg/ha rate in split dosages of 50% as base fertilizer + 50% topdressing at seedling stage. Local measurements were practiced to control the pests and weeds.

2.3 Sampling and measurement

2.3.1 Yield parameters

At maturity, 10 plants were chosen to measure the number of pods per plant, number of seeds per pod, 1000 seed weight and yield of both genotypes.

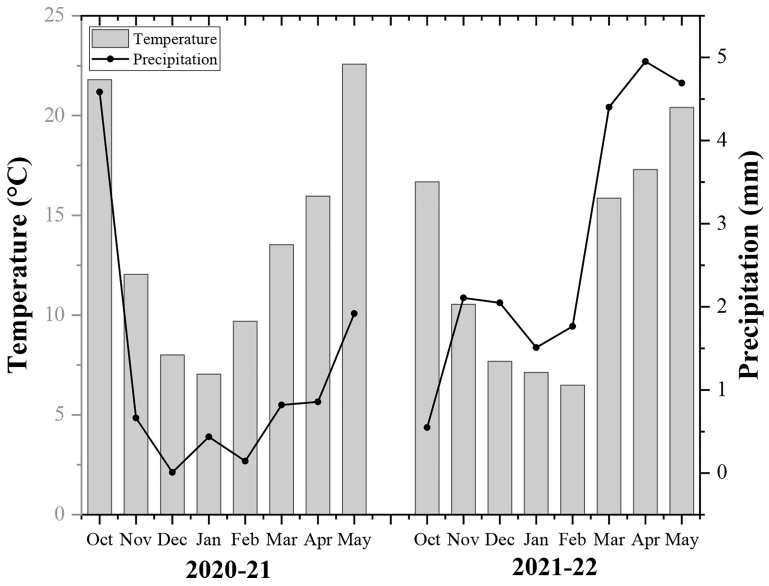

FIGURE 1
The monthly average temperature and precipitation of rapeseed growing seasons (2020-22).

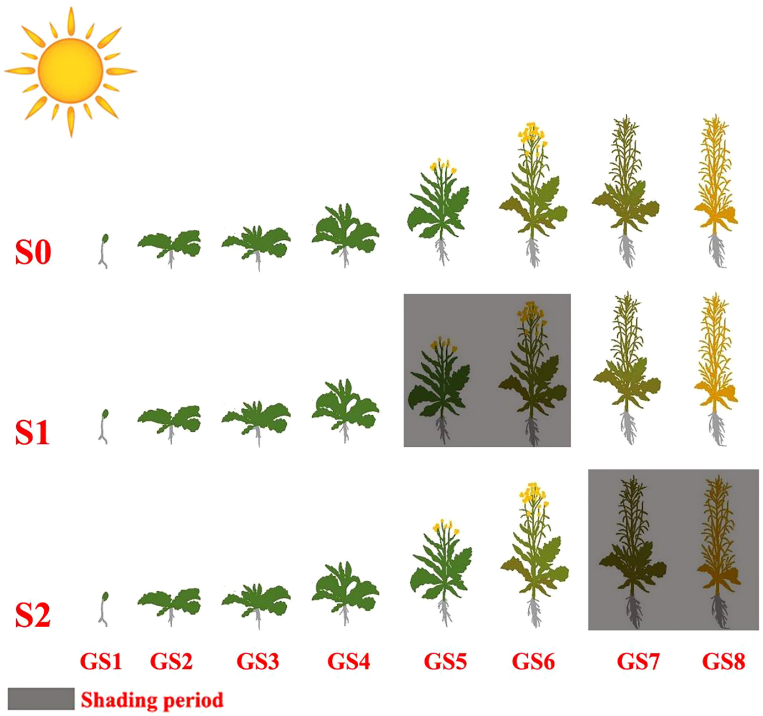

FIGURE 2
Diagrammatic representation of shading treatment at different growth stages of rapeseed. Control (ambient light) (S0); shade from GS5 to GS6 (S1); shade from GS7 to GS8 (S2); germination and emergence stage (GS1); leaf development stage (GS2); side-shoot development stage (GS3); stem prolongation stage (GS4); inflorescence emergence (GS5); flowering stage (GS6); pod development (GS7); harvesting stage (GS8).

2.3.2 Dry matter determination

At maturity stage, 10 plants were separated into stems, pod shells, and seeds to determine the dry matter. After that, samples were dried for 30 minutes at 105°C, followed by drying at 80°C, until a consistent weight was attained and the data was recorded as dry matter.

2.3.3 Determination of nitrogen content

The 6 plants were divided into stems, leaves, pod shells, and seeds at the GS6 and GS8 stages. Afterward, the samples were dried at 105°C for 30 minutes to stop the enzymatic activity, then dried at 80°C till a constant weight was obtained. Samples were then mashed with a mortar and sieved through a 0.5 mm sieve. The semi-automatic Kjeldahl nitrogen analyzer (FOSS 2300) was used to calculate total nitrogen content (Sparks et al., 2020). The following indices were calculated by following the previously published methods (Dordas and Sioulas, 2009; Gao et al., 2020):

$$\mathrm{NT\ (g\ plant^{-1})} = \mathrm{N\ at\ GS6} - \mathrm{N\ at\ GS8}$$
$$(\mathrm{stem + podshell + leaf\,(vegetativecomponents)})$$

$$\mathrm{NTE\ (\%)} = (\mathrm{NT/N\ content\ at\ GS6}) \times 100$$

$$\mathrm{NCP\ (\%)} = (\mathrm{NT/seed\ N\ at\ GS8}) \times 100$$

$$\mathrm{NHI\ (g\ plant^{-1})}$$
$$= \mathrm{Seed\ N\ at\ GS8/total\ N\ of\ above\text{-}ground\ biomass\ at\ GS8}$$

$$\mathrm{NA\ (g\ plant^{-1})} = \mathrm{Seed\ N\ at\ GS8/NT}$$

Note: N=nitrogen; GS6=pod development growth stage; GS8=harvesting stage; NT=nitrogen translocation; NTE=nitrogen translocation efficiency; NCP=nitrogen contribution proportion; NHI=nitrogen harvest index and NA=nitrogen assimilation.

The plants (3) with similar phenological characteristics of each plot were labeled with ^{15}N at GS5. Labeled plants of each plot were harvested at the end of GS7 and divided into leaves, stem, pod shell and grain. The samples were dried at 105°C for 30 minutes and then at 80°C in an oven (DHG-9423A Shanghai SANFA Scientific Instrument Co., Ltd.) to attain a constant weight. All of the samples were grounded into powder and sieved at 200 mesh. The enrichment of 15N in 4 mg powdered plant samples was determined using an isotope 100 mass spectrometer (Isoprime, Manchester, UK). The control treatment was

measured based on the plants without ^{15}N isotopes tracing. The accumulation of ^{15}N in organs was calculated as follows (Clay et al., 2016):

$$^{15}\text{Naccumulationofplantorgan} = \text{drymatterweight} \times \text{Nconcentration} \times ^{15}\text{Nenrichment}$$

2.3.4 Assay of NR, NiR, GS and GOGAT activities

Samples of fresh leaves and pods were collected in liquid nitrogen at 10-day intervals following shading to assess enzyme activity. According to previously described procedures, the enzymes nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT) were examined (LIANG et al., 2011; Majláth et al., 2016; Khan et al., 2020).

2.3.5 Total non-structural carbohydrates

The plant samples were oven-dried for 30 minutes at 105°C before being kept at 80°C till they reached a consistent weight. After that, samples were pulverized in an electric mortar and 0.1 g of powder was added to 6 mL of 80% ethanol, which was then put in water bath at 80°C for 40 minutes and centrifuged for 5 minutes at 5000 rpm. The supernatant was transferred to 50 mL tubes as the main solution, and the procedure was repeated twice. To make the primary solution 50 mL, 80% ethanol was added. For decolorization, 0.1 g charcoal solution was added to the primary solution, and the primary solution was filtered to use for the following analyses (Asghar et al., 2020).

2.3.5.1 Determination of sucrose

To determine sucrose content, 0.9 mL of primary solution was taken into test tubes and 0.1 mL of 2 M NaOH was added and placed in the water bath for 10 minutes. After heating, samples were allowed to cool at room temperature for 15 minutes. After that mixture was heated at 80°C with 3 mL of 10 M HCL and 1 mL of 0.1% resorcinol for 10 minutes. The supernatant was taken and absorbance was measured in a spectrophotometer at 480 nm (Spectra Max i3x from Austria) (Ghafoor et al., 2021).

2.3.5.2 Determination of reducing sugar

In 10 mL test tubes, 1.5 mL primary solution, 0.5 mL deionized H_2O, and 1.5 mL DNS solution were mixed to determine the reducing sugars. After that, the tubes were placed in 80°C water bath for 10 minutes. A spectrophotometer measured the absorbance at 520 nm in the supernatant (Spectra Max i3x from Austria).

2.3.5.3 Determination of soluble sugar

To measure the soluble sugar, 20 mL of test tubes were filled with 1 mL of primary solution and 4 ml of 0.2% sulfate anthrone combination. After that, samples were heated for 15 minutes in a water bath and cooled for 15 minutes at room temperature. The supernatant was measured at 480 nm in a spectrophotometer (Spectra Max i3x from Austria) (Raza et al., 2021).

2.4 Statistical analysis

The data was recorded and sorted out by Microsoft Excel 2019. SPSS 19.0 (SPSS, Chicago, IL, USA) software was used to statistically analyze all the data. To estimate the differences among treatments, ANOVA with three-way analysis of variance followed by least significant difference (LSD) at $p<0.05$ significance level was performed. Pearson correlation coefficient were calculated to determine the relationship between different parameters. All the tables and figures were shaped by Excel 2019 and Origin 2021 software (OriginLab Co., Northampton, MA, USA).

3 Results

3.1 Effect of shade on the yield attributes of rapeseed genotypes

Different shading treatments significantly altered the yield variables of both investigated rapeseed genotypes. In contrast to S0, the Chuannong genotype showed a decreased number of pods by 7.40 and 9.23% and Zhongyouza genotype experienced the 7.16 and 8.25% after S1 and S2 treatments as compared to S0, respectively. While the number of seeds per pod was lowered by 5.91 and 39.60% in Chuannong and 7.58 and 33.85% in Zhongyouza after the respective shading treatments. Under S1 and S2, the Chuannong exhibited 2.78 and 19.73% reduction and Zhongyouza showed 4.42 and 12.04% decline in 1000-seed weight following S1 and S2, respectively. In case of yield, the S1 and S2 declined the yield of Chuannong genotype by 13.31 and 50.03% and this reduction was 11.06 and 37.01% in Zhongyouza, respectively. Under various shading treatments, the aforementioned yield characteristics in both years demonstrated a similar trend, while 2020-21 year significantly exhibited higher yield in both genotypes (Figure 3). Additionally, S2 had a significant impact on all yield parameters. Taken altogether, it was observed that the Chuannong genotype was more shade-sensitive and showed lower yield than Zhongyouza under shade treatment.

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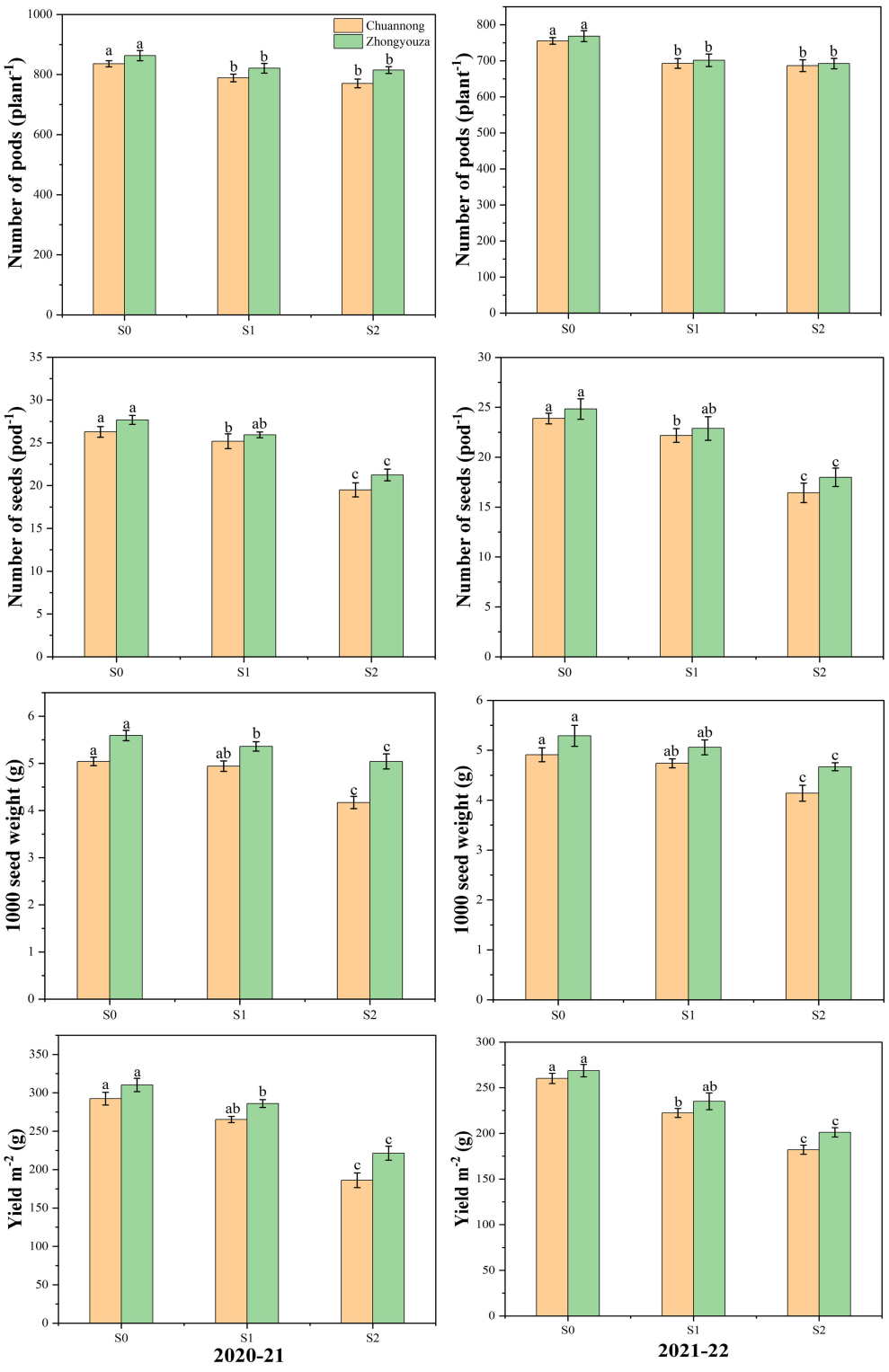

FIGURE 3
Effect of shading on yield parameters of rapeseed. S0= control (ambient light); S1= shade at the whole flowering stage and S2= shade at the start of pod development to pod maturity. Values were determined using the (n=10) LSD test, and various small letters denote the significance level of treatments at the 0.05 probability level (Duncan test).

3.2 Impact of shade stress on dry matter accumulation of rapeseed

Shade stress significantly reduced the dry matter of both rapeseed genotypes. According to two-year average data, the DM was reduced by 7.28 and 33.32% in Chuannong and 7.16 and 31.91% in Zhongyouza following S1 and S2 treatment as compared to S0, respectively. Shading at both growth stages disrupted the accumulation and distribution of DM in the organs of rapeseed. Under S2, a significant drop of DM was detected in the rapeseed organs. The seed weight was more affected by shading than stem and pod shells at the organ level under S2.

Contrary to S0, the Chuannong genotype showed the 8.96 and 58.34% decline in seed weights after S1 and S2 treatments as compared to S0, respectively, while Zhongyouza exhibited 22.9 and 49.63% inhibition after the respective shading treatments. The stem, pod shell, and seed weights of both genotypes under shading followed a similar decreasing trend: S2<S1<S0 (Figure 4). The DM of all organs followed the same reducing tendency in both years. but 2020-21 displayed higher dry matter than 2021-22 year. Aside from that, shade during the pod stage (S2) substantially impacted both cultivars' dry matter.

3.3 Shade-dependent changes in nitrogen accumulation and distribution in rapeseed

The differences in nitrogen accumulation and distribution were found under shading stress at distinct growth stages. The values in Tables 1 and 2 represent the mean value for two-year experiment. The total nitrogen (TN) of both genotypes was detected in the following increasing order: S0>S1>S2 at maturity stage. In contrast to S0, S1 and S2 treatments reduced the TN distribution of Chuannong by 17.84 and 73.29%, respectively, however this reduction was 8.47 and 40.27% in Zhongyouza, respectively (Table 1). Shading had an impact on rapeseed organs of both genotypes. For instance, S1 had lower nitrogen values for leaves and stems, whereas pod shells and seeds showed lower nitrogen values under S2. Regarding genotypes, a higher TN was observed in Zhongyouza. Moreover, shading treatments affected both genotypes' N contents of the leaves, stems, and pods (Table 1).

The lower value of N translocation (NT), N translocation efficiency (NTE) and N contribution proportion (NCP) was perceived in S1, whereas higher values were examined in S2 treatment. The NTE was 5.30 and 36.78% lower in Chuannong

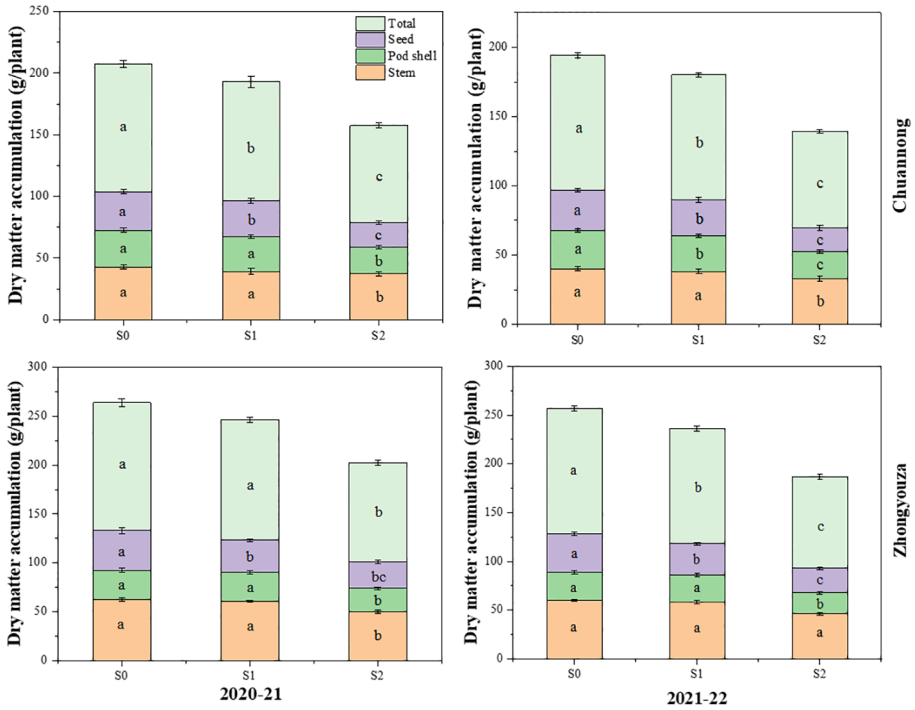

FIGURE 4
Effect of shading on dry matter accumulation in rapeseed. S0= control (ambient light); S1= shade at the whole flowering stage and S2= shade at the start of pod development to pod maturity. Values were determined using the (n=10) LSD test, and various small letters denote the significance level of treatments at the 0.05 probability level (Duncan test).

TABLE 1 Effect of shading on accumulation and distribution of nitrogen in rapeseed.

Varieties	Treatments	N accumulation at pod development (g plant^{-1})				N distribution at maturity (g plant^{-1})			
		Leaves	Stem	Pod	Total	Stem	Pod shell	Seed	Total
Chuannong	S0	2.02 ± 0.17b	1.23 ± 0.06a	2.13 ± 0.09a	5.39 ± 0.16b	1.74 ± 0.11b	1.08 ± 0.08a	5.35 ± 0.01b	8.18 ± 0.10b
	S1	1.13 ± 0.08c	0.26 ± 0.11c	1.33 ± 0.13b	2.72 ± 0.06de	0.48 ± 0.02d	1.02 ± 0.05a	5.04 ± 0.02c	6.54 ± 0.06d
	S2	2.29 ± 0.19ab	1.25 ± 0.22a	2.06 ± 0.03a	5.60 ± 0.03b	1.59 ± 0.15b	0.56 ± 0.02d	3.13 ± 0.03d	5.28 ± 0.10f
Zhongyouza	S0	2.55 ± 0.13a	1.42 ± 0.11a	2.10 ± 0.09a	6.08 ± 0.03a	1.95 ± 0.02a	0.89 ± 0.03b	6.14 ± 0.01a	8.97 ± 0.01a
	S1	1.09 ± 0.02c	0.56 ± 0.03bc	1.47 ± 0.03b	3.13 ± 0.01c	1.05 ± 0.03c	0.73 ± 0.01c	5.06 ± 0.01c	6.83 ± 0.03c
	S2	2.53 ± 0.14b	1.39 ± 0.06a	2.14 ± 0.04a	6.05 ± 0.16a	1.83 ± 0.02a	0.63 ± 0.03cd	3.12 ± 0.03d	5.57 ± 0.02e
Variance analysis	Y	**	**	**	*	**	**	**	**
	V	**	**	ns	**	**	**	**	**
	T	**	**	**	*	**	**	**	**
	Y×V	ns	ns	ns	ns	ns	ns	ns	ns
	Y×T	ns	ns	ns	ns	ns	ns	ns	ns
	V×T	*	ns	ns	*	**	**	**	**
	Y×V×T	ns	ns	ns	ns	ns	ns	ns	ns

S0, control (ambient light); S1, shade at the whole flowering stage and S2, shade at the start of pod development to pod maturity. Values were determined using the (n=6) LSD test, and various small letters denote the significance level of treatments at the 0.05 probability level (Duncan test). Y, V and T represent the year, variety and treatment, while **, * and ns denote the highly significant, significant and non-significant.

and 23.08 and 37.08% in Zhongyouza genotype under S1 as compared to S0 and S2, respectively. The N harvest index (NHI) and N assimilation (NA) values of both genotypes were lowest in S2 treatment (Table 2).

Shading decreased the distribution of ^{15}N isotopes in different organs of rapeseed (Figure 5). Compared to S0, the stem ^{15}N accumulation was declined in Chuannong genotype by 69.31 and 12.85% under S1 and S2, respectively, however, this change was 54.14 and 5.12% in Zhongyouza genotype. In Chuannong, the reduction of 7.17 and 72.35% in seeds ^{15}N accumulation was observed following S1and S2 relative to S0, respectively. While this inhibition was 15.31 and 86.38% for Zhongyouza. Leaf and stem ^{15}N accumulation of both genotypes displayed a increasing trend; S0>S2>S1. While pod shell and seeds exhibited a increasing trend; S0>S1>S2. The ^{15}N accumulation in the entire plant decreased under both shade tratements, compared to S0. In general, both rapeseed genotypes exhibited the following ^{15}N accumulation trend; S0>S1>S2. Taken altogether, it was noticed that the Zhongyouza displayed a higher accumulation of ^{15}N than Chuannong (Figure 5).

TABLE 2 Effect of shading on nitrogen translocation (NT), nitrogen translocation efficiency (NTE), nitrogen contribution proportion (NCP), nitrogen harvest index (NHI) and nitrogen assimilation (NA) in rapeseed.

Varieties	Treatments	NT(g plant $^{-1}$)	NTE(%)	NCP(%)	NHI (%)	NA (g plant $^{-1}$)
Chuannong	S0	2.56 ± 0.18d	47.44 ± 2.32c	47.84 ± 3.36b	0.65 ± 0.01d	2.79 ± 0.17b
	S1	1.22 ± 0.01e	45.05 ± 0.74c	24.28 ± 0.22c	0.77 ± 0.01a	3.81 ± 0.02a
	S2	3.45 ± 0.18ab	61.62 ± 3.04a	110.38 ± 5.92a	0.59 ± 0.03f	0.12 ± 0.18d
Zhongyouza	S0	3.24 ± 0.04c	53.27 ± 0.57b	52.78 ± 0.79b	0.68 ± 0.01c	2.89 ± 0.05b
	S1	1.35 ± 0.04e	43.28 ± 1.09c	26.75 ± 0.74c	0.74 ± 0.01b	3.70 ± 0.04a
	S2	3.59 ± 0.07a	59.33 ± 0.37a	115.30 ± 3.06a	0.56 ± 0.01g	0.18 ± 0.09d
Variance analysis	Y	**	**	**	**	**
	V	**	ns	*	*	ns
	T	**	**	**	**	**
	Y×V	ns	ns	ns	ns	ns
	Y×T	ns	ns	ns	ns	ns
	V×T	**	**	ns	**	ns
	Y×V×T	ns	ns	ns	ns	ns

S0, control (ambient light); S1, shade at the whole flowering stage and S2, shade at the start of pod development to pod maturity. Values were determined using the (n=6) LSD test, and various small letters denote the significance level of treatments at the 0.05 probability level (Duncan test). Y, V and T represent the year, variety and treatment. While **, * and ns denote the highly significant, significant and non-significant.

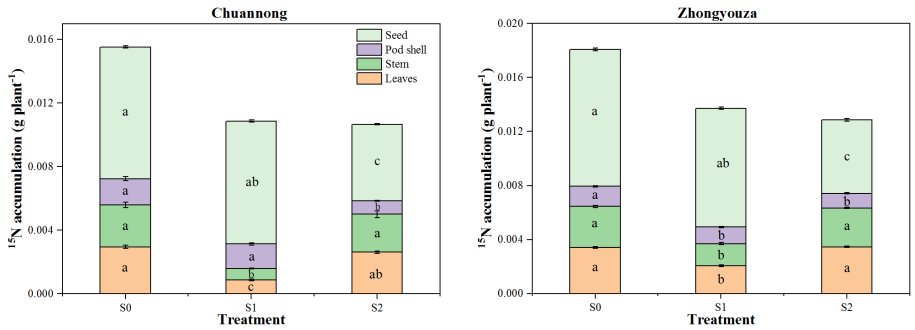

FIGURE 5
Distribution of ^{15}N to different plant organs of rapeseed at maturity under different shade conditions. S0= control (ambient light); S1= shade at the whole flowering stage and S2= shade at the start of pod development to pod maturity. Values were determined using the (n=3) LSD test, and various small letters denote the significance level of treatments at the 0.05 probability level (Duncan test).

3.4 Shade-induced modifications in enzymatic activities of rapeseed

The shading stress at both growth stages considerably influenced the enzymatic activities in the leaves and pod shells of both the studied genotypes. Relative to S0, the S1 reduced the NR, NiR, GS and GOGAT activities by 20.65, 8.60, 33.74 and 9.24% in leaves of Chuannong, respectively. Whereas the Zhongyouza experienced a 28.31, 12.96, 21.47 and 14.05% reduction following S1 as compared to S0, respectively (Figure 6).

In case of pod shell, the NR activity of Chuannong was reduced by 6.37 and 28.33% after S1 and S2 relative to S0, respectively, while this decline was 11.51 and 30% for Zhongyouza genotype. A decline of 7.75 and 11.47% was detected in NiR activity of Chuannong and 4.98 and 10.56% Zhongyouza genotypes under S1 and S2 when compared with S0, respectively. The S1 and S2 treatments also declined the pod shell GS activity of Chuannong (8.91 and 25.45%) and Zhongyouza (9.31 and 27.74%), respectively. Similarly, the pod shell GOGAT activity showed 15.05 and 24.67% decline in Chuannong and 6.50 and 16.46% in Zhongyouza genotype following S1 and S2, respectively (Figure 6). Comparing both genotypes, our findings unveiled that the Chuannong cultivar showed higher NR and GOGAT activity while Zhongyouza showed more NiR and GS enzymatic activities under S1 and S2 treatments. Furthermore, comparing S1 and S2, S2 significantly lowered all the enzymatic activities in both genotypes.

3.5 Shade-mediated modifications in carbohydrates accumulation at maturity

The changing trend of carbohydrates content of the both rapeseed genotypes was the same in both years. The shading treatment considerably reduced the sucrose, reducing sugar and soluble sugar contents of the stem and pod shell of both tested genotypes. The sucrose content of stem was declined by 14.08 and 41.28% in Chuannong and 6.87 and 35.36% in Zhongyouza, while pod shell showed a reduction of 16.45 and 35.18% in Chuannong and 14.19 and 37.99% in Zhongyouza following S1 and S2 treatments as compared to S0, respectively (average value based on two years). Generally, it was noticed that Zhongyouza genotype showed higher sucrose content than Chuannong genotype under all treatments.

Under various shading treatments, the reducing sugar content of Chuannong and Zhongyouza showed the following trend: S0>S1>S2 in both years. Compared with S0, the stem reducing sugar contents of Chuannong genotype experienced a decline by 15.21 and 76.66%, while this reduction for Zhongyouza genotype 10.71 and 51.21% after S1 and S2 treatments, respectively. In addition, the S1 and S2 decreased the reducing sugar of pod shell by 25.53 and 84.37% in Chuannong genotype and 15.52 and 55.81% in Zhongyouza genotype, respectively.

The soluble sugar content of Zhongyouza genotype was higher than that of Chuannong under all the treatments. Contrary to control, the stem soluble sugar of Chuannong genotype was inhibited by 10.52 and 46.72% and Zhongyouza genotype was reduced by 10 and 44.26% following S1 and S2 treatments, respectively. However, pod shell soluble sugar content showed a decline of 8.56 and 36.24% in Chuannong and 8.21 and 34.39% in Zhongyouza genotypes after the respective shading treatments. Moreover, the following inclination of carbohydrates was observed in both cultivars; S0>S1>S2 (Table 3). Furthermore, Zhongyouza showed significantly higher carbohydrates content in stem and pod shell and 2020-21 year showed higher values of carbohydrates as compared to 2021-22. Collectively, it was seen that the shade at the pod development stage (S2) significantly affected the carbohydrates content in both years.

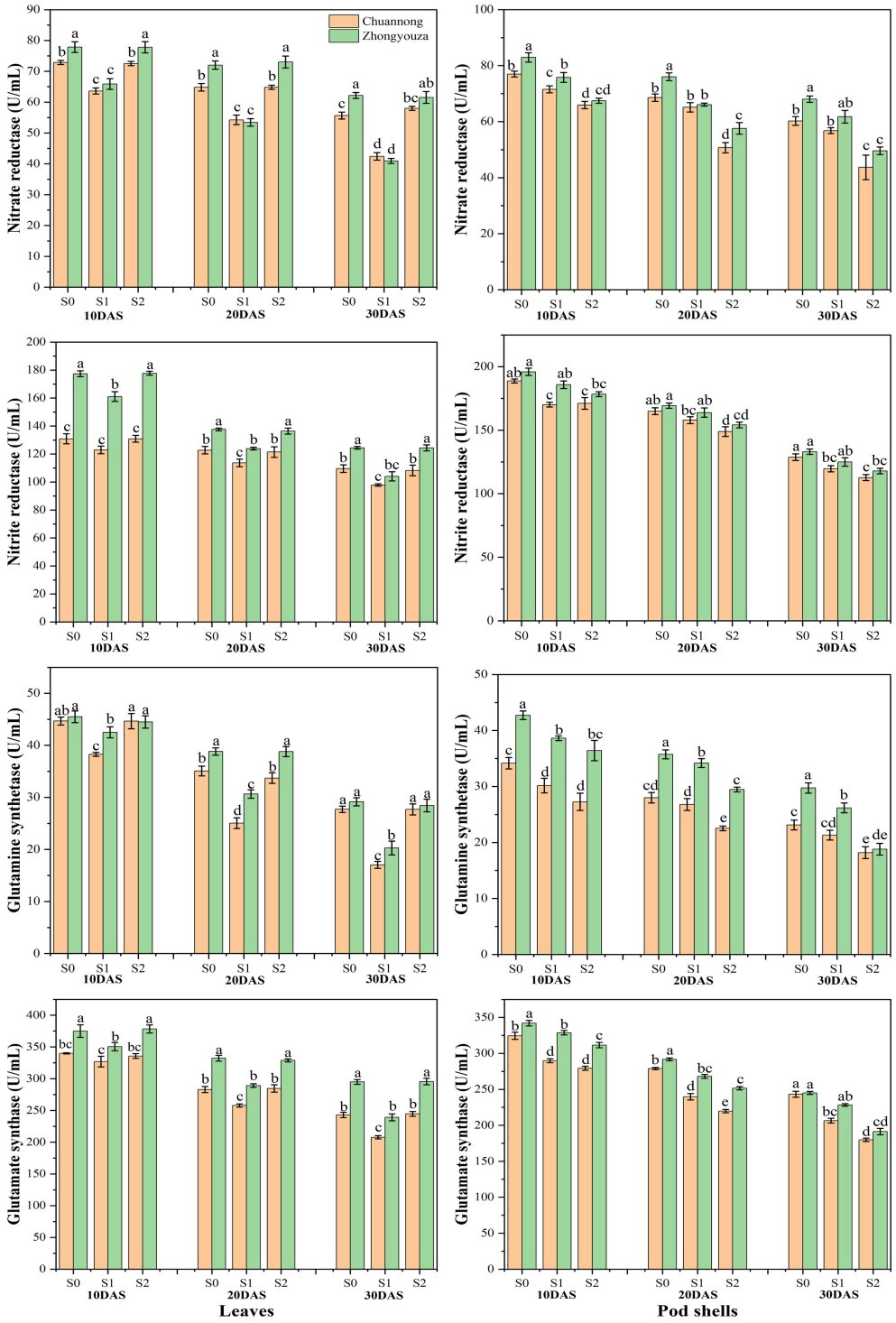

FIGURE 6
N mobilizing enzymatic activities under shade stress. S0= control (ambient light); S1= shade at the whole flowering stage and S2= shade at the start of pod development to pod maturity. Values were determined using the LSD test, and various small letters denote the significance level of treatments at the 0.05 probability level (Duncan test).

3.6 Correlation analysis

The current study's correlation analysis demonstrated that shade stress was substantially connected to yield metrics, nitrogen absorption and carbohydrates transportation. All the enzyme activities were significantly positive correlated with N transportation to different organs but a non-significant correlation of enzymatic activities with yield was observed. A negative correlation of NT, NTE, NCP and NA with carbohydrates were examined but carbohydrates exhibited positive correlation with yield parameters. Moreover, total dry matter and ^{15}N displayed a significantly positive correlation with seed yield (Figure 7).

4 Discussion

4.1 Response of yield parameters and dry matter under shade stress

Light is a critical environmental component impacting the growth and development of crops (Guoping et al., 2008; Zhong et al., 2014). Numerous studies have documented a decrease in yield due to shading stress (Cantagallo et al., 2004; Zhang et al., 2006; Acreche et al., 2009; Mu et al., 2010). In two-year studies, there were no significant declines in pods per plant but a significant fall in pod filling and grain weight (Wang et al., 2015). Previous researches have demonstrated that the drop in grain production was due to a reduction in grain number and weight (Acreche et al., 2009; Mu et al., 2010; Polthanee et al., 2011). Variations in ovule fertility and seed number per pod were impacted by changes in growth circumstances such as N availability, light and temperature (Bouttier and Morgan, 1992). Grain yield and spikelet filling had significant positive linear associations, while grain yield and grain weight showed a positive relationship (Wang et al., 2015). Shading reduced grain dry weight during grain filling, lowering grain yield (Ishibashi et al., 2014). To pinpoint crucial growth stage, most other reported field experiments have not used sufficiently defined durations of shading. For instance, (Habekotté, 1993) and (Iglesias and Miralles, 2014) both used shade (60% and 50%, respectively) for entire anthesis stage and resulting in yield losses of 50% and 15%, respectively, but no particular growth stage could be determined. Thus, to our knowledge, our study is among the fewer studies of rapeseed that has identified a relatively critical growth stage which affected by shading.

We found that shade in the beginning of the pod's development limited the assimilates transfer and decreased the

TABLE 3 Effect of shading on carbohydrates content of rapeseed at maturity.

			Stem carbohydrates at maturity (mg/g)			Pod shell carbohydrates at maturity (mg/g)		
Years	**Varieties**	**Treatments**	**Sucrose**	**Reducing sugar**	**Soluble sugar**	**Sucrose**	**Reducing sugar**	**Soluble sugar**
2020-21	Chuannong	S0	4.86 ± 0.02b	0.53 ± 0.01b	3.36 ± 0.03b	5.38 ± 0.01b	0.59 ± 0.01b	4.06 ± 0.05b
		S1	4.26 ± 0.03c	0.46 ± 0.01c	3.04 ± 0.03d	4.62 ± 0.04d	0.46 ± 0.02c	3.74 ± 0.04d
		S2	3.44 ± 0.09e	0.30 ± 0.01e	2.29 ± 0.01f	3.98 ± 0.03f	0.32 ± 0.01e	2.99 ± 0.02f
	Zhongyouza	S0	5.13 ± 0.04a	0.63 ± 0.01a	3.52 ± 0.01a	5.63 ± 0.02a	0.66 ± 0.01a	4.22 ± 0.02a
		S1	4.80 ± 0.03b	0.56 ± 0.01b	3.20 ± 0.01c	4.93 ± 0.02c	0.58 ± 0.01b	3.90 ± 0.01c
		S2	3.80 ± 0.03d	0.41 ± 0.01d	2.44 ± 0.02e	4.08 ± 0.05e	0.43 ± 0.01cd	3.14 ± 0.03e
2021-22	Chuannong	S0	4.1 ± 0.02b	0.49 ± 0.01b	3.05 ± 0.03b	4.61 ± 0.01b	0.52 ± 0.01b	3.74 ± 0.03b
		S1	3.50 ± 0.03c	0.42 ± 0.01c	2.73 ± 0.03d	3.85 ± 0.04d	0.39 ± 0.02c	3.42 ± 0.03d
		S2	2.67 ± 0.08e	0.26 ± 0.01e	1.97 ± 0.01f	3.20 ± 0.03f	0.25 ± 0.01e	2.67 ± 0.01f
	Zhongyouza	S0	4.37 ± 0.04a	0.58 ± 0.01a	3.21 ± 0.01a	4.86 ± 0.02a	0.59 ± 0.01a	3.90 ± 0.01a
		S1	4.04 ± 0.03b	0.51 ± 0.01b	2.89 ± 0.01c	4.16 ± 0.02c	0.51 ± 0.01b	3.58 ± 0.01c
		S2	3.04 ± 0.03d	0.37 ± 0.01d	2.13 ± 0.02e	3.31 ± 0.05e	0.36 ± 0.01d	2.82 ± 0.02e
Variance analysis		Y	*	**	*	*	**	*
		V	**	**	**	**	**	**
		T	*	**	*	*	**	*
		Y×V	ns	ns	ns	ns	ns	ns
		Y×T	ns	ns	ns	ns	ns	ns
		V×T	**	*	ns	**	*	ns
		Y×V×T	ns	ns	ns	ns	ns	ns

S0, control (ambient light); S1, shade at the whole flowering stage and S2, shade at the start of pod development to pod maturity. Values were determined using the (n=10) LSD test, and various small letters denote the significance level of treatments at the 0.05 probability level (Duncan test). Y, V and T represent the year, variety and treatment. While **, * and ns denote the highly significant, significant and non-significant.

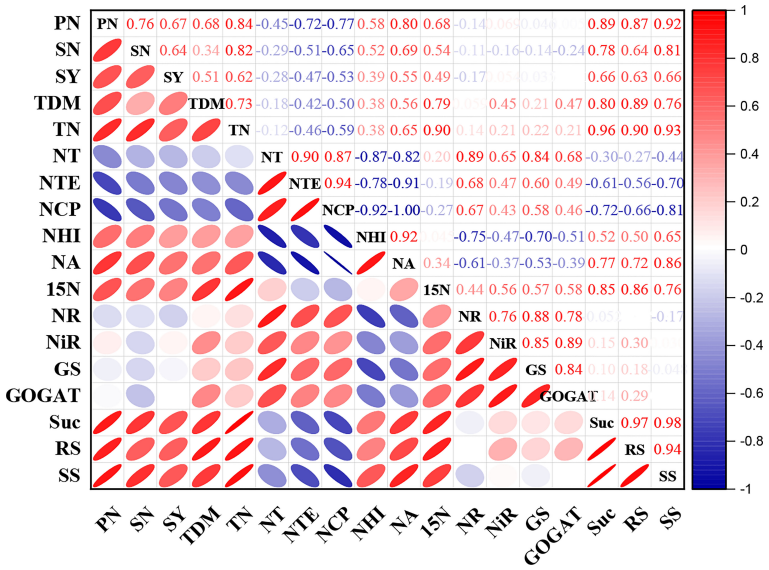

FIGURE 7
Correlation analysis between agronomic traits, nitrogen content, carbohydrates and yield. Red and blue color represents the positive and negative correlation. The size and intensity of color exhibited the significance of variables. PN, pod number; SN, seed number; SY, seed yield; TDM, total dry matter, TN, total nitrogen; NT, nitrogen translocation, NTE, nitrogen translocation efficiency; NCP, nitrogen contribution proportion; NHI, nitrogen harvest index; NA, nitrogen assimilation; 15N, ^{15}nitrogen isotope; NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; Suc, sucrose; RS, reducing sugar and SS, soluble sugar.

weight of the pod shell and the number of seeds per pod (Tayo and Morgan, 1979). In the current study, the S1 demonstrated a relatively high yield when the supply of pods and seeds resumes to normal levels after the shade has been removed, although a shortage of assimilates on flowers under shade is also damaging and diminishes the potential for compensatory growth. As previously discussed, I canola appears more vulnerable to severe temperatures and water deficits during late blooming and early pod set, aligning its sensitive periods with those of pulses rather than cereals (Sadras and Dreccer, 2015). Thus, they function similarly to the shade treatments applied in this study. In general, the observed correlations between the time of shade treatments and their effects on yield components and their relationships at maturity are similar to previously described physiological consequences of reduced assimilate supply (Tayo and Morgan, 1979; Keiller and Morgan, 1988; Diepenbrock, 2000). Dry matter production and accumulation are the primary determinants of crop yield, which are also limited by different environmental factors (CH, 1995). Shading stress greatly changed the physiology and morphology of the plant and eventually decreased the dry matter accumulation and distribution, resulting in decreased grain yield (Acreche et al., 2009; Li et al., 2010; Mauro et al., 2011). Dry matter accumulation in high-yield maize accounts for more than 60% of the total dry matter. Grain yield is influenced by the development and distribution of dry matter in vegetative organs such as the stem, leaf, and sheath (Huang et al., 2007). Our results demonstrated that S2 treatment considerably reduced the pod shell and seeds dry weight, which caused the yield drop in both rapeseed genotypes. We can conclude that shade at pod development stage (S2) is crucial to cause reduction in dry matter and yield.

4.2 N accumulation and distribution under shading conditions

Increasing biological yield is the foundation for increasing output; nutrient intake and distribution are key prerequisites for biological yield (Hirel et al., 2007). This study examined the changes in N accumulation and transportation under shade at various growth stages and deduced a portion of the mechanism underlying the grain yield response to N use. The remobilization of nitrogen in vegetative organs and the uptake of additional nitrogen throughout the grain-filling cycle provide grain N (Mueller and Vyn, 2016). Furthermore, nitrogen remobilization in the stem and leaf accounts for 69 to 80% of grain N (Subedi and Ma, 2005; Chen et al., 2014b). As a result, N accumulation and distribution in vegetative and reproductive organs play a pivotal role in dry matter weight at maturity and influence grain yield. According to our findings, shade declined the total N accumulation of rapeseed in the following order: S0>S1>S2>. The total N of the pod shell and seeds were significantly reduced by shade at pod development (S2)

compared to the flowering stage (S1). Furthermore, N buildup of S1 raised after the light was restored, but it did not return to normal levels (Table 1; Figure 5). The S2 inhibited the amount of N translocation towards pod shell and seeds compared to other treatments (Table 1), resulting in poorer yields (Chen et al., 2015b). When the accumulated N at pod development is smaller than the grain requirements, nitrogen transport rises (Chen et al., 2015b), as evidenced by our findings. Late-season shade (S2) reduced the N translocation towards economic organs (Table 1). As a result, we found that shading reduced N uptake distribution in all organs, resulting in a decrease in seed yield. In conclusion, shade reduced N buildup and impeded N transfer from vegetative organs to grain, such as leaves, stems, and pod shells. This study found that shading at pod developmental stage (S2) had a greater detrimental impact on N uptake than at flowering stage (S1), consistent with root shape and root physiology changes during shading (Figure 8) (Gao et al., 2017a). Shading altered the root structure, reducing root dry weight, absorption area, and active absorption area. Weather, climate, and air pollution contribute to shade, which is a challenging problem to solve in the manufacturing process. Changing sowing times is an excellent way to deal with low-light prone areas at later growth stage of crop, but it can be effected by temperature, soil moisture, and crop rotation as well (Gao et al., 2017a; Zhao et al., 2018).

4.3 Shade-dependent changes in N metabolizing enzyme activities

The leaf N content and enzyme activities are closely associated with each other (Sinclair et al., 2000). We observed that, shade inhibited the activity of NR, NiR, GS, and GOGAT, as was shown by the earlier research (Wang et al., 2020). GS and GOGAT are two essential enzymes involved in the N metabolism (Nigro et al., 2017). Due to shade, GS and GOGAT activities reduced gradually in the present study. This observation in grains was the same as in leaves (Wang et al., 2020). Wheat responds as a sensitive to ammonium nutrition at low light intensities, and its low GS activity is insufficient for ammonium assimilation. This occurrence apparently arose as a result of the significantly decreased light intensity in southern China, where plants face weak light stress during grain filling, which is relatable to our findings (Setién et al., 2013; Gao et al., 2017b). When plants were subjected to shade, nitrate delivery to the tops dropped considerably. The drop in NR activity resulted from the

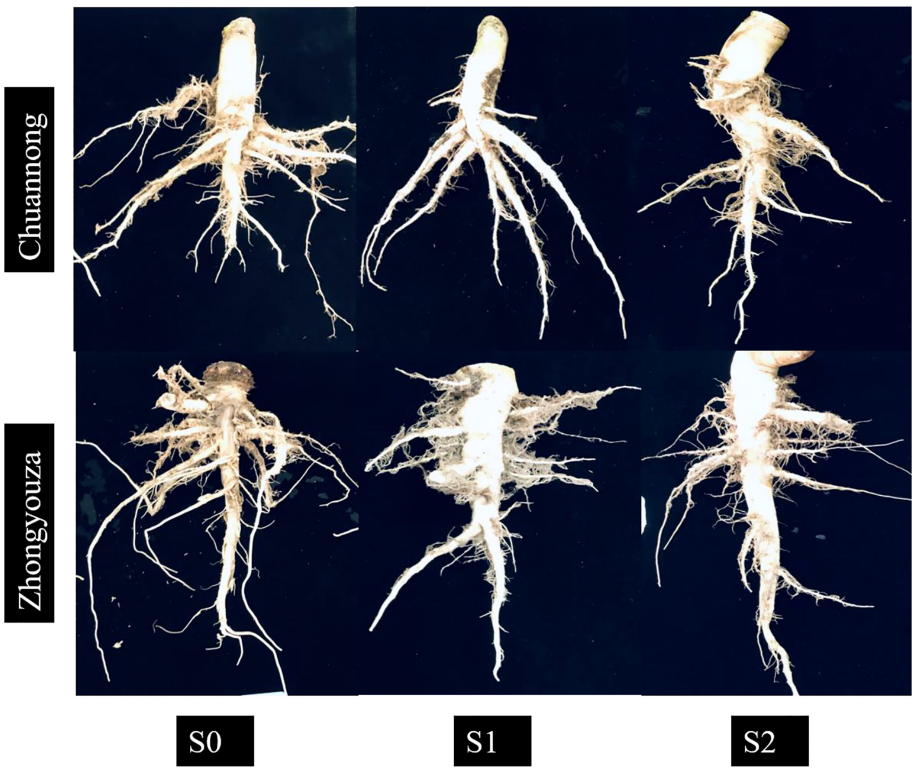

FIGURE 8
Effect of shading stress on the roots structure of two rapeseed genotypes.

reduction in nitrate concentration (Udayakumar et al., 1981). Reduced NR activities in shade-adapted plants make it easier for the plants to coordinate their N and carbon uptake across a variety of light conditions (Fredeen et al., 1991). The 50.3, 24 and 30.4% inhibition was also observed in NR, GS and GOGAT enzymatic activities following shade treatment (Yu et al., 2011). We concluded that leaves and pods' enzymatic activities are greatly reduced by shade. Among the shading treatments, the S2 treatment considerably declined the enzyme activities in the pods, which restricted the nitrogen transport towards seeds that led to low grain yield in both the investigated rapeseed genotypes.

4.4 Carbohydrates accumulation and distribution under shade

Shading limited the transformation of photosynthetic products. It accelerates the consumption of assimilates in leaves and stems and reduces grain yield (Li et al., 2013). Studies on different crops showed that the carbohydrate accumulation in leaves, stems, and roots decreased significantly under shading (Chen et al., 2014a; Hussain et al., 2021). As one of the main photosynthetic products, sucrose is significantly affected by light intensity and light cycle (Emerson, 1958). The decrease in sucrose content is closely related to light intensity. Shade reduce the output of leaves, the primary organ responsible for the formation of photosynthetic products, and eventually results in a decrease in sucrose content (Wu et al., 2017). The deleterious effect of whole-plant shade during grain filling on grain yield has been ascribed to photo-assimilate deficiency (Singh and Jenner, 1984; Okawa et al., 2003). However, there were differences in the accumulation and transport of carbohydrates under shading stress at different growth stages. This study showed that shading at pod stage (S2) had a more serious impact than shading at flowering stage (S1), which directly led to the reduction in grain yield. This could be because of leaf senescence, reducing photosynthetic potential, carbon fixation, and assimilates at pod development stage (Brouwer et al., 2012), which resulted in insufficient transportation of photosynthetic products. It was discovered that shaded wheat reduced grain output by speeding up the consumption of assimilates in the leaves and stems (Li et al., 2013). The authors further found that the carbohydrate of pod photosynthesis is mostly transported to the grain. In maize plants, the post-anthesis shading weakened the ability of nitrogen accumulation and stimulated the obvious re mobilization of carbohydrate reserves from stem to grain, but the decrease of grain filling rate eventually led to the decrease of grain yield (Reed et al., 1988). In this study, shading at flowering stage still reduced grain yield. The retardation can be attributed to the loss of non-structural carbohydrate transport to the kernel as a result of light deprivation (Mu et al., 2009) and kernel filling rate (Jichao and Zhiyong, 2005), which decreased the endosperm cell number and volume (Jia et al., 2011) and kernel set as a result of accelerated senescence. Starch deposition was decreased by shading, particularly under high shading. Additionally, ear shading decreased the kernel starch (Cui et al., 2012). Based on our findings, we can conclude that S2 treatment significantly reduced the carbohydrates translocation towards economic organs, leading to lower yields in both rapeseed genotypes.

5 Conclusion

Shading stress decreased DM and N accumulation, N transportation and distribution in multiple organs of rapeseed genotypes, and decreased the grain N content, which consequently reduced yield. The leaf and pod enzyme activities were also considerably influenced by the shade stress, which are associated with N accumulation and distribution. Relative to flowering stage, the shading at pod development stage significantly inhibited the carbohydrates transportation towards seeds. The Zhongyouza genotype outperformed Chuannong in all the aforementioned parameters under shade stress. Based on our findings, the current study provides the deeper insights into the effect of shade stress on the physio-biochemical mechanisms of rapeseed genotypes, which could be helpful for the management techniques of rapeseed grown under low light regions.

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

Conceptualization: Y-CW and HJ. Methodology: HJ, YH and WY. Data collection: HJ, JZ, XG, YH. Formal analysis and investigation: HJ, YH, JZ and XP. Writing - original draft preparation: HJ and MuAA. Writing - review and editing: HJ, MuAA, AG. Supervision: Y-CW. 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 γ-aminobutyric acid (GABA) mitigated salinity-induced impairments in mungbean plants by regulating their nitrogen metabolism and antioxidant potential

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Background: Increasing soil salinization has a detrimental effect on agricultural productivity.Therefore, strategies are needed to induce salinity-tolerance in crop species for sustainable foodproduction. γ-aminobutyric acid (GABA) plays a key role in regulating plant salinity stresstolerance. However, it remains largely unknown how mungbean plants (Vigna radiata L.) respondto exogenous GABA under salinity stress.

Methods: Thus, we evaluated the effect of exogenous GABA (1.5 mM) on the growth and physiobiochemicalresponse mechanism of mungbean plants to saline stress (0-, 50-, and 100 mM [NaCland Na2SO4, at a 1:1 molar ratio]).

Results: Increased saline stress adversely affected mungbean plants' growth and metabolism. Forinstance, leaf-stem-root biomass (34- and 56%, 31- and 53%, and 27- and 56% under 50- and 100mM, respectively]) and chlorophyll concentrations declined. The carotenoid level increased (10%)at 50 mM and remained unaffected at 100 mM. Hydrogen peroxide (H2O2), malondialdehyde (MDA), osmolytes (soluble sugars, soluble proteins, proline), total phenolic content, andenzymatic activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase(POD), glutathione reductase (GTR), and polyphenol oxidation (PPO) were significantlyincreased. In leaves, salinity

caused a significant increase in Na+ concentration but a decrease inK+ concentration, resulting in a low K+/Na+ concentration (51- and 71% under 50- and 100- mMstress). Additionally, nitrogen concentration and the activities of nitrate reductase (NR) andglutamine synthetase (GS) decreased significantly. The reduction in glutamate synthase (GOGAT)activity was only significant (65%) at 100 mM stress. Exogenous GABA decreased Na+, H2O2,and MDA concentrations but enhanced photosynthetic pigments, K+ and K+/Na+ ratio, Nmetabolism, osmolytes, and enzymatic antioxidant activities, thus reducing salinity-associatedstress damages, resulting in improved growth and biomass.

Conclusion: Exogenous GABA may have improved the salinity tolerance of mungbean plants by maintaining their morpho-physiological responses and reducing the accumulation of harmfulsubstances under salinity. Future molecular studies can contribute to a better understanding of themolecular mechanisms by which GABA regulates mungbean salinity tolerance.

KEYWORDS

Salinity, plant abiotic stress, plant growth regulators, phytohormones, physiologicalmechanism, antioxidant mechanism, nitrogen metabolism

1 Introduction

Soil salinity is a major abiotic factor that impedes agricultural productivity. Around 3600 million hectares (Mha) of arable land are degraded due to salinization, causing a loss of approximately USD 27.5 billion annually (Zhang and Shi, 2013; Qadir et al., 2014). As a consequence of global warming, environmental fluctuations, industrial pollution, unsustainable use of fertilizers, and irrigation using salt water, the problem of salinization is expected to worsen in the coming years (Zhu et al., 2019). The demand for food production will increase by 70% as the world population approaches 10 billion by 2050. This will put further pressure on the declining area of arable lands (Calone et al., 2021). Therefore, rapid salinization negatively impacts socioeconomic and ecological development (Sehrawat et al., 2018). Salt-affected soil results in the accumulation of toxic levels of salt ions in plants, for example Na^+ and Cl^- ions, resulting in osmotic stress, ionic toxicity, and nutrient deficiency (Munns and Tester, 2008; Polash et al., 2019; Rahman et al., 2019). Moreover, salinity hinders seed germination, seedling growth establishment (Ullah et al., 2022a; Saeed et al., 2022), nitrogen assimilation and anti-oxidant mechanism and several other key physiological processes (Ma et al., 2018; Ullah et al., 2022b).

The damages of salinity stress can be avoided by plants using several mechanisms, such as (i) the removal of toxic salt ions or their compartmentation within vacuoles or old tissues, (ii) the synthesis of compatible solutes, and (iii) the upregulation of non-enzymatic and enzymatic antioxidant defense mechanisms against salt-induced oxidative stress (Kaya et al., 2010a; Rahman et al., 2019; Mostofa et al., 2021). Salinity stress causes photoinhibition, which leads to an accumulation of untapped energy that destroys the photosynthetic apparatus, resulting in the formation of reactive oxygen species (ROS; Gururani et al., 2015; Polash et al., 2019; Ullah et al., 2022c). Increasing ROS levels weaken the antioxidant apparatus in cells, causing lipid peroxidation (MDA), which affects the membrane permeability and structure of the cell membrane, and causes damage to lipids, proteins, and nucleic acids (Tanveer, 2020). However, plants can upregulate their antioxidant mechanisms, including antioxidant enzymes [i.e. superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), glutathione reductase (GTR), and polyphenol oxidation (PPO), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR)] and metabolites [i.e. ascorbate (AsA) and glutathione

(GSH)] to reverse hydrogen peroxide (H_2O_2) (Kang et al., 2013; Kaya et al., 2020a). The anti-oxidant mechanism is, therefore, positively correlated with the salt tolerance of plants. Furthermore, plants use a greater fraction of their carbon and energy in stress-coping mechanisms, such as forming compatible solutes to maintain salinity homeostasis (Slama et al., 2015; Sami et al., 2016; Asrar et al., 2017).

These osmolytes prevent water loss and chlorophyll degradation, regulate cell division and expansion, stabilize proteins, eliminate excess ROS, maintain osmotic balance, prevent ionic toxicity, and regulate certain genes (Verdoy et al., 2006; Sami et al., 2016; Ahmad et al., 2016; Ullah et al., 2022b). Nitrogen (N) is an essential component of photosynthetic capacity, which enables plants to grow and develop optimally. Salinity impacts N metabolism by inhibiting its metabolizing enzymes such as nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthetase (GOGAT) enzymes (Debouba et al., 2006; Meng et al., 2016; Ullah et al., 2019; Ullah et al., 2022b). It is believed that N metabolism plays a profound role in the ability of plants to withstand salt stress. Nevertheless, the relationship between N metabolism and salinity is complex. It depends on several factors, including the level and duration of salt stress, the availability, type and source of N in the soil (Munns and Tester, 2008; Dai et al., 2015).

Mung bean (*Vigna radiata* L.) is a short-lived leguminous economic legume crop, rich in carbohydrates, protein, fibres, vitamins, fatty acids, minerals, and essential amino acids (Sehrawat et al., 2018). Approximately 3 million tons of mung beans each year, which accounts for 5% of the total production of pulses worldwide (World Vegetable Centre, 2018). Nevertheless, legumes such as mung beans are typically grown in arid and semi-arid regions where salt is a problem, thus making them salt-sensitive species (Sprent and Gehlot, 2010). Previous studies on mungbean plants reported that salinity affects their germination and seedling growth, plant growth and biomass, photosynthesis, nutrient acquisition, relative water content (RWC), ROS production, membrane stability, photosynthetic chlorophyll, and carotenoid pigments, root hair formation, nodule respiration and nodulation (review; Sehrawat et al., 2018). Moreover, its yield has been reported to drop to almost 70% under 50 mM NaCl, negatively affecting crop quality (Sehrawat et al., 2018). Considering the low productivity of mungbean, it is necessary to improve its salt tolerance to maintain its production in salt-affected soils. Therefore, it is imperative to examine its growth and adaptive physio-biochemical mechanism in response to salinity stress.

γ-Aminobutyric acid (GABA) is a nonprotein amino acid containing four highly water-soluble carbons. Mitochondria synthesizes GABA *via* the GABA shunt. Plants rapidly accumulate GABA in response to several abiotic stress factors (Mekonnen et al., 2016; Shelp et al., 2017; Sita and Kumar, 2020). Various plant growth regulators, including GABA, have been found to provide a level of tolerance to salinity through the modulation of physiological responses to the unfavorable environment (Ma et al., 2018; Jin et al., 2019; Khanna et al., 2021). Exogenous GABA has been demonstrated to enhance plant adaptation to abiotic stress conditions through improvements in growth, photosynthesis, enzymatic and non-enzymatic antioxidative defense mechanisms, and nitrogen metabolism (Beuve et al., 2004; Vijayakumari et al., 2016; Ma et al., 2018; Salah et al., 2019). Even though various studies have been conducted on the effects of salinity stress on mung beans, the effect of GABA application has largely been overlooked. For instance, a recent study on mung beans regarding GABA application under salinity focused only on its role in seed germination (Ji et al., 2020). Accordingly, GABA research needs to intensify the focus on legumes in general and mung beans in particular. We hypothesized that saline stress would hinder the metabolism of mungbean plants, but exogenous GABA may improve their morpho-physio-biochemical damages by reducing the adverse effects of saline stress. To test our hypothesis, we examined the effects of exogenous GABA application (1.5 mM) on growth, photosynthetic pigments, osmolytes, minerals regulation, nitrogen metabolism, lipid peroxidation, and antioxidant enzymes in saline-stressed (0-, 50-, and 100 mM) mung bean seedlings.

2 Materials and methods

2.1 Experiment design

The experiment was conducted in a greenhouse environment (controlled condition) at the Department of Botany, University of Peshawar, Peshawar, 25120 (34○15 North latitude and 71○42 East longitudes), KP Pakistan. Temperatures typically range between 5°C and 39°C from January to February and June to July, respectively, with an average rainfall of approximately 513 mm per year. The silt-loamy soil had pH 6.9, a bulk density of 1.55 g cm^{-3}, and EC 0.288 ds/m, collected from the experimental site. The seeds of local mungbean accession (*Vigna radiata* L.) were provided by Cereal Crop Research Institute (CCRI), Persabaq, Nowshera 24050, Pakistan. These seeds were sown in pots (15 cm in diameter) with an opening at the bottom (2 cm diameter) and filled with 2.5 kg of silt-loam soil.

2.2 Salinity treatments and γ-Aminobutyric acid (GABA) application

In the beginning, the seedlings were watered every three hours with tap water. At the first trifoliate leaf stage (three weeks after sowing), 48 pots (2 seedlings per pot) with uniform seedlings were selected and divided into six sets for the application of saline stress (SS; NaCl and Na_2SO_4, 1:1 molar ratio) and GABA application. There were three saline stress treatments: 0-mM (control), 50-mM, and 100-mM. The

remaining three groups were subjected to the same levels of saline stress but were given exogenous GABA solutions (1.5 mM, 200 ml; 100 ml GABA applied to each pot at 20- and 30-days of sowing, respectively). An initial seedlings growth experiment was conducted with GABA concentrations ranging between 0.25mM and 1.5mM (Supplementary Table 1). The optimal GABA concentration was selected based on the improved growth of mungbean seedlings subjected to 50 mM saline stress. Three replicates of each treatment were conducted. We harvested the 45-day-old mung bean plants and immediately froze them in liquid nitrogen before storing them at -80°C for physiologic analysis.

2.3 Measurement of plant growth and biomass

The stem height and root length were measured using a measuring tape. Next fresh and dry weights of the stem, leaves, and root of the seedlings were measured using an electric balance. For dry weight determination, the plants were oven-dried at 105°C for 30 min and then dried at 75°C until constant weight.

2.4 Measurement of photosynthetic pigments

We extracted photosynthetic chlorophyll pigments (0.1–0.3 g) from the fresh leaf samples (0.1–0.3 g) using ethanol (95%, vol/vol) following a standard method (Lichtenthaler and Buschmann, 2001). The absorbances were read at 665 nm and 649 nm using a spectrophotometer. Chlorophyll concentrations were calculated using the following equations (mg g^{-1} FW).

$$Chl\ a\ =\ 13.98\,A665\ -\ 6.88\,A649 \quad (1)$$

$$Chl\ b\ =\ 24.96\,A649\ -\ 7.32\,A665 \quad (2)$$

$$Chl\ a/b\ =\ Chl\ a\ /\ Chl\ b \quad (3)$$

$$Chl\ =\ Chl\ a\ +\ Chl\ b \quad (4)$$

2.5 Determination of mineral elements

We digested the leaf sample (0.05 g) with concentrated HNO_3 (3 mL). Afterwards, the extract was brought up and diluted to 15 mL with deionized water. An inductively coupled plasma-optical emission spectrometry (ICP-OES) was used to determine Na^+ and K^+ concentrations, following a standard method (Mostofa et al., 2015).

2.6 Determination of H_2O_2 and MDA concentration

A standard procedure (Patterson et al., 1984) was used to determine hydrogen peroxide (H_2O_2). Using a chilled mortar, fresh leaf samples (0.2 g) were homogenized in 5 ml of trichloroacetic acid (TCA) (0.1%) in an ice bath. In the following step, the extract was centrifuged at 5000 × g for 10 min (4°C). Next, the supernatant containing the titanium reagent (50 ml of 20% titanium tetrachloride in ammonia) and titanium reagent was centrifuged at 10,000 × g for 10 min. After five washes with acetone, the precipitate was centrifuged at 10,000 g for 10 min, followed by adding 3 ml of 1 M H_2SO_4.

For the evaluation of lipid peroxidation, malondialdehyde (MDA) concentrations were determined using the thiobarbituric acid (TBA) test (Heath and Packer, 1965). We homogenized fresh leaf samples (0.5 g) in 1ml of 5% TCA and centrifuged them for 10 min at 5,000 × g (4°C). We then added 4 ml of the supernatant to two ml of 20% TCA in a separate tube and heated this mixture at 100°C for 15 min before centrifuging it at 5,000 g (10 min). The absorbance at 450, 532, and 600 nm were read using a spectrophotometer, and MDA concentration was determined using the equation below.

$$MDA\ \left(mol\,g^{-1}FW\right)\ =\ =\ 6.45\,(A532\ -\ A600)\ -\ 0.56A450.$$

2.7 Antioxidant Enzyme Activities

A chilled mortar was used to grind and homogenize fresh leaf samples in a 0.1 M phosphate buffer (pH 7.3) solution and 0.5 mM ethylenediaminetetraacetic acid (EDTA). The Homogenates were centrifuged at 12000 ×g for 10 min (at 4° C). Following this, the supernatant containing enzyme extract was used for the assays. We determined the superoxide dismutase (SOD) activity using a standard method (Giannopolitis and Ries, 1977). Approximately 0.1 mL of enzyme extract was added to a reaction mixture of 50 mM phosphate buffer (pH 7.8), 130 mM of methionine, 2.0 mM of riboflavin, and 75 mM of nitro-blue tetrazolium (NBT). The activity of SOD was determined by measuring the decline rate of nitroblue tetrazolium at a 560 nm wavelength using a spectrophotometer. During the measurement of SOD activity, one unit corresponds to the amount of enzyme required to

inhibit 50% of NBT reduction at 560 nm.

Furthermore, POD activity was determined using standard methods (Wang et al., 2018) but with minor changes. An enzyme extract of 0.5 ml was mixed with 2 ml of buffer substrate (guaiacol and Na_3PO_4 pH 6.4), 24 mM H_2O_2, and 1 ml of buffer substrate. Measurement of absorbance at 460 nm was conducted twice at intervals of 1 min. Enzyme activity was calculated by increasing the absorbance of the reaction system by 0.01 up to a maximum of 1U per min, which was then converted to U/g·min^{-1}. The monitoring of H_2O_2 disappearance was used to determine CAT activity (Sabra et al., 2012). As a starting point, 50 ml of enzyme extract was dissolved in 1.5 ml of reaction mixture containing 50 mM K-phosphate buffer (pH 7.0) and 15 mM hydrogen peroxide. An absorbance measurement was conducted at 240 nm for 1 min using a spectrophotometer. The degradation of one mole of H_2O_2 per minute is equivalent to one unit of CAT. We determined the ascorbate peroxidase activity (APX) using a standard method. (Katsumi et al., 1994). Briefly, a 3 mL reaction mixture that contained 50 mM of phosphate buffer (pH 7.0), 1.0 mM of hydrogen peroxide, 0.25 mM of L-ascorbic acid, and 0.1 mL of enzyme extract was prepared. An increase in absorption, at 290 nm, was observed with a spectrophotometer following ascorbate oxidation. glutathione reductase (GR) activity was determined using a previous method (Carlberg and Mannervik, 1985). An enzyme extract was added to a reaction mixture of 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM GSSG, 3 mM MgCl2, and 0.2 mM NADPH. GSSG was added to initiate the reaction. A spectrophotometer was used to measure the absorbance at 340 nm. One unit of activity corresponds to the amount of glutathione reductase capable of catalyzing the oxidation of one mol of NADPH min$^{-1.}$ The Polyphenol peroxidase (PPO) activity was determined using a standard method (Cañal et al., 1988) with some modifications. We prepared a reaction mixture of 2.8 ml of 100 mM NaPi, pH 7.0, 0.1 ml of 25 mM pyrogallol, and 100 ml of enzyme extract. We maintained the mixture at 30°C for 30 min and measured the activity at 420nm after 30 minutes.

2.8 Determination of N-metabolizing enzymes

A sulfamate colorimetric method was used to determine nitrate reductase (NR) activity (Ullah et al., 2019). Briefly, fresh leaf samples (0.3 g) were homogenized in 200 mM KNO3, 5 mM EDTA, and 0.15 mM NADH in 100 mM phosphate buffer (pH 7.5), and the reaction mixture was incubated for 1 hour at 30°C. Next, the reaction mixture was centrifuged at 30,000 × g for 20 min. Next, 2 ml of sulfanilamide and N-naphthylamine reagents were added, and absorbance was read at 540 nm. Units of enzyme activity were expressed as µmol g^{-1} protein. We determined the glutamine synthetase (GS) activity using a standard method (Ullah et al., 2019). Fresh leaf samples (0.3 g) were homogenized in 2 ml of 50 mM Tris-HCl buffer (pH 7.8; containing 0.1% TritonX-100, 15% glycerol, 1 mM EDTA, and 14 mM 2-mercaptoethanol), and incubated at 37 °C for 30 min. Afterwards, the reaction mixture was centrifuged twice at 4°C for 10 min each time. Following this, 1 mL of ferric chloride reagent was added and centrifuged at 5,000°C for 10 min. At 540 nm, the absorbance was measured, and enzyme activity was expressed as µmol g^{-1} protein. The glutamine synthase (GOGAT) activity was determined according to a standard method (Ullah et al., 2019). The Assay mixture contained100 mM K^+-ketoglutaric acid, 20 mM L-glutamine, 3 mM NADH, and 10 mM KCl, in 25 mM Tris–HCl (pH 7.2). Next, the enzyme extract was added to initiate the reaction. An absorbance measurement at 340 nm continuously monitored the NADH oxidation. The oxidation of 1 µmol of NADH per minute was considered an enzyme unit (µmol g^{-1} protein).

2.9 Determination of biochemical parameters

We measured the soluble sugar concentration using the anthrone method (Yemm and Willis, 1954), and the standard was glucose. The concentration of proline was determined using a standard method (107). We homogenized 0.5 grams of fresh leaf samples in 3 percent aqueous sulfosalicyclic acid and then centrifuged at 5,000 × g (10 min). Next, the filtrate (2 ml) was mixed with 2 ml of glacial acetic acid and 2 ml of acid-ninhydrin in a test tube. Afterward, the reaction mixture was boiled at 100°C for 1 hour. Toluene was used to extract the reaction mixture. We aspirated and cooled the chromophore containing toluene. Finally, a spectrometer was used to measure absorbance at 520 nm. The soluble proteins were determined using 0.3 grams of fresh leaf samples (Bradford, 1976), standard bovine serum albumin. We extracted total phenolic content from dried leaf samples (0.3) in 80% methanol using the Folin-Ciocalteu colorimetric analysis (Scalbert et al., 1989). The absorbance was measured using a spectrophotometer at 765 nm. Phenolic content was expressed as mg g^{-1} DW, dry weight.

2.10 Analysis

Three replicates of the measurements were conducted. The descriptive statistics and one-way analysis of variance (ANOVA)

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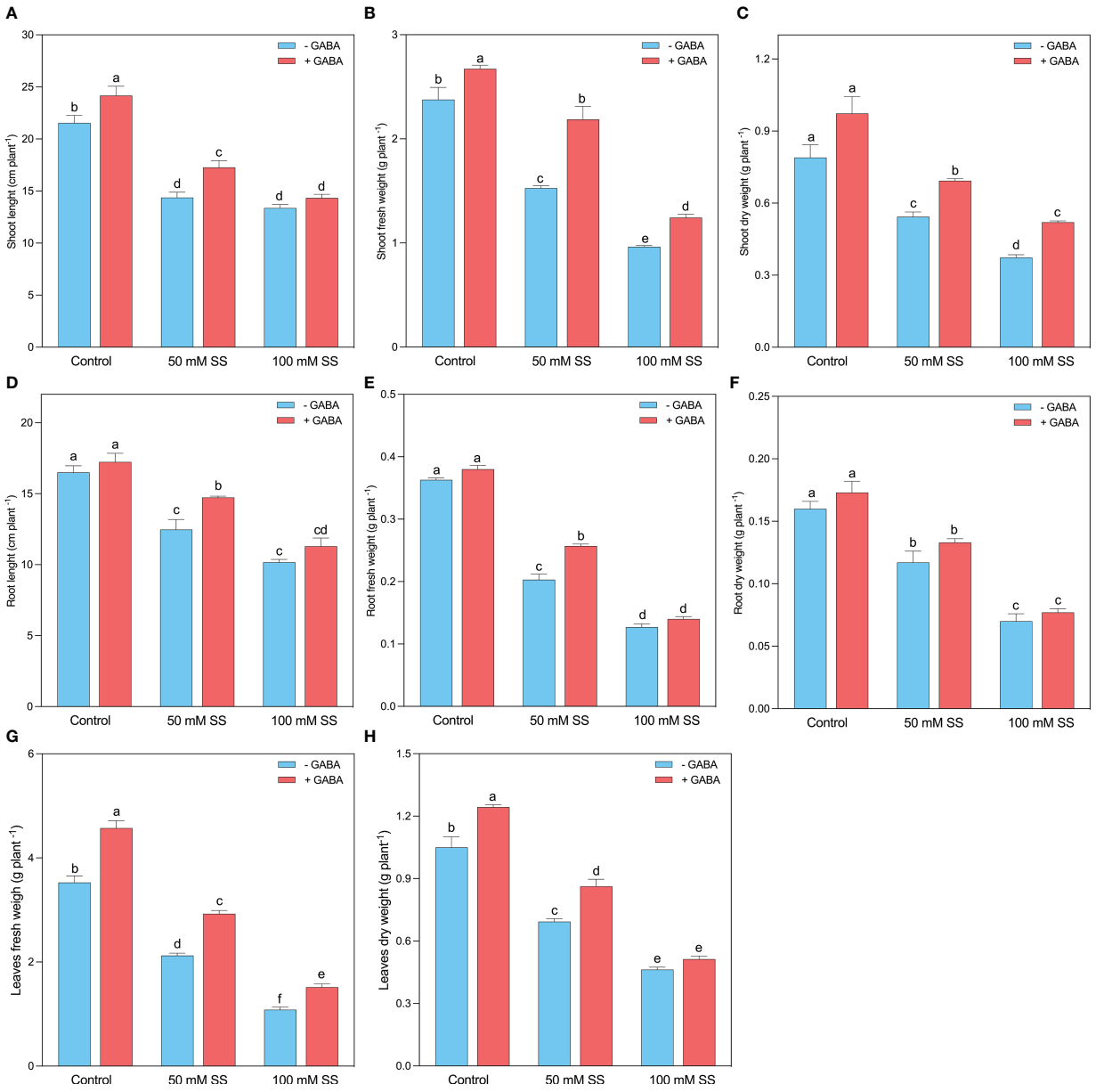

FIGURE 1
Changes in **(A)** shoot length, **(B)** shoot fresh weight and **(C)** shoot dry weight, **(D)** root length, **(E)** root fresh weight, **(F)** root dry weight, **(G)** leaf fresh weight, and **(H)** leaf dry weight of mungbean plants under saline stress (SS) and exogenous (γ-aminobutyric acid) treatments. The bars indicate the SE of the mean value, n=3. Different letters above the bars indicate significantly different values at $P<0.05$ following Duncan's method.

were conducted using SPSS version 16.0 (Chicago, IL, United States). At a significance level of $p<0.05$, Duncan's multiple range tests were used to compare means. The figures were created using GraphPad Prism 8. The growth parameters, chlorophyll pigment content, osmolytes, N metabolism, mineral nutrition, H_2O_2 and MDA levels, and antioxidant enzyme activity were examined using Pearson correlation analyses (Origin Lab Corporation, Northampton, MA, USA) for further interpretation.

3 Results

3.1 Changes in growth and biomass

Mungbean plants showed obvious differences in growth performance when treated with saline stress and GABA treatments. Both salinity levels (50 mM and 100 mM) substantially reduced the growth parameters of mungbean plants. The shoot length (SL), stem fresh weight (SFW), and

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stem dry weight (SDW) experienced a 33.3-, 35.8- and 31.2% inhibition following 50 mM SS, while 37.9-, 59.5- and 52.7% decline was found after 100 mM SS, respectively (Figures 1A–C). GABA, however, improved the SL, SFW, and SDW under 50 mM (20.2-, 43.2-, and 27.6%, respectively) and 100 mM (27.2-, 29.1-, and 39.3%, respectively) compared to untreated plants. This improvement was 12.2-, 12.5-, and 23.2% when GABA was applied under controlled conditions. Similar to the stem indices, the root length (RL), root fresh weight (RFW), and root dry weight (RDW) declined by 24.4-, 44.0- and 27.1% after 50 mM SS, while following 100 mM SS, this inhibition was 38.4-, 65.1- and 56.3% as compared to the control, respectively (Figures 1D–F).

Nonetheless, GABA application significantly improved RL and RFW under 50 mM (18.2- and 26.3%). Further, GABA caused a non-significant increment in RL and RFW under 100 mM and RDW under both stress conditions (Figures 1D–F). The leaf morphology was also substantially influenced by saline stress since a 39.9-, 34.0% and 69.3-, and 55.9% inhibition was recorded in leaf fresh weight (LFW) and leaf dry weight

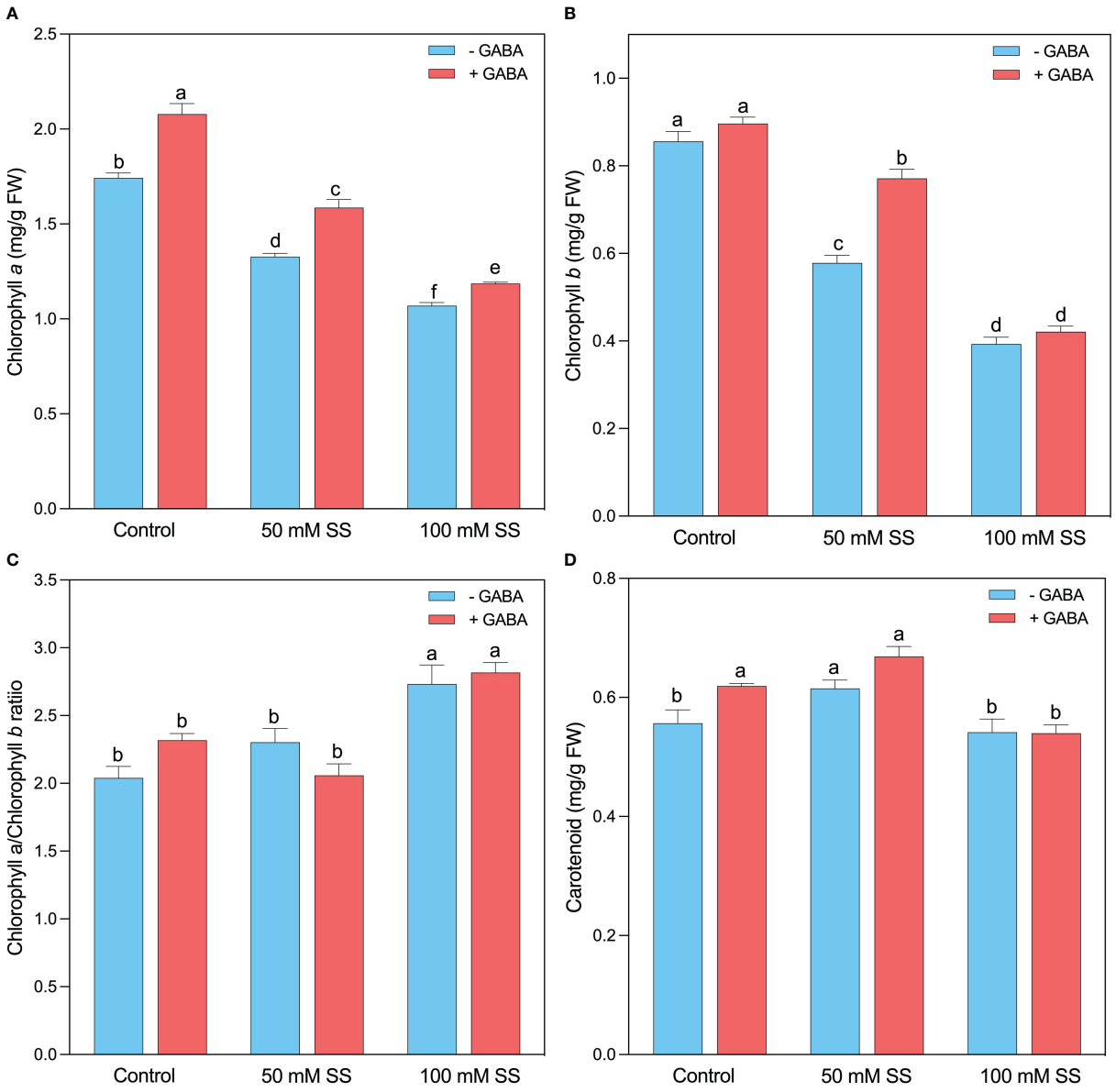

FIGURE 2
Changes in concentrations of **(A)** chlorophyll a, **(B)** chlorophyll b, **(C)** chlorophyll *a*/*b* ratio **(D)** of mungbean plants under saline stress **(SS)** and exogenous (γ-aminobutyric acid) treatments. The bars indicate the SE of the mean value, n=3. Different letters above the bars indicate significantly different values at $P<0.05$ following Duncan's method.

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(LDW) following the 50 mM and 100 mM SS, respectively. However, GABA significantly improved LFW, and LDW under 0 mM (29.7 and 18.4%, respectively), 50 mM (37.8 and 24.5%, respectively), and 100 mM SS (44.0-, and 10.8%, respectively), compared to untreated plants (Figures 1G, H).

3.2 Changes in photosynthetic pigments

The mungbean plants exhibited lower chlorophyll a (Chl a) and chlorophyll b (Chl b) content under salinity stress compared to the un-treated plants since a decline of 24-, 32- and 39- and 54%, following 50 mM and 100 mM, respectively (Figures 2A, B). Nonetheless, the supplementation of GABA to both saline levels considerably improved the Chl a and Chl b contents by 19.3-and 5-%; 19- and 33%; and 11- and 7%, respectively. Interestingly, the Chl a/b ratio showed a different behavior, and a significant increase was observed under both salinity levels (Figure 2C). A 13- and 34% rise was recorded under 50 Mm and 100 mM SS, respectively. GABA improved (21.6%) the Chl a/b ratio following 100 mM SS (Figure 2C). Carotenoid levels significantly increased at 50 mM and remained unaffected at 100 mM saline stress level. Exogenous GABA significantly increased carotenoid level under controlled condition, where it has no profound effect under salt conditions, compared to their untreated peers (Figure 2D)

3.3 Changes in Na^+ and K^+ concentrations

The excess Na^+ levels strongly indicate saline/alkaline stress persistence in plants. A Similar was the case in this study since a rapid enhancement was noticed after the induction of saline stress at both levels. This increment was 1.6- and 2.3-fold following 50 mM and 100 mM SS, respectively. Although the GABA could not profoundly reduce the Na^+ levels after 100 mM SS, however, significant inhibition was observed under 50 mM SS (Figure 3A).

The exposure to saline stress reduced N content, K^+ levels, and K^+/Na^+ ratio. The mungbean treated with 50 mM SS exhibited 38, 21, and 51% decreases in N content, K^+ levels, and K^+/Na^+ ratio, and this reduction was even greater (59, 33, and 71%) in the case of 100 mM SS, respectively (Figures 3B, C). Although, the exogenous application of GABA could help the mungbean plants in the considerable improvement of these parameters under 50 mM SS. Whereas the 100 mM saline-stressed plants did not display such an obvious promotion in these parameters after GABA application (Figure 3C).

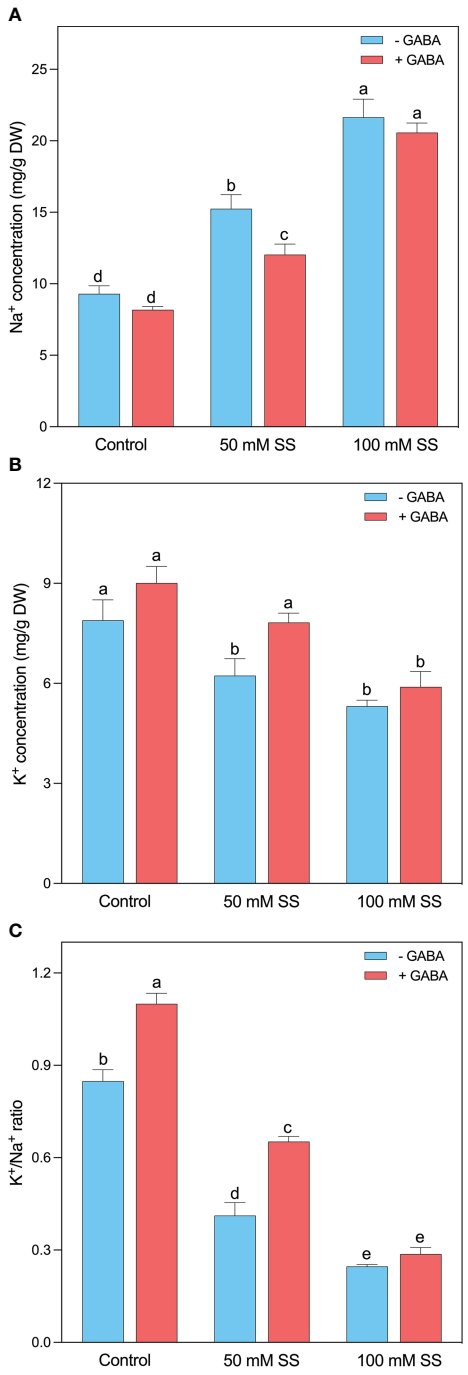

FIGURE 3
Changes in concentration of leaf **(A)** Na^+, **(B)** K^+, and **(C)** K^{+}/Na^+ ratio and root (f) Na^+, (g) Cl^- (h) K^+ (i) Mg^{++} and (i) K^{+}/Na^+ ratio of mungbean plants under saline stress (SS) and exogenous (γ-aminobutyric acid) treatments. The bars indicate the SE of the mean value, n=3. Different letters above the bars indicate significantly different values at $P<0.05$ following Duncan's method.

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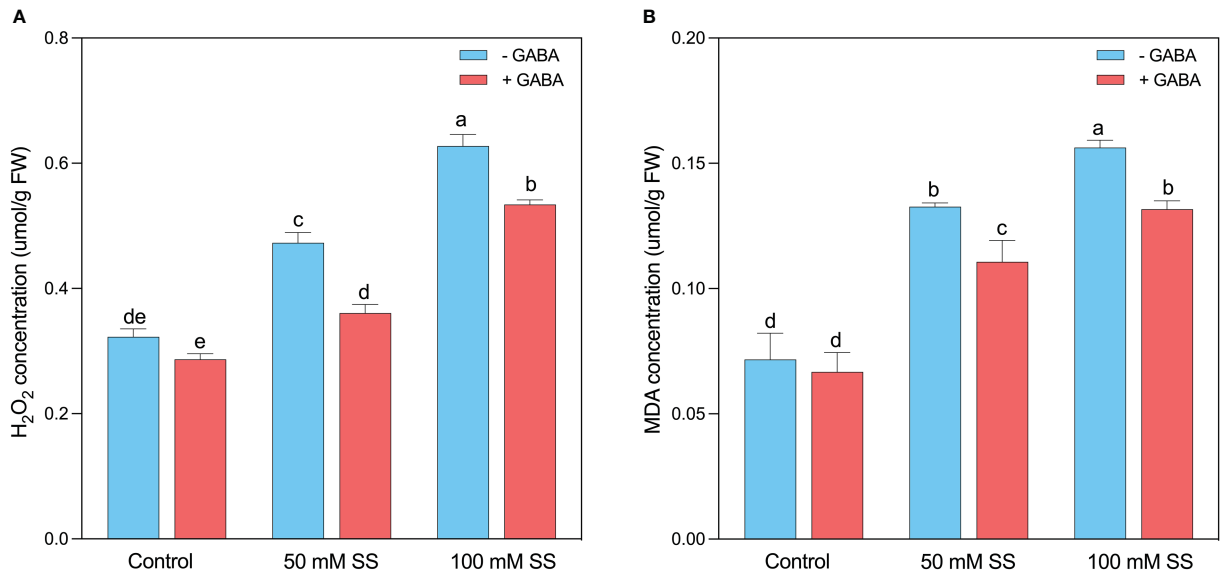

EFIGURE 4
Changes in concentrations of **(A)** H_2O_2 and **(B)** MDA of mungbean plants under saline stress (SS) and exogenous (γ-aminobutyric acid) treatments. The bars indicate the SE of the mean value, n=3. Different letters above the bars indicate significantly different values at $P<0.05$ following Duncan's method.

3.4 Changes in H_2O_2 and MDA concentration

The H_2O_2 and MDA are the major determinants of oxidative stress in plants since the production of these compounds is directly related to the increased cellular damage post oxidative stresses (Figures 4A, B). Similarly, in the current experiment, after the induction of oxidative stress caused by saline stress, the H_2O_2 and MDA levels were raised by 47 and 86% under 50 mM. This enhancement was 1.9- and 2.1-fold following 100 mM, respectively. Nevertheless, using exogenous GABA declined the H_2O_2 and MDA levels by 1.3- and 1.8-fold under 50 mM SS and 1.9- and 2.2-fold after100 mM SS, respectively (Figures 4A, B).

3.5 Changes in the enzymatic antioxidant system

Salt-induced higher concentrations of H_2O_2 and MDA, the mungbean plants tremendously stimulated their antioxidant potential to scavenge excessive ROS. Briefly, the SOD, POD, APX, PPO, and GTR activities were enhanced by 1.3-, 1.3-, 1.3-, 1.16-, and 2-fold when the plants were grown under 50 mM SS, while the 100 mM SS-treated mungbean plants displayed 1.3-, 1.3-, 1.4, 1.5-, and 2.4-fold stimulation after 100 mM SS, respectively (Figures 5A–E). The exogenous application of GABA further improved the antioxidant activities of POD and SOD by 38 and 35% at 50 mM SS levels, while the APX, PPO, and GTR increments were 1.5-, 1.4-, and 2.2-fold after 100 mM SS, respectively (Figures 5A–E). Intriguingly, the CAT activity significantly increased under 50 mM (30.4%) compared to the control condition. However, GABA application significantly enhanced under 0 mM and 50 mM (17.3- and 10.5%, respectively) compared to their untreated peers. However, SS and GABA had no significant effect on CAT activity (Figure 5F)

3.6 Changes in nitrogen metabolizing enzymes

The nitrogen (N) concentration significantly decreased under 50 mM and 100 mM by 38.3- and 58.9%, respectively, compared to the control (Figure 6A). Compared to untreated peers, exogenous GABA significantly increased N concentration under 0 mM and 50 mM (19 and 31.8%, respectively) but had no significant effect under 100 mM SS. Intriguingly, the NR (32-, 33.1%), GS (15- and 51%), and GOGAT (12.5- and 65%) were significantly reduced by the induction of 50- and 100 mM SS (Figures 6B–D). However, GABA significantly improved NR under 0- and 50 mM SS (16- and 27-%, respectively), whereas GS under 0-, 50- and 100 mM SS (28- and 37-, and 24.2%, respectively), compared to controlled conditions. Moreover, GABA had no significant effect on GOGAT under controlled and SS conditions (Figures 6B–D).

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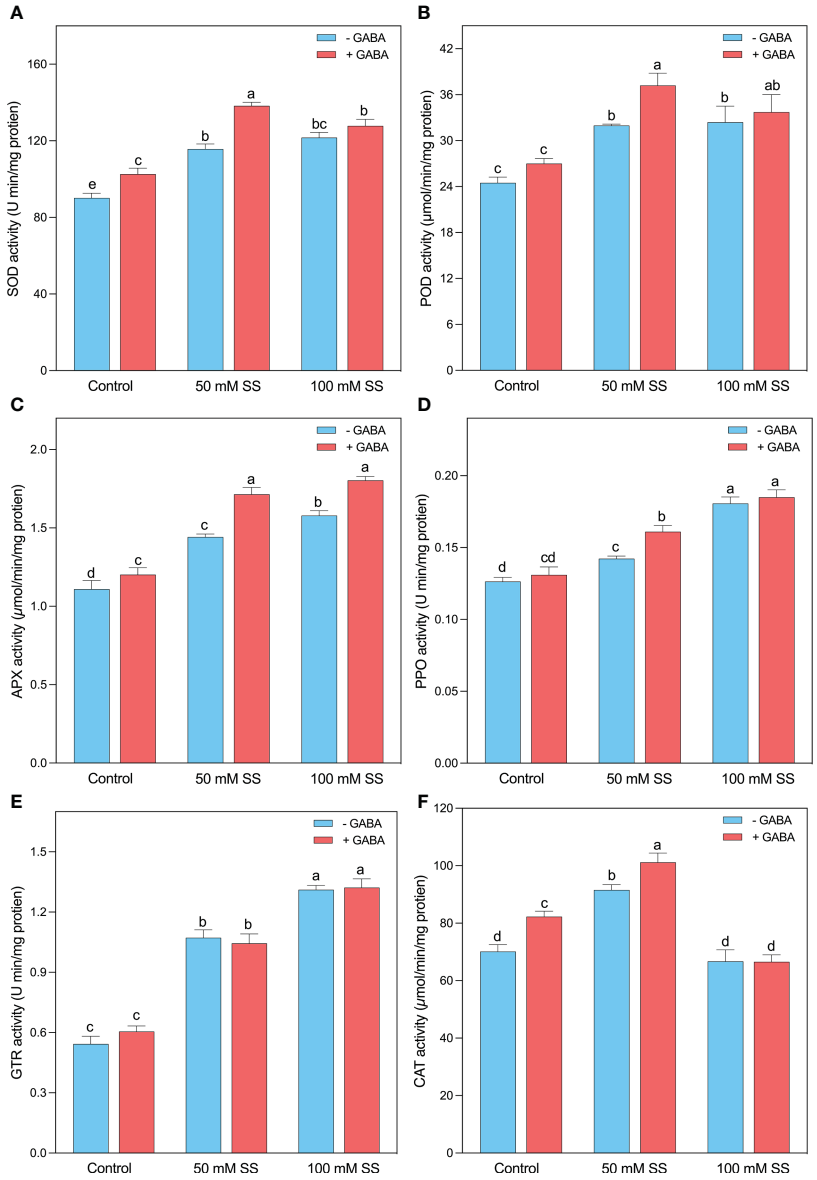

FIGURE 5
Changes in enzymatic activities of **(A)** SOD, **(B)** POD, **(C)** APX, **(D)** PPO, **(E)** GR, and **(F)** CAT of mungbean plants under saline stress (SS) and exogenous (γ-aminobutyric acid) treatments. The bars indicate the SE of the mean value, n=3. Different letters above the bars indicate significantly different values at $P<0.05$ following Duncan's method.

3.7 Changes in biochemical contents

The soluble sugar concentration increased under saline stress and GABA application. For instance, it increased by 29.2- and 6.5% under 50- and 100 mM SS (Figure 7A). Further, the GABA application enhanced soluble sugar by 30.3-, 13.3-, and 7.6% under 0-, 50-, and 100 mM SS. Interestingly, the protein content significantly improved at both GABA-treated and untreated treatments, irrespective of the salt stress. Generally, 44- and 28% of increment was observed after 50 mM of salinity stress in untreated and treated plants, respectively (Figure 7B). Similarly, both GABA-treated and untreated mungbean plants showed a 1.5- and 2-fold increase after 100 mM SS, respectively. Like enzymatic antioxidants, the non-enzymatic antioxidant compounds, such as proline, and phenol, were considerably influenced by the salt stress in mungbean plants (Figures 7C, D). When the plants were exposed to 50 mM SS, the proline, and total phenolic content, were enhanced by 88- and 36% and the GABA application further improved them by 68- and 66 4% under 50 mM SS, respectively. In addition, the mungbean plants

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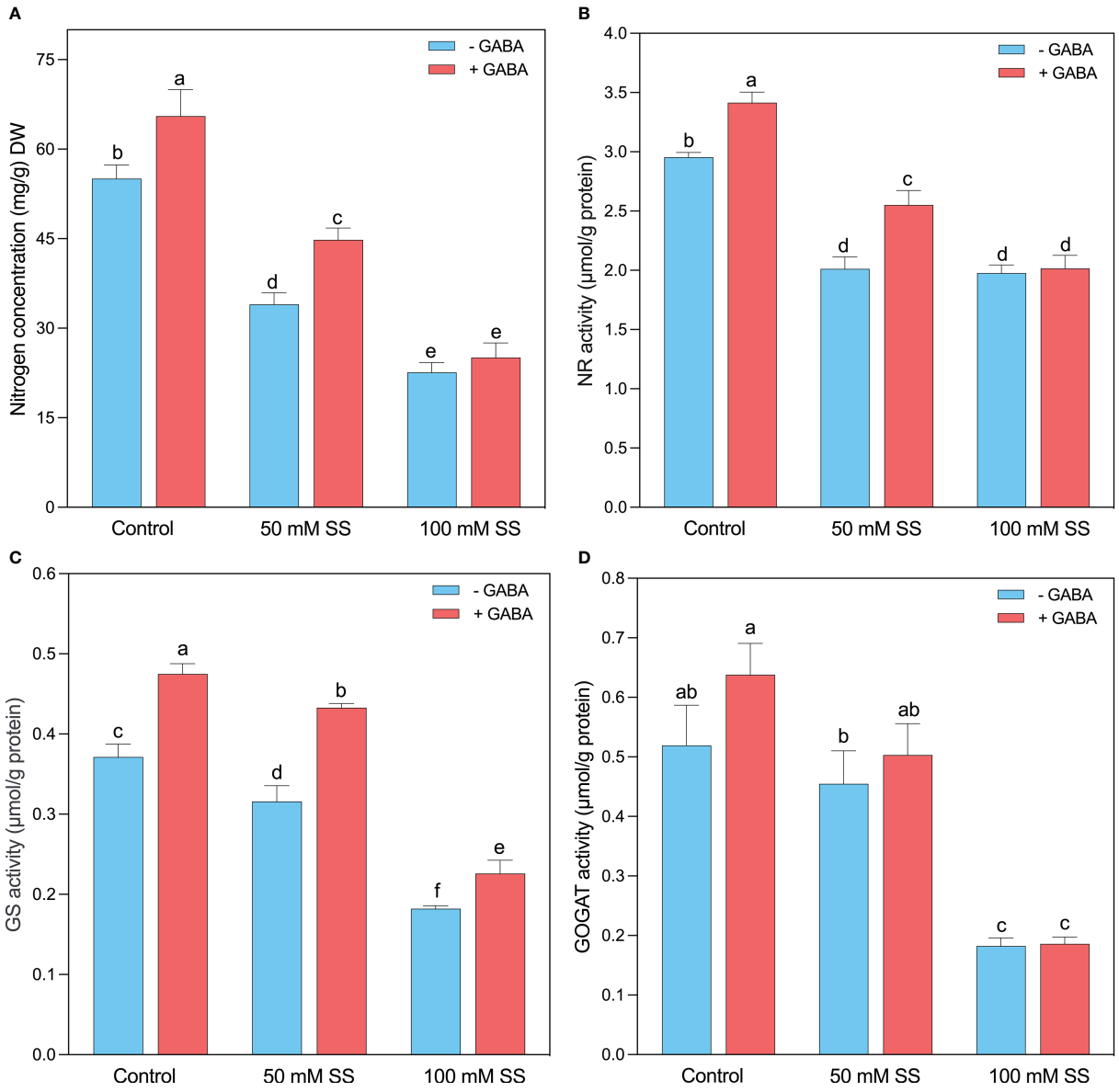

FIGURE 6
Changes in concentrations of **(A)** N concentration and enzymatic activities of **(B)** NR, **(C)** GS, and **(D)** GOGAT of mungbean plants under saline stress (SS) and exogenous (γ-aminobutyric acid) treatments. The bars indicate the SE of the mean value, n=3. Different letters above the bars indicate significantly different values at $P<0.05$ following Duncan's method.

under 100 mM salinity stress showed a 2.4- and 2- fold rise, and a further 2-and 1.9-fold increase was deployed by the GABA supplementation, respectively (Figures 7C, D).

3.8 Correlation analysis

The correlation analysis results displayed a significant positive relation between nitrogen related enzymes (NR, nitrate reductase; GS, glutamine synthetase; and GOGAT, glutamate synthase), chlorophyll content (chla, chlb), and biomass accumulation (fresh and dry biomass). It showed that the increasing activities of nitrogen metabolism-related enzymes prompted nitrogen uptake and result in maximum biomass production. On the other hand, antioxidant enzymes activities (POD, peroxidase; CAT, catalase; APX, ascorbate peroxidase; SOD, superoxide dismutase; PPO, polyphenol oxidases; GTR, glutathione reductase) and protein, proline, and total phenolic

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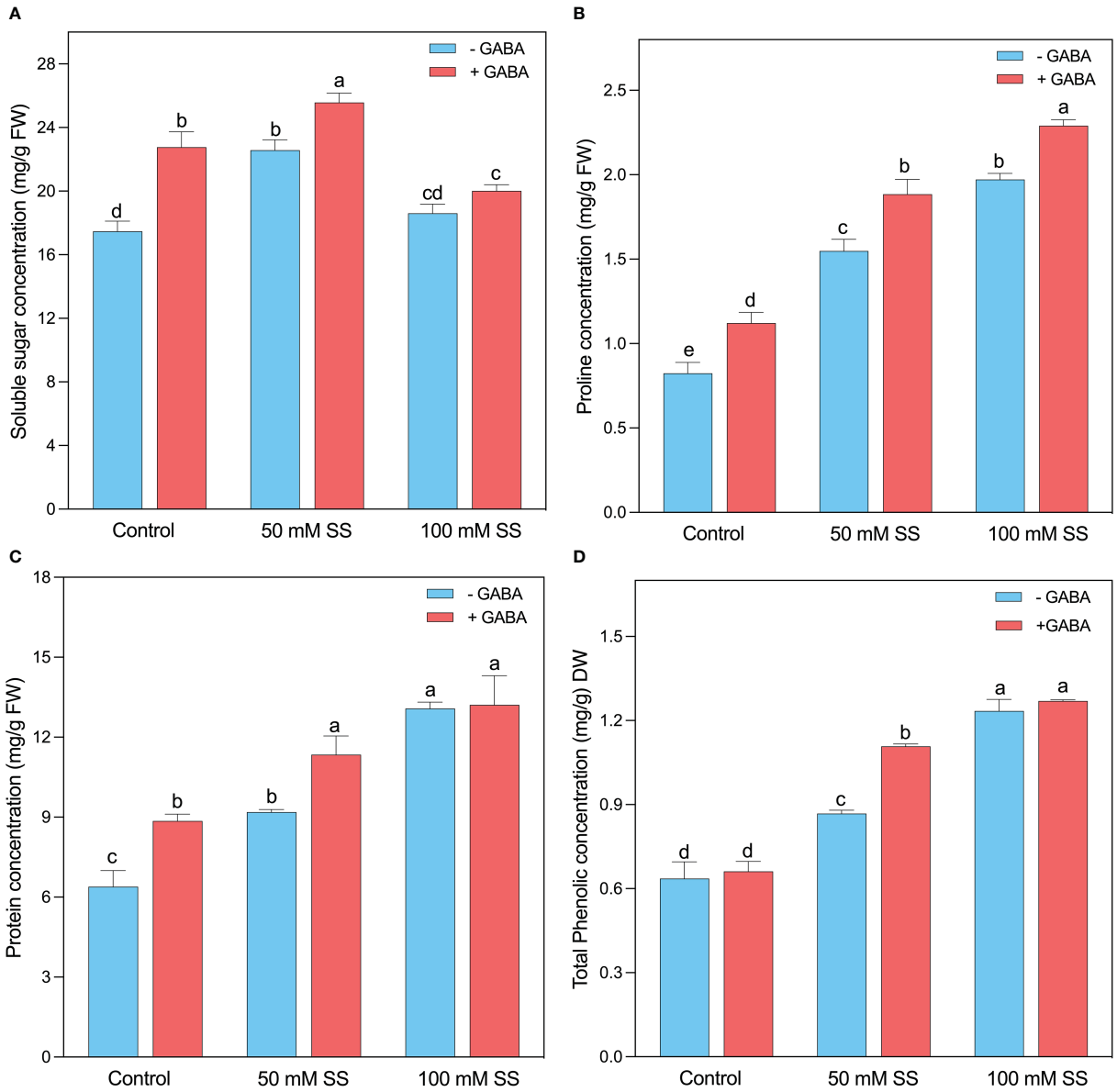

FIGURE 7
Changes in concentrations of **(A)** sugar, **(B)** soluble protein, **(C)** proline, and **(D)** total phenolic content of mungbean plants under saline stress **(SS)** and exogenous (γ-aminobutyric acid) treatments. The bars indicate the SE of the mean value, n=3. Different letters above the bars indicate significantly different values at $P<0.05$ following Duncan's method.

content (TPC) had s strong negative correlation with nitrogen related enzymes (Figure 8).

4 Discussion

4.1 Exogenous GABA improved growth features of mungbean plants under saline stress

Increasing soil salinity is a major global issue in sustainable agriculture as it disrupts cellular and physiological processes at all growth stages (Arif et al., 2020). Salinity-induced reduction in plant growth may be due to low uptake of mineral nutrients (Askari-Khorasgani et al., 2017), such as potassium (K^+), as a result of excess Na^+ accumulation (Chakraborty et al., 2016). In the present study, a similar phenomenon was observed, as increased saline stress (SS) led to impaired growth features (stem height, root length, leaf area, stem-leaf-root fresh and dry weight; Figures 1A–H) and chlorophyll pigments (Chl a, Chl b; Figures 2A, B) of mungbean plants, suggesting hindrance of cell division. The decline in growth and biomass is correlated negatively with increased Na^+ levels and excessive H_2O_2 production (Figure 8). Excessive H_2O_2 disrupts the

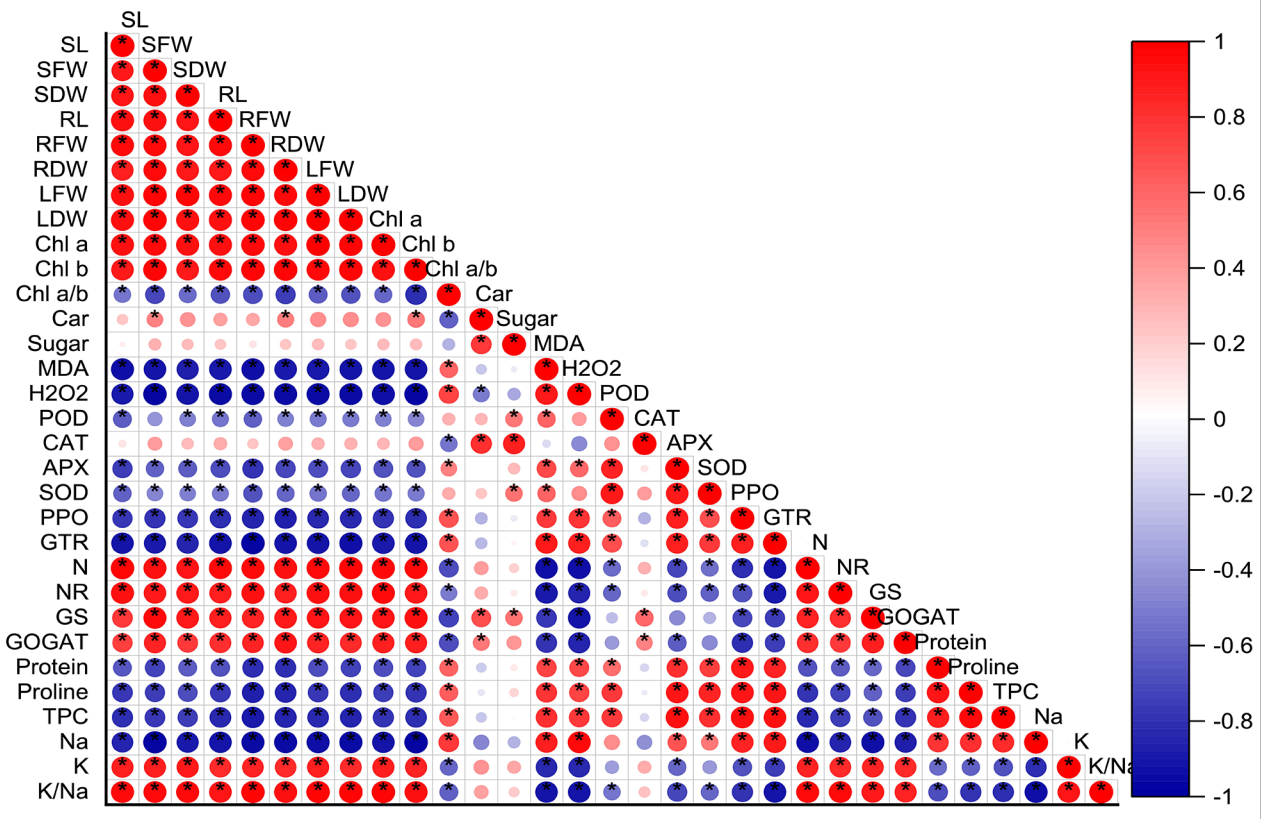

FIGURE 8
Correlation analysis between all the studied parameters. Red and blue color represent the positive and negative correlation. The size and intensity of color exhibited the significance of variables, and * represents the significance level at $P<0.05$. SL, stem length; SFW, stem fresh weight; SDW, stem dry weight; RL, root length, RFW, root fresh weight; RDW, root dry weight, LFW, Leaf fresh weight; LDW, leaf dry weight; Chl, chlorophyll; Car, carotenoids; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; POD, peroxidase; CAT, catalase; APX, ascorbate peroxidase; SOD, superoxide dismutase; PPO, polyphenol oxidases; GTR, glutathione reductase; N, nitrogen; NR, nitrate reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; TPC, total phenolic content; Na, sodium and K, potassium.

plasma membrane causing ionic imbalance, inhibiting metabolic processes, eventually declining plant growth (Mittler et al., 2011; Kaya et al., 2019; Ullah et al., 2022b; Ullah et al., 2022c). In the present study, gamma-aminobutyric acid (GABA) was applied under SS to investigate whether or not it can mitigate salinity-induced adverse effects on mungbean growth and metabolism. We found that exogenous GABA relieved salt-induced reductions in plant growth features (Figures 1A–H). So, GABA might be an active growth regulator involved in the salinity-tolerance of mungbean plants, as has been reported in previous studies (Jin et al, 2018; Wang et al., 2015; Kalhora et al., 2018; Wu et al., 2020), which corroborated our findings. Hence, we suggest that exogenous GABA could increase the salinity-tolerance of mungbean by improving their growth under SS.

4.2 Exogenous GABA improved photosynthetic pigments under saline stress

The reduction in salinity-induced inhibitions of the photosynthetic pigments primarily inhibits plant growth. During chlorophyll biosynthesis in plants, a series of reactions take place, and when any of these reactions are disrupted, chlorophyll biosynthesis is affected (Min et al., 2006; Ali et al., 2022). In our study, increased SS significantly inhibited chlorophyll (Chl a and Chl b) concentration, which could be attributed to a specific symptom of SS-induced oxidative damage (Taïbi et al., 2016) and chlorophyllase inhibition/degradation (Chakhchar et al., 2018). An overabundance of H_2O_2 in mungbean plants under SS may also have contributed to the decrease in chlorophyll concentrations (Kaya et al., 2018). However, exogenous GABA improved chlorophyll concentration in SS-stressed plants, suggesting that GABA can mitigate the damaging effects of SS on photosynthetic pigments by reducing the production of H_2O_2 and improving plant growth. Similarly, GABA enhanced chlorophyll synthesis in maize plants under SS (Aljuaid and Ashour, 2022). Moreover, carotenoid concentrations significantly increased under 50 mM SS. Carotenoid is an auxiliary pigment that can dissipate excess light energy, making it an adaptive trait for dealing with salt stress (Duan et al., 2012). Exogenous GABA under 0 mM and 50 mM SS resulted in a significant increase in carotenoids

compared to GABA-untreated counterparts (Figure 2D). Exogenous γ-aminobutyric acid (GABA) has been demonstrated to alleviate salinity-induced inhibition of plant growth by reducing chlorophyll degradation and enhancing photosynthetic capacity. For instance, in lettuce plants treated with exogenous GABA, salinity was less toxic to fresh and dry shoot masses than those untreated with GABA (Luo et al., 2011; Kalhora et al., 2018). So, GABA might be an active growth regulator involved in the salinity tolerance of mungbean plants by promoting their photosynthetic pigments and growth under SS.

4.3 Exogenous GABA alleviated Na^+ toxicity by regulating Na^+ concentration under saline stress

The excessive accumulation of Na^+ caused by salinity stress leads to a decline in the uptake of essential nutrients like K^+ from roots to leaves (Zhang et al., 2020). Increasing levels of Na^+ and decreasing K^+ in photosynthetic leaves may degrade chlorophyll and disrupt thylakoids (Bose et al., 2017). Plant salinity tolerance is characterized by its ability to lessen the accumulation of toxic Na^+ ions in sensitive shoots (Niu et al., 2018). Hence, Na^+ accumulation in tissues is often considered an indicator of the severity of salinity stress damage. In our study, increased salinity stress resulted in a significant increase in Na^+ and a decline in K^+ concentration in the leaves. Similar findings were reported for maize (Kaya and Ashraf, 2020; Aljuaid and Ashour, 2022), barley (Akhter et al., 2021), canola (Naveed et al., 2020), and soybean (Ullah et al., 2019). Further, salinity-induced decline in K^+ ion resulted in low K^+/Na^+ ratio (Figures 3B, C). High salinity causes high pH in the rhizosphere, which reduces the availability of ions of nutrient elements, including K (Yang et al., 2007). Further, low K^+ levels may also be caused by stress-induced repression of K^+ absorption. Consequently, the low K^+ and K^+/Na^+ ratio may also be attributed to K^+ and Na^+ competing for binding sites for cellular functions (Azooz et al., 2015; Ullah et al., 2019). In our study, the declined mungbean growth could be attributed to cellular membrane damage caused by osmotic, ionic, and pH-induced damage associated with a rise in Na^+ ions and a decrease in beneficial K^+ ions as a result of saline stress (Zhu, 2001; Chartzoulakis, 2005; Ullah et al., 2019; Aljuaid and Ashour, 2022; Noor et al., 2022). Several studies have been conducted on the effect of exogenous GABA on Na^+ concentrations in plants, but whether GABA can directly reduce Na^+ accumulation is unclear. Nevertheless, it has been demonstrated that *Arabidopsis* mutants with high GABA levels in their roots have significantly lower Na^+ than mutants with low GABA levels (Su et al., 2019). In the present study, exogenous GABA treatments reduced Na^+ levels in the leaves of mungbean plants, compared to their untreated GABA peers (Figure 3A). A study by Wu et al., 2020 found that exogenous GABA inhibits the absorption of Na^+ ions by roots and reduced their transport to leaves. Further, GABA has been shown to improve plant salt tolerance by influencing ion membrane potential differences and osmoregulation, thereby enhancing ion transport (Seifikalhor et al., 2019). We, therefore, believe that exogenous GABA alleviates saline stress damage in mungbean plants by reducing Na^+ in the leaves (Figure 3A). Numerous studies have demonstrated that GABA accumulation reduces Na^+ uptake and reactive oxygen species (ROS) concentration, activates H^+ ATPase, and inhibits K^+ loss under stress conditions (Shi et al., 2010; Li et al., 2019, 2020; Su et al., 2019; Wu et al., 2021; Aljuaid and Ashour, 2022). Therefore, we suggest that GABA-induced reduction in Na^+ concentration was associated with increased K^+ levels and a high Na^+/K^+ ratio, which is beneficial for salinity adaptation.

4.4 Exogenous GABA improved salinity tolerance by regulating the antioxidant potential of mungbean plants

The osmotic and oxidative stresses caused by salinity impair plant metabolism (Ma et al., 2018), leading to the accumulation of lipid peroxides and membrane damage (Mittler et al., 2004). Compared to controls, we observed significantly higher levels of MDA and H_2O_2 during all levels of saline stress. Similar results were reported for chufa (Ullah et al., 2022b), maize (Aljuaid and Ashour, 2022), barley (Akhter et al., 2021), canola (Naveed et al., 2020), and pepper (Kaya et al., 2020b). It is well known that plants possess a highly specialized antioxidant defense system that is capable of scavenging reactive oxygen species (ROS) under stress conditions, including salinity. in the present study, saline-treated mungbean plants displayed significantly greater levels of antioxidant enzymes, compared to control mungbean plants. For example, compared to the control, the activities of SOD, POD, APX, PPO, and GTR, increased with increasing saline stress levels, indicating that it is a solid antioxidant defense mechanism for detoxifying the excessive ROS (Munns and Tester, 2008; Polash et al., 2019). Several studies have reported the upregulation of SOD, POD, APX, PPO, GTR, and CAT in plants under salinity stress (Luo et al., 2011; Weisany et al., 2012; Farhangi-Abriz and Torabian, 2017; Aljuaid and Ashour, 2022), which corroborate our findings. It is suggested that salinity-treated mungbean plants upregulate their antioxidant enzymes to scavenge ROS at the expense of growth and biomass reduction as more energy has been devoted to antioxidant mechanisms instead of organ development. Further, we found that exogenous GABA significantly reduced H_2O_2 and MDA under both saline stress levels, where its effect on antioxidant enzymes was only significant under 50 mM saline stress. It has been demonstrated that GABA exhibits ROS

scavenging potentials and can contribute to stress mitigation (Liu et al., 2011; Aljuaid and Ashour, 2022). For instance, GABA inhibits MDA, an indicator of lipid oxidation (Deng et al., 2010; Aljuaid and Ashour, 2022). However, it is unclear whether GABA can directly scavenge ROS to alleviate stress. Still, several studies reported that exogenous GABA plays a key role in scavenging ROS and modulating the activity of antioxidant enzymes (Renault et al., 2010; Barbosa et al., 2010; Aljuaid and Ashour, 2022). We suggest that exogenous GABA protected mungbean plants from salinity-associated oxidative stress damage and enhance their tolerance by reducing lipid peroxidation and upregulating enzymatic antioxidant mechanisms.

4.5 Exogenous GABA improved nitrogen concentration by regulating N-metabolizing enzymes in mungbean plants

Regulation of nitrogen (N) metabolism is essential for salt tolerance, and salinity and N nutrition interact intricately (Läuchli and Lüttge, 2002). In this study, mungbean plants subjected to saline stress had lower N content and, along with declined activities of nitrate reductase (NR), glutamine synthetase (GS), and glutamine oxoglutarate aminotransferase/glutamate synthetase (GOGAT) (Figure 6A-D). The salinity-induced decline in nitrogen assimilation has been reported for tomato (Debouba et al., 2006), Chinese cottonwood (Meng et al., 2016), soybean (Ullah et al., 2019), and chufa (Ullah et al., 2022b), which corroborate our findings. We suggest that salt ions impaired nitrogen metabolism in mungbean plants by inhibiting NR activity which prevents GS/GOGAT from supplying NH_4^+ to the amino acid synthesis pathway (Debouba et al., 2006; Meng et al., 2016). The decrease in N assimilation and the synthesis of amino acids and proteins can reduce plants' dry weight (Queiroz et al., 2012). However, exogenous GABA treatments improved nitrogen concentration and activities of NR, GS and GOGAT under salt and controlled condition, compared to their untreated peers. However, the effect was only significant under 50 mM saline stress. GABA has been shown to play a role in buffering N metabolism (Salah et al., 2019). For example, GABA may influence plants' growth and stress responses by regulating their carbon and nitrogen metabolism (Chen et al., 2020). A recent study found that plants treated with GABA displayed increased nitrogen metabolism, as illustrated by increased nitrogen concentration and its metabolizing enzymes (Khanna et al., 2021), which support our findings. Therefore, we suggest that exogenous GABA enhances salinity stress tolerance of mungbean plants by regulating the key enzymes of N assimilation and increase N concentrations, resulting in improved growth and biomass.

4.6 Exogenous GABA, regulated biochemical changes in mungbean plants

In the present study, significantly higher concentration of soluble sugar recorded at 50 mM, compared to control condition. Under stress condition, plants use soluble sugars to maintain osmotic and ionic homeostasis, limit chlorophyll destruction and water loss, remove excess ROS, stabilize proteins and membrane structures, regulate cell division, and control gene transcription (Sami et al., 2016). Moreover, there was a significant increase in proline, soluble protein and total phenolic concentration with increasing saline stress levels compared to the control condition. The accumulation of proline might have contributed to the protection of photosynthetic apparatus elimination of excessive ROS to stabilize membranes, enzymes, and proteins in salinity-treated mungbean plants (Verdoy et al., 2006; Ahmad et al., 2016). In other studies, proteins have also been shown to act as osmotins and contribute to salt stress tolerance (Zhang et al., 2013). Furthermore, plants have the ability to upregulate small molecules of protein that can be used for storing N and can be mobilized rapidly during a period of stress relief (Singh et al., 1987). Osmotic adjustment may also be mediated by these proteins (Ashra and Harris, 2004). In addition, phenolic compounds play an important role in protecting plants from both biotic and abiotic stress (Ma et al., 2018; 2019). The application of exogenous GABA further increased soluble sugar, proline, soluble protein, and total phenolic content, suggesting that the GABA protected the mungbean plants from salt stress and thus enhanced their salinity tolerance.

5 Conclusion

Exogenous GABA was used to test whether it could mitigate the adverse effects of saline stress on mungbean plants' growth and physio-biochemical attributes. Increased saline stress adversely affected the growth, biomass, and physiological metabolism of our test species. However, exogenous GABA decreased the damage caused by salinity stress on mungbean plants by promoting growth and physiological metabolism. For example, exogenous GABA causes (i) reduced Na^+ concentration, (ii) increased K^+, which resulted in a higher K^+/Na^+ ratio, (iii) promoted photosynthetic pigment biosynthesis, (iv) reduced H_2O_2 and MDA concentration by upregulating enzymatic antioxidant potential (v) improved N concentration and activities of N-metabolizing enzymes and (vi) enhanced osmolytes accumulations. Our findings suggest that GABA can mitigate the salinity-associated morpho-physio-biochemical damages in mungbean plants. Therefore, applying GABA to mungbean plants could effectively reduce salinity-induced adverse effects on the growth and yield of mungbean plants in

salinity-affected areas worldwide. Future research should investigate whether exogenous GABA can positively influence the nutrient composition of mungbean at deeper molecular levels to offer solutions to its low productivity in salt-affected areas.

Data availability statement

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

Author contributions

Conceptualization: AU, SU, Data curation: AU. Formal analysis: AU, MA, IA, SB, SE and JN. Investigation: AU, JN. Methodology: AU and SU. Project administration: SU. Resources: AU and SU. Software: AU, MA, KS, JN, and HJ. Supervision: SU. Validation: AU, SU, and Visualization: JN, KS, KS, HJ, and MA. Writing - original draft: AU, JN, IA, SB, SE and HA: Writing - review and 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.

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Supplementary material

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Instigating prevalent abiotic stress resilience in crop by exogenous application of phytohormones and nutrient

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In recent times, the demand for food and feed for the ever-increasing population has achieved unparalleled importance, which cannot afford crop yield loss. Now-a-days, the unpleasant situation of abiotic stress triggers crop improvement by affecting the different metabolic pathways of yield and quality advances worldwide. Abiotic stress like drought, salinity, cold, heat, flood, etc. in plants diverts the energy required for growth to prevent the plant from shock and maintain regular homeostasis. Hence, the plant yield is drastically reduced as the energy is utilized for overcoming the stress in plants. The application of phytohormones like the classical auxins, cytokinins, ethylene, and gibberellins, as well as more recent members including brassinosteroids, jasmonic acids, etc., along with both macro and micronutrients, have enhanced significant attention in creating key benefits such as reduction of ionic toxicity, improving oxidative stress, maintaining water-related balance, and gaseous exchange modification during abiotic stress conditions. Majority of phytohormones maintain homeostasis inside the cell by detoxifying the ROS and enhancing the antioxidant enzyme activities which can enhance tolerance in plants. At the molecular level, phytohormones activate stress signaling pathways or genes regulated by abscisic acid (ABA), salicylic acid (SA), Jasmonic acid (JA), and ethylene. The various stresses primarily cause nutrient deficiency and reduce the nutrient uptake of plants. The application of plant nutrients like N, K, Ca, and Mg are also involved in ROS scavenging activities through elevating antioxidants properties and finally decreasing cell membrane leakage and increasing the photosynthetic ability by resynthesizing the chlorophyll pigment. This present review highlighted the alteration of metabolic activities caused by abiotic stress in various crops, the changes of vital functions through the application of exogenous phytohormones and nutrition, as well as their interaction.

KEYWORDS

abiotic stress, phytohormone, nutrient, signaling, antioxidant, gene expression

1 Introduction

Feeding the global population rise which is soon to reach 2.3 billion by 2050 is a challenging task in every way, so a considerable increase in grain productivity to at least about 70% is the need to accomplish this global challenge efficiently (Tilman et al., 2011). However, the major drawback in achieving this objective is the frequent occurrence of abiotic stress which affects the plant's metabolic activities and triggers the biosynthetic pathways ultimately reflected in the reduction in quality and yield loss. Plants show their own mechanism to overcome the period of abiotic stress, for which maximum of their energy synthesized by the plant becomes diverted towards creating resistance or tolerance to the stress condition. The abiotic stress includes drought, cold, salinity, heat, water logging, metallic stress, etc. in plants transferring the energy to prevent the plant from such stresses and maintain normal growth. In the current scenario, these abiotic stressors are the major factors affecting production and productivity. Amongst various abiotic stresses, high temperature, water scarcity, and salinity are the most widespread and significant ones (Wani et al., 2013).

Plant body is a complex of several biomolecules, among them phytohormones are the molecules produced in very low concentrations, however, they show their active participation in regulatory activities (Shabir et al., 2016). The cellular activities are mostly regulated by the chemical communication inside the plant body with low-volume phytohormones (Vob et al., 2014). Phytohormones are most important to regulate various signal transduction pathways during abiotic-stress response. They regulate external as well as internal stimuli (Kazan, 2015). Auxin, cytokinin (CK), gibberellic acid (GA), ethylene, abscisic acid, brassinosteroids, salicylic acid, jasmonates, and strigolactones are the major phytohormones that have the major network in plant growth and development as well as in alleviating abiotic stress in plants. Nutrients are another crucial component that can minimize the effect of abiotic stress in plants by maintaining the inner homeostasis of the cell. Plant nutrients are considered the available form of food for plants for their normal growth and development. The plant nutrients are grouped into primary nutrients like nitrogen (N), phosphorus (P), and potash (K); secondary nutrients like calcium (Ca), magnesium (Mg)and sulfur (S); micronutrients like boron(B), zinc (Zn), iron (Fe)conditions, copper (Cu); and other beneficial nutrients like cobalt (Co), selenium (Se), silicon (Si). Due to global climate change, plant suffers a lot from nutrient deficiency. It was also noted that nutrient deficiencies are the major cause of yield loss during abiotic stress. Hence, proper nutrient management can elevate abiotic stress conditions in plants to some extent. Plant nutrients can mitigate stress also by activating stress resistance genes, enhancing antioxidant enzyme activity, creating osmoprotectant in cells, synthesizing heat shock proteins and other proteins related to stress tolerance, decreasing ROS activities, creating membrane stability, repairing DNA, enhancing chlorophyll content in leaves, reducing the uptake of heavy metals in the plant.

2 Effects of abiotic stress on plants

Abiotic stresses cause disorders in plants like osmotic stress in cells, retardation in cell development, reduced photosynthetic activity, seed dormancy, and late reproduction, and eventually show a negative effect on yield (Table 1). Among different types of abiotic stresses, water-deficit stress is most frequent in nature and causes ample of damage to crop plants. The rigorous impact of water deficit stress is due to reduced plant relative water content which causes osmotic and oxidative stress (Diouf et al., 2018). This condition occurs in salinity stress also and further triggers the same effect as drought (Munns and Tester, 2008). Both the drought and salinity stress the most menacing global abiotic stresses, which force a series of morphological, physiological, and molecular changes in plants, and in order to survive they require osmotic adjustment, ROS detoxification stomata closure, and cellular signaling (Diouf et al., 2018). Among the other stressors, high temperature can impact plants' hormone production, nutrient uptake, stomatal conductance, transpiration rate, photosynthetic activities, enzymatic activity, antioxidants level, membrane stability index and reactive oxygen species (ROS) production (Hussain et al., 2018). Similarly, chilling stress in plants can also affect by putting impacts on tissue water content, membrane fluidity, and chlorophyll content (Zhang et al., 2012).

TABLE 1 Common responses of plants under abiotic stress conditions.

Types of stress	Effect on plant	References
Drought	Increases in leaf yellowing and senescence, leaf drooping, wilting, scorching of leaves, leaf rolling and brittleness, closed flowers and flower sagging, leaf etiolation, and premature fall of leaves.	Ruehr et al., 2019
Salinity	Ion toxicity, osmotic stress, nutrient deficiency, oxidative stress on plants, leaf area and chlorophyll content reduction, altered stomatal conductance, limited water uptake, and cell death,	Shrivastava and Kumar, 2015
Water logging	Inhibition of root respiration, blocked gas exchange between soil and atmosphere, accumulation of toxic substances, leaf stomata closure, chlorophyll degradation, leaf senescence, and yellowing, the decline in photosynthetic rate, inhibition of germination, nutrient deficiencies, inadequate ATP production, ROS production, chlorosis and necrosis in waxy leaves and yield reduction	Jiawei et al., 2021
Chilling/ frost injury/ cold stress	Reduced water potential, ice crystal formation leads cell and plant death, membrane destabilization, altered membrane permeability, destruction or degradation of chlorophyll, photosynthetic inhibition, cell expansion inhibition, cell death, tissue browning, blackening, wilting or curling of leaves and stems, disruption of conversion of starch to sugar, decrease CO_2 exchange, disturbed mating system and yield reduction.	Mayland and Cary, 1970; Salvi et al., 2021
High temperature/ heat stress	Inhibition of seed germination, increased oxidative stress, water loss, alteration in phenology, improper growth and development, alteration in photosynthesis, pollen grain sterility, improper seed setting, reduced shoot, and root growth scorching of leaves, branches and stems leaf senescence and abscission, fruit discoloration, and altered dry matter accumulation, reduced yield in plants	Hasanuzzaman et al., 2013

2.1 Drought stress

About half of the global arid and semi-arid regions are affected by drought stress. Under the conditions of drought stress, photosynthesis, growth, and physio-biochemical processes of plants are highly disrupted, which inhibits plant growth and development and results in yield loss. A significant loss in total biomass and productivity has resulted due to water stress conditions. Many researchers have reported that oxidative stress from excessive ROS i.e. superoxide, hydroxyl ions, nitric oxide, singlet oxygen production, and nutrition imbalance, altered cell membrane balance and biomolecules like DNA, proteins, and lipids, imbalanced photosynthetic efficiency reduced turgor pressure, and alterations in leaf gas exchange rates as some of the harsh impacts due to drought (Perveen and Hussain, 2020; Sofy et al., 2021; Alam et al., 2021; Zandi and Schnug, 2022). Numbers of morphological characteristics of plants, including seed germination, plant height, relative root length, root diameter, the total biomass of leaves and roots, number of leaves/plants, number of branches/plants, etc. are negatively impacted by drought stress (Table 2) which are more or less observed in every crops. Among the physiological impacts, crop plants experience partial stomatal closure and an increase in photorespiration due to an imbalance in carbon metabolism during water stress (Hu et al., 2019). Additionally, during stress, plants produce more reactive oxygen species (ROS), which harms chloroplasts through oxidation. All of these factors work together to limit photosynthates, which eventually lowers agricultural productivity. In response to the deadly impacts of water stress, plants activate their natural defense systems including various morphological, physiological, and biochemical adaptations, leaf rolling, altered leaf angle, deep root system, drought-resistant epigenetic phenotypic plasticity and gene activation, production of osmolytes, soluble proteins, proline, soluble sugars, and glycine betaine, etc. (Ozturk et al., 2021; Ghafar et al., 2021). While considering the effect of drought on phytohormones, the impact of stress depends on balancing of IAA and ABA content (Krishnan and Merewitz, 2014). Rapid ABA accumulation has also been observed under salinity and heat stress (Xiong et al., 2001). Experimental evidence regarding the exposure of moderate drought on *Triticum aestivum* and *T. spelta* showed initial increased accumulation of ABA and SA, decreased level of GA_3 and IAA, alteration of CKs in roots and shoots (Kosakivska et al., 2022). ABA and ethylene significantly reduced gas exchange parameters, chlorophyll a and b content in cotton (Pandey et al., 2003).

2.2 Salinity stress

Saline soil having a high concentration of soluble salts with an ECe value of 4 dS/mL or higher in the soil. Salinity in the soil make it harder for roots to absorb water, and make it hazardous for plants. Salinity-resistant plants display morphological, biochemical, and physiological adaptations in an effort to maintain their life cycles. It's estimated that 50% of cultivated agricultural lands will be under salt stress by 2050 (Shrivastava and Kumar, 2015; Salts of NaCl and Na_2SO_4 are the main reasons affecting the salinity of agricultural lands (Pessarakli and Szabolcs, 2010). Germination and early seedling stages are the most susceptible stages to soil salinity (Munns and Gilliham, 2015). By disrupting ionic and osmotic equilibrium, salinity creates stress, which ultimately causes physiological drought in plants.

TABLE 2 Impacts of drought stress on some major crops.

Crop	Effect	Reference
Wheat	Spikelet fertility and grain filling reduced crop yields and quality	Grzesiak et al., 2019
	Reduced leaf area	Naz and Perveen, 2021
Rice	Poor seedling germination	Liang et al., 2021
	Reduced leaf area	Naz and Perveen, 2021
Pea	Poor seedling germination	Al-Quraan et al., 2021
	Reduces nitrogen fixation	Gonzalez et al., 2001
Maize	Seedling germination	
	Reduced number of leaves	Ahmad et al., 2019
	Reduced hypocotyl length and fresh and dry weight of roots	Hu and Chen, 2020
	Decreased seed oil content	Ali et al., 2010
Phaseolus vulgaris	Drop in the dry weight of the shoot	Widuri et al., 2018
Soybean	Reduces nitrogen fixation	Serraj, 2003
	Decreased oil content up to 12.4%, reduction in oleic acid content	Dornbos and Mullen, 1992
Common bean	Altered Fe, Zn, P, and N nutrient concentrations, decreased in total protein content	Ghanbari et al., 2013
Chickpea	Altered ABA levels and seed-filling rate	Sehgal et al., 2018
Nicotiana tabacum	Chlorophyll pigments affected	Hu et al., 2018

Salt stress causes a number of cellular and metabolic changes such as cellular growth and expansion disruption, plant membrane instability, ion toxicity, altering metabolism, inhibited seed germination, reduced photosynthesis, and reduced shoot, root, and leaf development in various crops (Table 3).

Along with the aforesaid effects, there is a fall observed in osmotic potential which ultimately reduced the uptake of nutrients and water by salinity stressed roots (Jose et al., 2017). Salinity induced stomata closure led to the inhibition of CO_2 fixation and destruction of photosynthetic pigments (Qados, 2011), which adversely affected the photosynthetic processes, and electron carrier (Sudhir et al., 2005). Salinity stress has a negative impact on plants considering the hormonal level as well as nutrient level. It causes a hormonal imbalance of the ABA, and IAA levels in stressed plants as reported (Wu et al., 2005). Further, salts of NaCl increases concentrations of Na^+ and Cl^- ions which put forward the ionic stress by getting in to competition with essential nutrients such as K^+, $Ca^{2+,}$ and Mg^{2+} leading a nutrient deficiency condition in plants (Botella et al., 2007). The aforesaid negative implications of NaCl salt will gradually lead to decreasing photosynthetic activity, generation of ROS, and programmed cell deaths (Serrano et al., 1999).

3 Response of phytohormones during abiotic stress

Low molecular weight phytohormones are considered to be the most important endogenous compounds having a crucial role in regulating physiological reactions of helps plants to heal in adverse environmental stress condition. (Khan et al., 2013). Reduced seed germination and plant growth have been linked to lower endogenous levels of phytohormones which can further be aggravated by various abiotic stresses (Iqbal et al., 2006). Stress can induce and activate various plant endogenous phytohormonal activities which further help in expression of various beneficial plant genes and proteins (Hamayun et al., 2010). Exogenous phytohormone application has also been proposed as a useful tactic to address various abiotic stresses, such salinity, drought, etc. (Iqbal et al., 2006), and also associated with several studies in reducing the negative impacts of abiotic stressors (Sharma et al., 2013; Iqbal and Ashraf 2013a, b; Amjad et al., 2014). The primary location for auxin production is in the apical meristem of shoots, immature leaves, and seeds. They contribute to phyllotaxis, apical dominance, root formation, embryogenesis, and reaction catalysis. In the molecular mechanism of auxin production, the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family, YUCCA gene families are the most important contributors. Majorly *YUC* gene family from which YUC flavin monooxygenases (YUC1, YUC2, YUC4, and YUC6) play essential roles in its auxin biosynthesis and plant development (Cheng et al., 2006).

The main cytokinins found in higher plants are zeatin, isopentenyl adenine, and dihydrozeatin, however zeatin is the most common cytokinin (Kieber and Schaller, 2018). The inhibition of lateral root initiation (Bielach et al., 2012), differentiation of phloem and metaxylem in roots (Bishopp et al., 2011), differentiation of photomorphogenic cells in expanding leaves and shoots (Efroni et al., 2013), and inhibition of leaf senescence are just a few examples of the significant regulatory functions of cytokinins at the tissue and organ levels (Zwack and Rashotte, 2015). The phytohormone has a good control over cell division (Miller et al., 1955), cell homeostasis, and adaptation of plants to climate change (Landrein et al., 2018). ABA is also known as stress hormone as whilst under stress, plants build up ABA, which sets off a reaction to deal with the adverse environment (Mahajan and Tuteja, 2005). It is a signaling molecule for regulation of seed germination and plant growth and development and seed maturation (Yan and Chen, 2016). From seed germination until senescence, the physiological and developmental processes of plants are thought to be significantly regulated by ethylene (Pierik et al., 2006). It plays a part in the regulation of photosynthesis, the metabolism of nutrients and proline and the antioxidant defense mechanism that shields plants from environmental stressors. Numerous studies have shown both benefits as well as negative impacts of the phytohormone. while in corn, *Arabidopsis*, tomato,

TABLE 3 Impacts of salinity stress on some major crops.

Crop	Effect	Reference
Rice	Excessive accumulation of Na+ ion in the root, reduction in the plant root and shoot growth, fresh weight, poor development of spikelets and panicle sterility, and loss of grain yield	Kazemi and Eskandari, 2011; Hussain et al., 2017; Munns, 2002; Hussain et al., 2019
Wheat	Decrease in seed germination, reductions in the growth and development of shoot and roots, leaves, and cells, decreases in ion transfer, gaseous exchange, decrease in the photosynthetic ratio and yield loss	Wahid et al., 2006; Motos et al., 2017; Zhang et al., 2017
Maize	Hampered seed germination, decrease in shoot growth, necrosis	Khodarahmpour et al., 2012; Farooq et al., 2015
Sorghum	Mineral deficiency, ion toxicity, decrease in plant stem yield and photosynthates	Netondo et al., 2004; Almodares et al., 2014
Cotton	Leaf area reduced, reduced plant growth, root and shoot growth, decreases in photosynthetic activity, Fiber quality, metabolic activities, decrease in fiber quality	Muhammad et al., 2018; Hussain et al., 2019
Coconut palm	Reduction in CO_2 permeability, photosynthetic inhibition,	Gomes and Prado, 2007
Medicago truncatula	Damaged Photosystem II, reduction in photosynthesis rate, inhibition of gaseous exchange	Najar et al., 2018

and grapevines, ethylene and its precursor ACC (1-aminocyclopropane-1-carboxylate) helps to tolerate environmental adversities; in *Cucurbita pepo*, tomato, *Arabidopsis*, and tobacco ethylene claimed its negative impact on plant growth (Lin et al., 2012; Yang et al., 2013; Freitas et al., 2017; Gharbi et al., 2017; Xu et al., 2019; Cebrián et al., 2021). The genetic basis unravels the APETALA 2/ethylene-responsive element binding factor (AP2/ERF) which is a plant specific transcription factor family is an important ethylene biosynthesis factor. It has four major subfamilies: DREB (Dehydration Responsive Element-Binding), ERF (Ethylene-Responsive-Element-Binding protein), AP2 (APETALA2) and RAV (Related to ABI3/VP), and Soloists (few unclassified factors). These subfamilies act as crucial regulators in a variety of biological and physiological processes, including signal transduction, regulator of plant morphogenesis, stress-response mechanisms, and metabolic activities (Li et al., 2020). Gibberellins (GA) are growth regulators that are particularly effective for seed germination, stem lengthening, enlarging fruit, and inducing blooming (Camara et al., 2018). Gibberellins' main function is to promote cell elongation, which in turn promotes cell division, accelerating both the vegetative and reproductive stage of plant growth (Colebrook et al., 2014; Kang et al., 2014). Exogenous GA treatment has also several benefits like it promotes early and large number sprouting in potato tuber (Alexopoulos et al., 2017), further it can improve the amount of viable seeds and antioxidant enzyme activity, increases the weight of individual fruits (Zang et al., 2016). Among the other phytohormones, brassinosteroid (BR) which was initially discovered in pollen of *Brassica napus* (Saini et al., 2015) was reported to be involved in root extension, maintenance of meristem size, initiation of lateral roots, creation of root hairs, mycorrhiza, and nodule formation (Mc Guinness et al., 2019; Wei and Li, 2016). Further during stress condition, crops like maize, soybean and banana are benefitted from methyl jasmonic acid in terms of increasing photosynthetic rate, grain yield, and drought tolerance (Anjum et al., 2016; Yu et al., 2019) Stress responses are essentially driven differently by different phytohormones and their crosstalk, that leads to transcriptional reprogramming in plants' response. The pivotal roles of phytohormones can be manipulated for mitigating the effect of the stressor.

4 Response of nutrients during abiotic stress

All the seventeen essential nutrients of plants are more or less responsible for abiotic stress alleviation in their own way. The most important plant nutrients, nitrogen (N), have an impact on physiology, growth, the reduction of biotic and abiotic stress, and structural integrity (Karim et al., 2016). However, it has a significant impact on crop plants' ability to effectively use solar energy, increase photosynthetic activity, and synthesize chlorophyll (Waraich et al., 2011). Phosphorus not also improves root architecture and proliferation in the soil even in soil drying conditions, but also stimulates root volume and hydraulic conductivity (Tariq et al.,2017). The modulation of numerous morphological, physiological, and biochemical processes by phosphorous within the plant system helps them to withstand stress better. Plant growth and development under stress are strongly and positively correlated with the use of phosphoric fertilizers. Ge et al. (2012) reported that potassium is another crucial nutrient for many fundamental physiological and metabolic processes including photosynthesis, stomatal control, photosynthesizing, carbohydrate metabolism, preservation of cell turgidity, enzyme activations, etc. Potassium is also essential for improving crops' tolerance to various abiotic stresses (Danial et al., 2010).

Calcium (Ca), an important secondary nutrient, acts as a signaling molecule in a number of physiological and biochemical processes that are necessary for a plant to develop stress tolerance (Ahmad et al., 2015). Magnesium (Mg) is essential for the conformational stabilization of macromolecules such as nucleic acids, proteins, cell membranes, and walls and is a structural component of the ribosome (Marschner, 2012). Its absence can have an impact on photosynthesis because it is a crucial element of the chloroplast, which controls photosynthetic activity. In the abiotic stress response, cellular acclimatization, and adaptability to challenging circumstances, sulfur performs protective roles (Cao et al., 2014). According to reports, an exogenous dose of sulfur increases crop productivity while maintaining regular metabolic processes that enable plants survive in harsh settings (Hasanuzzaman et al., 2013). The micronutrients like boron, zinc, iron, and copper reduce environmental stress through a variety of mechanisms, including glucose metabolism and transport, production of cellular integuments, preservation of membrane integrity, and activation of numerous enzymes. The structural role of selenium (Se) in the synthesis of glutathione peroxidase (GPX), which protects plants from the damaging effects of ROS, is also well documented (Lobanov et al., 2008). An adequate supply of Zn shields plants from the damaging effects of heat stress because it plays a significant role in maintaining membrane permeability (Peck and McDonald, 2010). The plant nutrients can be very much effective similar to the phytohormones for alleviation of various negative impacts of abiotic stresses. A brief account of abiotic stress alleviation using plant nutrients has been depicted in Figure 1. It is observed that in response to several abiotic stresses, major nutrients like N can enhance the photosynthesis of plant, phosphorus can be able to produce proliferate and strong root system, calcium can enhance the membrane stability and cellular integrity in plant, the micronutrients can able to regulate the cellular activity and mitigate abiotic stress by activating numerous enzyme and selenium can protect the plant from ROS activities.

5 Phytohormones and their effect on abiotic stress

During abiotic stress, it was observed that the phytohormones levels are altered; majorly ABA and ethylene level enhanced along with reduction of auxin and cytokinin are seen in a number of crops. The phytohormone works both in the response to stress as well as works for alleviation of stress. Both endogenous and exogenous level of phytohormones is showing equal importance in alleviation by regulating the internal and external stimuli in plants. The genes

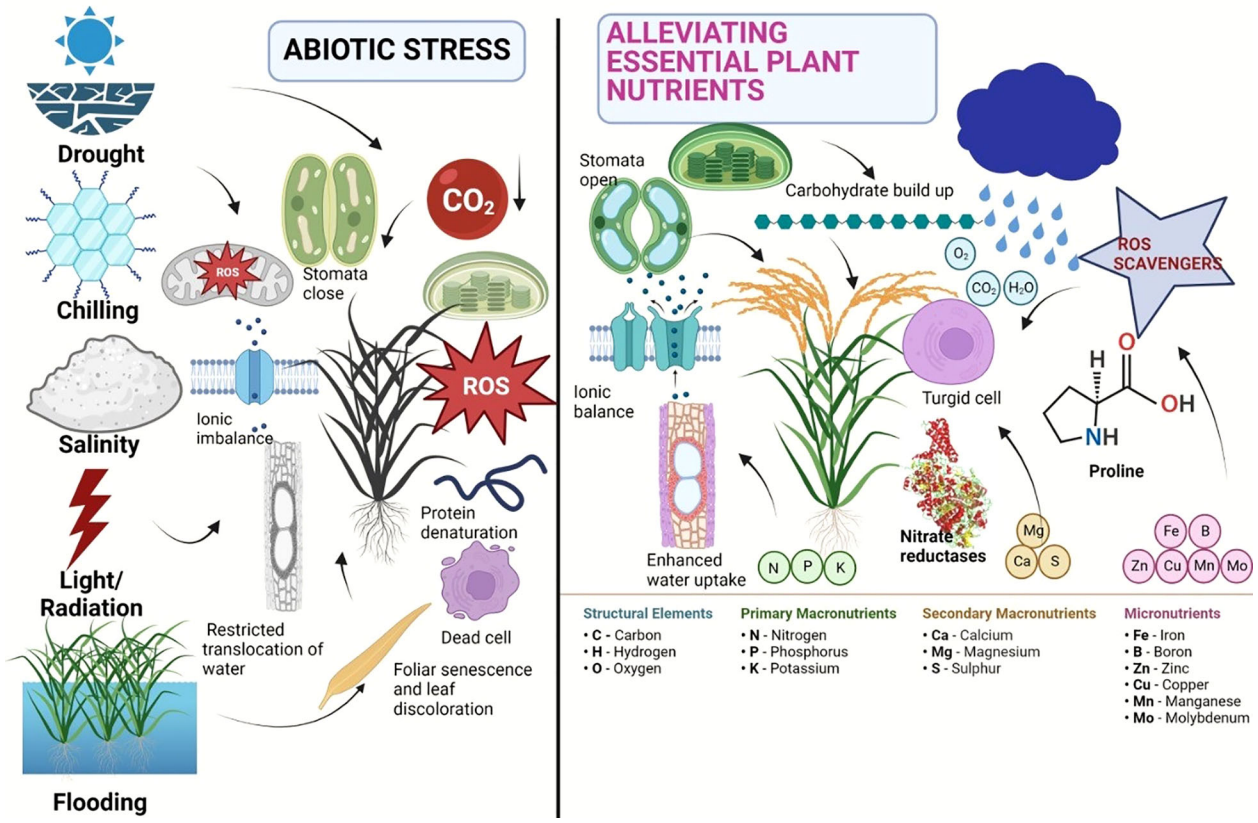

FIGURE 1
Negative impacts of abiotic stress and their alleviation using plant nutrients in plant.

responsible for the phytohormone level regulation are activated and their upregulation can be helpful for enhancing stress tolerance in plant. When plants are affected by several stresses, especially water deficit, plant hormones play vital roles in their growth and development (Raza et al., 2021). Several plant growth regulators, including salicylic acid, gibberellins, auxins, cytokinin, and abscisic acid, have reacted to drought (Chen et al., 2019). Phytohormones regulate internal and external stimuli, as well as signal transduction pathways, in addition to stress responses. Water logging or flood is a major constraint in low land conditions, the use of phytohormones signaling pathway can lead to a better way to alleviate the stress and achieve higher yield. Cold stress is a major problem in tropical and subtropical crops, whereas heat stress in temperate crops hampers crop production and productivity. It was observed that endogenous phytohormones level like gibberellic acid, brassinosteroids, cytokinins, abscisic acid, salicylic acid, jasmonic acid, and, auxin modified and regulates plant growth. A number of genes are activated during the exogenous application of plant hormones as a result tolerance can be created in the plants. So, studies on gene regulation and translation mediated by phytohormones can unlock a new way to recover low-temperature stress in plants.

5.1 Effect of auxin on stress

On exposure to drought, the plasticity of the plant root is affected that is regulated by the auxin. Auxin buildup in the root system reduces daytime and nocturnal water use and modifies hydraulic characteristics to allow the expression of water-saving features in wheat, maize, and sorghum yields during droughts. (Shao et al., 2017; Li et al, 2012; Rama Reddy, 2014). The exogenous application of auxins has shown to be effective in managing drought stress in plants. Indole-3-acetic acid (IAA) is the most common plant hormone of the auxin class and is mainly synthesized from the amino acid tryptophan (Trp). IAA triggers the activation of other stress-responsive hormones as well as the production of ROS. ROS production molds several physiological processes in a plant in response to water deficit stress. Discovery and characterization of numerous auxin-responsive genes in a number of plant species including rice, soybean, and Arabidopsis has paved the way for exploiting the genes to induce stress response (Hagen and Guilfoyle, 2002). A membrane-bound transcription factor NTM2 was used for auxin signaling controls for seed germination in salinity stress (Jung and Park 2011; Park et al.,2011). The number of genes like TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family, YUCCA gene family is the most important contributors to auxin biosynthesis (Cheng et al., 2006) that controls several metabolic activities in the drought-affected crop plants. Table 4 highlighted the impact of auxin-linked genes on the stress response.

Auxins have significant involvement in temperature-related stress. Shibasaki et al. (2009) performed a direct transport assay using an auxin-responsive marker (*IAA2-GUS*) on cold stress and concluded that the intracellular auxin efflux carriers are inhibited in plants due to cold stress. In high temperature, the plant mainly suffers

TABLE 4 Impact of auxin-linked gene on stress response.

Crop	Gene	Physiological impact	Reference
Transgenic rice	Expression of auxin-coding genes *OsIAA6*	Tillering behavior	Jung et al., 2015
Transgenic poplar and potato	Overexpression of YUC6	Faster shoot growth and retarded main root development with enhanced root hair formation, reduced levels of ROS production, higher photosystem II efficiency, and less membrane permeability	Ke et al., 2015
Tomatoes	Auxin-responsive genes (WRKY108715, MYB14, DREB4, and bZIP 107)	Increased root density and growth, maintained chlorophyll content, and increased soluble sugar content	Bouzroud et al., 2018; Zhang et al., 2020
White clover	Up-regulated auxin responsive genes (GH3.1, GH3.9, IAA8), drought stress-responsive genes (bZIP11, DREB2, MYB14, MYB48, WRKY2, WRKY56, WRKY108715 and RD22), and down-regulated leaf senescence genes (SAG101 and SAG102)	Increased stem dry weight, chlorophyll content, delayed senescence	Zhang et al., 2020
Arabidopsis	Expression of auxin responsive IAA5/6/19	Maintained level of glucosinolates (GLS), regulation of stomatal closure and ROS production	Salehin et al., 2019
Wheat	TAA family gene TaTAR2.1-3A overexpression	Increased grain yield under various nitrogen supply levels, high lateral root branching	Shao et al., 2017
Sorghum	IAA-amido synthetase gene GH3.5	Stay green	Rama Reddy et al., 2014
Tobacco seedlings	Initial elevated DR5: GUS gene expression levels and later decreased expression levels	Lateral root branching	Wang et al., 2018a

due to reduction of pollen viability in major crop, affecting seed set and eventually reducing yield. In crops like wheat and barley, it was observed that the initial pollen development stage is majorly hampered due to the reduction of pollen auxin concentration at high temperatures (Tadashi et al., 2010). They concluded that tissue-specific auxin concentration reduction can lead to pollen abortion during high-temperature stress. An analogous study was reported by Zhang et al. (2018) through an experiment in rice by exposing the rice spikelet to high temperature which drastically reduced the spikelet fertility and mitigated its effect by the application of NAA (1-naphthaleneacetic acid). Through the application of NAA in rice crops, auxin concentration was increased and leads to the proper development of pollen tube growth, elongation in the pistil, stylar length of the flower, and ultimately the pollen behaves normally under high temperatures leading to proper pollination and fertilization. In wheat, the effect of the exogenous application of auxin was estimated under heat stress conditions and found that the application of 1 μM of IAA can enhance higher grain number and yield by 6% – 8% under heat stress conditions (Abeysingha et al., 2021).

5.2 Effect of abscisic acid (ABA) on stress

Abscisic acid is a signaling molecule in plants in responses to stress conditions and noted as second group of phytohormone. ABA is a 15-carbon atom compound that belongs to a group of metabolites, known as isoprenoids or terpenoids, which are synthesized in the plastids (Xiong and Zhu, 2003; De Ollas et al., 2013). Under optimal conditions, ABA is expressed at low concentrations in plants (Parveen et al., 2021) and the concentration increases with the signal of stress to plants. In drought conditions, ABA alteration of guard cell ion transport regulates stomatal opening that reduces water loss (Kim et al., 2010). Upon exposure to drought stress, ABA is synthesized in roots and translocated to leaves wherein mesophyll cells are the predominant location of ABA synthesis (McAdam and Brodribb, 2018). Biosynthesis of ABA triggers drought adaptation mechanisms in the plants such as growth retardation, stomata closure and activation of several drought-responsive genes (Qi et al., 2018; Wilkinson and Davies, 2010). ABA regulates turgor by decreasing transpiration as well as by increasing water influx into roots (. Root-specific ABA signaling helps in balanced root growth toward soil exploration which regulates the transpiration and increases water influx into roots (Glinka and Reinhold, 1971, Duan et al., 2013). Considering the molecular activities of the phytohormone, in drought tolerant transgenic *Arabidopsis* overexpression of IbARF5 gene up-regulates the ABA biosynthetic genes (IbZEP, IbNCED, and IbABA2) was reported (Kang et al., 2018). Various transcription factors such as DREB2A/2B, AREB1, RD22BP1, and MYC/MYB are known to be the regulator of the ABA-responsive gene expression (Tuteja, 2007). SAPK2 ((Stress-Activated Protein Kinase) of SnRK2s (Sucrose nonfermenting1–Related protein Kinase 2) family which is an important ABA regulator, upregulates the expression of several drought responsive genes, including OsLEA3, OsOREB1, OsRab16b, OsRab21, and OsbZIP23 (Lou et al., 2017). From the aforesaid reported studies, it has been clear about the roles of endogenous ABA regulator genes and their role in the drought alleviation. Similarly, the exogenous application of ABA during drought in maize seedlings can also play role in activating antioxidant enzymes which in molecular basis regulates expression of ASR1, and endogenous ABA level, as well as reduce oxidative damage (Yao et al., 2019).

Similar to drought, salt-responsive genes' expression is also known to be regulated by both endogenous and exogenous ABA-mediated signaling when the soil is affected by salinity (Wang et al., 2001, Narusaka et al., 2003). Zhang et al. (2006) found a proportionate link between plants' exposure to salinity and their ABA content. Both endogenous ABA and its exogenous application demonstrate a critical role in preserving ionic balance in plants, as shown by their ability to prevent chloride toxicity in citrus leaves, avoid Na^+ and Cl^-, and maintain K^+/Na^+ ratio response in rice, K^+ and Ca^+ homeostasis (Gomez et al., 2002; Bohra et al., 1995; Gurmani et al., 2013). In addition to stomatal regulations, this phytohormone also aids in the osmoprotectants like proline (Iqbal et al., 2014) and dehydrins in response to ROS production during salinity-induced dehydration (Szabados and Savoure, 2009; Kim and Wang 2010; Hara 2010; Gurmani et al. 2013). Sripinyowanich et al. (2013) reported that, the expression of the OsP5CS1 gene increased proline accumulation and increased the survival rate (20%) of *Indica* rice seedlings by exogenous application of 100 M ABA. Shi and Zhu (2002) noted that ABA induced AtNHX1 expression in barley in response to salt stress. Keskin et al. (2010) stated that, ABA treatment caused quicker expression of MAPK4-like genes (TIP1 and GLP1) in wheat crops under salinity. Apart from drought and salinity, the exogenous application of abscisic acid (ABA) and ethylene also plays role in controlling other abiotic stresses. In green under greenhouse conditions the above hormones inhibit the suppression of photosynthesis in waterlogging by rejuvenating several factors like photosynthetic rate, transpiration rate, stomatal conductance, chlorophyll content and leaf water potential (Ahmed et al., 2002).

In case of cold stress, the effect of ABA application was studied on bermudagrass at 4 ^{0}C with application of 100 µM ABA which showed increased levels of chlorophyll content, maintained cell membrane stability, improved the performance of photosystem II, and altered expression of ABA or cold-related genes, including ABF1, CBF1, and LEA developing cold resistance in the grass (Huang et al., 2017). When wheat was exposed to low temperatures (0°C, −10°C, −20°C, and −25°C) application of exogenous ABA decreases the amount of H_2O_2 and relative conductivity (Jing et al., 2020). ABA was found to enhance cold tolerance in both leaves and rhizomes at −10°C and −20°C by increasing ROS production (Jing et al., 2020). In the same way, the ABA application was studied for its effect on heat tolerance in two rice germplasm lines. Li et al. (2020) used rice germplasm lines having flat leaves called wild type (WT) and others having rolling leaves with high-temperature sensitivity (*hts*) lines exposed to high temperature. The high-temperature lines showed a higher respiration rate with high tissue temperature and lower transpiration rate and stomatal conductivity but the WT line showed increased carbohydrate content, dry matter increased production of heat shock proteins (HSP71.1 and HSP24.5) under high-temperature stress. Through ABA application in these two lines, it was observed that thermo-tolerance was increased in the wild type but tolerance was reduced in *hts* plants (Li et al., 2020).

5.3 Effect of cytokinins on stress

Many drought-related processes are mediated by the stress hormone (ABA) as well as cytokinins (CKs). When plants are under drought stress, their CK content falls, and this increase in ABA responses causes the stomata to close and impede photosynthesis (Rivero et al., 2010). Stomatal conductance and transpiration are increased by CKs' ability to keep the stomata open (Lechowski, 1997). These CKs and ABA alterations brought on by stress encourage early leaf senescence and hormonal adjustments that cause leaf abscission, which results in a smaller canopy and less water loss. Pospisilova et al. (2000) observed that the expression of a cytokinins biosynthetic gene isopentenyltransferase (IPT), catalyzes the rate-limiting step in CK synthesis. Overexpression of IPT enhances the antioxidant system activity and increases drought tolerance by improving root growth in plant (Xu et al., 2016). Hormones like cytokinin enhance primary root growth in Arabidopsis by giving positive signaling to plant (Naulin et al., 2020). I In transgenic barley (*Hordeum vulgare*) and in tobacco it was observed that root specific reduction of cytolinin led to the enlarged root system under stress situations (Werner et al., 2010; Pospíšilová et al., 2016). It has also been shown that the increased transcriptional level of *CKX* genes and/or CKX activity was due to the exogenous application of cytokinin. It was observed that due to oxidase/dehydrogenase (CKX) which catalyzes CK and the overexpression and breakdown of CKX in Arabidopsis carried out as a result endogenous CK contents is decreased in plant (Werner et al., 2010). The abnormal expression of *CKX* in barley *via* maize *β-glucosidase, a* mild root-specific promoter has also been found to alter root architecture leading to lignification of the root tissue as well as activation of flavonoids biosynthesis (Vojta et al., 2016). Plant shows a higher level of accumulation of CK in root tissues due to a decrease in the activity of CKX, during drought stress (Havlova et al., 2008). The plant growth rate was slow down and elevate the content of protective compounds due to overexpression of CKX, which finally gives rise to increased drought tolerance in *Arabidopsis*, tobacco, and barley (Macková et al., 2013; Nishiyama et al., 2011; Pospíšilová et al., 2016).

Salt-sensitive plants' development was negatively impacted by salinity by lowering CK levels, indicating genotypic specificity (Kuiper et al., 1989). After being exposed to salinity, the amounts of CKs such as zeatin (Z), zeatin riboside (ZR), and isopentenyl adenine (iP) in the shoots and roots of barley cultivars drastically decreased (Kuiper et al., 1990). The negative effects of salt on plant growth are also known to be mitigated by CKs (Barciszewski et al., 2000; Fahad et al., 2014). Plant resistance to salt stress was reported to enhance with seed primingB with CKs (Iqbal et al., 2006). Iqbal et al. (2006) reported that CKs operate as ABA antagonists and IAA antagonists/synergists in a variety of plant processes and assist reduce salinity stress (Iqbal et al., 2014). Under exogenous application of CKs, it enhanced salt resistance *via* increased proline levels in brinjal (Wu et al., 2013). Plant hormones, particularly CKs, control the expression of a large number of stress-induced genes. Merchan et al. (2007) reported that the changes in osmotic circumstances also affect the expression of CKs receptor genes, showing that these receptors may have a similar function in the osmotic stress response despite the lack of a clear mechanism.

In waxy corn, exogenous application of 6-benzyl adenine (BA) in water logging conditions noted that not-treated plants showed chlorosis and necrosis in leaves, inhibiting growth and leading to the accumulation of O_2, H_2O_2, and MDA-like reactive oxygen species (ROS) but in treated plants, the reduction of ROS accumulation and

increase of enzyme activities like ascorbate peroxidase, glutathione reductase, dehydroascorbate reductase, and monodehydroascorbate reductase (Wang et al., 2021). Hence, the application of exogenous BA can alleviate water-logging-induced damage and improve water logging tolerance in waxy corn *via* the activation of the AsA-GSH cycle system and the elimination of ROS. The application of BA in waterlogged maize crops showed enhanced grain filling by improving grain weight and volume, which was beneficial to yield increase as compared to the untreated plant (Baizhao et al., 2019). It was recorded that the application of exogenous BA alleviated endogenous hormone levels of IAA, zeatin, and $GA_{3,}$ and at the same time, ABA content was decreased during grain-filling periods of waterlogged summer maize. The foliar application of CK and GA_3 under waterlogged conditions revealed that growth and biomass were enhanced, which was associated with increased levels of photosynthetic rate and pigments in the plant (Islam et al., 2022). It was reported that the accumulation of ROS and malondialdehyde levels is reduced during the water logging condition by application of CK and GA_3. Therefore, a better osmotic adjustment was carried out through proline and TSS level improvement in plants. Both CK and GA_3 were effective in water-stressed plants, however, CK was considered more effective than GA_3 (Islam et al., 2022). Prerostova et al. (2021) identified two genes which to be associated with cytokinin metabolism in plant, i.e., CK biosynthetic gene isopentenyl transferase (DEX: IPT) and CK degradation gene HvCKX2 (DEX: CKX). They observed that plants containing the DEX: IPT gene showed better stress tolerance with increased production of CK and SA levels in shoots and also auxin in the apex. At the same time plant containing the DEX: CKX gene and control plants showed weaker stress tolerance with lowered levels of CKs and auxins in cold conditions.

5.4 Effect of ethylene on stress

Ethylene (ET) has a significant role in fruit softening along with a vital role in mitigating the harmful impact of stress conditions due to abiotic factors (Pech et al., 2018; Wang et al., 2020). In diverse range of abiotic and biotic stress condition it was found that ET has a major role in nodule formation and nodule signaling (Khalid et al., 2017). Furthermore, it also enhances root emergence from nodal region which leads to retardation in development of nodal root and ultimately give rise to a negative effect on root-lodging resistance in *Zea mays* (Shi et al., 2019). Drought induces ethylene synthesis in shoots, by up-regulating the synthesis and xylem transport from roots to shoots of the ethylene precursor ACCs (Sobeih et al., 2004). It was found from research that adventitious root initiation sites in *Arabidopsis* hypocotyls are controlled by ethylene (Rasmussen et al., 2017). The overexpression of ethylene response factor such as GmERF3 of AP2/ERF gene family, leads to improvement in proline content, soluble sugar, and decreases in the accumulation of malondialdehyde to improve drought tolerance in the tobacco plant (Zhai et al., 2017). Further, SlERF5 of the aforesaid transcription family in over-expressing transgenic tomato plants resulted in high tolerance against drought (Pan et al., 2012). It was also found that gene 269 AP2/EREBP in cotton showed water stress response in plant (Liu and Zhang, 2017). Ethylene application response was also studied under water logging stress in soybean. It was noted that after the application of ETP (ETP; donor source of ethylene) in soyabean under water logging stress, the chlorophyll content significantly enhanced, and also cellular gibberellic acid is increased in the treated plant as compared to untreated plants (Yoonha et al., 2018). The amino acid content was also found appreciably higher in ETP-applied soybean plants than in the control. Several adventitious roots were induced in the plant after ETP application which enhance the root surface area and considerably amplified the expressions of glutathione transferases which that control ROS under water stress (Yoonha et al., 2018).

In the case of *Arabidopsis thaliana*, it was observed that freezing tolerance decreases by the introduction of the *ethylene overproducer1* gene and by the application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid but the freezing tolerance enhanced when ethylene biosynthesis inhibitor amino-ethoxyvinylglycine was applied (Shi et al., 2012). Shi et al. (2020) thus suggested from their research that ethylene can negatively regulates cold signaling through the direct transcriptional control of cold-regulated *CBFs* and type-A ARR genes. Sun et al. (2016) found a positive correlation between ethylene (ET) and cold stress was studied in grapevine. The treatment of exogenous 1-aminocyclopropane-1-carboxylate a form of ethylene was able to mitigate the cold stress in crops compared to the application of ET biosynthesis inhibitor amino-ethoxyvinylglycine which reduced the cold tolerance of grapevine. It was also observed that overexpression of gene 'VaERF057' enhances cold tolerance in *Arabidopsis* and ethylene is associated with the signaling of this gene. Thus, the research concluded that ET positively regulates cold tolerance in grapevine by regulating the expression of VaERF057 gene associated with cold tolerance (Sun et al., 2016). Wang et al. (2021) reported in case of apple seedlings, when treated with 1-aminocyclopropane-1-carboxylate (an ethylene precursor) and amino-ethoxyvinylglycine (an ethylene biosynthesis inhibitor), it was observed that the cold tolerance was increased and decreased respectively in the crop. They reported that during low-temperature treatment, ethylene level enhanced which leads to the over expression of MdERF1B significantly, increasing the cold tolerance of apple planting materials (seedlings and calli) as well as in *Arabidopsis* seedlings by mediating ethylene signaling pathway. Furthermore, molecular analysis proved that MdERF1B interacted with the promoters of two ethylene biosynthesis genes, i.e., MdACO1 and MdERF3. Wang et al. (2021) result thus concludes that MdERF1B–MdCIbHLH1 is a potential regulatory pathway that integrates the cold and ethylene signaling pathways in apples by up-regulating ethylene production under cold stress. While under high-temperature stress or heat stress ethylene is found to affect the pollen viability and sterility in plants similar to the auxins. In research conducted by Jegadeesan et al. (2018), it was observed that tomato pollen sterility can be overcome by the application of ethylene hormone (ethephon) during heat stress conditions. A protein analysis conducted during the study showed pollen development was hampered during heat stress due to the degradation of some proteins responsible for pollen development, pollen tube germination, and tube growth under the pistil surface. Jegadeesan et al. (2021) reported that ethylene hormone had a positive impact on pollen viability and germination, and the ability to increase the overproduction of heat tolerance genes like *SlHSP17*, SlHSP*101*,

SlMBF1 in tomatoes, when applied exogenously reducing the harmful effects of heat stress in due course. Another study in wheat showed ethylene again plays a vital role by regulating the biosynthesis of proline and modifying the antioxidative mechanism under heat stress. Application of 200 µL of ethephon and 50mM of proline showed improved tolerance of wheat in heat stress by activation of defense mechanism and protecting the photosynthetic pigment by enhancing the photosynthetic gene expression in crops (Sehar et al., 2022).

5.5 Effect of gibberellins on stress

On drought stress conditions, down regulation of GA could be a major target in making drought-tolerant plants. Nir et al. (2014) reported that the transgenic plants with the lower GA level tend to produce high stomatal intensity, lower stomatal conductance, and smaller leaves, which reduces the transpiration rate in stress. Further, the overexpression of SlDREB of the AP2/ERF family down-regulates GA biosynthetic genes in tomatoes. In tomato internode elongation and leaf expansion is reduced as a result of lower GA level in plant which ultimately create drought tolerance mechanism in plant (Li et al., 2012). Further studies confirmed water deficiency leads to downregulation of GA biosynthesis genes GA20 oxidase1 (GA20ox1) and GA20ox2 and induce the GA deactivating gene GA2ox7 in guard cells and leaf tissue, resulting in reduced levels of bioactive Gas in tomato (Shohat et al., 2021). Moreover, the over-expression of another transcription factor PtGA2ox1 decreases the GA level in the roots, stems, and leaves of the tobacco plant to promote drought tolerance (Zhong et al., 2014). In addition to maintaining protein and RNA levels, higher water level was also credited with GA's beneficial effects under salinity stress (Yamaguchi 2008). Maggio et al. (2010) reported that the application of GA to tomato plants reduced stomatal resistance and increased plant water usage effectiveness at lower salinity levels. Under salinity, the root and leaf cell nitrogen and magnesium are increased due to GA application (Tuna et al., 2008). Multiple factors, including an increase in reducing protein synthesis, activity of enzymatic antioxidants, sugars, and decreased activity of ribonuclease and polyphenol oxidase, contributed to GA3's beneficial effects on salt-stressed mung bean seedlings (Mohammed, 2007). Modulation of ions absorption and partitioning (inside shoots and roots) as well as hormonal homeostasis brought on by GA3 priming under salinity (Fahad et al., 2014). Through changed GA levels, the seed germination rate is enhanced due to the salt-inducible DDF1 gene (dwarf and delayed flowering 1) in high saline stress condition.

The applications of gibberellins in soybean plants found to reduce chlorophyll damage and also enhance the endogenous level of GA1 and GA4, and jasmonic acid in the plant along with the reduced level of ABA under flooding conditions (Muhammad et al., 2018). The research reveals that exogenous application of GAs during short-term waterlogging could enhance the transcriptional pathways and biochemicals which are majorly needed for maintaining plant growth during stress. Calvin et al. (2019) reported that the application of GA3 (200 ppm) in combination with salicylic acid (150ppm) on the soybean plant provides better mitigation effects by improving the number of pod and seed, chlorophyll content in waterlogged conditions. Gibberellins were found to be extremely sensitive towards cold stress and several GA metabolic genes, GA3ox1, GA20ox1, and GA2ox1 were found to be activated during cold temperatures (Ding et al., 2015). GA3 treatment has also improved fruit storage under low temperatures by decreasing malondialdehyde content and electrolyte leakage, increasing proline content and improving antioxidant enzyme activities as compared to untreated conditions (Ding et al., 2015). Shashibhusan et al. (2021) observed that pre-treatment of plants with 1gm, 2gm, and 3 gm of GA3 promotes plant growth and other yield-attributing traits in cold stress conditions in rice. Whereas, GA3 was found to have no direct role in heat tolerance but rather be associated with cell expansion gene activation and also positively affect the test weight of the seed in wheat (Nagar et al., 2021). They also noted that the application of paclobutrazol showed a thermo-tolerance effect rather than GA3 biosynthesis inhibition in wheat. Guo et al. (2022) suggested that gibberellins can mitigate the effects of heat stress response in plants by providing evidence obtained in tomatoes. They concluded from the result that exogenous application of gibberellic acid (GA_3) of 75 mg/L can mitigate heat stress by improving the plant growth, morphology, and physiological characteristics of tomatoes.

5.6 Effect of brassinosteroids on stress

Under stress condition, BRs increase Rubisco and the water usage efficiency of leaves hence improving CO2 assimilation and leaf water economy (Farooq et al., 2009). Several studies have also revealed that brassinosteroids s play a beneficial function in drought-stressed *Brassica napus*, wheat and *Arabidopsis* (Kagale et al., 2007). Exogenous 24-epibrassinolide treatment raises BRs content while lowering ABA and ROS levels, which further aids in increasing stomatal hole for water stress resistance (Nie et al., 2019; Tanveer et al., 2019). Unraveling the molecular basis of BRs control, three WRKY transcription factors—WRKY46, WRKY54, and WRKY70—have been identified as crucial signaling components that play oppositely positive and negative roles in BRs-regulated growth and drought responses (Chen et al., 2017). It was reported that the overexpression of a BRs biosynthetic gene AtDWF4, isolated from *Arabidopsis* in applied in transgenic *Brassica napus* results in improved drought tolerance (Sahni et al., 2016). BRs along with ABA showed a major role in drought stress in plants.

The negative effects of salt on plant growth performance are also known to be mitigated by BRs (Zhu 2002; Krishna 2003; Zhang et al., 2007; Kartal et al., 2009; Wang et al., 2011). By restoring pigment levels and elevating nitrate reductase activity, application of BRs through exogenous application reduced the negative effects of salt stress on root elongation, seed germination, and subsequent growth of rice (Anuradha and Rao 2001). Krishna (2003) found that barley leaf segments pre-incubating with BRs prior to exposure to salinity was successful in minimizing the cells' ultra-structures, such as their nucleus and chloroplasts. Under salinity, treatment of seed with BL considerably improved the accumulation of dry matter and antioxidant enzyme activity in lucerne (Zhang et al., 2007). In rice, Arabidopsis, and brassica, treatment with 24-epibrassinolide significantly increased seed germination, seedling growth, antioxidant system, and proline content, while reducing lipid peroxidation under salinity stress (Ozdemir et al., 2004, Kagale et al., 2007, Divi et al., 2010).

5.7 Effect of jasmonate on stress

Jasmonic acid (JA) encourages plant water uptake and methyl JA encourages increased osmoprotectant and compatible solute accumulation to increase chlorophyll content, antioxidant activity, and leaf gas exchange to trigger stomatal closure and improved water usage efficiency (S'anchez-Romera et al., 2014). There were negative impacts during drought stress; it also modifies polyamine and endogenous phytohormones (Xiong et al., 2020). It has been shown that exogenous administration of 0.5 mM methyl JA can preserve wheat growth and output during water deficit stress (Anjum et al., 2016). The application of 10 M methyl JA to sugar beet decreases the negative impacts of severe drought (Fugate et al., 2018). Kang et al. (2005) reported the comparison of salt-sensitive and tolerant rice cultivars and observed that salt-tolerant rice cultivars have a much higher concentration of JA. A critical component of the barley response to salt was thought to be the induction of JA-responsive genes (Walia et al., 2006). Endogenous JA contents in barley leaf segments that were subjected to sorbitol or mannitol osmotic stress increased significantly (Kramell et al., 2000).

JA is considered to have a major role in alleviating heat and light stress damage in the plant. A study conducted in the *Arabidopsis* crop showed a combination of high light and high heat (HL+HS) stress causing major damage to photosynthetic pigments and reducing the D1 protein level in plants with the same time accumulation of jasmonic acid that may provide tolerance in plant (Balfagón et al., 2019). They found that the plant deficient in jasmonic acid is highly sensitive to heat and light stress. Convergent study was conducted in Ryegrass; a temperate grass is sensitive to high temperatures. In this study impact of jasmonic acid on ryegrass was studied, it was observed that methyl jasmonic acid (MeJA) has a positive effect on augmenting tolerance in plants to a high temperature by altering the antioxidant defense mechanism, decreasing chlorophyll loss due to heat, maintaining good water balance in plant and lowering electrolyte leakage in the crop (Su et al., 2021). Along with that also the plant oxidize activity was enhanced by exogenous MeJA treatment which can increase the scavenging ability of ROS produced during heat stress and leads to alleviating the oxidative damage caused by heat stress and production of more heat shock proteins may be expressed in the plant during heat stress condition. (Su et al., 2021).

5.8 Effect of salicylates on stress

A phytohormone called SA is produced by chloroplasts (Dempsey and Klessig, 2017). According to reports, SA treatments sustain the cell's turgor pressure by increase the amount of osmolyte and proline in the root and shoot without affecting the other metabolic processes. Further, when SA is applied exogenously to canola, it increases the number of pods and seed output and is also involved in cell division and expansion (Keshavarz and Sanavy, 2018). Additionally, its use on marigolds under drought stress boosts bioproduction, enhances a number of physiological processes, and lessens the detrimental effects of water stress (Abbas et al., 2019). When crop plants under drought stress, such as wheat, saffron, and *Brassica rapa*, are exposed to it, SA activates nonenzymatic defensive mechanisms like sugar accumulation for energy saving and osmoregulation and lowers their malondialdehyde and free radical contents (Chavoushi et al., 2019; Ilyas et al., 2017). Through redox homeostasis and proline metabolism in agricultural plants, SA treatment increases drought-stress resistance (Chavoushi et al., 2019; Ilyas et al., 2017); La et al., 2019). By accumulating endogenous SA, the Arabidopsis loss of function lines cpr5 and acd6 demonstrated a drought tolerance mechanism (Miura et al., 2013). It has been revealed that in Arabidopsis, the SIZ1-mediated buildup of endogenous SA improves drought tolerance and encourages stomatal closure (Miura et al., 2013). It was observed that the osmolyte content in the vegetative phase of barley, safflower and corn has been increased by triggering multiple defense mechanisms, along with the antioxidant system through exogenous administration of SA which improve drought tolerance in those plants (Abdelaal et al., 2020; Chavoushi et al., 2019). Thus, a potential transgenic strategy for making plants resistant to drought would be to target genes involved in triggering the effect of drought resistance in response to the exogenous administration of SA.

SA's salt-ameliorating effects have also been widely reported in various crops including bean (Azooz 2009), wheat (Sakhabutdinova et al., 2003), barley (El-Tayeb, 2005), and mung bean (Khan et al., 2010; Syeed et al., 2011). Another study indicated that SA treatment of salt-stressed maize and mustard increased their ability to tolerate salt by speeding their photosynthesis and carbohydrate metabolism (Khodary, 2004; Nazar et al., 2011). Bastam et al. (2013) applied an exogenous treatment of SA to enhance the salt tolerance of pistachio seedlings. Palma et al. (2009) reported that under salinity stress, SA activates the antioxidant systems and is also attributed to the buildup of suitable solutes like proline and glycine betaine (Nazar et al., 2011). In addition, plants treated with SA showed reduced levels of membrane permeability and lipid peroxidation, which were otherwise rather significant under salinity (Horvath et al., 2007). Salicylic acid (SA) application was studied under waterlogging conditions in wheat crops revealing that lateral roots development was enhanced along with the emergence of surface adventitious roots which originate from the basal stem nodes of wheat, but root elongation was hindered, leading to the development of a shallow root system able to survive in water logging condition. (E scholar encyclopedia, 2022). The effects of salicylic acid become more apparent in plants under stress conditions. In maize crops, application of 0.5mM of salicylic acid improves the growth rate of plant under hydroponic conditions under cold stress (Janda et al., 1997; Janda et al., 1999). It was observed that SA application reduced electrolyte leakage and improves CAT activity with a level of enhancement in the activities of glutathione reductase and guaiacol peroxidase. Application of SA in normal conditions may cause deleterious effects on plants but in stress conditions, it can act positively (Waraich et al., 2011). Likewise in the wheat crop that treatment with salicylic acid at the rate of 0.5 mM can mitigate heat stress damage by increasing the production of proline and reducing the activities of proline oxidase (PROX) which finally leads to maintaining osmotic potential and photosynthetic activities in the plant (Khan et al., 2013). From the result, it was observed that plant tolerance was created SA through interacting with proline activity and ethylene formation and eventually leads to alleviating the photosynthetic damage caused by heat stress in wheat.

6 Nutrients and their effect on abiotic stress

Nitrogen is a major component of all cellular and metabolic activities in crop plant as it is a major element of proteins, chlorophyll, nucleic acids, amino acids, plant hormones, enzymes, and osmolytes, all of which are involved in plant abiotic stress tolerance mechanisms through different pathways (Arghavani et al., 2017; Singh et al., 2019). The application of N enhances the plasticity and water extraction capacity of plant roots from the soil, which helps to maintain optimal relative leaf water content and increase water use efficiency in environments with limited moisture (Yang et al., 2012; Tran et al., 2014). Nitrogen supplementation was able in alleviating NaCl-induced toxicity in tomato seedlings which up-regulate the AsA–GSH cycle, K^+, and K^+/Na^+ ratio, which resulted in better growth performance (Nazar et al., 2011). In *Brassicas it was found that* application of N may improves a lot of cellular activities and also prove to be mitigate the ill effects of salt stress in plant. Under the salinity stress condition application of N can improve growth attributes, physio-biochemical parameters, nutritional enrichment, and yield attributes in brassicas (Siddiqui et al., 2010). Application of nitrogen fertilizer to crops promotes antioxidative defense mechanisms and reduces leaf senescence. These processes include carbon partitioning, carbohydrate buildup, cellular membrane stability, and osmoregulation (Saneoka et al., 2004; Saud et al., 2017), cell synthesis and expansion of plant cells (Li et al, 2012), increased photosynthetic capacity (Gessler et al., 2017). N can boost the root system in crops including rice, wheat, rapeseed, and pearl millet as well as improve xylem transport, photosynthetic enzyme activity, antioxidant defense, delay cell senescence, control stomata, increase proline accumulation, and encourage profuse branching (Rostamza et al., 2011; Albert et al., 2012; Tran et al., 2014; Abid et al., 2016.). Under drought conditions, phosphorus promotes root architecture and proliferation in the soil, which increases root volume and hydraulic conductivity (Jin et al., 2015). Application of phosphorous during the early stages of the wheat crop boosted root growth and establishment (Ahmed et al., 2018). The application of P reduces the formation of ROS caused by drought by energizing enzymatic antioxidants as POD, CAT, APX, SOD, and monodehydroascorbate reductase (MDHAR), which consequently increases resistance to stress (Meng et al., 2021). Sardans and Penuelas (2012), P treatment has also been linked to the remodeling of nitrogenous compounds in terms of buildup and absorption of NH_4 + and NO_3 in water-stressed agricultural plants. Phosphorus fertilization significantly increased all growth parameters, chlorophyll content, nucleic acid content and minerals content of the common bean plants under salinity stress (Mohamed et al., 2021). Protective effect of potassium application on salt stress in two tomato genotypes (Nasir and Skyland-II) more dry biomass production, shoot K^+ concentration, chlorophyll contents, stomatal conductance, and K^+/Na^+ ratio under saline condition (7.5 dSm^{-1}) (Muhammad et al., 2020). Exogenous K fertilizer treatment of 160 kg/ha under water stress enhances grain yield, harvest index, and other physiological indicators in rice (Zain et al., 2014). K can increase the photosynthetic process and glucose metabolism in a stressed cotton crop (Zahoor et al., 2017). In order to reduce abiotic stress in plants, secondary nutrient like calcium is also necessary for food uptake, enzymatic and hormonal up-regulations, and stabilization of cell membranes (Rahman et al., 2015), improves the ability to preserve water (Shao et al., 2008). Ca^{2+} alters the plasma membrane's level of hydration, which enhances the cohesiveness of the cell walls and raises the viscosity of the protoplasm, enhancing the resistance of cells to dehydration (Ma et al., 2009). Xu et al. (2013) reported that the application of 10 mM Ca in drought conditions caused the production of more root and shoot biomass and dry weight. Magnesium can produce photosynthetic pigments, accumulate higher proline content in mungbean, and encourage better root proliferation in rice (Thalooth and Tawfik Mohamed, 2006; Ding and Xu, 2011). Min et al. (2016) reported that Sulfur helps to nullify the oxidative stress produced due to drought stress by increasing the activities of ROS scavengers like CAT, SOD, and APX; higher H_2S and soluble sugar contents along with reducing H_2O_2. Boron promotes the resistance of crop plants by improving hormone synthesis, lipid metabolism, pollen formation, sugar transport, photosynthetic efficiency, seed germination, flower retention, and seed yield during drought stress (Michael et al., 2016). Under water scarce conditions, B improved water uptake, and nutritional status from the rhizospheric soil by enhancing the growth of more root hairs and mycorrhizae, ROS detoxification process in chloroplasts preventing photooxidative damage hence establishes membrane integrity and improves drought tolerance in plants (Venugopalan et al., 2021). Zn as an important micronutrient has been observed to improve the synthesis of IAA and gibberellic acid (GA3) like plant hormones under moisture stress conditions and thereby improving plumule length and increase shoot dry weight under drought stress. Zn application also helps in a significant expansion in leaf surface area, stomatal conductance, relative leaf water content, and improvement in chlorophyll and accumulation of osmolyte, thus resulting in enhancing cellular growth, plant harvest and prevention the destructive impacts on leaf cell due to moisture deficiencies (Hassan et al., 2020). Spraying with Fe reduces oxidative stress by depleting H_2O_2 content along with breakdown of lipid peroxidation activities by accelerating the enzymatic antioxidant mechanisms (CAT, SOD, and GPX) under water scarce situations and also showed a major impact in triggering the quality and resistance of protein under drought stress (Baghizadeh and Shahbazi, 2013; Afshar et al., 2013). While going for role of copper under drought condition, Copper chlorophyllin (Cu-chl) has been proved to be an important modified water-soluble and semi-synthetic bio-stimulant that helps to improve the antioxidative capacity which leads to decreased oxidative stress in plant (Kamat et al., 2000). Cobalt imparts drought tolerance in plants by increasing water use efficiency by reducing the rate of transpiration, further it activates the antioxidant defense mechanisms in plants (Banerjee et al., 2021).

Among the other nutrients, there are several elucidations of the alleviation effects of Si in salt-induced osmotic stress (Zhu et al., 2015) and oxidative stress (Yin et al., 2019). Si-mediated up-regulation of aquaporin gene expression and osmotic adjustment play important roles in alleviating salinity-induced osmotic stress (Zhu et al., 2019). Further foliar application of micronutrients could be useful for improving the nutrient status, root features, and physiological performance of wheat plants (Fouly et al., 2011). Nutrients in combination with phytohormones, it was noted that many plant nutrients can also alleviate water-logging stress and temperature stress. For example, it is reported that application of boron can improves the activity of the

antioxidant system significantly and which leads to nullify the toxic effects of ROS produced by heat stress (Waraich et al., 2011). Similarly, selenium (Se) is known for its major role in synthesis of glutathione peroxidase (GPX) and ultimately prevents the plants from the negative impact of ROS (Lobanov et al., 2008). Also, Zn micronutrients can be used to maintain the permeability of cellular membrane and the optimum dose of Zn can mitigate plants from the devastating impacts of heat stress (Peck et al., 2010). Tables 5–7 highlighted the nutrient application in the alleviation of abiotic stresses in plant systems.

7 Crosstalk with abiotic stress, phytohormones and nutrients

Crosstalk among and between the phytohormones and nutrients has been reported to have important role in abiotic stress alleviation. Auxin being an important phytohormone enhances drought resistance by interacting with other phytohormones. During drought stress, auxin regulates various members of the ACS (1-aminocyclopropane-1-carboxylate synthase) gene family, which is a rate-limiting enzyme in ethylene biosynthesis further increasing resistance against the stress in plants (Colebrook et al., 2014). It was reported that the exogenous application of IAA can enhance ABA and JA content and it can promote the up-regulation of over expression of drought stress-responsive genes (WRKY2, WRKY56, bZIP11, MYB14, DREB2, MYB48, WRKY108715, and RD22), auxin-responsive genes (GH3.9, GH3.1, IAA8) and down-regulation of leaf senescence genes (SAG101 and SAG102) and auxin responding genes (GH3.3, GH3.6, IAA27) which ultimately improves the plant tolerance towards drought stress in white clover (Zhang et al., 2020). Further, during drought ABA accumulation maintains maize primary root elongation by restricting the production of ethylene (Spollen et al., 2000). Furthermore, in drought stress endogenous CK level reduction in the roots also leads to higher concentrations of macro- and micro-elements, such as manganese (Mn), phosphorous (P), or zinc (Zn) (Ramireddy et al., 2018; Nehnevajova et al., 2019). ABA-activated type-A ARR5 magnifies the ABA-mediated response to stress e. Simultaneously, restricts plant growth by repressing CK signaling *via* a negative feedback loop in Arabidopsis (Huang et al., 2018). Further osmotic stress trigger synthesis of CK which down-regulate the genes of ABA synthesis and ABA-mediated responses, which reduces the damage caused by ROS and lipid peroxidation, reduce the senescence ability of leaves and thus improves the abiotic stress tolerance ability of plant and plant growth (Gujjar and Supaibulwatana, 2019). Further, ABI1 and ABI2 which negatively

TABLE 5 Reports of nutrients involved in mitigating stress in plant.

Types of nutrients	Crop	Impact on plant	References
Nitrogen	Winter rapeseed (*Brassica napus* L.)	Application of nitrogen in winter rapeseed in water logging can avoid the degradation of photosynthetic pigments and ultimately the dry matter accumulation is enhanced	Men et al., 2020
calcium nitrate, potassium nitrate, and tricyclazole	Canola	Application of calcium nitrate, potassium nitrate, and tricyclazole in water logging conditions can enhance the dry weight of plants along with the length of shoots and roots were increased	Habibzadeh et al., 2013
Phosphorus	Wheat	The application of phosphorus in a waterlogged condition is able to increase root establishment and growth under water stress conditions.	Ahmed et al., 2018
Potash	Cotton (*Gossypium hirsutum* L.)	Application of potash in water logging conditions in plants can show improved growth of plants, enhanced photosynthetic pigments, and photosynthetic capacity. It also enhances the uptalking capacity of nutrients in waterlogged plants	Ashraf et al., 2011
	Rice	In water logging, condition higher concentration of K showed improved photosynthetic pigments, non-structural carbohydrates (NSC) contents, and higher activities of antioxidant as well as reduces the activity of lipid peroxidation in waterlogged rice.	Hasanuzzaman et al., 2018
CR urea	Wheat	According to one research in Australia, it was revealed that the application of Controlled Released urea can avoid waterlogging effects of wheat, and grain yield is increased by approximately 20%	Manik et al., 2019
FYM		Application of farmyard manure in waterlogging conditions can enhance grain Fe, Zn, and Cu concentration of paddy which is essential to prevent water stress in plant	Masunaga and Marques Fong, 2018
Boron	Maize	According to research, it was found that Foliar application of boron can able to improve plant growth and mitigate the deleterious effect of maize under waterlogging	Sayed, 1998
Calcium	Rice-Rape rotation field	In Waterlogging condition of rice–rapeseed rotation field production of rapeseed was particularly reduced and it can be mitigated by the application of Calcium peroxide which after reacting with water releases oxygen, which can serve as an excellent supply of oxygen in redox zone.	Wang et al., 2022
Sulphur	Peach	Application of Hydrogen Sulfide (sulfur source) in waterlogging conditions can reduce the damage occured in Peach Seedlings by improving the activities of antioxidation and reducing Ethylene Synthesis	Xiao et al., 2020
Calcium	Pepper	Application of Ca^{2+} in pepper plants improve the photosynthetic capacity, and root growth, and ultimately the biomass is increased in water logging condition along with enhanced antioxidant enzyme and alcohol dehydrogenase activities.	Yang et al., 2016

TABLE 6 Reports of nutrients involved in mitigating cold stress in plant.

Types of nutrients	Crops	Effect	References
Hydrogen Sulfide	Cucumber	Application of sodium hydrosulfide (NaHS, an H_2S donor) develops cold stress tolerance of cucumber seedlings also the level of auxin is enhanced in the crop.	Zhang et al., 2021
Potash	Carnation	Application of K in high concentrations with irrigation water prevents plant stem damage during low night temperatures in carnation plants.	Kafkafi, 1990
	Potato	In potato plants, during cold stress decreased yield and increased leaf damage were found which can be mitigated by the application of potash in plants.	Grewal and Singh, 1980
	Tomato, Pepper, and Brinjal	Through the application of K, it was observed that total plant yield was increased by 2.4-fold in tomato, 1.9-fold in pepper, and 1.7-fold in brinjal.	Hakerlerler et al., 1997
Phosphorous	Lowland rice	Application of exogenous phosphorus can alleviate low-temperature stress along with p deficiency also it was helpful in shortening day to heading in early and intermediated transplanting crop of rice	Andrianary et al., 2021
Boron	cucumber, cassava, sunflower	Application of boron during cold stress can alleviate the effect of chilling-induced reduction in, membrane fluidity, plasmalemma hydraulic conductivity, root pressure, and water channel activity which leads to a improving in hydrolic conductance of root, uptake of water and nutrient in plant	Huang et al., 2005
Magnesium	Tomato	During low temperatures and high concentrations of K, the risk of Mg deficiency in tomatoes is high. So, the application of magnesium can achieve the normal growth of plants during cold-stress conditions.	Li et al., 2018

regulate ABA signaling interact with BIN2 and regulate BRs signaling, which ultimately shows stress responses in Arabidopsis (Wang et al., 2018a; Wang et al., 2018b).

The crosstalk is also having an important place in dealing with salinity resistance in plants. The effect of salt stress can be nullified by seed priming with IAA on wheat seed germination and growth *via* regulation the biosynthesis of free salicylic acid induced by auxin and maintaining ionic homeostasis in leaves (Iqbal and Ashraf 2007). Fahad and Bano (2012) observed that during salinity stress plant can produce significant amount of IAA and reduce the synthesis of ABA in maize plants; however, the application of salicylic acid can significantly increase the IAA. Application of auxin restricts the nodes of tiller in rice by biosynthesis of cytokinin in nodes along with down-regulating OsIPT expression (Liu et al., 2011) during salinity stress. CKs play an important role by acting as a bridge in showing the protective role of epibrassinolide and methyl jasmonate in wheat under salinity (Shakirova et al., 2010). Iqbal and Ashraf (2013a) reported a non-consistent effect of GA_3 priming (150 mg L^{-1}) on auxin concentration in wheat genotypes under salinity stress. GA improved the growth of soybean by regulating the level of other phytohormones under salinity (Hamayun et al., 2010), and increased levels of bioactive GA1 and GA4 showed a concurrent decrease in the level of ABA and SA. In brassica, the application of GA in conjunction with nitrogen was helpful in alleviating salinity stress (Siddiqui et al., 2010). Moreover, BRs-mediated stress tolerance in Arabidopsis was linked with ABA, SA, and ETHY pathways (Divi et al., 2010). The BRs act as synergists to GA and IAA during the hypocotyl elongation of Arabidopsis (Tanaka et al., 2003). ABA acts as an antagonist as it repressed the BR-enhanced expression (BEE1, BEE2, and BEE3) proteins (Friedrichsen et al., 2002). Exogenous application of jasmonates (JA) may change the endogenous ABA, which provides a

TABLE 7 Reports of nutrients involved in mitigating heat stress in plant.

Types of nutrients	Crops	Effect	References
Magnesium	maize and wheat	Application of Magnesium during heat stress of wheat and maize plants can nullify the damage effect by decreasing oxidative cellular damage caused by ROS.	Mengutay et al., 2013
Nitrogen	Spinach	It was observed in spinach both the photosynthetic activity and the light collection ability of the plant is reduced due to low nitrogen content.	Verhoeven et al., 1997.
	Bean	nitrate-grown bean plants had higher tolerance to photodamage than ammonium-grown ones.	Zhu et al., 2000
	Tomato	plant with ammonium application show better tolerance to heat stress than nitrate-applied plants due to the assembly of proline and quaternary ammonium compounds in tomato plant	Rivero et al., 2004
K+Zn+B	Cotton	In cotton increased ability of TNBPP, NSBPP, TSP, RWC, fiber length, fiber strength and fiber fineness were observed due to foliar application of K and Zn followed by B.	Sarwar et al., 2022
Mg	Bean	The antioxidant activities and antioxidant molecules are increased in bean due to the application of Mg	Cakmak and Marschner, 1992
	Maize	The antioxidant activities and antioxidant molecules are increased in maize due to the application of Mg	Tewari et al., 2004
	Pepper	The antioxidant activities and antioxidant molecules are increased in peach due to the application of Mg	Anza et al., 2005
	Mulberry	The antioxidant activities and antioxidant molecules are increased in mulberry due to the application of Mg	Tewari et al., 2006

significant hint for understanding the protection mechanisms against salt stress (Kang et al., 2005). Furthermore, foliar application of N fertilizers at the reproductive stage, particularly in leguminous crops, significantly slows the synthesis of abscisic acid with an enhance synthesis of cytokinin production, which promotes cell elongation, nodulation, shoot development, apical dominance, photosynthetic activity, and assimilates translocation to the sink organs under drought conditions (Vries et al., 2016). Likewise, the synergistic regulation of H_2S with phytohormones such as abscisic acid, ethylene, and salicylic acid can able to regulate the plant stress response (Zhang et al., 2021). It was observed that a balanced application of nutrients can be useful to mitigate cold stress by protecting the cell against freeze-dry death for a limited period of time (Huixia et al., 2018). The plant supplemented with potassium and magnesium provides better protection during a cold injury in the plant. The application of potassium can regulate the closing of stomatal cells, improves water balance, and prevents uncontrolled water loss through the leaves (Danilova et al., 2016m). Also, Magnesium promotes root growth up to a deeper zone of soil and therefore helps ensure that plants can still absorb water from deeper soil layers *via* a well-developed root system, even when the soil is slightly frozen (Danilova et al., 2016m). Whereas, Auxin a plant growth that promotes its synthetic pathway can create thermo-tolerance in crops. During heat and moisture stress conditions, soil cobalt application combined with foliar K and B sprays manifested immense potential to achieve higher black gram production (Banerjee et al., 2021). A similar study was also carried out in *Lathyrus sativus* by the authors that showed the combined application of N, P, and K with Mo improved growth, physiological efficiency, nutrients uptake, and yield ameliorate heat and moisture stress (Banerjee et al., 2021). Combined application of Zn, B, and Si increased plant height, shoot dry weight, number of stems per plant, leaf relative water content, leaf photosynthetic rate, leaf stomatal conductance, chlorophyll content, and tuber yield in potato during salinity stress condition (Mahmoud et al., 2020). Co-application with other plant nutrients like N, P, K, Zn, Si, etc. can be proven beneficial in alleviating salinity, heat, and moisture stress in plants (Akeel et al., 2020). Application of nutrients like K and Ca improves root growth and improve the uptake of water which leads to regulating the stomatal cell and maintaining the plant body temperature during heat stress. The application of micronutrients like B, Mn, and Se can alter the physical, biochemical and metabolic processes in plants in a positive direction to alleviate the adverse effects of heat stress. Combine application of Selenium (Se) and Salicylic acid (SA) can improve tolerance in crops by activating antioxidant production which can eliminate the ROS and make the plant free from membrane damage (Kumari et al., 2022). It was concluded that hormonal balance and their cross-talk with themselves and the nutrients are critical regarding signal perception, transduction, and mediation of stress response in plants.

8 Conclusion

In this current review highlighted the comprehensive information on the response of phytohormones, nutrients application and their interaction in crops grown under various abiotic stress conditions. Majority of phytohormones control and sustain the homeostasis inside the cell by detoxifying the ROS and enhancing the antioxidant activities during varied abiotic stress and can enhance tolerance in plants. In drought condition, application of IAA can trigger the activation of other stress-responsive hormones as well as the production of ROS. Enhanced level of ABA in drought condition can alter the guard cell ion transport and stomatal opening which leads to reduced water loss. Cytokinin application increases transcriptional level of *CKX* genes leads to enhanced CKX activity in many plants. Proline activity is enhanced by applying CKs to create salt resistance in plant. It was also concluded that endogenous hormone levels of IAA, zeatin, and GA_3 is enhanced by application of that exogenous application of BA. In water logging condition, the accumulation of ROS and malondialdehyde levels is reduced by application of CK and GA_3. The overexpression of ethylene response factor such as GmERF3 of AP2/ERF gene family, leads to improvement in proline content, soluble sugar, and decreases in the accumulation of malondialdehyde to improve drought tolerance in plant. During heat stress, the pollen sterility is the major cause of yield loss, which can be overcome by application of application of ethylene hormone (ethephon) during heat stress conditions. In saline condition by altering the GA levels can enhance seed germination by overproduction of the salt-inducible DDF1 gene (dwarf and delayed flowering 1). It was estimated that application of GA_3 (200 ppm) in combination with salicylic acid (150ppm) on the soybean plant provides better mitigation effects by improving the number of pod and seed, chlorophyll content in waterlogged conditions. Also, it was observed that methyl jasmonic acid (MeJA) has a positive effect on augmenting tolerance in plants to a high temperature by altering the antioxidant defense mechanism, decreasing chlorophyll loss due to heat, maintaining good water balance in plant and lowering electrolyte leakage in the crop. It was also revealed that application of 0.5mM of salicylic acid improves the growth rate of plant under hydroponic conditions under cold stress condition. Besides, the application of plant nutrients like N, K, Ca, and Mg are also found to reduce the ROS activities through elevating antioxidants quantity that can scavenge the ROS effect and finally leading to the reduction in cell membrane leakage and increase the photosynthetic ability in the plant by recuperating the chlorophyll cells. Hence, it is concluded that the crosstalk with phytohormones and nutrients can complement each other streamlining the antioxidant activities or ROS signaling pathway in cells and improving the tolerance of crop plants. More amalgamated and detailed research is needed with the combined application of hormones and nutrients to precisely understand the mechanism involved.

Author contributions

RS, writing and conceptualization of the manuscript. SS, drafting of the manuscript, preparing the table and figure. MB, drafting the manuscript, preparing the different tables. GR, editing and critical reviewing of the final version. All authors contributed to the article and approved the submitted drafted version.

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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Phytohormones unlocking their potential role in tolerance of vegetable crops under drought and salinity stresses

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Globally, abiotic stresses are drastically reducing the productivity of vegetable crops. Among abiotic stresses, drought and salinity are more challenging constraints for the sustainable production of vegetables. A great variety of vegetables are facing dry and hot summer spells, poor water availability, and higher salinity mainly due to irrigation with brackish water. Vegetables are considered higher water-dependent crops, requiring water for proper growth and yield. Drought and salinity impair plant metabolism. The disruption in plant metabolism leads to a reduction in growth, developmental processes, and ultimately crop yield. Appropriate management measures are needed to cope with the adverse effects of drought and salinity. Different agronomic and molecular approaches contributed to improving tolerance. Therefore, the present review significantly explores the impact of phytohormones on vegetable crops under drought and salinity stresses. Phytohormones (salicylic acid, melatonin, jasmonates, Brassinosteroids, ascorbic acid, and numerous others) can be sprayed for improvement of plant growth, yield, and photosynthetic pigments by modulation of physiological and biochemical processes. In this manner, these phytohormones should be explored for sustainable production of vegetable crops growing under abiotic stress conditions.

KEYWORDS

metabolic mechanisms, irrigation practices, sustainable yield, water needs, crops

Introduction

Phytohormones are considered plant-protecting hormones under abiotic stress conditions. Different phytohormones are well-known management strategies, which act as stress-relieving bioactive compounds in vegetable crops (Fahad et al., 2015; Altaf et al., 2022a). The exogenous spray of numerous phytohormones can reduce the drought and salinity stresses and also improve the plant defense mechanism focusing on sustainable production. Fascinatingly, phytohormones are more effective for the reduction of challenges that occur from stressful conditions at any growth or developmental phases even from germination to plant senescence. These hormones are contributing to numerous signaling

and transduction pathways through hormonal reception and regulatory actions (Hu et al., 2012). The membrane receptors, ionic networks, reactive oxygen species (ROS) indications, and mitogen-activated protein kinase (MAPK) indications are noticeable to numerous fundamental utilities in the synchrony by phytohormones to cope with the negative effects of abiotic stress. Understanding the interactive mechanism of phytohormones and transcriptomics can be effective for the development of tolerant germplasm of vegetables (Diao et al., 2015; Mangal et al., 2022). Modulation of physiological and photosynthetic pigments was found to be helpful for an increase in plant yield growing under water stress and saline conditions. The susceptible germplasm can also become higher yielding by sufficient use of phytohormones based on genetic makeup and climatic conditions of the characterized germplasm, as reported by Forni et al. (2017). However, the impact of phytohormones on the regulation of secondary metabolites and other signaling molecules needs further investigation for better understanding. For the development of tolerant germplasm, traditional breeding ways are time-consuming and not specific. However, the application of phytohormones is more effective for the alleviation of abiotic stress tolerance.

Plants growing under field conditions could be exposed to multiple stresses, which can damage the crops' yield (Glick, 2012). Severe climatic conditions in summer, irregular nutrition management, and unavailability of irrigation water are causing stunted growth and poor crop yield. Sustainable agricultural crop production is drastically affected by numerous biotic stresses (such as insects, pests, and disease) and abiotic stresses (like drought, salinity, temperature extremes, humidity, light, ultraviolet radiations, mineral nutrition deficiencies, and heavy metals) (Akram et al., 2017; Shakoor et al., 2017). Drought and salinity are considered more destructive conditions, extensively affecting growth, developmental stages, and yield. Plants can change their defense system against stressful conditions to regulate metabolism, growth, and development (Ahmad et al., 2008). Vegetable crops are potentially growing under diverse environmental conditions by natural acclimation, as well as numerous adaptation strategies. However, these approaches may not be sufficient to reduce losses from variations in climate change (Shahid et al., 2021; Zhang et al., 2022a). The severity of abiotic stress is mainly based on the type of species and intensity and duration of stress (Zhang et al., 2022b). Stressful conditions cause variations in plant physiological and biochemical processes, either reversible or irreversible. However, these constraints affect vegetable crops primarily, which are susceptible to abiotic stress (Parveen et al., 2020). Presently, vegetable crop demand is higher; therefore, it is necessary to develop some excellent approaches or tolerant germplasm to tackle the severity of drought and salinity stresses. Drought and salinity stresses are critical global concerns and harm the sustainable production of crops. Irrigation water resources are depleting due to climate change, urbanization, and industrialization (Gruda et al., 2019). Soil salinity is also increasing, mainly due to irrigation with poor-quality and brackish water. The unavailability of quality water in various regions is causing salt accumulation in the soil, which further translocates toward the root zone of vegetable crops. It has been estimated that approximately 20% of global land is negatively affected by salt extremes (Forni et al., 2017).

Vegetables are considered an essential source of the human diet because they are rich in dietary fibers, vitamins, antioxidants, and minerals. Their consumption is also due to good taste, excellent texture, and religious value (Gamalero and Glick, 2022). Global vegetable production in 2020 increased by nearly 66%, from 447 to 1,130 Mt (FAO, 2021). Farmers are investing considerable efforts in improving vegetable production and nutritional aspects under stressful environments (Gruda et al., 2019). The severity of drought and salinity is mainly based on different climatic constraints like the distribution of solar radiation, the need for evapotranspiration, and the retention of soil moisture content (Sabir et al., 2022). Hence, numerous agricultural practices and breeding approaches can be employed for the alleviation of tolerance in vegetable crops against drought and salinity.

Plant researchers urge sustainable management practices to increase vegetable production under drought and salinity stresses (Ahmad et al., 2010; Checker et al., 2018). The exogenous application of phytohormones is a more promising approach to cope with the adverse effects of drought and salinity for sustainable vegetable production. The involvement of phytohormones is attracting much attention from plant researchers due to their multifunctioning behavior against drought and salinity stresses. However, their utilization is still limited in vegetable crops growing under drought and salinity. Therefore, the present study elaborates on the utilization of phytohormones in vegetable crops under drought and salinity stresses. Deep insights into physiological, biochemical, and molecular basis were also explored in the vegetables to cope with the adverse effects of drought and salinity.

Phytohormones are major modulators of plant responses to drought and salinity

Vegetable production is low in different growing areas due to water deficit and salinity. Restricted growth and low yield are due to the unavailability and shortage of water and excessive salt accumulation in the root zone of plants. The higher uptake of Na^+ through roots by xylem vessels resulted in restriction in the uptake of nutrients and minerals necessary for sufficient growth and yield (Maksimovic and Ilin, 2012). Salinity, sodicity, and water stress revealed adverse effects on the growth, yield, and quality of vegetable crops. Higher accumulation of salts disturbed the soil structure, texture, porosity, and permeability of water, which ultimately reduces the productivity of vegetable crops (Malhi et al., 2021). Soil provides better anchor and acts as a reservoir of mineral nutrients necessary for better growth, development, and yield. Therefore, the development of mechanistic approaches is needed to minimize the damaging effects of drought and salinity in vegetable crops. Drought and salinity affect vegetable crops, causing restriction in growth with poor yield (Hossain et al., 2022). Drought stress and excessive Na^+ accumulation are causing a disturbance in the metabolism of vegetable crops. Moreover, the osmotic potential of plants is also adversely affected due to drought and excessive salt accumulation in different plant cells and compartments (Zaidi et al., 2015). Alterations in metabolism and disturbances in osmotic

potential are the leading causes of restricted growth and low yield and sometimes complete or partial death of a plant (Neha et al., 2021).

In addition to supporting signaling pathways, endogenous plant hormones are critical in the response to drought and salinity. Phytohormones play a major role in mediating how plants respond to osmotic adjustment under stress conditions. Small signaling molecules called phytohormones have a significant impact on almost every aspect of the development of plants. The methods of action taken by different hormones for various activities may be very different. Furthermore, it is well recognized that even a single hormone can have an impact on a wide range of cellular and developmental processes or that multiple hormones can regulate a single function concurrently. Phytohormones protect and control plants from biotic and abiotic stresses. As a result, phytohormone application aims to expand crop stress research in the future (Ahmad et al., 2008).

General signs and ion toxicity under drought and salinity stresses

Drought and salinity stress decrease the uptake of Ca^{2+} and K^{+} in vegetable crops, which is the primary reason why nutritional imbalances occur in plants. Plant physiology and morphology are also affected by numerous stresses and thus are susceptible to drought and salinity stresses (Rodrıguez et al., 2005). The initial response of vegetable plants under drought and salinity is the dropping of leaves or the initiation of leaf senescence. After that, a reduction in fresh and dry weights may also be considered an early response of plants growing under water shortage and salinity stress conditions (Zhu, 2002). The decline in fresh and dry weights ultimately reduces the plant yield. Yield reduction is evident in vegetable crops growing under drought and salinity stresses (Alian et al., 2000). However, a reduction in yield can also be a responsive mechanism, especially in aerial plant parts (Sharma et al., 2011) (Figure 1).

Plants have been categorized into two main groups, halophytes and glycophytes. It has been reported that halophytes are more tolerant than glycophytes (Zhang et al., 2017). The potential of halophytes was much imperative, and higher survival and reproduction rates were observed as compared to glycophytes due to improved root architecture, regulation in stomatal conductance, balanced nutrition, improved metabolism, and distinctive genetic makeup (Gao et al., 2018). Halophytes can tolerate approximately 200 mM of NaCl because, at this level, glycophytes cannot survive. Furthermore, the halophyte group constitutes a 1% proportion of global flora, and the individuals of this group were grown naturally (Patane et al., 2013). Leaf growth, especially leaf area, is also considered an initial response in stressful conditions within plant cells and compartments. Numerous other signs include leaf scorching from tip and margins, yellowing and bronzing, leaf dropping of leaves, dieback in twigs, necrosis, blackening, and burning (Bernstein et al., 2004).

Different ion movements continue within plant organelles and compartments under normal conditions. Higher regulation of cytosolic K^{+} and Na^{+} ratio was recorded in the vegetable crops grown under favorable conditions (Zhu and Gong, 2014). Under salinity and drought conditions, ion balances are disturbed, and abnormal movements of ions continue until the availability of favorable conditions. Water-deficit conditions increased the accumulation of salts in the root zone (Sattar et al., 2021). Excessive Na^{+} in the root zone and its translocation to other plant parts are also improved. Na^{+} and K^{+} channels are also present in the xylem vessels. The discrimination of both ions is necessary, although both are similar in power to hydrated ions, and their discrimination is difficult for plants. However, some transporters of ions with high-affinity potassium transporters (HKTs) are more effective for the discrimination and movement of ions through xylem vessels in all plant parts. Furthermore, some proteins, such as integrated membrane proteins, are also involved in the regulation of solute movements within plant cells and compartments (Ahmad et al., 2008). Moreover, these transporters and proteins are specific for ion regulation; for example, some are specific for the discrimination of Na^{+} and others for the discrimination of K^{+}. Hence, it has been reported that regulation of Na^{+} and K^{+} is

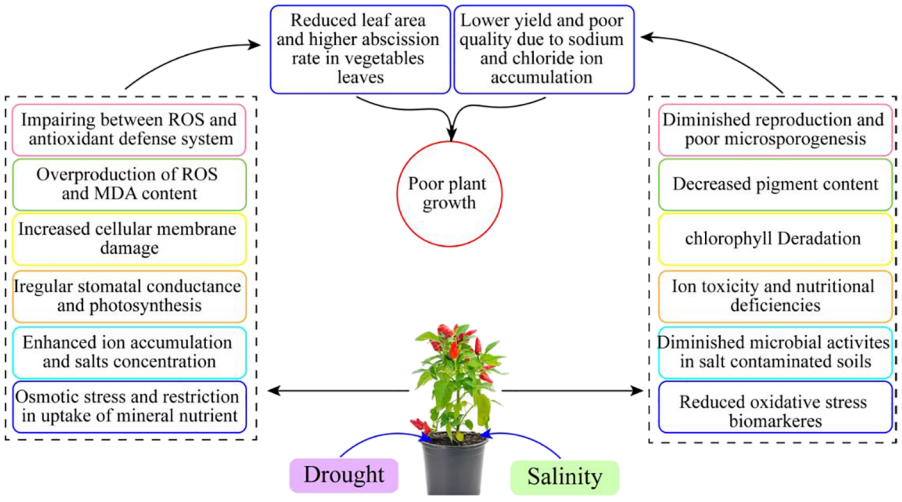

FIGURE 1
Adverse effects of salinity in vegetable crops.

necessary for sufficient plant growth, development, and yield of potatoes (Kamran et al., 2021).

Recently, vegetable crops are facing numerous biotic and abiotic stresses; however, a single abiotic stress is also sufficient for the drastic reduction in crop yield. The water shortage and excessive salt concentrations show a direct effect on the reduction in vegetable crop yield (Lin et al., 2006). Any plant parts, even underground or aerial parts, can be damaged due to low soil moisture levels and excessive salts (Li et al., 2022). Under stressful conditions, vegetable plants and their response to drought and salinity are mainly based on the type of species, cultivars, and even landraces. It has been studied that Cl^- ions are effective for the catabolism of numerous enzymatic and non-enzymatic activities, and these are also known as co-factors for the regulation of the photosynthesis process (Rodríguez-Delfín et al., 2011). The behavior of sensitive and tolerant germplasm of vegetable crops toward Cl^- is more different. The excess of Cl^- is toxic; however, Na^+ is more toxic than Cl^-. Numerous genes are also involved in regulating Cl^- produced in plants. Aquaporin has also been involved in the characterization of numerous genes that contributed to the regulation of Cl^- efflux, which has significant involvement in the sustainable production of crops.

Avoidance mechanism of vegetable crops against stressful conditions

Salt exclusion and excretion restrict the salt's access to the xylem vessels of vegetable crops. The exclusion of salts like Na^+ and Cl^- *via* roots revealed that the storage of Na^+ and Cl^- in leaves is not at a toxic level (Andre et al., 2009). However, their increased concentration disturbed physiological mechanisms, further resulting in leaf drop (Sobhanian et al., 2011). Grafting will be successful in numerous vegetable families and species like Solanaceae and Cucurbitaceae. Rootstock and scion combination contributed to the avoidance of salt mechanism in vegetable crops (Colla et al., 2010). Excessive salts in the root zone further translocated toward other plant parts. However, salt translocation can be reduced and not transported toward leaves. The rootstock's basal portion can absorb the salts (Giordano et al., 2021). Therefore, it has been distinguished that the rootstock and scion combination is most necessary for alleviating salinity in vegetable crops (Figure 2).

Salt exclusion is a variety-specific character in vegetable crops, and the higher exclusion of salts is the capability of a specific variety. However, this mechanism does not reveal the tolerance mechanism in vegetable crops. The avoidance mechanism of drought and salinity is also based on the root architecture of vegetable crops. Zhang et al. (2019) reported that grafting improves plant performance under drought and salinity stress in tomatoes. Similarly, phytohormones could improve the Solanaceae vegetable crop performance.

Phytohormones and gene expression under drought and salinity stresses

Expression of genes related to drought and salts is a more imperative utilization for the development of tolerant germplasm. Different genes and their expression in agronomic crops are widely discussed in the literature; however, in vegetable crops, this molecular phenomenon is still in progress. Most functional markers are related to numerous genes involved in the stress tolerance mechanism of vegetable crops (Ahmad et al., 2008). Gene expression potentially contributes to the development of salt-tolerant germplasm. Characterization of drought- and salt-tolerant and susceptible germplasm is a prerequisite for the sustainable production of vegetable crops (Malhi et al., 2021). Wild germplasm had more significant variation in genetic makeup and novel alleles, which can be explored to develop salt-tolerant germplasm. Numerous resistant genes can be identified, and further genome editing and transformation can be helpful for the development of tolerant germplasm in vegetable crops (Malhi et al., 2021). However, the expression of genes can be regulated by exogenous and endogenous improvements of phytohormones for the increase of tolerance against stress drought and salinity. Phytohormones are involved in the upregulation of transcriptomics of ATPase. Moreover, they were

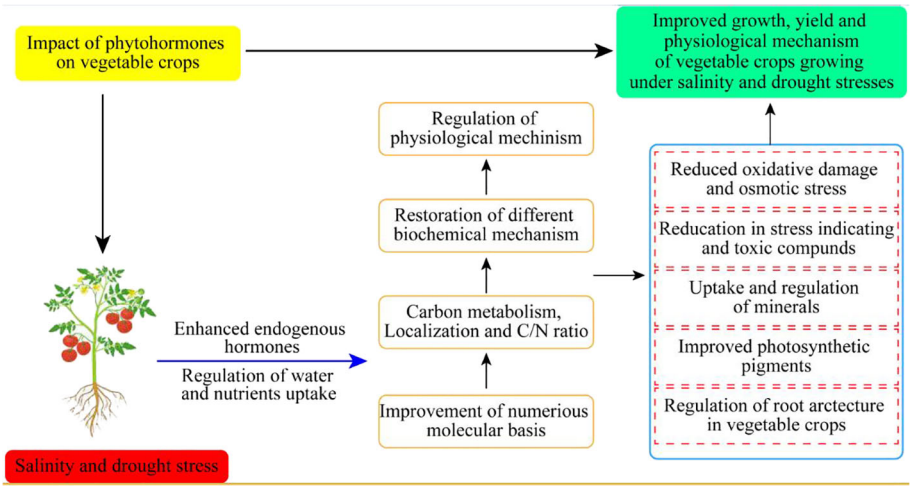

FIGURE 2
Critical stages of irrigation water at different growth stages of vegetable crops.

also involved in the reduction of the expression level of *PpATG* for the regulation of numerous morpho-physiological and biochemical activities in cucumbers. Moreover, similar findings were also reported by Parveen et al. (2021) for the expression of genes related to the tolerance mechanism.

Management approaches for mitigation of drought and salinity in vegetables

Different management approaches comprised of proteomics, marker-assisted selection, genome characterization, genome editing, genome mapping, quantitative trait locus (QTL) mapping, genomic editing, and genomic transformation are promising molecular bases for salinity and drought tolerance in vegetable crops (Saidi and Hajibarat, 2020). Furthermore, the molecular bases can be utilized for the backcrossing of genes present in wild species toward offspring or landraces. The first genome map was developed in the 1980s on potatoes in relation to sexual recombination regularities. Plant breeders have successfully characterized disease-tolerant genes in potatoes (Byun et al., 2007). Moreover, numerous economic traits were detected in potatoes. Furthermore, "NL25" is one of the functional markers with excellent capability to identify candidate genes related to tolerance characteristics of potato warts (Saidi and Hajibarat, 2020).

Phytohormones and vegetable crops under drought and salinity stresses

Phytohormones have the potential to enhance vegetables' growth and development by interacting with numerous processes responsive to stressful conditions (Groppa and Benavides, 2008). Phytohormones have the capability to improve the defense system of vegetable crops growing under drought and salinity stresses. Plants activate their defense system against adverse climatic conditions for their survival (Choudhary et al., 2012). Therefore, supplementation of phytohormones boosts the immune system of plants growing under drought and salinity stresses. Plant defense system comprises the activation of enzymatic compounds (i.e., superoxide dismutase (SOD), peroxidase (POD), and catalases (CATs), non-enzymatic activities (i.e., ascorbic acid (AsA), phenolic content, and different sugars), osmolytes (i.e., glycine betaine (GB), ascorbate peroxidase (APX), and proline), and oxidative stress-indicating activities (ROS, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) (Table 1). Therefore, the impact of phytohormones on vegetable crops is imperative and needs more investigation on the molecular level to enhance plant tolerance (Diao et al., 2015).

Brassinosteroids

These are more emerging, eco-friendly, and multifunctional plant hormones involved in regulating physiological mechanisms occurring within the plants (Mumtaz et al., 2022). Plant researchers and physiologists are working on utilizing these plant hormones for sustainable crop production. Alhaithloul et al. (2020) reported that Brassinosteroids (BRs) are more effective for plants growing under drought and salinity stress environments. It has been studied that BRs enhanced seed germination, root growth, seedling development, cell expansion and differentiation, ripening of fruits, leaf senescence, and reproduction of floral parts of vegetable crops (Bhandari and Nailwal, 2020). Moreover, in the findings of Kaya (2021), it has been discovered that BRs can improve growth traits, mineral content, antioxidant activities, and osmolytes and protect from membrane injury. Similarly, Shahid et al. (2011) evaluated that BRs elevated pea productivity against drought and salinity. Thus, it has been confirmed that BRs effectively elevate salinity tolerance in vegetable crops. BRs are effective for amelioration of drought and salinity tolerance in numerous vegetable crops like tomatoes (Jangid and Dwivedi, 2017), cucumber (Jakubowska and Janicka, 2017), and radish (Ramakrishna and Rao, 2015). Furthermore, elevated enzymatic activities like SOD,

TABLE 1 Role of different antioxidant activities in drought and salt tolerance mechanism of vegetable crops.

Bioactive molecules	Key findings	References
ROS, MDA, and H_2O_2	The activation of these activities is more toxic for plants. Chances of membrane injury increased under stress.	Sobhanian et al. (2011)
Electrolyte leakage	Membrane injury increased due to stress conditions because membrane damage enhanced due to the production of lipid peroxidation.	Zhang et al. (2013)
SOD, POD, and CAT	Toxic ROS, MDA, and H_2O_2 scavenging are made by CAT activity naturally. These are scavengers of toxic compounds, and their activation also improved the defense system of plants growing under stressful environments. These are helpful to disturb the O_2 to form H_2O_2 and remove the harmfulness of superoxide anion.	Zhang et al. (2013)
APX and glutathione	Ascorbate activity enhanced the plants' tolerance mechanism. These are effective to decrease the H_2O_2 production in vegetables against osmotic stress and oxidative injury. H_2O_2 and its derivatives are rapidly decreased by glutathione. These have better scavenging capability under stress conditions.	Wu et al. (2018)
Proline and GB	These osmolytes are considered signaling molecules against stress conditions. Proline and GB are known as antioxidant profiling that improves drought and salt tolerance in vegetables. Proline may act as a signaling molecule in order to maintain osmotic regulation. Oxidative injury is regulated by the production of proline and GB.	Zhang et al. (2013)

ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; APX, ascorbate peroxidase; GB, glycine betaine.

POD, CAT, and improved metabolites were recorded with exogenous application of these phytohormones. Moreover, improved physiological systems and reduction in oxidative injury were also observed in numerous vegetables, i.e., tomatoes (Jordan et al., 2020), peppers, and cucumbers (Per et al., 2017; Fahad et al., 2019), by application of BRs. Abiotic stress tolerance can be mitigated in the radish by supplemental use of BRs. Reduction in the over-generation of ROS, MDA, H_2O_2, and electrolyte leakage indicated that BRs are stress-relieving compounds for radish plants growing under stressful conditions as studied by Ramakrishna and Rao (2015). Furthermore, the increase in plant defense indicated that BRS is effective for the improvement of the plant immune system against harsh environments. In another study by Jakubowska and Janicka (2017), it has been indicated that BRs are much more effective for abiotic stress tolerance, as a similar tolerance mechanism was reported in the cucumbers. The exogenous spray of 24-EBRs on cucumbers improved the gaseous exchange processes and all its related traits, chlorophyll fluorescence, starch, soluble sugars, and rubisco activities. Therefore, it is much more effective for higher-yielding vegetable crops growing under normal and abiotic stress conditions. Similarly, in the other research by Choudhary et al. (2012), it has been studied that free radicle-scavenging potential in radishes was improved with enhanced antioxidant potential along with improvements in morphological traits of roots under heavy metal (copper) excess. From previous literature, it has been indicated that BRs are the more effective, eco-friendly, naturally occurring substances that might be extensively utilized for the reduction of drought and salinity stresses in vegetables.

Jasmonates

This group is comprised of methyl jasmonate (MeJA) and jasmonic acid (JA), which have been explored for their impacts on vegetable crops (Dar et al., 2015). Deprivation of photosynthetic pigments and tuber formation can be regulated under the exogenous application of JAs, as studied by Viswanath et al. (2020). The exogenous spray of this plant hormone improved sugar beet growth and defense system under drought (Ghaffari et al., 2019). Importantly, the exogenous JA application improved the endogenous production of JAs, and consequently, it can be used for hormonal regulation (Shahzad et al., 2015). MJ improved the drought resistance in cauliflower by improving oxidative bioactive compounds (Wu et al., 2012). Therefore, it has been exhibited that vegetable production can be increased with JA supplementation. JAs strengthen the defense system against environmental stresses in horticultural crops (Dar et al., 2015). These are significant for horticultural crops growing in areas with drought (Ge et al., 2010) and salinity (Pedranzani et al., 2003). Environmental threats can be regulated by the application of JAs. Similarly, Zou et al. (2017) revealed that the defense mechanism of plants was improved under environmental stresses like waterlogged conditions in peppers. JAs have good potential as a regulatory mechanism of vegetable crops against drought and salinity stresses. Abouelsaad and Renault (2018) reported that ROS mediation can be improved with JA because ROS is an indication of stress occurrence in tomato plants. Manan et al. (2016) reported that MeJA had the good capability to enhance the yield-related traits of tomato cultivars growing under elevated salinity as evaluated by Manan et al. (2016). The exogenous spray of MeJA on peas growing under stressful situations results in the improvement of indigenous hormonal levels of JA (Shahzad et al., 2015). Cauliflower grows under water-deficit conditions, facing challenges in growth at the seedling stage and poor yield at the reproductive stage. Wu et al. (2012) examined whether MeJA potentially triggered both oxidative and non-oxidative activities. Absorption and uptake of heavy metals were decreased in eggplant through the exogenous application of MeJA as supplementation (Yan et al., 2015). Seed priming is also an effective way to reduce challenges due to stress conditions. Therefore, it has been recorded that JA contributed to the increase in the germination of okra seeds, increase of seedlings, improved level of osmoprotectants, defense activities, photopigments, ROS reduction, lessening of H_2O_2, and low MDA level against salinity as studied by Iqbal et al. (2022). Exogenous application of MeJA on peppers improved osmolyte generation, oxidative and non-oxidative bioactive molecules, and metabolism and also improved the uptake of minerals *via* roots. Furthermore, decreases in MDA, H_2O_2, electrolyte leakage, and ROS were also reported by supplemental application of MeJA in the peppers. Therefore, it has been considered that JAs are suitable phytohormones for the mitigation of adverse effects of salinity and water-deficit conditions in horticultural crops.

Salicylic acid

This is a phenolic-based hormone that contributes to the elevated growth and yield of vegetable crops grown under drought and salinity environments, mainly by improving the plant defense system (Khan et al., 2015). Similarly, in another vegetable crop (pea), different concentrations of salicylic acid were applied exogenously (nearly 1–4 mM) under salinity conditions (50, 100, and 150 mM of NaCl) (Saidi and Hajibarat, 2020). In this study, it has been noted that salicylic acid improved pea growth, yield, enzymatic and non-enzymatic activities, and osmolytes. Salicylic acid (SA) (300 ppm) improved the mineral content in garlic and decreased Na^+ uptake and translocation to other plant parts. Therefore, it has been considered that SA is helpful for vegetable crops growing under drought and salinity stresses (Shama et al., 2016). Similarly, in another study, nearly 0.11 mM of SA improved the tolerance of potatoes against abiotic stress (chilling). Priming seeds with salicylic acid at 100 mg/L is an effective strategy for the mitigation of adverse effects of salinity in cucumber (Rehman et al., 2011). The use of salicylic acid is an effective strategy for tolerance of abiotic stresses in vegetable crops, i.e., potatoes (Li et al., 2019), bell pepper (Zhang et al., 2020), spinach (Gilani et al., 2020), and peppermint (Ahmad et al., 2018). Spraying 1 mM of SA on tomatoes growing under heat stress resulted in an improved process of gaseous exchange, good water use potential, enzymatic activity generation, non-oxidative activation, and reduced oxidative stress conditions as studied by Zulfiqar et al. (2021). Moreover, biomass reduction on a fresh or dry basis was decreased, ultimately reducing the yield because of salinity and drought stresses. Furthermore,

disruption in photopigments, photosynthesis disturbances, and irregularities in the functions of stomata are causes of osmotic stress. Therefore, it has been explored that regularities in the process of photosynthesis and stomatal function are important by application of different levels of SA. Similarly, in other findings, nearly 0.1 mM of SA enhanced the fresh and dried biomass, regulated photosynthesis, generation of oxidative and non-oxidative compounds, regulation in electrolyte leakage, protection from membrane injury, efficient water use potential, and excellent anatomical responses (Galviz et al., 2021). Moreover, it has been recorded that SA had the capability to mitigate challenges that occur from drought and salinity stresses by reduction of oxidative and osmotic injuries (Kaya, 2021).

Polyamines

The polyamine (PA) group from phytohormones primarily comprised spermidine, putrescine, and spermine having a lower molecular weight (Ahmad et al., 2012). Several physiological and biochemical processes were administered through polyamines by improving root, leaf differentiation, pollen viability, flower development, fruit growth, gene transcription, morphogenesis, embryo-genesis, leaf senesce, organogenesis, embryogenesis, and fruit maturation of the respective vegetable crop (Chen et al., 2019). Multiple abiotic stresses can be regulated by the application of varying concentrations of polyamines in horticultural crops, especially vegetable crops. Abiotic stresses can be regulated by the alteration of numerous processes of plants with a spray of polyamines available in the markets globally, as reported by Kamran et al. (2019). Moreover, the exogenous application of spermidine revealed good outcomes for tomato seedlings grown under stressful conditions. Moreover, the application of spermidine also enhanced the concentration of polyamine compounds within cells and compartments, especially in the root zone of tomato seedlings. The higher concentration of spermidine can be effective for tomato plants growing under saline conditions. The differentiation of ions and their translocation to other plant parts can be improved by supplementing polyamines (Hu et al., 2012). Exogenous application of spermidine is found to be effective for the improvement of plant growth, chlorophyll content, proline level, and different sugars, as reported by Zapata et al. (2004). Furthermore, it has also been reported that the reduction in ROS, MDA, and H_2O_2 was also measured in tomato plants. Pepper seeds were treated with different polyamines (spermine, putrescine, and spermidine), and it has been studied that the improved rate of germination, higher germination index, and early germination were recorded in treated seeds as compared to non-treated seeds. Similarly, in another study by Wu et al. (2018), it was revealed that the application of polyamines in cucumber seedlings improved crop performance under stressful conditions. Ormrod and Beckerson (1986) reported that polyamines are stress-relieving molecules as in the tomato for higher yield. The reduction and balance in the generation of oxidative stress markers, i.e., ROS, H_2O_2, MDA, free radicles, and movement of electrons, indicate the reduced stress in plants. Therefore, it has been studied that PA is an appropriate hormone for the improvement of endogenous hormones and also improved the activation of scavengers of toxic compounds.

Ascorbic acid

This contributed to the regulation of biosynthesis of ascorbates within the plant body. It is involved in the detoxification and compartmentation of H_2O_2 and MDA activities. Ascorbic acid is an important phytohormone necessary for sustainable vegetable production globally, grown under drought and salt stress conditions. The increased concentration of ascorbic acid on lettuce revealed that ascorbic acid is also effective for increasing the fresh and dry weights of lettuce and the number of leaves, which are considered yield-contributing factors against salinity. Seed germination is disturbed due to stressful conditions. Therefore, the exogenous application of AsA significantly enhanced seed germination with the endogenous improvement of ascorbates, which further activates the scavengers of toxic bioactive molecules within the plant cells. Hence, the initiation of seed germination is regulated with supplemental AsA as studied by Akram et al. (2017). Moreover, its application had a good role in the balance and neutralization of free radicals and toxic ROS generated within the plant cells. The exogenous application of ascorbic acid effectively improves the endogenous ascorbic acid content.

Abscisic acid

Its production is enhanced due to low moisture availability in the root zone of plants (Seiler et al., 2011; Lim et al., 2015). The enhanced production of abscisic acid (ABA) adversely affected plant growth and yield by producing nutritional imbalances (Sreenivasulu et al., 2012). The optimum production of ABA regulates the osmotic stress conditions (Ali et al., 2021a; Ali et al., 2021b). Induced levels of Na^+ and ABA are the main causes of nutrient uptake restriction and nutrient translocation from roots toward leaves for food synthesis (Soma et al., 2021). Vegetables faced a reduction in stem and leaf, cell membrane injury, chlorophyll instability, lipid peroxidation, low water potential, degradation of photosynthetic pigments, poor gas exchange, higher Na^+, Cl^-, ABA, reduced K^+, turgidity in leaf, osmolyte generation, and ROS scavenger production under stress (Malhi et al., 2021).

Melatonin

Melatonin (MLE) is a stress-reducing molecule by exogenous supplementation. It is involved in the improvement of seed germination, the proliferation of roots, better flowering, fruit set and enlargement, fruit ripening, shelf life, and quality as studied by Wang et al. (2021). Drought and salinity stresses can be mitigated by the supplemental spray of MLE on many vegetable crops because of MLE's multifaceted functions (Wang et al., 2013; Qi et al., 2018; Altaf et al., 2022b). MLE has the good capability to scavenge toxic ROS, MDA, H_2O_2, and electrolyte leakage as studied by Wang et al. (2012). The enhanced level of endogenous MLE has the capability to mitigate challenges that occur from drought and salinity in agricultural crops as reported by Nawaz et al. (2018). Restricted translocation of minerals (macronutrients and micronutrients) is upregulated by supplemental application of MLE. Moreover, the uptake and absorption of minerals by roots are regulated due to the application of appropriate melatonin

levels. Oxidative and osmotic stresses are relieved by MLE due to the regulation of endogenous hormones and activation of scavengers of oxidative stress markers (Neha et al., 2021). The morphology of roots is improved regarding uptake, absorption, and further translocation toward other plant parts by supplemental MLE. Drought and salinity are involved in the disruption of plant metabolism. Therefore, disturbance in the plant metabolism is an indication of a stress situation. The activation of oxidative stress markers like ROS, H_2O_2, and MDA is reduced, and their scavengers (enzymatic, non-enzymatic, and osmolytes) are activated. Therefore, the availability of nutrients to plants is imperative especially when growing under stressful conditions. Hence, MLE can be a suitable method for the alleviation of drought and salinity in vegetable crops.

Hormonal regulations are necessary to enhance vegetable crop tolerance against drought and salinity stress conditions (Figure 3). Different concentrations of antioxidant sprays improved crop performance by modulation of physiological and biochemical mechanisms in sweet potatoes (Lin et al., 2006) (Table 2).

Conclusion and prospects

In the present study, it has been explored that modulation of physiological and biochemical mechanisms is necessary for sustainable production of vegetable crops growing under drought and salinity stresses. Exogenous application of phytohormones is necessary for the improvement of vegetable growth, yield, photosynthetic pigments, minerals nutrient content, and defense-related characteristics. It has been concluded that phytohormones are necessary for the sustainable production of vegetable crops.

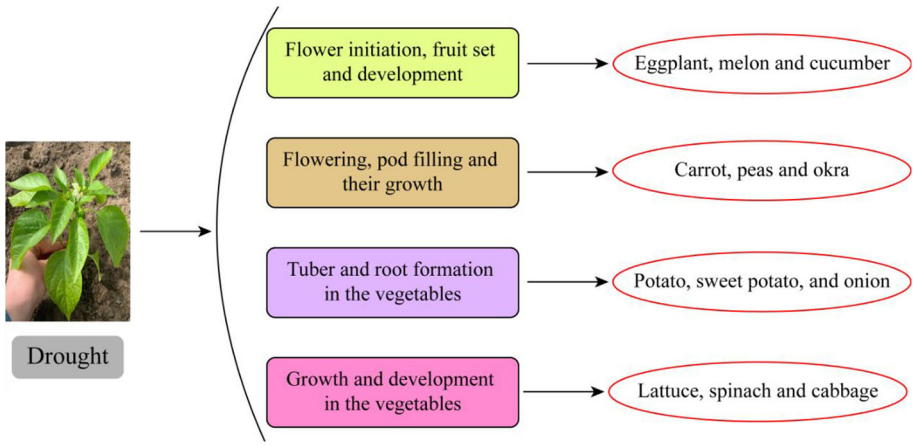

FIGURE 3
Impact of phytohormones on different vegetable crops growing under drought and salinity stresses.

TABLE 2 The role of exogenous phytohormones against drought and salinity stresses in vegetable production.

Stress type	Phytohormones	Vegetable crop	Key findings	References
Salinity	Salicylic acid	Pea	0.4 mM of salicylic acid enhances growth by improving the defense system	Jangid and Dwivedi (2016)
Salinity	Ascorbic acid	Lettuce	0, 100, 200, 300, and 400 mg/L improved lettuce performance with improved yield	Jangid and Dwivedi (2016)
Drought	Osmoprotectants	Tomato	50%–57% of field capacity also increased the level of salts in plants. Germination was very poor in seeds	Jangid and Dwivedi (2016)
Drought	Salicylic acid	Tomato	10–5 M improved seedling growth under 10 days of water-holding capacity	Hayat et al. (2008)
Drought	Melatonin	Cucumber	100 µM significantly improved growth, yield, and defense mechanism	Zhang et al. (2013)
Salinity	Polyamines (spermidine)	Cucumber	Adverse effects due to 50 mM of NaCl can be regulated by the application of polyamines such as spermidine	Duan et al. (2008)
Drought	Methyl jasmonate	Sweet potatoes	13 µM/L of jasmonates improved growth, yield, and quality characters	Yoshida et al. (2020)
Drought	Jasmonic acid	Potato	Overexpression of StJAZ1 resulted in decreased relative leaf water potential in the plants MDA and lipid peroxidation were enhanced. Jasmonic acid is effective to reduce the production of MDA and lipid peroxidation	Jing et al. (2022)
Drought	Mannitol and methyl jasmonate	Pepper	Regulates signaling and antioxidant defense potential in plants	Ma et al. (2021)

MDA, malondialdehyde.

* Climate change, urbanization, and industrial zones are depleting and polluting water resources. Water shortage is going to worsen. To feed a huge population, it is necessary to develop management approaches to obtain higher vegetable production with limited water resources.
* Elevated drought and salinity conditions severely affect the productivity of vegetable crops. In this situation, phytohormones are considered a supportive strategy for the sustainable production of vegetable crops in the current scenario.
* To achieve zero hunger, it is necessary to elevate drought and salinity tolerance in vegetables. Moreover, the development of tolerant landraces is also a present need.
* Exploration of molecular basis, i.e., genome characterization, QTL mapping, marker-assisted selection (MAS), genome editing, genetic transformation, and genome sequencing are also imperative for the development of tolerant germplasm of vegetable crops.

Author contributions

JC: conceptualization, literature survey, writing major original draft, and review structure. XP: literature survey, writing—review and editing, and figure designing. 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 application of salicylic acid improves freezing stress tolerance in alfalfa

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Freezing stress is one of the most detrimental environmental factors that can seriously impact the growth, development, and distribution of alfalfa (*Medicago sativa* L.). Exogenous salicylic acid (SA) has been revealed as a cost-effective method of improving defense against freezing stress due to its predominant role in biotic and abiotic stress resistance. However, how the molecular mechanisms of SA improve freezing stress resistance in alfalfa is still unclear. Therefore, in this study, we used leaf samples of alfalfa seedlings pretreatment with 200 μM and 0 μM SA, which were exposed to freezing stress (-10°C) for 0, 0.5, 1, and 2h and allowed to recover at normal temperature in a growth chamber for 2 days, after which we detect the changes in the phenotypical, physiological, hormone content, and performed a transcriptome analysis to explain SA influence alfalfa in freezing stress. The results demonstrated that exogenous SA could improve the accumulation of free SA in alfalfa leaves primarily through the phenylalanine ammonia-lyase pathway. Moreover, the results of transcriptome analysis revealed that the mitogen-activated protein kinase (MAPK) signaling pathway-plant play a critical role in SA alleviating freezing stress. In addition, the weighted gene co-expression network analysis (WGCNA) found that *MPK3*, *MPK9*, *WRKY22* (downstream target gene of *MPK3*), and TGACG-binding factor 1 (*TGA1)* are candidate hub genes involved in freezing stress defense, all of which are involved in the SA signaling pathway. Therefore, we conclude that SA could possibly induce *MPK3* to regulate *WRKY22* to participate in freezing stress to induced gene expression related to SA signaling pathway (NPR1-dependent pathway and NPR1-independent pathway), including the genes of non-expresser of pathogenesis-related gene 1 (*NPR1)*, *TGA1*, pathogenesis-related 1 *(PR1)*, superoxide dismutase (*SOD)*, peroxidase *(POD)*, ascorbate peroxidase (*APX)*, glutathione-S-transferase *(GST)*, and heat shock protein *(HSP)*. This enhanced the production of antioxidant enzymes such as SOD, POD, and APX, which increases the freezing stress tolerance of alfalfa plants.

KEYWORDS

candidate genes, freezing stress, *Medicago sative*, salicylic acid, signal transduction

1 Introduction

Alfalfa (*Medicago sative* L.) is one of the most important forage crops cultivated worldwide (Chen et al., 2021c), whereas the extreme environmental conditions can seriously impact alfalfa growth and development, especially sudden freezing stress, not only reducing biomass productivity but also restricting the geographical distribution of alfalfa (Chen et al., 2015; Adhikari et al., 2021; Song et al., 2021). Therefore, it is important to study the mechanisms regulating freezing stress responses in alfalfa.

Freezing stress can cause negative changes at physiological, biochemical, and molecular levels, and preliminarily leads to inhibition of photosynthetic activity, ion permeability, nutrition uptake (Huang and Cheung, 2021), increased oxidant stress due to Reactive oxygen species (ROS) accumulation and induced redox homeostasis and other changes (Cui et al., 2019). At low temperatures, the formation of ice crystals in plants directly result in cellular dehydration and membrane damage (Knight and Knight, 2012). Plants have evolved numerous regulatory mechanisms to cope with the damage caused by freezing stress (Shu et al., 2017). To scavenge ROS, plants trigger their antioxidant system to synthesize antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) (Wang et al., 2009a; Dong et al., 2014; Zhuo et al., 2018; Zaid and Wani, 2019). To survive, plants have evolved precise thermos sensory system to trigger signal transductions and provoke numerous freezing-related genes to improve freezing tolerance (Ding et al., 2022). Signal transductions include the calcium signal transduction and the mitogen-activated protein kinase (MAPK) signal module. Örvar et al. (2000) found that low temperatures stimulated calcium signal transduction in alfalfa. MAPK cascades have been reported to play an important role in regulating downstream biotic and abiotic stress-related genes (Chen et al., 2021a), including MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MPKK), and MAPK.

In addition, freezing stress can increase endogenous phytohormones, including abscisic acid (ABA), polyamines (PAs), and SA, which enhances freezing tolerance (Zhang et al., 2011). SA is a natural phenolic phytohormone and is a vital hormone that participates in numerous plant physiological processes, including seed germination, vegetative growth, nodulation in legumes, and stomatal closure. Furthermore, SA plays a critical role in the resistance to biotic and abiotic stress, such as freezing stress (Ding et al., 2016; Khokon et al., 2017; Wassie et al., 2020). In plants, there are two distinct pathways to generate SA: one is the isochorismate synthase (ICS) pathway, which is in the chloroplast. Isochorismate is synthesized from chorismic acid and transforms to SA *via* isochorismate pyruvate layse (Dong et al., 2014). Previous reports have found that the *ICS1/ICS2*, Enhanced disease susceptibility *(EDS)5*, and GH3.12/avrPphB susceptible 3 (*PBS3*) genes play a critical role in the ICS pathway of SA biosynthesis, and that 90% of SA was synthesized through the ICS pathway during pathogen attacks (Ding and Ding, 2020; Mishra and Baek, 2021). The second pathway is phenylalanine ammonia-lyase (PAL). In the cytoplasm, the phenylalanine is deaminated by PAL and converted to trans-cinnamic acid, while the other bifurcate is oxidized to trans-cinnamic acid and converted to benzoic acid (BA). BA is hydroxylated by BA 2-hydroxylase (BA2H) to synthesize SA (Dong et al., 2014). Interestingly, *PAL* genes in *Arabidopsis* play a vital role in the PAL pathway to against environmental stress (Huang et al., 2010). Although multiple pieces of evidence demonstrated that those two pathways were activated under abiotic stress, it is unclear how they have been activated and regulated by the related genes. With increasing researches have assessed the mechanism of SA defense against biotic and abiotic stress, the SA mechanism in plant pathogen resistance is well known. In *Arabidopsis*, SA binds to receptor non-expresser of pathogenesis-related gene 1 (NPR1) to regulate pathogenesis-related (PR) genes and other SA-induced genes to resist disease (Wang et al., 2020a; Chen et al., 2021b). Since NPR1 has no DNA binding domain, Zhou et al. (2000) demonstrated that NPR1 can activate PR-1 genes through TGACG-binding motif (TGA) 2 and TGA3 binding to the activating sequence-1 (as-1) element. Fan and Dong (2002) demonstrated that overexpressed TGA2.2 decreased SA- and PR-related genes in tobacco, while TGA2 is an SA-responsive and NPR1-dependent activator in tobacco. Thus, TGA interacts with NPR1 differently in different species.

Some WRKY genes binding to the W-box motif (TTGACC/T) participated in regulating the NPR1-dependent PR genes to resist pathogen attacks, except for TGAs (Chen et al., 2021b). The *WRKY18*, *WRKY40*, and *WRKY60* triple mutant increased the expression of PR-1 genes to resist bacterial pathogen attacks (Xu et al., 2006), *WRKY53*, *WRKY54*, and *WRKY70* were expressed in active downstream-related genes against pathogen infections (Wang et al., 2006; Zhou et al., 2018). WRKY could be phosphorylated and inactivated by a MAPK cascade during a pathogen attack, which would depend on MAPK and control the activity and subcellular location of *WRKY22* and *WRKY29* (Asai et al., 2002). Activating the expression of *AtMPK4* could reduce the accumulation of SA to against *Pseudomonas syringae* pv tomato strain DC3000 (Pst DC3000) (Liang et al., 2013), while the MEKK1-MKK1/2-MPK4 cascade negatively regulated genes based on MEKK1 or MPK4, which decreased SA and PR genes (Rodriguez et al., 2010). Automatically, MAPK cascades are not only mediated by freezing stress but also by SA signaling. In addition to the NPR1-dependent pathway, SA also induced the expression of glutathione peroxidase (*GPX*, Li et al., 2013), glutathione-S-transferase (*GST*, Blanco et al., 2005), heat shock proteins (HSPs, Jumali et al., 2011), *POD*, *SOD*, and *APX* (Per et al., 2017) as NPR1-independent pathway-related genes to enhance antioxidant and non-antioxidant activities against stresses. Intriguingly, Olate et al. (2018) reported that the signaling of cold and pathogen response was crosstalk, including the SA signal pathway. Automatically, PRs as important genes in SA signal pathway also play an important role in freezing stress. However, it remains unknown how SA application improves freezing stress resistance (Yeh et al., 2000; Griffith and Yaish, 2004). Recently, numerous researches have

reported that exogenous SA could improve the freezing tolerance through physiological changes in many plants (Saleem et al., 2021b), including alfalfa (Miura and Tada, 2014). And Wang et al. (2022) have found that SA could play a vital role in the freezing stress of alfalfa leaves, while the mechanism of SA in freezing stress requires further investigation.

In our study, we tested the effect of SA on the freezing resistance of alfalfa and performed a transcriptome analysis to explore the molecular mechanism of SA under freezing stress. Our data reveal that pretreatment of SA could protect alfalfa from freezing stress and proposed a working model on how SA might regulate freezing stress to tolerance in alfalfa. This study contributes to a better understanding of the molecular mechanisms of SA-induced alfalfa freezing tolerance, which provides a cost-effective way of reducing freezing stress damage in alfalfa.

2 Material and methods

2.1 Plant materials and treatments

Seeds of the alfalfa cultivar 'WL326GZ' (fall dormancy score 3.8) were obtained from Zhengdao Ecological Technology Co. (Beijing, China). The healthy seeds were sown on arenaceous quartz in a growth room at 20°C with 60%-80% humidity under a 14h light cycle with 200 μmol m^{-2} s^{-1} photosynthetic photons flux density and 10h dark at 18°C regimens. The seedlings were grown hydroponically in half-strength Hoagland solution, which was applied daily after the seeds germinated.

In this study, 200μM SA was used based on preliminary experiments that showed the most effective concentration to improve freezing stress. For SA application and abrupt freezing stress, three-week-old alfalfa seedlings were divided into two groups with WCK (control) and T (200μM SA treatment). The T group was watered with 20ml of SA solution with Ph 6-8 for five days before freezing stress while the WCK group was watered with 20ml pure water. Subsequently, the seedlings were transferred to a growth chamber at -10°C for 0h, 0.5h, 1h, and 2h for freezing stress. Finally, they were allowed to recover in a growth chamber with normal temperature (20°C/day, 18°C/night) for 2 days. A total of 30 leaf samples were harvested for use in this study. Detailed sample names are as follows: WCK01-03, WCK11-13, WCK21-23, WCK31-33, and WCK41-43 represent three samples of 'WL326GZ' exposed to -10°C at 0, 0.5, 1, 2 h, and recover for 2 days, respectively; T01-03, T11-13, T21-23, T31-33, and T41-43 represent three samples of SA treated 'WL326GZ' exposed to -10°C at 0, 0.5, 1, 2h, and recover for 2 days, respectively.

2.2 Enzyme extraction and assays

To investigate the effects of SA on antioxidant activities, we measured SOD, POD, and APX using the method described by Giannopolitis and Ries (1977) and Chance and Maehly (1955). First, we used fresh leaves of approximately 0.1g that were homogenized into a 2 ml tube with potassium phosphate buffer (50 mM, pH 7.0), and obtained the upper supernatant to measure the antioxidant activities *via* centrifugation for 20 min at 12000 r min^{-1} and 4°C. We then used the upper supernatant to measure the antioxidant activities using the method outlined by Wassie et al. (2020) with minor modifications.

2.3 Hormone assay content

To analyze changes in hormones in fresh leaves, we used high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) to measure the SA contents as described by Pan et al. (2010). Briefly, only 50 mg of each fresh sample was ground into a powder with liquid nitrogen and SA was twice extracted with acetonitrile to purify on the Poroshell 120 SB-C18 column (2.1×150,2.7 um). Finally, we measured the contents of SA by HPLC-MS/MS (Agilent 1290, SCIEX-6500Qtrap).

2.4 RNA extraction and Illumina sequencing

Total RNA was isolated from the leaf samples with a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. And thirty libraries were contrasted and sequenced using Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China). To obtain high-quality clean reads, we used raw reads to remove reads containing adapters, more than 10% of unknown nucleotides, and more than 50% of low-quality (Q-value ≤ 20) bases through fastp (version 0.18.0) (Chen et al., 2018). Furthermore, we removed rRNA mapped reads, leaving only final clean reads for further assembly and calculation using the short reads alignment tool Bowtie 2 (version 2.2.8) (Langmead and Salzberg, 2012). Finally, paired-end clean reads were mapped to the alfalfa reference genome (https://figshare.com/projects/whole_genome_sequencing_and_assembly_of_Medicago_sativa/66380) using HISAT2. 2.4 with "-rna-strandness RF" and other parameters set as a default for further assembly (Kim et al., 2015).

2.5 Differential expression genes identification

For the quantity of each transcript's expression and variations, we used FPKM (fragment per kilobase of transcript per million mapped reads) values and the RSEM software for calculations. Furthermore, the differential expression analysis was performed by DESeq2 between two different groups (WCK01-03 vs. WCK11-13, WCK01-03 vs. WCK21-23, WCK01-03 vs. WCK31-33, WCK01-03 vs. WCK41-43, T01-03 vs. T11-13, T01-03 vs. T21-23, T01-03 vs. T31-33, T01-03 vs. T41-43, WCK01-13 vs. T01-03, WCK11-13 vs. T11-13. WCK21-23 vs. T21-23 and WCK41-43 vs. T41-43). The

transcripts with false discovery rate (FDR) < 0.05 and |log2 (fold change) | ≥1 were used as DEGs.

2.6 Gene function annotation

To analyze DEG functions, we used all DEGs mapped to Gene Ontology (GO) terms in the Gene Ontology database (http://www.geneontology.org/) to classify the genes to the three ontologies and significantly enriched (FDR ≤ 0.05) GO terms in DEGs, which were compared to the genome background and defined by a hypergeometric test. Furthermore, as the Kyoto Encyclopedia of Genes and Genomes (KEGG) is the major public pathway-related database outlining the genes' biological function, we overlapped the DEGs with a pathway enrichment analysis and pathways with FDR ≤ 0.05 were defined as significantly enriched reactions in DEGs.

2.7 Weighted gene co-expression network analysis analysis, gene network co-expression, and visualization

WGCNA is a method of clustering modules of highly correlated genes among multiple genes. Co-expression networks were constructed using the WGCNA (v1.47) package in R, and 31,310 genes were used to analyze unsigned by WGCNA. However, the other parameter of WGCNA as power is 12, merge cut height is 0.75 and the minor module size is 50.

2.8 qRT-PCR analysis for candidate gene validation

To verify the efficacy of RNA-seq, we verified 6 genes by Quantitative Real-time PCR (qRT-PCR): MS.gene21548, MS.gene02035, MS.gene004340, MS.gene035685, MS.gene05280, and MS.gene017127. The cDNA was obtained from reverse transcription with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., CA, USA) and the primers were designed by Primer 5.0, which is listed in Table S1. The qRT-PCR reaction was performed through Universal SYBR Green Master supermix (Roche, Shanghai, China), and then performed on the Applied Biosystems 7500/7500 fast Real-time PCR (BIORAD). There were three technical replicates of each sample. Finally, we used the $2^{-\Delta\Delta Ct}$ method to analyze the expression of all genes based using the UBL-2a as the reference gene (Castonguay et al., 2015).

2.9 Statistical analysis

The all data in our study were assessed by one-way analysis of variance (ANOVA) with SPSS 20.0 and performed in Excel 2019 software. The values represent the mean ± STEDV (n=3). Different letters indicate represent values that were significantly different between treatments at $p<0.05$. The networks of key modules and hub genes were visualized using Cytoscape v.3.3.0.

3 Results

3.1 SA treatment improved alfalfa survival under freezing stress and enhanced related antioxidant enzymes

To assess whether SA treatment improves alfalfa's survival rate under freezing stress, we used 200 μM SA (treatment) and 0 μM SA (control) pretreatment on three-week-old alfalfa for five days, including exposure to -10°C for 0, 0.5, 1, 2h to observe changes in phenotypes. Obviously, alfalfa samples with 200μM SA treatment had a higher survival rate than the control under exposure to -10°C for 2h (Figure 1A). To exhibit the phenotypic changes, we subjected all alfalfa to growth chamber with normal temperature for 2 days (Figure 1B). Interestingly, 14.83% of treatment samples survived, while only 7.17% survived in the control group (Figure 1B), which indicates that SA treatment could improve alfalfa survival rate under freezing stress conditions.

To measure the effects of SA treatment on alfalfa leaves under freezing stress, we analyzed the antioxidant enzymes of SOD, POD, and APX (Figure 2). The results demonstrated that the changes in SOD activity between the CK and SA treatments dramatically decreased, and then significantly increased as the duration of freezing stress increased. Interestingly, the SOD activity of SA treatment was greater than in CK at different times except for exposure to freezing stress at 0.5h (Figure 2A). In the CK, POD activity dramatically decreased at 0.5h and there were no significant changes as the time of freezing stress increased. Similar to SA treatment, POD activity increased at 0.5h, and no remarkable changes were observed at different freezing stress times (Figure 2B). The variation in APX activity was similar to POD, but that of CK decreased dramatically at 0.5h and 1h (Figure 2C). All leaves of POD and APX activity of SA treatment were higher than in CK (Figures 2B, C). Therefore, SA treatment could enhance the leaves of SOD, POD, and APX activity enzymes to defend against freezing stress.

3.2 Identification of DEGs in different treatments and related DEGs, including GO functional annotation and KEGG pathway analysis

To explore the mechanism of SA treatment in alfalfa under freezing stress, we used RNA-seq to analyze all the leaf samples in this study. We generated 5,929,423,457-7,218,419,876bp clean data reads, which were cleaned by GC<42%, Q20>97%, and Q30>93% (Table S2). All raw data have been updated to National Center for Biotechnology Information (NCBI) under accession number PRJNA867517.

Furthermore, we identified 126,974 genes (Table S3) from clean reads after mapping to an alfalfa reference genome more than 93%

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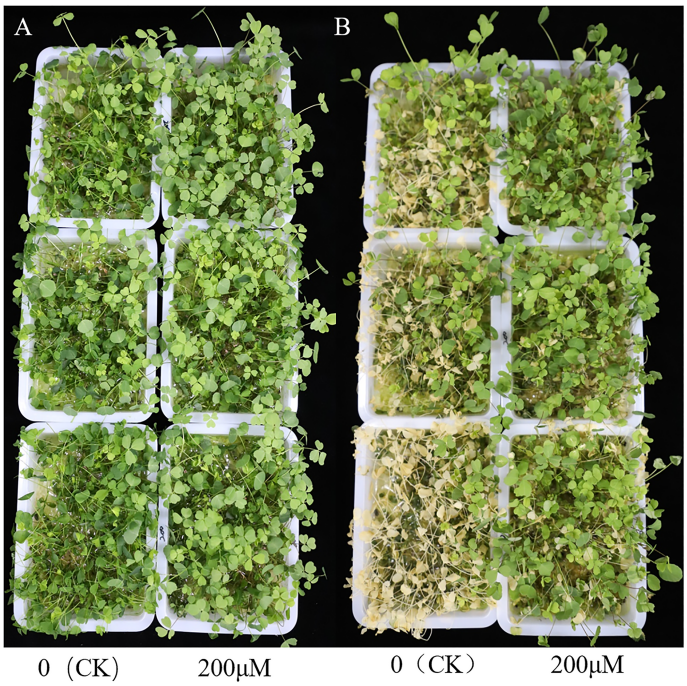

FIGURE 1
Phenotypes of 0 **(CK)** and 200 μM SA treatment alfalfa leaves to freezing stress. **(A)** The phenotype of 0 (CK) and 200 μM SA treatment "WL326GZ" leaves to -10°C for 2h. **(B)** The phenotype of the **(A)** growth under normal temperatures for 2 days.

(Table S4). Finally, we constructed thirteen comparisons to identify 41,175 DEGs, as seen in Figure S1. In detail, most DEGs were upregulated in WCK-0-vs-WCK-4 (7,957 DEGs) and T-0-vs-T-4 (7,601 DEGs), and most DEGs were downregulated in WCK-0-vs-WCK-3 (7,023 DEGs) and T-0-vs-T-3 (6,725 DEGs) (Figure S1).

To better understand the function of the DEGs, we mapped those DEGs to the GO database and the KEGG database (Figure S2). In WCK-0-vs-WCK-4, T-0-vs-T-4, WCK-0-vs-WCK-3, and T-0-vs-T-3, the results showed that the most abundant biological processes were metabolic processes and cellular processes; in cellular components, the most DEGs were enriched in the cell and cell part terms; in molecular function, the most DEGs were enriched in catalytic activity and binging terms (Figure S2A1–Figure S2D1). Meanwhile, in KEGG analysis, the DEGs are most enriched in the biosynthesis of the secondary metabolite pathway and metabolic pathways. There were some differences in DEGs enriched in the significant pathways. In WCK-0-vs-WCK-4, and T-0-vs-T-4, DEGs were significantly enriched in the biosynthesis of the secondary metabolite pathway and metabolic pathways. Similar to WCK-0-vs-WCK-3 and T-0-vs-T-3, DEGs were significantly enriched in the MAPK signaling pathway-plant, during biosynthesis of the secondary metabolite pathway and the plant-pathogen interaction pathway (Figures S2A2–S2D2). Therefore, it is possible that the MAPK signaling pathway-plants play a vital role in SA defense against freezing stress after alfalfa seedlings are exposed to -10°C for 2h.

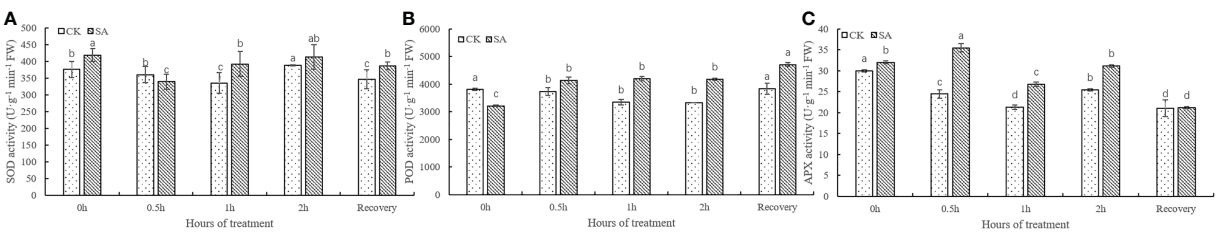

FIGURE 2
Effects of SA application on the activities of SOD **(A)**, POD **(B)**, and APX **(C)** in alfalfa leaves under freezing stress. The column chart represents the mean ± STEDV (n=3). The different letters represent values that were significantly different under CK or SA application to "WL326GZ" under freezing stress at different times ($p<0.05$, Duncan's test).

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3.3 Comparative analysis of DEGs in SA treatment at different times

To explore the potential difference between SA treatment on DEGs and the control, we used 4 Venn diagrams to overlap the DEGs. The results showed that there were 1,420 DEGs in common between (WCK-0-vs Wck-1) and (T-0-vs-T-1) (Figure 3A1). In GO analysis, the circle diagram showed that the most DEGs of the top 20 most significant GO terms enriched were GO:0050896 (528, response to stimulus), GO:0042221 (382, response to chemical), and GO:0006950 (357, response to stress) (Figure 3A2). In KEGG pathway analysis, most DEGs were significantly enriched in the MAPK signaling pathway, and during biosynthesis of secondary metabolites and plant-pathogen interaction pathway (Figure 3A3). Moreover, there were 7,544 unique DEGs in (WCK-0-vs Wck-1) VS (T-0-vs-T-1), which could be due to SA treatment on the leaves. Most DEGs in the top 20 significant GO terms enriched were GO:0044238 (2845 primary metabolic process), GO:0044763 (2541, single-organism cellular process), and GO:0044444 (1852, cytoplasmic part) (Figure 3A4). The most significant KEGG pathway was enriched in the fatty acid elongation pathway, biosynthesis of secondary metabolites, and biosynthesis of amino acids pathway (Figure 3A5).

As in the freezing stress for 1h (WCK-0-vs Wck-2 VS T-0-vs-T-2), there were 4,295 DEGs in common between the SA treatment and control groups (Figure 3B1). In GO analysis, the most DEGs in the top 20 GO terms enrichment were enriched in GO:0050896 (1,431, response to stimulus), GO:0042221 (992, response to chemical), and GO:0050794 (906, regulation of cellular process) (Figure 3B2). In the KEGG pathway analysis, the most significant DEGs were enriched in the plant-pathogen interaction pathway, the MAPK signaling pathway-plant pathway, and the plant hormone signal transduction pathway (Figure 3B3). In addition to the shared DEGs, based on the SA application, there were 4,483 unique DEGs in this phase (Figure 3B1). The most DEGs of the top 20 significant GO terms were enriched in the GO:0003824 (1,819, catalytic activity), GO:0044710 (1,223, single-organism metabolic process), and GO:0044281 (652, small molecule metabolic process) (Figure 3B4). In the KEGG pathway, the most significant KEGG enrichment pathway was the biosynthesis of secondary metabolites, metabolic pathway, and biosynthesis of amino acids pathways (Figure 3B5).

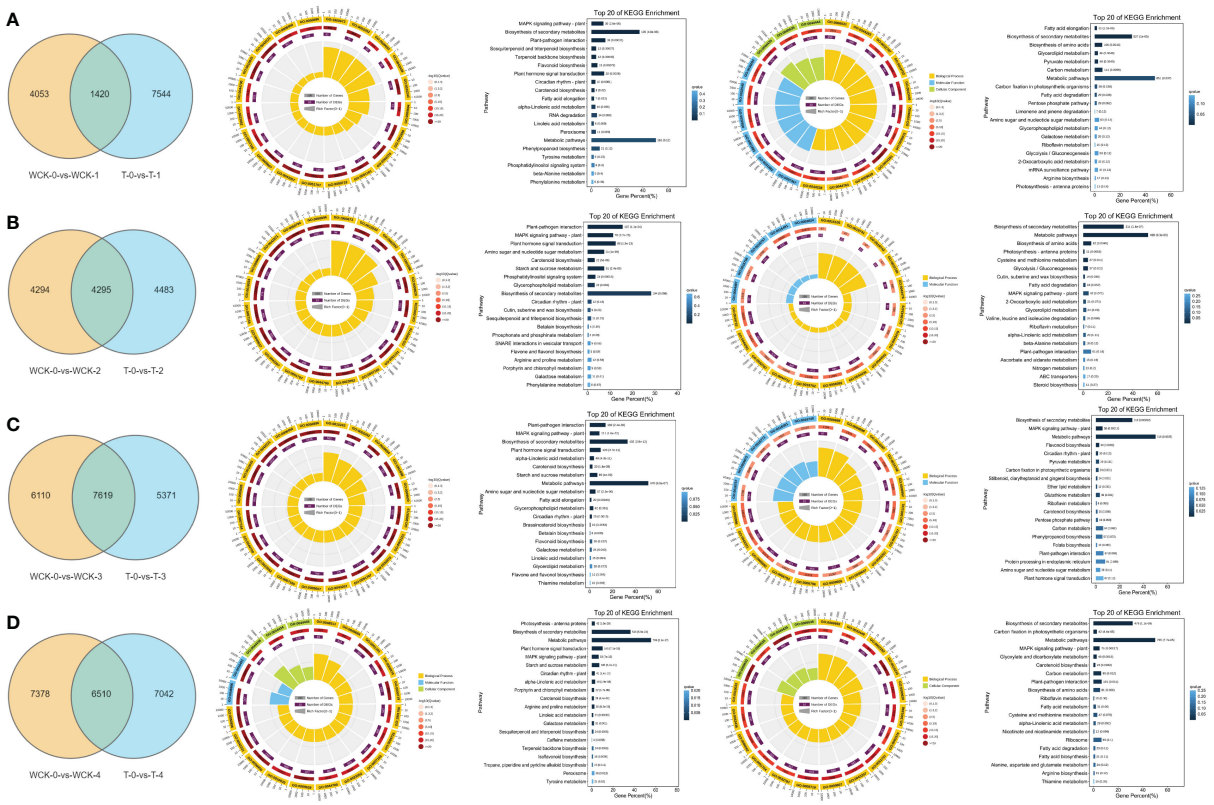

FIGURE 3
Comparison of DEGs between SA pretreatment and the control in alfalfa exposed to freezing stress for different periods. **A1, B1, C1**, and **D1** represent the Venn diagram of DEGs between SA pretreatment and the control in alfalfa exposed to freezing stress at 0.5, 1, and 2h, and allowed to recover for 2 days, respectively. **A2, B2, C2**, and **D2**; **A3, B3, C3**, and **D3** represent the common DEGs between SA pretreatment and control in alfalfa exposed to freezing stress at 0.5, 1, 2h, and allowed to recover for 2 days mapped to GO and KEGG database, respectively. **A4, B4, C4**, and **D4; A5, B5, C5**, and **D5** represent the especially DEGs of SA pretreatment alfalfa exposed to freezing stress at 0.5, 1, 2h, and allowed to recover for 2 days mapped to GO and KEGG database, respectively.

As the duration of freezing stress increased, more DEGs (7,619) were shared between the SA treatment and control (WCK-0-vs WCK-3 VS T-0-vs-T-3 Figure 3C1). The most DEGs of the top 20 significant GO terms were enriched in GO:0050896 (2,438, response to stimulus), GO:0042221(1,614, response to chemical), and GO:0006950 (1,577, response to stress) (Figure 3C2). The 7,619 DEGs enriched in the KEGG of the top 3 pathways were plant-pathogen interaction, MAPK signaling pathway-plant, and biosynthesis of secondary metabolites pathways (Figure 3C3). Furthermore, there were 5,371 unique DEGs in the SA treatment group under freezing stress for 2h (Figure 3C1). Most DEGs of the top 20 significant GO terms coincided with shared DEGs, including GO:0050896 (1,460, response to stimulus), GO:0042221(888, response to chemical), and GO:0006950 (901, response to stress) (Figure 3C4). The results of the KEGG pathway analysis differed in metabolic pathways instead of the plant-pathogen interaction pathway (Figure 3C5).

Finally, we contrasted the DEGs in the WCK-0-vs Wck-4 VS T-0-vs-T-4. The results revealed there were 6,510 DEGs in common (Figure 3D1). The GO analysis results were kept within the unique DEGs in WCK-0-vs WCK-3 VS T-0-vs-T-3 (Figure 3D2). However, in the KEGG pathway analysis, the top 3 significant pathways were photosynthesis-antenna proteins, biosynthesis of secondary metabolites, and metabolic pathways (Figure 3D3). Based on the SA application, there were 7,024 unique DEGs in this comparison (Figure 3D1). The most DEGs of the top 20 significant GO terms were GO:0009536 (1,053, plastid), GO:0010033 (883, response to organic substance), and GO:0006082 (801, organic acid metabolic process) (Figure 3D4). In KEGG pathway analysis, the most significant DEGs were enriched in the biosynthesis of secondary metabolites, carbon fixation in photosynthetic organisms, and metabolic pathways (Figure 3D5).

Shared DEGs were highly related to freezing stress, and the terms GO:0050896 (response to stimulus), GO:0042221 (response to chemical), and GO:0006950 (response to stress) could play vital roles in freezing stress response, while the plant-pathogen interaction pathway, the MAPK signaling pathway-plant pathway, the plant hormone signal transduction pathway, and the biosynthesis of secondary metabolites pathways also play important roles in freezing stress. While the unique DEGs could have resulted in SA application, many GO terms interact to play a role in resisting freezing stress. In addition, the KEGG pathway of fatty acid elongation, biosynthesis of secondary metabolites, biosynthesis of amino acids, metabolic pathway, and MAPK signaling pathway-plant pathway could play critical roles in reducing freezing stress damage after SA application. Interestingly, the MAPK signaling pathway-plant pathway could play an important role in freezing stress and SA treatment to alleviate damage due to freezing stress.

3.4 Co-expression network analysis, GO and KEGG classification

To validate potential candidate genes related to SA application and reduce damage due to freezing stress, we chose 31,310 DEGs for WGCNA and divided them into 18 modules (Fig 4A1). Then we further analyzed the typical differences in expression modules at the same time as freezing stress. The results showed that the 'darkgreen' (2602), 'darkgrey' (2555), 'salmon' (2327), and 'magenta' (2292) modules were highly related to SA application under freezing stress (Figure 4). In the GO analysis (Figure S3A1), the most DEGs in the darkgreen module were enriched in the GO:0044464 cell, the GO:0005622 intracellular, and GO:0044424 intracellular parts. In KEGG pathway analysis (Figure S3A2), the most significant DEGs were enriched in the autophagy - other eukaryotes, ubiquitin-mediated proteolysis, and photosynthesis pathways.

In the darkgrey module, the most DGEs in GO terms were enriched in the GO:0044238 primary metabolic process, GO:0050896 response to stimulus, and GO:0051179 localization (Figure S3B1). Furthermore, in KEGG analysis (Figure S3B2), the DEGs were significantly enriched in the circadian rhythm – plant, ribosome biogenesis in eukaryotes, and plant hormone signal transduction pathways.

In the GO analysis in the salmon module (Figure S3C1), the results showed that the most DEGs were enriched in the GO terms of GO:0050896 (response to stimulus), GO:0042221 (response to chemical), GO:0006950 (response to stress). Moreover, in the KEGG pathway analysis (Figure S3C2), the DEGs were significantly enriched in the MAPK signaling pathway - plant, proteasome, and plant-pathogen interaction pathways.

In the magenta module, the GO analysis results showed that the most DEGs were enriched in the GO terms of GO:0044424 intracellular part, GO:0005737 cytoplasm, and GO:0044444 cytoplasmic part (Figure S3D1). In the KEGG pathway analysis, the DEGs were significantly enriched in the photosynthesis, ribosome, and photosynthesis - antenna proteins pathways (Figure S3D2).

To validate the candidate hub genes in those related modules, we used the All.kWithin value to measure the candidate genes in the module. The higher the value was, the more important the gene was in the module (Table 1). The candidate 10 genes of the four modules are shown in Figure 4, including MPK 3, MPK 9, and some transcription factors (transcription factor TGA1, transcription factor MYC2, WRKY transcription factor 22).

3.5 Analysis of candidate genes related to SA biosynthesis and content changes of SA in alfalfa

In plants, there are two pathways related to the synthesis of SA *via* chorismite, which are a product of the shikimate pathway (Kumar, 2014). One pathway used cinnamate through PAL, and the other pathway used isochorismate (IC) through ICS. To analyze which pathway is dominant during freezing stress, we screened 14 *PALs*, 3 *ICSs* in the WGCNA analysis, and 22 *PALs* and 0 *ICSs* in all DEGs. Furthermore, we overlapped the genes to find out the 14 *PALs* from the WGCNA analysis were DEGs. The heatmap of the 14 *PALs* is shown in Figure 5. However, there were no *ICS* DEGs in our study, which suggests that the PAL pathway could predominate in the SA synthesis of leaves under freezing stress.

TABLE 1 The details of hub genes of four modules.

Gene ID	Module	All.kWithin	Symbol	Description
MSTRG.7811	darkgreen	211.23	CNOT9	CCR4-NOT transcription complex subunit 9 isoform X2 [Medicago truncatula]
MS.gene58025	darkgreen	184.89	SPS	sucrose-phosphate synthase [Medicago sativa]
MS.gene69726	darkgreen	180.04	VPS29	vacuolar protein sorting-associated protein 29 [Medicago truncatula]
MS.gene015744	darkgreen	176.66	ALDH2C4	aldehyde dehydrogenase family 2 member C4-like [Abrus precatorius]
MS.gene045075	darkgreen	175.60	TGA1	transcription factor TGA1 isoform X1 [Medicago truncatula]
MS.gene57768	darkgreen	166.73	CNGC5	probable cyclic nucleotide-gated ion channel 5 [Medicago truncatula]
MS.gene066083	darkgreen	161.86	TPS5	alpha,alpha-trehalose-phosphate synthase [UDP-forming] 5 [Medicago truncatula]
MS.gene23948	darkgreen	154.52	PLD1	phospholipase D alpha 1 [Medicago truncatula]
MSTRG.85009	darkgreen	152.18	At3g26720	alpha-mannosidase At3g26720 [Medicago truncatula]
MS.gene033282	darkgreen	148.15	RH30	DEAD-box ATP-dependent RNA helicase 20 [Medicago truncatula]
MS.gene56484	darkgrey	133.77	STP-1	alpha-1,4 glucan phosphorylase L-2 isozyme, chloroplastic/amyloplastic isoform X1 [Medicago truncatula]
MS.gene067596	darkgrey	127.17	STP-1	alpha-1,4 glucan phosphorylase L-2 isozyme, chloroplastic/amyloplastic isoform X1 [Medicago truncatula]
MS.gene20434	darkgrey	126.98	SS2	granule-bound starch synthase 2, chloroplastic/amyloplastic [Medicago truncatula]
MS.gene52552	darkgrey	126.66	MIOX2	inositol oxygenase 2 [Medicago truncatula]
MS.gene006839	darkgrey	119.90	STP-1	alpha-1,4 glucan phosphorylase L-2 isozyme, chloroplastic/amyloplastic isoform X1 [Medicago truncatula]
MS.gene06703	darkgrey	118.44	SS1	starch synthase 1, chloroplastic/amyloplastic isoform X1 [Medicago truncatula]
MS.gene043386	darkgrey	117.64	Mpv17l2	protein SYM1 [Medicago truncatula]
MS.gene015369	darkgrey	116.60	TIFY10A	protein TIFY 10a isoform X1 [Medicago truncatula]
MS.gene02685	darkgrey	114.89	FDH	beta-ketoacyl CoA synthase 10 [Medicago sativa]
MS.gene057285	darkgrey	114.77	KCS2	3-ketoacyl-CoA synthase 11 [Medicago truncatula]
MSTRG.54558	magenta	145.15	MPK9	mitogen-activated protein kinase 9 isoform X1 [Medicago truncatula]
MSTRG.87802	magenta	142.34	LSM2	sm-like protein LSM2 [Medicago truncatula]
MSTRG.49053	magenta	141.40	ALATS	alanine–tRNA ligase [Medicago truncatula]
MS.gene025934	magenta	131.93	UBC28	Ubiquitin-conjugating enzyme E2 [Zostera marina]
MS.gene21844	magenta	125.89	OASA1	cysteine synthase [Medicago truncatula]
MS.gene065561	magenta	120.15	PSBR	photosystem II 10 kDa polypeptide, chloroplastic [Medicago truncatula]
MSTRG.52727	magenta	119.69	ALATS	alanine–tRNA ligase [Medicago truncatula]
MS.gene028410	magenta	117.81	RPL44	60S ribosomal protein L44 [Medicago truncatula]
MS.gene32893	magenta	114.60	TAF4B	transcription initiation factor TFIID subunit 4b [Medicago truncatula]
MSTRG.56164	magenta	114.30	PAHX	phytanoyl-CoA dioxygenase domain protein [Medicago truncatula]
MS.gene57961	salmon	395.72	GAE1	UDP-glucuronate 4-epimerase 1 [Medicago truncatula]
MS.gene46308	salmon	392.05	AUX22E	auxin-induced protein 22e-like [Trifolium pratense]
MS.gene020318	salmon	385.57	AUX22E	auxin-induced protein 22e-like [Trifolium pratense]
MS.gene055478	salmon	380.16	GAE1	UDP-glucuronate 4-epimerase 1 [Medicago truncatula]
MS.gene65266	salmon	377.60	MPK3	LOW QUALITY PROTEIN: mitogen-activated protein kinase 3 [Medicago truncatula]
MS.gene006867	salmon	375.77	At4g26390	pyruvate kinase, cytosolic isozyme [Medicago truncatula]
MS.gene08572	salmon	375.66	MPK3	LOW QUALITY PROTEIN: mitogen-activated protein kinase 3 [Medicago truncatula]
MS.gene048435	salmon	366.46	MYC2	transcription factor MYC2 [Medicago truncatula]

(Continued)

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TABLE 1 Continued

Gene ID	Module	All.kWithin	Symbol	Description
MS.gene29459	salmon	356.49	CML23	calcium-binding protein CML24 [Medicago truncatula]
MS.gene79681	salmon	355.37	WRKY22	WRKY transcription factor 22 [Medicago truncatula]

kWithin Value: The gene connectivity within each module.

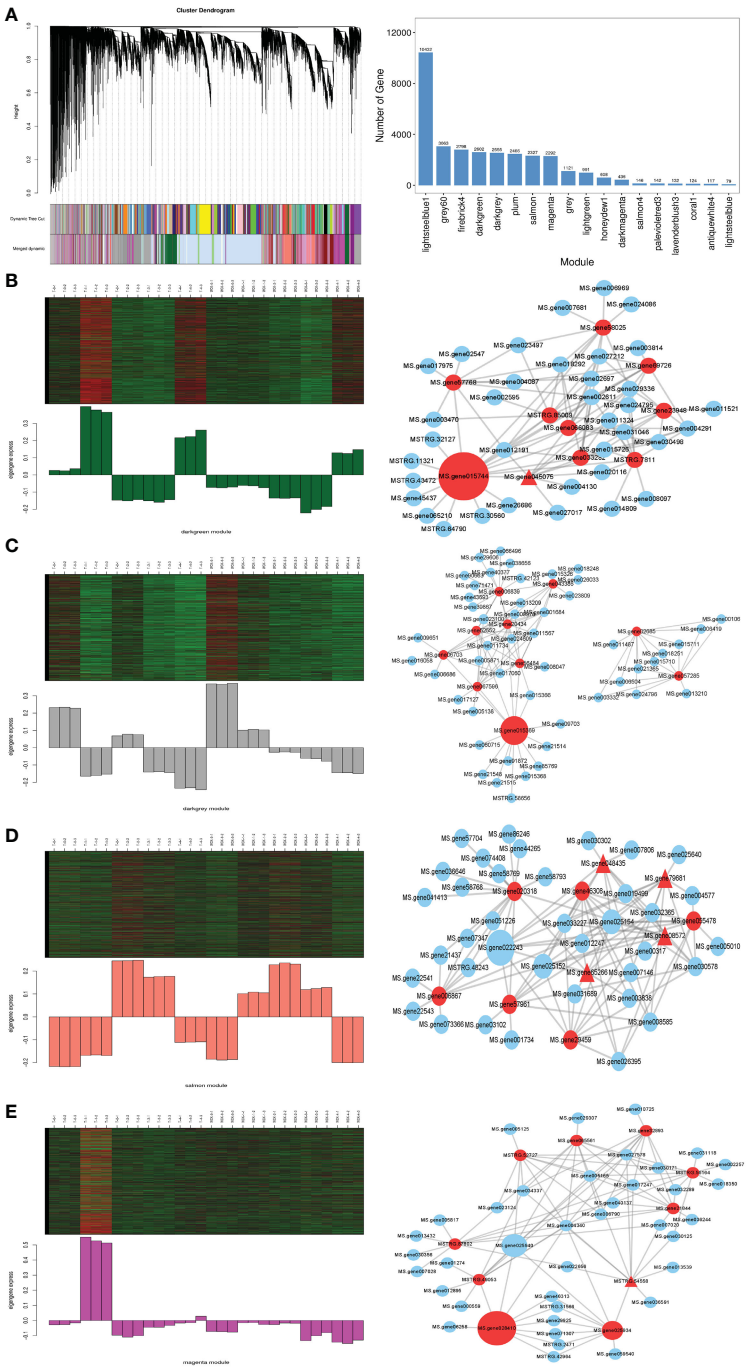

FIGURE 4
Weighted gene co-expression network analysis (WGCNA) of 31,310 genes. **(A1)** Cluster dendrogram; **(A2)** Number of genes of 18 modules; **(B1, C1, D1, E1)** The four modules of most related SA pretreatment alfalfa seedlings increased freezing stress; **(B2, C2, D2, E2)** The correlation networks of the top 10 hub DEGs corresponding to the four modules. The networks only showed the top 10 weight values of each hub gene, the different types of nodes represent the value of betweenness centrality is higher, the type of the node is bigger, and the triangle of the node represents the transcription factor.

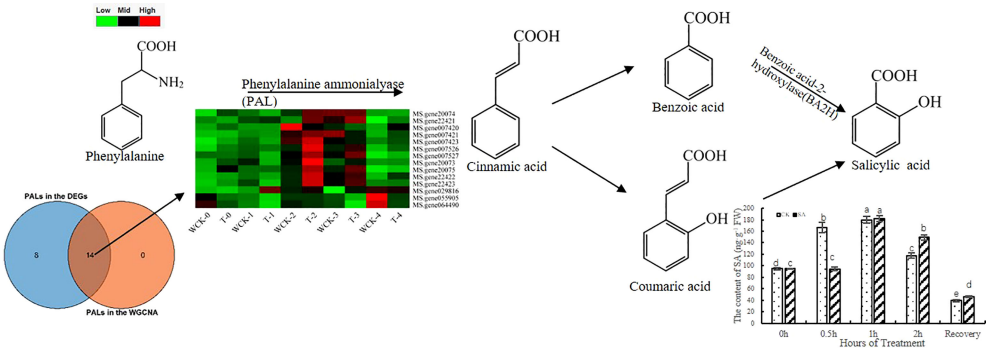

FIGURE 5
Analysis of the *PALs* expression related to increasing biosynthesis of SA.

To analyze the changes in endogenous SA content, we used HLPC-MS/MS to detect the contents of all sample leaves. The results (Figure 5) showed that the SA content increased as the duration of freezing stress increased until 1h and then decreased. Interestingly, the content of CK increased earlier than in the exogenous SA. The CK could be activated earlier as a protection system to defend against freezing stress.

3.6 SA induced large numbers of genes in the SA signaling pathway to defend against freezing stress

In our study, there were 43 *MAPKKKs* (Figure S4A), 9 *MKKs* (Figure S4B), and 23 *MAPKs* (Figure S4C) that could play a role in transmitting SA signaling transduction. All genes reported in the NPR1-dependent pathway could participate in freezing stress. In the NPR1-dependent pathway, the application of SA during freezing stress induced the expression of 134 *WRKYs* (Figure S4H), and promoted the expression of 4 *NPR1* genes (Figure S4D). Interestingly, the expression of the *NPR1s* increased as the duration of freezing stress increased, and then decreased. There were 21 *TGAs* (Figure S4E), which as key NPR1 activate transcript factors, were also found in this experiment. Most *TGAs* in the SA application were more highly expressed than in the CK during freezing stress. Moreover, as the time of freezing stress increased, the expression of 11 *TRx hs* increased, and most *TRx h* genes in SA were more highly expressed than in the CK at 0.5 and 1h, while the opposite occurred at 2h (Figure S4F). The expression trend of 4 *PR1* genes was similar to *TRx h* genes, but the inflection point was at 0.5h of freezing stress. The expression of most *PR1* genes was higher than CK under freezing stress at 2h (Figure S4G).

Except for the NPR1-dependent pathway genes take a vital role against freezing stress, there have many NPR1-independent pathway genes expressed in this experiment, including *SOD*, *POD*, *APX*, glutathione S-transferases (*GST*), and *HSPs*. Fortunately, most of the 9 *SOD* genes were increased with the duration of freezing stress and then decreased, and the expression of *SOD* genes in SA application was higher than that of CK after freezing stress at 0, 0.5h, whereas opposite after freezing stress at 1 and 2h (Figure S4I). Among the 10 *POD* genes, except for the expression of *POD* genes in SA application after freezing stress at 2h were higher than that of CK, the others were lower than that of CK (Figure S4J). As in 12 *APX* genes, nearly most of them in SA application had higher expression than in CK after freezing stress at 0.5h, while the others were not more than in CK (Figure S4K). Moreover, SA and freezing stress could induce GSTs, and SA application had higher expression than CK during all freezing stress processes (Figure S4L).

High SA concentrations have been reported to induce HSPs (Jumali et al., 2011). In our research, there were 124 different types of HSP genes induced (Figure S4M), including *HSP 1* (4 genes), *HSP 15* (4 genes), *HSP 17* (8 genes), *HSP 18* (17 genes), *HSP 21* (2 genes), *HSP 22* (5 genes), *HSP 23* (1 genes), *HSP 70* (72 genes), *HSP 83* (5), *HSP 90* (6 genes). SA could induce HSP-related genes in freezing stress.

3.7 qRT-PCR validation of the expression profiles of the candidate genes

To validate the reliability of the transcriptome data, we randomly monitored the expression of 6 DEGs through qRT-PCR analysis: MS.gene 21548 (Rop-interacting receptor-like cytoplasmic kinase 2), MS.gene 02035 (regulatory protein NPR1 isoform X1), MS.gene 004340 (GDSL-like lipase/acylhydrolase), MS.gene 035685 (pathogenesis-related protein PR-1), MS.gene 05280 (mitogen-activated protein kinase 9), and MS.gene 017127 (mitogen-activated protein kinase 10 isoform X1) (Figure S5). Fortunately, the trends of gene expression by the qRT-PCR analysis were similar to the FPKM in transcriptome data analysis.

4 Discussion

4.1 SA effectively induced physiological response in leaves under freezing stress

SA is a potent phenolic signaling biomolecule and a highly potent plant growth regulator that can decrease crop biomass losses

due to its cheap, biodegradable, and positive response to biotic and abiotic stresses (Arif et al., 2020; Zaid et al., 2022). Freezing stress is one of the most significant abiotic stresses that seriously impacts crop biomass (Barnaby et al., 2020), and can cause oxidative damage by excess ROS accumulation in plants (Wei et al., 2021). Pretreatment of SA in plants could increase the ability of the antioxidant system to defend against freezing stress (Arif et al., 2020). Similar to rice, soaking in SA solution increased the SOD, CAT, APX, and GR activities against freezing stress (Pouramir-Dashtmian et al., 2014), while Hossain et al. (2015) reported that the SA-induced antioxidant system decreases freezing stress by maintaining the integrity of the plasma membranes and other physiological functions. Simultaneously, exogenous 0.1 mM SA increased the activities of SOD, POD, and CAT under freezing stress in barley (*Hordeum vulgare* L.) (Mutlu et al., 2016). Interestingly, in our study, pretreatment of 200 μM SA increased SOD, POD, and APX activities against freezing stress (Figure 2), which was similar to rice and barley. Furthermore, Wang et al. (2012) reported that exogenous SA could improve the activities of CAT and POD at low temperatures, while the activity of SOD does not significantly change to CK. This could be because the SA pretreatment method is different. Therefore, exogenous application of 200 μM SA could improve the activities of SOD, POD, and APX in alfalfa under freezing stress.

4.2 SA induced differentially expressed genes in freezing stress

To elucidate the possible mechanism behind how SA application improved freezing stress in alfalfa, we transcribed RNA from alfalfa leaves pretreated with 0 and 200 μM SA, which were exposed to -10°C for 0, 0.5, 1, 2h, and allowed to recover to growth temperature for 2 days. We identified 126,974 genes by RNA-seq from the alfalfa reference genome (Table S3); these results were more effective than other research on alfalfa freezing stress (Song et al., 2016; Shu et al., 2017; Xu et al., 2019; Wang et al., 2021). To better understand the function of SA application in freezing stress, we constructed 13 comparisons to identify 41,175 DEGs for further analysis. The results demonstrated that the plant-pathogen interaction pathway, the MAPK signaling pathway-plant pathway, the plant hormone signal transduction pathway, and the biosynthesis of the secondary metabolites pathway could play a vital role in freezing stress (Figure S2). Wang et al. and Song et al. found that all pathways, except for the MAPK signaling pathway-plant that is predominant in alfalfa, confer freezing stress (Song et al., 2016; Wang et al., 2021). The MAPK signaling pathway has been reported as important in freezing signal transduction in many plants (Cowan and Storey, 2003; Liu and Zhou, 2018; Wu et al., 2022), which means it could have a similar function in our study. As for SA application, the domain KEGG pathways were fatty acid elongation, biosynthesis of secondary metabolites, biosynthesis of amino acids, metabolic pathway, and MAPK signaling pathway-plant. In bananas, Chen et al. (2020) found that SA could improve chilling stress *via* metabolic pathways and enhance energy charge by synthesizing fatty acids and amino acids (Chen et al., 2020). Many reports suggest that SA is involved in the induction of secondary metabolites (Khan et al., 2015), and thus pathway of biosynthesis of secondary metabolites pathway could be one of the domain pathways. SA is known as the primary signaling hormone that is always in coordination with other signal transduction - MAPK signaling pathways to participate in complex transduction networks to enhance abiotic tolerance in plants (Singh and Jwa, 2013; Chai et al., 2014; Liu et al., 2022). Moreover, the MAPK signaling pathway is dominant in defense signaling (Shan et al., 2021), and is involved in freezing stress and SA application.

4.3 MAPK cascades could play a dominant role in transmitting SA signals or SA-dependent related genes to defend against freezing stress

MAPK cascades are highly conserved signaling components in all eukaryotic organisms (Zheng et al., 2018) and play an indispensable role in plant growth, development, and biotic and abiotic stresses responses, (Mohanta et al., 2015), including in regulating hormonal responses (Long et al., 2020). MAPK cascades include MAPKKK, MPKK, and MPK (MAPK). In *Arabidopsis thaliana*, there were 60-80 MAPKKKs, 10 MAPKKs, and 20 MAPKs (Pitzschke, 2015; Saucedo-García et al., 2021). In our study, we identified 43 MAPKKKs, 9 MKKs, and 23 MAPKs as DEGs that belong to MAPK cascades (Figure S4). Moreover, the evidence showed that SA could induce BaMKK9, BaMPK1, 2, BaMKK2, 4, 5, and BaMPK3, 6 integrated into the SA signaling pathway to regulate defense genes in canola (*Brassica napus* L.) (Liang et al., 2013). AtMKK4/MKK5 is essential for the induction of SA-mediated defense response (Liu et al., 2021), while MPK3/MPK6 positively regulates SA-dependent defense responses (Bartels et al., 2009). Therefore, the MAPK cascades could take part in transmitting SA signaling or direct the SA-dependent related genes downstream against freezing stress.

We obtained three hub genes of SA application to alleviate the damage of freezing stress as MAPKs through WGCNA (Table 1). Two of them belong to MPK3, and the other is MPK9. Much research has found that MPK3 in different species plays an important role in SA mediating plant defense responses (Long et al., 2020). While AtMPK3 has been reported to direct regulated SA-dependent related genes against freezing defense and is related to SA biosynthesis (Bartels et al., 2009), Guan et al. (2020) revealed that NtMPK3 could interact with the SA signaling pathway in triclosan stress. Recently, evidence has found that MPK3 could mediate SA-dependent genes, such as PR-1 expression to defense response, and SA-independent genes to defense response (Genot et al., 2017). Consequently, the detailed function of the MPK3 remains unknown, though MPK3 occupied a predominant role in SA application against freezing stress. In addition, studies have demonstrated that MPK9 can be expressed in guard cells (Jammes et al., 2009) and positively regulated ABA signaling mediates stomatal closure (Liu et al., 2010). Moreover, Khokon et al. (2017) found that AtMPK9 positively regulates SA signaling by closing stoma in

Arabidopsis. Therefore, we hypothesize that the function of alfalfa MPK9 is similar in *Arabidopsis* to defense freezing stress.

4.4 PAL pathway could be the dominant method of synthesizing SA in alfalfa against freezing stress

Plants use two independent pathways (ICS and PAL pathways) to synthesize SA. In *Arabidopsis*, Xu et al. (2017) demonstrated that the ICS pathway is the most common way to synthesize SA against biotic and abiotic stress, especially in pathogen-induced SA synthesis (Ding and Ding, 2020). Furthermore, ICS1 has been reported to play a more critical role than ICS2 in SA synthesis (Huang et al., 2020). In soybean, the PAL and ICS pathways contributed equally to SA biosynthesis (Shine et al., 2016). Ogawa et al. (2006) revealed that the PAL pathway was the primary route to the synthesis of SA in tobacco by investigating ICS and PAL gene expression. Therefore, the dominant pathway involved in SA synthesis depends on the plant species. In our study, we investigated the expression of ICS and PAL genes based on the RNA-seq database and WGCNA results. Unfortunately, there were no DEGs as ICS genes (Figure 5), which could be because the freezing stress time was too short. However, 11 PALs were identified as DEGs. Automatically, the PAL pathway could be dominant in alfalfa leaves under freezing stress, leading to the biosynthesis of SA and SA accumulation.

4.5 NPR1-dependent and independent pathway-related genes possibly participate in SA to resist freezing stress in alfalfa

Exogenous application of SA improving cold stress tolerance has been reported in different species, including maize, potato, *Aradibopsis* (Saleem et al., 2021b), rice (Wang et al., 2009b), wheat (Wang et al., 2021), cucumber, pepper, banana (Hara et al., 2011), and barley (Mutlu et al., 2016). However, numerous studies focused on the physiological changes and the expression of cold signaling genes (Miura and Tada, 2014), though how the SA signaling pathway regulates related genes to improve cold stress is unknown. SA signaling could regulate NPR1-dependent and NPR1-independent pathway-related genes to participate in resistance to biotic and abiotic stress (Li et al., 1999; Yu et al., 2001; An and Mou, 2011), and the NPR1-dependent pathway regulated more than 98% of SA-responsive genes (Saleem et al., 2021a). With NPR1 as the SA receptor, SA and the transcriptome coactivator can regulate SA-dependent gene expression against biotic and abiotic stress (Wu et al., 2012). In an NPR1-dependent pathway, SA could modulate signaling transducers, including MAPK signal cascades, to contribute to WRKY transcript factors binding to the W-box to promote the expression of NPR1 genes (Chai et al., 2014). As the concentration of SA increased, redox changes occurred and thioredoxin (TRx h) monomerized NPR1 to reduce NPR1 (Saleem et al., 2021a). When NPR1 enters the nucleus, it could bind TGA or WRKY transcript factors with the as-1 element or W-box to regulate SA-related gene expression (Wang et al., 2006; Saleem et al., 2021a). We identified 134 WRKY genes, 4 NPR1 genes, 21 TGA genes, 11 TRx h genes, and 4 PR1 genes in our study (Figure S4). Furthermore, to analyze which gene is dominant in this pathway, we used WGCNA to identify possible WRKY 22 and TGA1 that play vital roles in the SA signaling pathway. Numerous studies demonstrated that WRKY22 could be induced by abiotic and biotic stresses, including cold and pathogen stress (Zhang et al., 2015; Kloth et al., 2016; Li et al., 2018a; Li et al., 2019; Chung et al., 2020). Therefore, signal transduction pathways could convert common regulators-WRKY22. Previous studies have found that VvWRKY22 participates in sugar accumulation in grapes (Huang et al., 2021). WRKY22 of LA Hybrid Lily not only regulates gibberellin signaling but is also involved in low-temperature signals (Li et al., 2019), and Ms WRKY22 has been identified in pathogen attacks (Zhang et al., 2022). In *Arabidopsis*, *AtWRKY22* was involved in Pathogen-associated molecular patterns (PAMP)-triggered MAPK cascades (AtMEKK1-AtMKK4/5-AtMPK3/6), which induced the SA signaling pathway to regulate PR1 expression and resulted in expression of genes related to plant growth and cell-wall loosening (Zipfel et al., 2004; Kloth et al., 2016). Furthermore, Kloth et al. (2016) reported that abiotic stress induced WRKY22 by MAPK cascades or indirectly by SA accumulation. Therefore, WRKY22 is involved in the SA signaling pathway and the function of WRKY22 in alfalfa was similar to that in *Arabidopsis*.

TGA transcription factors bind to NPR1 to regulate the expression of PR genes (Kesarwani et al., 2007). In *Arabidopsis*, 10 TGAs were identified (Li et al., 2018b), while only TGA1-TGA7 could interact with NPR1. Furthermore, TGA2, TGA3, TGA5, TGA6, and TGA7 have been identified to interact with NPR1 in yeast and planta, but only TGA1 and TGA4 bind to NPR1 in SA-induced planta leaves (Despres, 2003; Fobert and Després, 2005; Kesarwani et al., 2007). Therefore, TGA1 was induced in our study (Table 1). Budimir et al. found that TGA1 was expressed in SA-induced NPR1-dependent genes (Budimir et al., 2020). Moreover, TGA1 interacts with NPR1 in SA, which regulates the expression of PR genes (Budimir et al., 2020). Sun et al. found that TGA1 regulates SA biosynthesis (Sun et al., 2017), and Kesarwani et al. (2007) found that TGA1 plays a dominant in basal resistance, which coincides with our results.

Except for the NPR1-dependent pathway, SA could induce the expression of NPR1-independent pathway-related genes against stress (Uquillas et al., 2004), including *GPXs*, *GSTs*, *HSPs*, *PODs*, *SODs*, *APXs*, GSHs, and *LEAs* (Aftab and Yusuf, 2021; Saleem et al., 2021b). *SODs*, *PODs*, *APXs*, *GSTs*, and *HSPs* were induced in our study (Figure S4). In numerous studies, SA could induce *SOD*, *POD*, APX, and *GST* encoding genes to upregulate the activities of related enzymes and alleviate freezing stress damage (Wang et al., 2020b; Serna-Escolano et al., 2021). In addition, the *WRKY* gene plays an important role in SA's improved encoding genes of antioxidant enzymes in freezing stress (Yokotani et al., 2013; Wang et al., 2020b). Wan et al. (2009) found that low-temperature stress HSP73 could increase the accumulation of

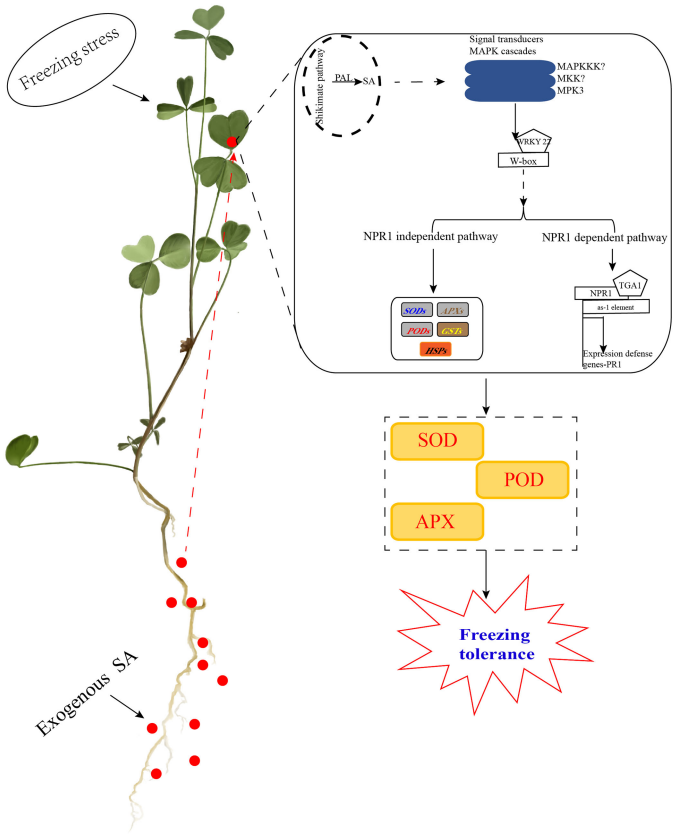

FIGURE 6
Working diagram for the mechanism of SA application improved alfalfa freezing tolerance in SA signaling transduction. Exogenous SA could improve the free SA in alfalfa leaves after freezing stress primarily through the PAL pathway. Moreover, SA induced MAPK cascades to regulate WRKY22 to participate in the SA signaling pathway, including the NPR1-dependent pathway and NPR1-independent pathway. We hypothesize that the hub gene MPK3 contributes to WRKY22 binding to the W-box to promote the expression of NPR1 genes downstream and NPR1-independent related genes against freezing stress. In the NPR1-dependent pathway, NPR1 enters the nucleus, which could bind to TGA1 with the as-1 element to regulate the expression of *PR1* genes to defend against freezing stress. In the NPR1-independent pathway, WRKY22 could directly regulate the expression of *SODs*, *PODs*, *APXs*, *GSTs*, and *HSPs*. Collectively, SA enhanced the antioxidant enzymes of SOD, POD, and APX to improve freezing tolerance in alfalfa.

SA, while HSP70 and HSP90 have been demonstrated to accumulate under cold temperature stress and SA application. *HSPs* could prevent the proteins from denaturing and misfolding (Lopez-Matas et al., 2004). Therefore, SA application on alfalfa under freezing stress induces the expression of *SODs*, *PODs*, *APXs*, *GSTs*, and *HSPs* genes.

5 Conclusion

In conclusion, exogenous SA can improve the accumulate of free SA in alfalfa leaves primarily through the PAL pathway under freezing stress. Moreover, SA induced MAPK cascades to regulate WRKY22 to participate in the SA signaling pathway, including the NPR1-dependent pathway and the NPR1-independent pathway, to regulated related genes against freezing stress. Interestingly, the transcriptome analysis and WGCNA database found that MPK3 could contribute to WRKY22 binding to the W-box and promote the downstream expression of NPR1 genes and NPR1-independent related genes to defend against freezing stress. In the NPR1-dependent pathway, NPR1 enters the nucleus, which could bind to TGA1 with the as-1 element to regulate the expression of PR1 genes to defend against freezing stress. In the NPR1-independent pathway, WRKY22 could directly regulate the expression of *SODs*, *PODs*, *APXs*, *GSTs*, and *HSPs*. Collectively, SA enhanced the antioxidant enzymes of SOD, POD, and APX to improve the survival of alfalfa under freezing stress. Furthermore, MPK9 can positively regulate SA signaling transduction to defend against freezing stress (Figure 6). Our study provides new insights into the mechanism of how exogenous SA can improve defense against freezing stress in alfalfa.

Data availability statement

The data presented in the study are deposited in the NCBI repository, the accession number is PRJNA867517.

Author contributions

SS and XW conceived and designed research; XW, JM and WK conducted experiment; XW and JM analyzed data; XW, and WK wrote manuscript; XW, JM and SS finalized 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1091077/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Numbers of differential upregulated and downregulated expressed genes in alfalfa samples. WCK-0, WCK-1, WCK-2, WCK-3, and WCK-4 represent the samples of 0 μM SA pretreatment seedlings exposed to -10°C at 0, 0.5, 1, 2 h, and allowed to recover for 2 days, respectively. T-0, T-1, T-2, T-3, and T-4 represent the samples of 200 μM SA pretreatment seedlings expose to -10°C at 0, 0.5, 1, 2 h, and allowed to recover for 2 days, respectively.

SUPPLEMENTARY FIGURE 2
The results of the DEGs in most upregulated (WCK-0-vs-WCK-4, T-0-vs-T-4) and downregulated (WCK-0-vs-WCK-3, T-0-vs-T-3) DEG comparisons mapped to the GO and KEGG databases. A1 and A2 represent the results of all the DEGs in the WCK-0-vs-WCK-4 comparison mapped to the GO and KEGG databases, respectively. B1and B2 represent the results of all the DEGs in the T-0-vs-T-4 comparison mapped to the GO and KEGG databases, respectively. C1 and C2 represent the results of all the DEGs in the WCK-0-vs-WCK-3 comparison mapped to the GO and KEGG databases, respectively. D1and D2 represent the results of all the DEGs in the T-0-vs-T-3 comparison mapped to the GO and KEGG databases, respectively.

SUPPLEMENTARY FIGURE 3
The GO and KEGG enrichment of the darkgreen **(A1, A2)**, darkgrey **(B1, B2)**, salmon **(C1, C2)**, and magenta **(D1, D2)** modules.

SUPPLEMENTARY FIGURE 4
The heatmap of expressed genes in the SA signaling pathway. **(A)** the expression genes of *MAPKKKs*; **(B)** the expression genes of *MKKs*; **(C)** the expression genes of *MPKs*; **(D)** the expression genes of *NPR1s*; **(E)** the expression genes of *TGAs*; **(F)** the expression genes of *TRx hs*; **(G)** the expression genes of *PR1s*; **(H)** the expression genes of *WRKYs*; **(I)** the expression genes of *SODs*; **(J)** the expression genes of *PODs*; **(K)** the expression genes of *APXs*; **(L)** the expression genes of *GSTs*; **(M)** the expression genes of *HSPs*.

SUPPLEMENTARY FIGURE 5
Expression profile of 6 candidate genes by qRT-PCR and RNA-seq. Error bar represents STEDV of each treatment.

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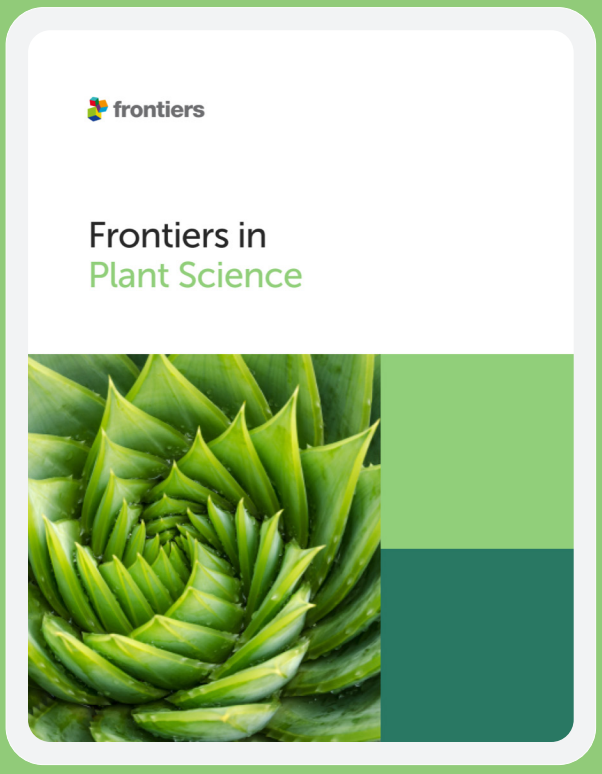

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