Physiological and transcriptomic analysis of antioxidant mechanisms in sweet sorghum seedling leaves in response to single and combined drought and salinity stress

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ABSTRACT

The effects of drought, salinity, and combined stress on ROS and ROS metabolic physiology and transcriptomics in sweet sorghum seedling leaves were evaluated. The results showed that drought stress had little effect on photosynthesis, while the SOD activity, CAT activity, and the expression of their related genes were elevated in leaves, but no excessive accumulation of \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), or \(-\text{OH}\) was observed. Under salinity stress, photosynthesis was inhibited, the \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \(-\text{OH}\) contents increased significantly, and the SOD, POD, CAT activities and the expression of their related genes in leaves were elevated. Under combined stress, photosynthesis was significantly inhibited, the highest accumulation of \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \(-\text{OH}\) contents occurred, and the SOD and POD activities and the expression of related genes in leaves were significantly increased, but the CAT was significantly decreased. These results collectively indicate that oxidative damage to sweet sorghum seedling leaves was higher with combined stress than with either drought or salinity stress alone. Under combined stress the SOD and POD activities were increased, but the CAT activity in the AsA-GSH cycle was severely reduced, demonstrating that antioxidant mechanisms in seedlings did not play a normal protective role, leaving the plants severely damaged by oxidative stress.

Introduction

ROS are partially reduced or excited forms of atmospheric oxygen, a general term for oxygenates with extremely active properties and high oxidative capacity, including \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), \(-\text{OH}\), and \( ^1\text{O}_2 \) (Mittler 2017). ROS play a dual regulatory role in plant growth and development as a byproduct of aerobic plant metabolism. Low levels of ROS can act as signaling molecules, regulating physiological processes such as systemic signaling, PCD, and abiotic stress responses. However, excessive ROS can disrupt intracellular redox homeostasis and cause damage to proteins, lipids, carbohydrates, and DNA, ultimately leading to oxidative stress (Mittler 2017; Soares et al. 2019; Zhang et al. s2020). ROS play an important role in damaging, protective, or signaling in cells depending on the delicate equilibrium between ROS production and effective scavenging (Gill and Tuteja 2010). To maintain the balance between ROS production and scavenging, plants possess a powerful and multifaceted antioxidant system composed of enzymatic and nonenzymatic antioxidant mechanisms, which are involved in sensing, detoxifying, eliminating and/or neutralizing ROS overproduction (Soares et al. 2019; García-Caparrós et al. 2021). Nonenzymatic components include a variety of low-molecular-weight metabolites, such as AsA, GSH, Pro, α-tocopherol, carotenoids, and flavonoids. Enzymatic components include SOD, POD, CAT, APX, DHAR, MDHAR, GPX, and GR within the AsA-GSH cycle (Gill and Tuteja 2010; Hasnuzaman et al. 2019). Enzymatic and nonenzymatic components act synergistically to provide a complex and multifaceted ROS scavenging mechanism for plants to prevent and mitigate oxidation-induced damage in plant cells (Soares et al. 2019).

Soil drought and salinity are two critical abiotic stresses experienced during plant growth and development, that reduce the average quality and yield of crops worldwide and seriously threaten the sustainability of modern agriculture (Zandalinas et al. 2018; Phour and Sindhu 2022). Under natural conditions, due to reduced rainfall or
excessive fertilization in arid areas, the soil salinity concentration increases, stressing the growth of crops by the combination of drought and salinity (Alvarez and Sánchez-Blanco 2015; Lu et al. 2021). Recent studies have shown that it is challenging to predict the response of plants to a combination of several abiotic stresses based on the response of plants to the same stress applied individually. Combinatorial stress treatments should be considered a new type of stress requiring new defenses or acclimation regimens (Suzuki et al. 2014; Prasch and Sonnewald 2015; Zandalinas et al. 2018). Therefore, unlike the mitigation of single stress, combined stress requires the tailoring of individual metabolic and signaling treatments to protect antioxidant mechanisms, photosynthesis, hormone signaling, and osmolyte synthesis (Zandalinas et al. 2018).

Sweet sorghum (Sorghum bicolor (L.) Moench) is a C₄ plant crop with high resistance to adversity, fast growth rate, and high biomass, which makes it an excellent cereal, fodder, and energy crop (Kanbar et al. 2021). Due to its ability to combat various harsh conditions at successive developmental stages and grow on marginal lands, sweet sorghum is well suited for arid and semiarid areas and saline lands (Mansour et al. 2021; Zheng et al. 2021; Abreha et al. 2022). It is well documented that drought and salinity stress can lead to an imbalance of ROS metabolism in plants, with excess ROS leading to peroxidation of the cytoplasmic membrane and impairing plant growth and development (Gill and Tuteja 2010). To date, a large number of studies on ROS metabolism in sweet sorghum under drought and salinity stress have been conducted (Sui et al. 2015; Yan et al. 2015; Nxele et al. 2017; Li et al. 2020; Punia et al. 2021; Wang et al. 2022). However, there are few studies on the formation and scavenging of reactive oxygen species in sweet sorghum seedling leaves under combined drought and salinity stress. Therefore, we used physiological and transcriptomic approaches to study ROS metabolism and its related gene responses in sweet sorghum leaves under drought, salinity, and both stresses combined. The aims of this study were to identify the antioxidative mechanisms of ROS scavenging in sweet sorghum seedling leaves under drought, salinity, and combined stress and to provide essential reference data for studying the regulatory mechanisms of sweet sorghum in response to abiotic stress.

Materials and methods

Plant materials and treatment methods

Liaotian No. 1 sweet sorghum seeds were provided by the Institute of Sorghum Research, Liaoning Academy of Agricultural Sciences. Fifteen sweet sorghum seeds were evenly sown in plastic pots filled with an equal weight of quartz sand (diameter approximately 0.3 cm) and incubated in an indoor intelligent artificial climate chamber (BIC800, Shanghai) with a light cycle of 12 h (25°C)/12 h (20°C), and the Hoagland nutrient solution was changed once a week during the incubation. When they were four weeks old, eight even seedlings were retained per pot and subsequently subjected to four experimental conditions (control (CK): Hoagland nutrient solution + 100 g·L⁻¹ PEG-6000, salinity(S): Hoagland’s nutrient solution + 9 g·L⁻¹ NaCl, drought + salinity(D_S): Hoagland’s nutrient solution + 100 g·L⁻¹ PEG-6000 + 9 g·L⁻¹ NaCl). The relevant parameters were measured after 48 h of stress when the variations in the physiological responses of the plants were more pronounced.

Measurement of growth parameters and biochemical staining

The seedlings were selected from each treatment and photographed using a digital camera to observe morphological differences between the treatments. Whole seedlings were washed with distilled water. The fresh weight of the seedlings was measured using absorbent paper to remove water from the seedlings, and the dry weight of the seedlings was measured after drying in an oven at 80°C. The topmost, fully expanded leaves of the seedlings were removed and immediately weighed to determine the FW and SW. This was determined by immersing samples in distilled water and storing them at 4°C in darkness (to minimize respiration losses) until they reached a constant weight, and finally, the leaves were oven dried at 70°C for 24 hours to determine DW. RWC was calculated using the following equation: RWC = (FW-DW)/(SW-DW) × 100% (Flexas et al. 2006). Each measurement was repeated six times (biological repetition).

The samples were washed with distilled water to remove any extraneous material associated with the leaf tissues. Then, the samples were immersed in NBT or DAB staining solution to detect O₂⁻ or H₂O₂, respectively. The procedures for NBT and DAB staining were performed according to Zhang et al. (2011). The samples were photographed with a digital camera.

Determination of photosynthetic parameters and chlorophyll fluorescence parameters

The topmost, fully expanded leaves of similar size were selected to receive uniform illumination. The net Pn, Gs, and Tr were measured using a Li-6400 portable photosynthesis system (Lincoln, United States) at 9:00-10:00 am. The temperature was set at 25°C, flow at 500 μmol·s⁻¹, CO₂ at 400 μmol·mol⁻¹, and light intensity at 1,000 μmol·m⁻²·s⁻¹. After leaves had been dark-adapted for one night, chlorophyll fluorescence parameters, Fv/Fm, ETR and ΦPSII, were determined using an integrating fluorescence fluorometer (LI-COR Biosciences, Lincoln, United States). Each measurement was repeated three times (biological repetition).

Determination of physiological parameters

Chlorophyll content was determined by reference to the method of Sui et al. (2015) using a spectrophotometer to determine absorbance at 645 and 663 nm. Kits produced by Solarbio Science & Technology, Co. Ltd. (Beijing, China) were used to determine the O₂⁻ and H₂O₂ contents and -OH as well as the MDA content. The RMP was measured using an EC215 electrical conductivity meter (DDS-307A, INESA, China). RMP was calculated with the following equation: RMP (%) = S1/S2*100%, where S1 and S2 refer to the conductivity of leaves and boiled leaves, respectively (Gao et al. 2020). The activities of SOD, POD, and CAT were determined using the methods reported in Deng (2015) (Deng et al. 2015). The contents of compounds associated with antioxidant mechanisms, such as the AsA-
GSH, including reduced AsA, DHA, GSH, and GSSG, and the activity of related enzymes, including MDHAR, DHAR, APX, GR, and GPX, were determined using kits produced by Suzhou Comin Biotechnology Co., Ltd. (Jiangsu, China) and Solarbio Science & Technology Co., Ltd. (Beijing, China). Three biological replicates were used for each sample.

**Transcriptome analysis**

Sweet sorghum leaf samples were sent to Shanghai Majorbio (Shanghai, China) for transcriptome analysis. cDNA library construction was performed using the Illumina TruSeq TM RNA Sample Prep Kit. Then, the libraries were paired-end sequenced with the Illumina HiSeq X™ Ten platform.

To checking the quality of the sequenced reads. The raw data were preprocessed on SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle). Mapping of these high-quality reads and transcriptome assembly were carried out by HISAT2 (McCormick et al. 2018; Kim et al. 2019). The expected FPKM number for each gene was calculated based on the length of the gene and the number of reads mapped to that gene (Trapnell et al. 2010). RSEM (Version 1.3.1) was used to quantify gene abundance (Li and Dewey 2011). Differential expression analysis of samples was conducted using the DESeq2 R package (Version 1.2.4) (Love et al. 2014). The adjusted $p$ value for each gene was calculated using the Benjamini & Hochberg (BH) method (Benjamini and Hochberg, 1995). Only those genes with a value of $|\log_{2} \text{fold change}| > 1.0$ and an associated adjusted $p$ value $< 0.05$ were considered as DEGs. Gene expression values from the RNA-Seq data were uploaded to the majorbio cloud platform (https://cloud.majorbio.com/) for this analysis.

**Validation of DEGs by qRT–PCR**

To validate the RNA-seq results, 11 DEGs were selected for quantitative reverse transcription qRT-PCR on three biological replicates of the samples used for RNA-seq. The amplification conditions consisted of a first step of 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 40 s at 72°C. Relative gene expression was calculated with the $2^{- \Delta \Delta CT}$ method using the Hemp actin gene as the internal reference gene to normalize the data (Livak and Schmittgen 2001). To assess the consistency between RNA-seq and qRT-PCR analyses, a linear regression were performed using Microsoft Excel. The experiments were repeated in triplicate. Specific primers for qRT-PCR are listed in Table S1.

**Statistical analysis**

Each experiment was performed three times independently and all data are presented as the mean and standard deviation (SD). The statistical analysis was performed using a T test and Duncan’s tests of one-way analysis of variance (ANOVA) in SPSS 20.0 software (IBM Corp., Armonk, NY, USA), with the significance level set at $P < 0.05$ for all tests. The GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and TBtool (Chen et al. 2020) were used to construct graphs.

**Results**

**Changes in plant phenotype and growth parameters**

Figure 1A shows that sweet sorghum seedlings under drought, salinity, and combined stress exhibited different phenotypic changes. Compared to the control, seedling leaf fresh weight, leaf dry weight and relative water content decreased by 11.38% ($P < 0.05$), 6.95% and 9.02%, respectively, under drought stress, 22.31% ($P < 0.05$), 7.12% and 13.87% ($P < 0.05$), respectively, under salinity stress, and 29.91% ($P < 0.05$), 13.91% ($P < 0.05$), respectively, under combined stress. Moreover, the relative water content decreased by 29.91% ($P < 0.05$), 13.29% ($P < 0.05$), and 20.73% ($P < 0.05$), respectively, and the overall performance of seedling leaves under combined stress was leaf yellowing, especially when some of the old leaves wilted. These results indicate that all three stresses can inhibit the growth of sweet sorghum seedlings, where the degree of inhibition is combined stress > salinity stress > drought stress.

**Changes in ROS content, MDA content, and RMP**

The NBT and DAB staining results showed that the blue spots on the leaves indicated the accumulation of superoxide under stress, and the brown spots on the leaves indicated the accumulation of hydrogen peroxide under stress. The blue and brown spots on the leaves increased slightly under drought stress and increased obviously under salinity stress and combined stress compared with the control, where the area of blue and brown spots under combined stress was more significant than those under salinity stress (Figure 1B-C).

As shown in Figure 1G-I, the O$_2^•$, H$_2$O$_2$, and -OH contents of leaves were slightly (but not significantly) increased under drought stress compared to the control; were increased under salinity stress compared to the control by 133.92% ($P < 0.05$), 59.23% ($P < 0.05$) and 72.38% ($P < 0.05$), respectively, and under combined stress, it increased 187.77% ($P < 0.05$), 127.07% ($P < 0.05$) and 183.79% ($P < 0.05$), respectively, compared to the control. MDA and RMP are important indicators of cell membrane damage. As shown in Figure 1J-K, compared to the control, MDA and RMP of leaves increased by 20.55% and 18.09% under drought stress, 29.91% ($P < 0.05$), and 13.29% ($P < 0.05$), and under combined stress, increased 83.72% ($P < 0.05$), 13.87% ($P < 0.05$), and 6.95% and 9.02%, respectively, under salinity stress and under combined stress, respectively. These results indicate that the combined stress caused the most severe oxidative damage to sweet sorghum seedlings, followed by salinity stress alone, with the lowest oxidative damage caused by drought stress alone.

**Transcriptome sequencing**

To investigate the changes in gene expression in sweet sorghum seedlings under different treatments, RNA-seq was performed on seedling leaves under CK, D, S and D+S treatments, respectively. Twelve cDNA libraries were created by RNA-seq. Twelve samples were filtered with RNA-seq data ranging from 47065594-60983370, with Q30 values exceeding 96.45% and GC content ranging from 54.58%–56.99%. The clean reads of each sample were sequenced against the
designated reference genome separately, and the comparison rate ranged from 95.88% to 96.78% (Table S2).

PCA and Pearson correlation analysis of gene expression between samples showed high correlation coefficients between the treated replicate samples (Figure 2A-B). This finding indicates that the transcriptome sequencing data in this study are of high quality and reproducible between samples, which is suitable for further analysis. To verify the accuracy of gene expression in transcriptome sequencing results, 11 DEGs were randomly selected for qRT–PCR validation under different treatments. Figure 2C shows that the correlation coefficient between qRT–PCR and RNA-Seq results is 0.8853 ($P < 0.0001$), and the results confirm the accuracy of RNA-Seq in this study.

The RNA-Seq results showed that 1897, 3761 and 6285 DEGs were identified under drought, salinity stress and combined stress, respectively (Figure 2D-F). Moreover, we identified 975, 1,714 and 3,200 DEGs that were upregulated and 922, 2,047 and 3,085 DEGs that were downregulated by the three kinds of stress treatments. In addition, Venn analysis revealed the presence of 159, 516, and 2555 specific genes under the three stresses, respectively (Figure 2G).

Changes in chlorophyll content, photosynthetic parameters, chlorophyll fluorescence parameters, and gene expression in the photosynthetic pathway

As shown in Figure 3A-B, both the Chl a and Chl b contents of sweet sorghum seedling leaves under drought stress were slightly reduced compared to the control (but not significantly). Chl a and Chl b under salinity stress were reduced by 18.43% and 16.94%, respectively, compared to the control ($P < 0.05$). Chl a and Chl b under combined stress were reduced by 22.54% ($P < 0.05$) and 36.10%, respectively, compared to the control ($P < 0.05$). Compared with the control, $Pn$, $Gs$, $Tr$, $Fv/Fm$, $ETR$ and $\Phi_{PSII}$ decreased under drought stress, but only $ETR$ and $\Phi_{PSII}$ were significantly different ($P < 0.05$). $Pn$, $Gs$, $Tr$, $Fv/Fm$, $ETR$ and $\Phi_{PSII}$ decreased under salinity stress by 24.57% ($P < 0.05$), 25.74% ($P < 0.05$), 8.07%, 0.27%, 38.41% ($P < 0.05$) and 36.57% ($P < 0.05$), respectively. $Pn$, $Gs$, $Tr$, $Fv/Fm$, $ETR$ and $\Phi_{PSII}$ were reduced by 30.15% ($P < 0.05$), 32.30% ($P < 0.05$), 17.09% ($P < 0.05$) and 0.46% under combined stress compared to the control ($P < 0.05$), 38.41% ($P < 0.05$) and 38.17% ($P < 0.05$) (Figure 3C-H), respectively.
Transcriptome data showed that 47 DEGs were associated with photosynthesis, including 6 DEGs in LHC I, 8 DEGs in LHC II, 10 DEGs in PS II, 2 DEGs in Cyt b6/f, 1 DEG in PC, 10 DEGs in PS I, 5 DEGs in Fd, and 2 DEGs in FNR (Figure 3I). The expression of all 47 DEGs was downregulated by drought, salinity and combined stress compared to the control, with genes downregulated obviously less under combined stress than under single drought or salinity stress, and most genes downregulated less under salinity stress than under drought stress. These results indicated that the highest inhibition of photosynthesis in sweet sorghum seedlings was caused by combined stress, followed by salinity stress, with the lowest impact caused by drought stress.

**Activity of SOD, POD and CAT and related gene expression**

As shown in Figure 4B-D, compared with the control, the SOD, POD and CAT activities of leaves increased by 23.09% ($P < 0.05$), 15.07% and 21.04% ($P < 0.05$) under drought stress; the SOD, POD and CAT activities of leaves increased by 24.17% ($P < 0.05$), 31.51% ($P < 0.05$) and 68.49% ($P < 0.05$) under salinity stress; and the SOD, POD and CAT activities of leaves increased by 24.17% ($P < 0.05$), 31.51% ($P < 0.05$) and 68.49% ($P < 0.05$) under combined stress, while the dual stress decreased CAT activity by 23.06% ($P < 0.05$).

Transcriptome data showed that genes for SOD (SOD[Cu-Zn], SOD[Fe], SOD[Cu-Mn]), POD (POD-2F, POD-2C) and CAT (CAT-1, CAT-2, CAT-3) were upregulated under drought and salinity stress, and most genes had the highest upregulated expression under salinity stress. The SOD and POD genes were upregulated, and the CAT genes were downregulated under combined stress (Figure 4A). The above results indicated that the leaves of sweet sorghum seedlings under drought, salinity, and combined stress could scavenge excess ROS by SOD and POD; CAT scavenging was significantly enhanced under drought or salinity stress imposed separately, while the scavenging ability was significantly reduced under combined stress.

**AsA-GSH cycle and related gene expression**

As shown in Figure 4E-H shows that the APX, AsA, MDHAR and DHAR activities of leaves under drought and salinity stress were significantly increased ($P < 0.05$) compared to the control, with the increase caused by salinity stress being more pronounced than that caused by drought stress. Under combined stress, APX, AsA, MDHAR, and DHAR activities decreased by 25.46% ($P < 0.05$), 20.69% ($P < 0.05$), 29.30% ($P < 0.05$), and 10.60% ($P < 0.05$), respectively, compared to the control, but DHA activity increased by 28.79%. As shown in Figure 3K-O, GSH, GSSG, GR and GPX activities in the leaves increased by 19.85%, 16.59%, 19.32% and 10.79%, respectively, under drought stress compared to the control and increased by 87.54% ($P < 0.05$), 48.50% ($P < 0.05$), 76.85% ($P < 0.05$) and 31.86% ($P < 0.05$) under salinity stress. However, the reduction under the combined stress was 29.88% ($P < 0.05$), 14.23% ($P < 0.05$), 18.12% ($P < 0.05$), and 0.31%, respectively. AsA/DHA increased by 2.97% and 6.19% under drought and salinity stress, respectively, compared to the control and decreased by 33.33% ($P < 0.05$) under combined stress. GSH/GSSG increased by 3.91% and 44.33% ($P < 0.05$) under drought and salinity stress, respectively, compared to the control and decreased by 17.98% ($P < 0.05$) under combined stress.

Transcriptome data showed that genes for APX (APX-1, APX-2, and APX-8) were all upregulated under all three stresses compared to the control, with combined stress causing the highest upregulation and drought stress the lowest upregulation, which was not entirely consistent with the changes in APX activity (Figure 4A). The MDHAR, DHAR, GR, and GPX genes were upregulated to different degrees under drought, salinity, and combined stress.
degrees under all three stresses, and the overall expression of these genes was approximately the same as the enzyme activities (Figure 4A). The above results indicate that sweet sorghum seedlings maintained normal AsA-GSH cycle efficiency under drought stress, enhanced AsA-GSH cycle efficiency under salinity stress, and reduced AsA-GSH cycle efficiency in seedlings under combined stress.

**Discussion**

**Effects of drought, salinity and combined stress on the growth and photosynthesis of sweet sorghum seedlings**

Growth and morphological changes are the most visual indication of the magnitude of plant injury by adversity stress. The results of this study showed that the fresh weight, dry weight, and relative water content of seedling leaves under drought or salinity stress alone were reduced compared to the control. All seedling growth parameters under salinity stress were more affected than those under drought stress, indicating that salinity stress was more detrimental to seedling growth than drought stress. The results also showed that the growth parameters of seedlings under combined stress were significantly lower than those of the control and were all smaller than those of single drought or salinity stress, indicating that the combined stress is more detrimental to sweet sorghum seedling growth than single drought or salinity stress. This indicates morphological changes in sweet sorghum seedlings in response to the combined treatment exhibiting antagonistic effects between the two stresses. This is consistent with the results of most studies (Álvarez and Sánchez-Blanco 2015; Sahin et al. 2018). In this study, transcriptome sequencing revealed that the number of DEGs under combined stress was 6,285, while 3,761 DEGs were found under salinity stress, and 1,897 DEGs were found under drought stress (Figure 2D-F). Previous studies have suggested a possible link between the number of responsive genes and the complexity and intensity of their applied stress treatments (Osthoef et al. 2019). For example, experiments in which soybeans were exposed to different levels of water deficit showed that more severe
stress treatments resulted in an increase in the number of DEGs (Song et al. 2016). Thus, combined stress had a more significant effect on the overall transcriptome changes in sweet sorghum seedling leaves than single drought or salinity stress alone.

Excess electrons generated by photosynthetic and respiratory electron transport chains can attack intracellular free O2, increasing O2 production. Inhibiting CO2 fixation and photophosphorylation can also cause a loop in electron transport, resulting in an ROS outbreak. Excess ROS can obstruct the normal flow of photosynthetic electrons by attacking electron transporters, impairing plants’ capacity to photosynthesize (Mittler et al. 2004; Ruban et al. 2012; Jia et al. 2020; Zhang et al. 2020). There were no significant changes in the chlorophyll content, Pn, Gs, ETR, or ΦPSII and significant increases in O2- content under salinity stress. In addition, significant reductions in chlorophyll content, photosynthetic parameters, and chloroplast fluorescence parameters were also noticed. The O2- content was significantly increased under combined stress, implying that ΦPSII activity was decreased, electron transport in ΦPSII was blocked, and photosynthetic carbon assimilation and respiration were inhibited. Meanwhile, transcriptome analysis of the expression of photosynthesis-related genes was consistent with the results of the photosynthetic efficiency of seedlings measured by the photosynthetic apparatus. The above results indicated that combined stress had the highest inhibition of photosynthetic capacity of sweet sorghum seedlings, followed by salinity stress, with the least impact caused by drought stress alone.

Figure 4. SOD activity, POD activity, CAT activity, AsA-GSH cycle, and related gene expression. A: Heatmap of the expression of genes related to the AsA-GSH cycle; B: SOD activity; C: POD activity; D: CAT activity; E: APX activity; F: AsA content; G: MDHAR activity; H: DHA content; I: DHAR activity; J: AsA/DHA; K: GSH content; L: GSSG content; M: GR activity; N: GSH/GSSG; O: GPX activity.
Role of SOD, POD and CAT in ROS scavenging by sweet sorghum seedling leaves under drought, salinity, and combined stress

SOD is considered the first line of enzymatic defense against oxidative stress in plants and is present in every cell. The primary function of this enzyme is to convert or dissociate toxic O$_2$ into H$_2$O$_2$ and molecular oxygen; thus, SOD has a pivotal role in ROS detoxification by affecting the levels of both O$_2$ and H$_2$O$_2$ and preventing toxicity associated with O$_2$ (García-Caparrós et al. 2021). In plants, SODs have been classified into three groups based on the type of prosthetic metal: Cu/Zn-SOD, Mn-SOD and Fe-SOD (Wang et al. 2017). Overexpression of the SOD gene (EC: 1.15.1.1) has been shown to enhance ROS scavenging in Arabidopsis (Wang Y. et al. 2021), wheat (Wang et al. 2016), cotton (Zhang et al. 2021), and tomato (Sun et al. 2020) under stress. Wang et al. (2021) found that the SOD activity of walnut (Juglans regia L.) seedling leaves was significantly elevated under drought, salinity, and combined stress, and the SOD activity under combined stress was significantly higher than that under single drought or salinity stress alone. The SOD activities of sweet sorghum seedling leaves under drought, salinity, and combined stress were significantly higher than those of the control, and Cu/Zn-SOD, Mn-SOD and Fe-SOD showed significantly upregulated expression (Figure 4A-B) in the study. This indicates that the SOD of seedling leaves can eliminate O$_2$ production in response to the three stresses. There was no significant change in the O$_2$ content of seedling leaves under drought stress, but the O$_2$ content of seedling leaves under salinity and combined stress was significantly higher than that of the control (Figure 1G). These results indicate that SOD of sweet sorghum seedling leaves under drought stress can effectively scavenge O$_2$ and that the efficiency of O$_2$ production under salinity and combined stress is greater than the scavenging ability of SOD, which in turn leads to excessive accumulation of O$_2$.

H$_2$O$_2$ is the most stable ROS transported actively across membranes by aquaporins. Low concentrations of H$_2$O$_2$ can act as signaling molecules to enhance plant tolerance to adversity, while excess H$_2$O$_2$ can damage DNA, proteins, carbohydrates and lipids in cells, causing progermed cell death (Cuypers et al. 2016; García-Caparrós et al. 2021). H$_2$O$_2$ can also generate highly reactive and destructive -OH through Haber–Weiss or Fenton reactions, which can reduce the function of PSII by destroying the constituent subunits of the oxygen-emitting complex and hindering its turnover (Zhang et al. 2020). H$_2$O$_2$ scavenging in plant cells is dependent on POD and CAT and APX and GPX in the AsA-GSH cycle (Foyer et al. 2017; Rahantaniaina et al. 2021). It has been reported that adversity stress can induce POD overexpression in tomato (Manaa et al. 2011), mulberry (Zhang et al. 2020) and rice (Cheng et al. 2009). Changes in CAT activity are usually associated with the establishment of oxidative stress conditions, and overproduction of ROS leads to reduced CAT activity in plants (Borges et al. 2018; Soares et al. 2019). In this study, the genes encoding POD were slightly upregulated in the leaves of sweet sorghum seedlings under drought stress, and there was no significant change in POD activity. However, the genes encoding CAT were upregulated in expression and significantly elevated CAT activity. Both genes encoding POD and CAT were upregulated in seedling leaves under salinity stress, and both POD and CAT activities were significantly elevated. The expression of POD activity and related genes was significantly increased in seedling leaves under combined stress, but the expression of CAT-1 and CAT-2 genes was downregulated, and CAT activity was significantly decreased. This may be because the ROS content of seedling leaves under combined stress was significantly higher than that under drought stress or salinity stress alone, thus explaining the reduction in CAT activity and expression in sweet sorghum seedling leaves under combined stress. Based on these results, we propose that sweet sorghum seedlings under drought stress can scavenge H$_2$O$_2$ through the CAT pathway, and seedlings under salinity stress can scavenge H$_2$O$_2$ through the POD and CAT pathways, while the CAT activity of seedling leaves under combined stress is inhibited and POD has an enhanced ability to scavenge H$_2$O$_2$.

Role of the AsA-GSH cycle in ROS scavenging from sweet sorghum seedling leaves under drought, salinity and combined stress

In plant cells, the AsA-GSH cycle is the primary antioxidant defense pathway to detoxify H$_2$O$_2$ (Rahantaniaina et al. 2017). In the AsA-GSH cycle, APX catalyzes the disassociation of H$_2$O$_2$ to H$_2$O and MDHA by using the reducing power from AsA. Therefore, APX has a vital role in ROS scavenging and defense in higher plants (García-Caparrós et al. 2021). APX gene overexpression has enhanced abiotic stress tolerance in Arabidopsis (Guan et al. 2015) and rice (Zhang et al. 2013). Verma et al. (2022) showed that drought and salinity stress could induce the expression of APX-related genes in mustard and oilseed rape. APX is sensitive to excess ROS generated under extreme adversity and is susceptible to blunting, and severe oxidative stress significantly reduces APX activity (Viaea Dos Santos and Rey 2006; Zhang et al. 2020). Sharma and Dubey (2005) found that the APX activity of rice seedlings increased under moderate drought stress but significantly decreased under severe drought stress (Sharma and Dubey 2005). In this study, APX-related genes were found to be upregulated in the leaves of sweet sorghum seedlings under both drought and salinity stress, and APX activity was significantly increased, indicating that the ability of APX to scavenge H$_2$O$_2$ increased under drought or salinity stress. However, the APX activity in the leaves of seedlings under combined stress was significantly lower than that of the control, indicating that the ability of APX to scavenge H$_2$O$_2$ was reduced under combined stress, which may be due to the overproduction of ROS in seedling leaves under combined stress. However, the expression of APX in the leaves was significantly increased. Mano et al. (2001) proposed that excessive accumulation of H$_2$O$_2$ and lack of AsA may lead to APX inactivation. Therefore, the decrease in APX activity and the increase in APX expression under combined stress may be associated with higher H$_2$O$_2$ content and lower AsA/DHA ratio. This is similar to the results of Zhang et al. (2020) in mulberry seedlings.

In the AsA-GSH cycle, AsA acts as an electron donor for APX to scavenge H$_2$O$_2$, and the oxidized MDHA can be reduced by MDHAR to AsA or by DHAR to DHA to reduce AsA, thus regenerating AsA (Meng et al. 2021). Stevens et al. (2008) showed that MDHAR activity has an essential role in
regulating AsA content. Sharma et al. (2012) found that DHAR activity contributes to the regulation of cellular redox homeostasis and promotes AsA regeneration. Overexpression of the MDHAR and DHAR genes can improve tobacco tolerance to drought and salinity stress (Eltayeb et al. 2006; Eltayeb et al. 2007). In this study, sweet sorghum seedling leaf MDHAR and DHAR activities were significantly higher under drought and salinity stress and significantly lower under combined stress. Similar results were observed for the genes encoding MDHAR and DHAR changes. Among them, only the change in DHAR activity was the same as the change in AsA content. Thus, these data suggest that elevated DHAR activities can increase the AsA content in the leaves of sweet sorghum seedlings. Additionally, we found that AsA, DHA and their reducing power (AsA/DHA) in sweet sorghum seedling leaves were significantly elevated under drought and salinity stress but decreased under combined stress. Based on these results, we propose that sweet sorghum seedlings under drought and salinity stress mitigate the damage to seedlings by maintaining a high AsA content and reducing power (AsA/DHA). In contrast, the combined stress caused more severe damage to seedlings, resulting in reduced AsA and DHA synthesis in seedling leaves, decreased reducing power and an inability to perform normal protective functions.

GR is a relevant component of the AsA-GSH cycle that catalyzes the reduction of GSSG to GSH, thereby maintaining GSH levels and the GSH/GSSG ratio (Hasanuzzaman et al. 2019; Zhang et al. 2020). GPX catalyzes the production of GSSG from GSH and promotes the catabolism of H2O2, thereby reducing lipid and organic hydrogen peroxide accumulation (Bela et al. 2015; Hasanuzzaman et al. 2019). Previous studies showed that GR overexpression resulted in plant cells exhibiting higher GSH levels and GSH/GSSG ratios, which enhanced stress tolerance in plants (Yin et al. 2017). ElSayed et al. (2021) found that salinity stress could increase GR activity in Phaseolus vulgaris L. leaves, reducing ROS-induced oxidative damage. GPX gene expression and GPX activity are usually increased under oxidative stress conditions (Bela et al. 2015). However, it has also been shown that high salinity treatment significantly inhibits the expression of GPX activity and its related genes in plants (Zheng et al. 2016; Aazami et al. 2021). The present research showed that the GR and GPX activities of sweet sorghum seedling leaves did not change significantly under drought stress, while they were significantly increased under salinity stress, GR activity was significantly reduced, and GPX did not change significantly under combined stress. Both GR and GPX genes were upregulated in seedling leaves under drought or salinity stress, with the upregulation under salinity stress being higher than that under drought stress. However, GR and GPX gene expression was downregulated in seedling leaves under combined stress. Based on these findings, we hypothesize that drought-stressed sweet sorghum seedlings can maintain the stability of GSH/GSSG by modulating GR and GPX activities. Under salinity stress conditions, seedlings control GSH/GSSG levels by drastically increasing GR and GPX activities and associated gene expression to fight oxidative damage. ROS and lipid peroxidation levels were significantly increased in seedling leaves under combined stress. This results in a decrease in GR and GPX activity and a significant decrease in GSH/GSSG levels in seedling leaves, resulting in a significant decrease in seedling leaves’ antioxidant capacity, which is severely impaired.

Conclusions
In conclusion, combined stress resulted in significantly more severe oxidative damage in sweet sorghum seedling leaves than the single stress of drought or salinity. No significant accumulation of ROS products was observed in the leaves of sweet sorghum seedlings under drought stress. This may be due to increased SOD and CAT activities in seedlings, expression of their related genes, and the AsA-GSH cycle continuing to function efficiently. Under salinity stress, the antioxidant enzyme activities and AsA-GSH cycle function in sweet sorghum seedlings were significantly enhanced but the scavenging of ROS was not adequate, and accumulated ROS caused damage to the seedlings. Under combined stress, although the SOD and POD activities of sweet sorghum seedlings were increased, CAT activity and the AsA-GSH cycle were significantly inhibited, leaving the antioxidant mechanisms in the seedlings unable to maintain their normal protective role and allowing ROS to accumulate in large quantities that caused the seedlings to be severely damaged.

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Data availability
The raw sequence data of the libraries are accessible at NCBI under bioprojects (PRJNA664417).

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