SALICYLIC ACID PRIMING PROMOTES SORGHUM GERMINATION UNDER DROUGHT STRESS: EVIDENCE FROM COMPARATIVE METABOLOMICS ANALYSIS

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Abstract. Drought can significantly hinder seed germination and seedling emergence, even in drought-tolerant crops like sorghum. Seed priming can improve seedling emergence under drought stress. Here, we sought to elucidate the mechanism by which seed priming with salicylic acid (SA) improves the drought tolerance of sorghum seedlings. We studied changes in morphology, antioxidant status, and primary metabolites in response to drought stress in both primed and unprimed seedlings. The experiment consisted of three treatment groups: normal germination (CK), unprimed seeds germinated under drought stress (D), and 0.1 mM SA-primed seeds germinated under drought stress (D-SA). The results showed that drought stress severely restrained sorghum seed germination, although SA mitigated the effects of drought stress. Compared to D, the shoot and root length increased 3.72-fold and 4.28-fold in D-SA, respectively. SA priming significantly increased the antioxidant enzyme activity and decreased the content of malondialdehyde (MDA) and H_2O_2 in sorghum seedlings under drought stress. A total of 518 metabolites were identified by ultra-high-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS), including 122 phenolic acids, 119 lipids, 89 amino acids and derivatives, 70 organic acids, 52 nucleotides and derivatives, and 66 others. Enrichment analysis showed that these metabolites were mainly involved in the stilbene, diarylheptane, and gingerol biosynthesis and flavonoid biosynthesis pathways. Among 68 core metabolites, phenolic acids and other substances within the phenylpropane biosynthesis pathway were enriched by seed priming with SA, including coniferin, chlorogenic acid, and sinapinaldehyde, among others. The phenolic acids may be the core metabolites influenced by exogenous SA, and may be at least partly responsible for the increased drought resistance exhibited by primed sorghum seedlings. The results of this study present an important step toward understanding the mechanism by which seed priming with SA increases the drought resistance of sorghum seedlings. It should be pointed out that the effect of SA priming on drought resistance of sorghum at other growth stages still needs further research.
Keywords: *sorghum, salicylic acid, seed priming, drought stress, metabolomics*

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a staple food for millions of people worldwide, particularly for those living in arid and semi-arid locations (Chadalavada et

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al., 2021). However, although sorghum is generally considered drought tolerant, the germination stage can be particularly sensitive to drought (Li et al., 2009). In most agricultural systems, timely and uniform germination and seedling emergence are key determinants of crop yield (Ashraf and Foolad, 2005). For example, in China, insufficient soil moisture during spring sowing can lead to low germination rates, extended emergence times, and uneven emergence in sorghum. Because drought conditions occurring during germination can significantly limit sorghum production (Wu et al., 2016), improving the drought resistance of sorghum during germination is crucial to ensure food security.

Seed priming is a technique used to improve seed water uptake and reduce dryout (Heydecker, 1974). This technique is very promising for both enhancing abiotic stress tolerance and stabilizing germination rate (Wang et al., 2016). Because seed priming is economical, convenient, and efficient, this technique has been widely utilized to mitigate drought stress during germination (Gammoudi et al., 2020; Hameed et al., 2020; Ocvirk et al., 2021; Rhaman et al., 2020). Recent research into the mechanism of seed priming-induced drought resistance suggests that seed priming can improve nutrient mobilization, induce osmolyte accumulation (e.g., glycine betaine, proline, polyols, and sugars), and enhance the activity of endogenous antioxidant enzymes (e.g., superoxide dismutase [SOD] and peroxidase [POD]) (Khan et al., 2021; Marthandan et al., 2020; Sharma et al., 2020). In addition, seed priming also promotes pre-germination metabolic processes in preparation for radicle protrusion, repairs membranes, develops immature embryos, and reduces physical resistance during embryo growth (Ibrahim and Ehab, 2016).

Because it provides an unbiased, sensitive, and accurate characterization of the total metabolite pool of a plant tissue, metabolomics has been widely applied in studies of plant-environment interactions. Furthermore, metabolomic technologies allow the comprehensive analysis of plant responses to stress in order to gain insight into stress resistance mechanisms (Wang et al., 2021, 2019). Specifically, metabolomic studies have revealed some of the physiological mechanisms of plant resistance to drought stress. For example, Zhao et al. analyzed the metabolite profiles of drought-stressed Jerusalem Artichoke (*Helianthus tuberosus* L.) seedlings and found that the processes of glycolysis, phenolic metabolism, tricarboxylic cycle, glutamate-mediated proline biosynthesis, urea cycle, amino acid metabolism, unsaturated fatty acid biosynthesis, and met salvage pathway are all involved in the plant response to drought stress (Zhao et al., 2021). Sun et al. found that exogenous fulvic acid significantly enhanced the drought tolerance of tea plants (*Camellia sinensis* (L.) O. Kuntze) by improving ascorbic acid metabolism, glutathione metabolism, and flavonoid biosynthesis (Sun et al., 2020).

Although it is clear that metabolomic analysis is an effective method for studying crop responses to drought stress, this technique has not been extensively employed to study the metabolic responses of crops to seed priming. Our previous work suggests that exogenous salicylic acid (SA) can improve the drought resistance of sorghum (Zhang et al., 2022). However, SA-induced stress resistance involves an array of complex physiological processes (Jisha et al., 2013), many of which are poorly characterized. Here, we have sought to test the hypothesis that SA priming improves the drought tolerance of sorghum seedlings by regulating antioxidant metabolism and secondary metabolite biosynthesis. Specifically, we evaluated the morphological, metabolic, and endogenous antioxidant responses of drought-stressed sorghum seedlings to seed

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priming with SA. The results of this study will improve our understanding of the effects of seed priming with salicylic acid on the drought tolerance of sorghum seedlings, and will be applicable to the sustainable production of this important staple crop in arid and semi-arid regions.

Materials and methods

Seed materials and growth conditions

The experiment was carried out in the laboratory of the Industrial Crops Research Institute of Shanxi Agricultural University (China) in 2021. The sorghum used in this study, 'Jinza 22'(JZ22), was developed and provided by the Shanxi Agricultural University Sorghum Research Institute. This study consisted of three treatment groups: normal germination (CK), unprimed seeds germinated under drought stress (D), and 0.1 mM SA-primed seeds germinated under drought stress (D-SA). To simulate drought stress, 150 mM polyethylene glycol (PEG) was used. The experiment used a completely randomized design, with three replicates per treatment, and 50 seeds per plate.

Only sorghum seeds with full grains and uniform size were used for germination experiments. Exactly 150 pre-screened seeds were randomly selected, sterilized for 5 min with 0.5% sodium hypochlorite, and then rinsed five times with distilled water. For the D-SA group, the sterilized seeds were treated with 0.1 mM SA at a 1:5 (w/v) ratio and incubated in the dark for 10 h at 25°C, stirring gently 3-4 times during the incubation period. Subsequently, the seeds were quickly air-dried to their original moisture content (13%, w/w). The CK and D group seeds were sterilized in the same manner and placed in petri dishes (15 cm diameter) with double a layer of sterile filter paper and 10 ml of either distilled water or 150 mM PEG, respectively. The petri dishes were placed in a dark incubator at 25 ± 1°C and 70% relative humidity. The 150 mM PEG solution in the petri dishes was refreshed daily to maintain a stable concentration.

Morphological index determination

72 h after treatment, 5 sorghum seedlings were randomly selected from each petri dish for determination of shoot and root length. Three biological replicates were samples from each treatment, for a total of nine samples. Samples were immediately flash-frozen in liquid nitrogen and stored in 15 mL cryovials at -80°C prior to further analyses.

Antioxidant activity determination

The method of Loreto et al. (Loreto and Velikova, 2001) was utilized to measure the hydrogen peroxide (H_2O_2) content of seedlings, which was recorded as μmol g^{-1} fresh weight (FW). The trichloroacetic acid (TCA) and thiobarbituric acid (TBA) method of Yadav et al. (2020) was utilized to measure the malondialdehyde (MDA) content of seedlings, which was recorded as mmol g^{-1} FW. The method of Zhang et al. (2019) was utilized to measure the SOD activity of seedlings, with one unit (U) of SOD activity defined as the amount that inhibited the photoreduction of nitro blue tetrazolium (NBT) by 50%. The method of Wang et al. (2018) was utilized to measure the POD activity of seedlings, and was recorded as U min^{-1} g^{-1} FW.

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Extraction of primary metabolites

Metabolite extraction, identification, and quantification were performed at MetWare Biotechnology Co., Ltd (www.metware. cn). Briefly, samples were vacuum freeze dried with a biological lyophilizer (Scientz-100F) and the lyophilized samples were ground to a powder at 30 Hz using a grinder (MM 400, Retsch). Each 100 mg subsample of power was dissolved in 1.2 mL of 70% methanol, vortexed 6 times for 30 s at 30 min intervals, and places in a 4°C refrigerator overnight. The next day, the samples were centrifuged (12,000 rpm for 10 min), the supernatant was aspirated, and the samples were filtered through a microporous membrane (0.22 μm pore size) and subsequently stored in a sample vial for UPLC-MS/MS analysis.

Chromatography and mass spectrometry

Metabolites were analyzed using Ultra Performance Liquid Chromatography (UPLC) (SHIMADZU Nexera X2, https://www.shimadzu.com.cn/) and Tandem Mass Spectrometry (MS/MS) (Applied Biosystems 4500 QTRAP, http://www.appliedbiosystems.com.cn/).

For UPLC, an Agilent SB-C18 chromatographic column (1.8 μm, 2.1 mm × 100 mm) was used with ultrapure water (with 0.1% formic acid) as the mobile phase A and acetonitrile (with 0.1% formic acid) as phase B. At 0.00 min, the elution gradient consisted of 5% phase B. The proportion of phase B increased linearly to 95% within 9.00 min, and maintained at 95% for 1 min. From 10.00-11.10 min, the proportion of phase B decreased to 5%, with the 5% equilibration continuing to 14 min. The flow rate was 0.35 mL min^{-1}, the column temperature was 40°C, and the injection volume was 4 μL.

For MS, linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on an AB4500 triple quadrupole linear ion trap mass spectrometer (Q TRAP) UPLC/MS/MS system equipped with an ESI Turbo. The ion spray interface was controlled by Analyst 1.6.3 software (AB Sciex) to run positive and negative ion modes. The ESI source operating parameters were as follows: ion source, turbo spray; source temperature, 550°C; ion spray (IS) voltage, 5500 V (positive ion mode)/-4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set to 50, 60, and 25.0 psi, respectively; collision-induced ionization parameter was set to high. Instrument tuning and mass calibration were performed with 10 and 100 μmol L^{-1} PEG solutions in QQQ and LIT modes, respectively. QQQ scans used multiple reaction monitoring (MRM) mode and the collision gas (nitrogen) was set to medium. Through further declustering potential (DP) and collision energy (CE) optimization, the DP and CE of individual MRM transitions were completed. A specific set of MRM transitions was monitored in each epoch based on the metabolites eluted in each epoch.

Metabolomic data analysis

Mass spectral data processing was performed using Analyst 1.6.3 software (AB Sciex). Quantitative and qualitative analysis of metabolites based on MS was performed using the MetWare Biotechnology metabolic database. In order to maximize the distinction between groups and thus aid in identifying differential metabolites, partial least squares discriminant analysis (OPLS-DA) was used to analyze metabolomic data. The OPLS-DA analysis was performed by the metabo analyst R package in R ver. 1.0.1. Based on the OPLS-DA results, the obtained multivariate analysis of the OPLS-DA model's Variable Importance in Projection (VIP) was used to preliminarily screen out

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differential metabolites. The differential metabolites were further screened by combining the p-value or the fold change (FC) value of the univariate statistical analysis. Metabolites with FC ≥ 2 and ≤ 0.5, and VIP ≥ 1, were considered significantly different. Principal component analysis (PCA) was performed by the base package in R ver. 3.51. Hierarchical cluster analysis (HCA) was performed by the complex heatmap package in R ver. 2.8.0. Differential metabolite volcano graphs and differential metabolite bar graphs were plotted by the gglot2 package in R ver. 3.3.20. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed by base package and gglot2 package in R ver. 3.3.0.

Statistical analysis

All morphological data analyses were performed using Microsoft Excel and SPSS ver. 19.0 (SPSS Inc., USA) via ANOVA followed by Duncan's significant difference test at the $p \leq 0.05$ level. The results were expressed as the mean ± standard deviation (SD) of three biological replicates. Graphing was performed using Origin 8.0 (OriginLab, USA).

Results

SA improves drought stress-induced morphological abnormalities

Overall, drought stress severely impaired sorghum germination (*Fig. 1*). Under drought conditions, shoot length and root length were decreased by 69.06% and 46.27%, respectively, compared to CK. However, the effects of drought stress were mitigated by seed priming with SA. Compared to D, seedling height and root length increased 3.72-fold and 4.28-fold in the D-SA group.

SA improves the antioxidant status of drought-stressed seedlings

To examine the effect of seed priming with SA on drought-induced oxidative stress, we measured the contents of H_2O_2 and MDA in sorghum seedings. Drought stress increased the accumulation of H_2O_2 and MDA in sorghum seedings by 340.22% and 203.79%, respectively, compared to CK (*Fig. 2A, B*). However, seed priming with SA significantly reduced the contents of H_2O_2 and MDA. Compared with D, the contents of H_2O_2 and MDA decreased by 50.82% and 42.12%, respectively, in the D-SA group. Furthermore, compared with CK, the activities of SOD and POD increased by 44.45% and 18.05%, respectively, in the D-SA group.

Metabolite profiling of sorghum seedlings

To study the effect of seed priming with SA on the metabolome of drought-stressed sorghum seedlings, we performed metabolomic analyses on germinated seeds and seedlings. Metabolite profiling was performed with three biological replicates on a total of nine samples. UPLC (Shim-pack UPLC SHIMADZU CBM30A, http://www.shimadzu.com.cn/) and MS/MS (Applied Biosystems 6500 QTRAP, http://www. appliedbiosystems.com.cn/) were used to obtain mass spectrometric data. Based on a combination of the self-established Metware database (MWDB), the samples were qualitatively and quantitatively analyzed. A total of 518 metabolites were identified, including 122 phenolic acids, 119 lipids, 89 amino acids and derivatives, 70 organic acids, 52 nucleotides and derivatives, and 66 others (*Table 1*).

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Table 1. *Metabolite profile of sorghum seedlings*

Metabolite category	Number	Percentage (%)
Phenolic acids	122	23.55
Lipids	119	22.97
Amino acids and derivatives	89	17.18
Organic acids	70	13.51
Nucleotides and derivatives	52	10.04
Others	66	12.74

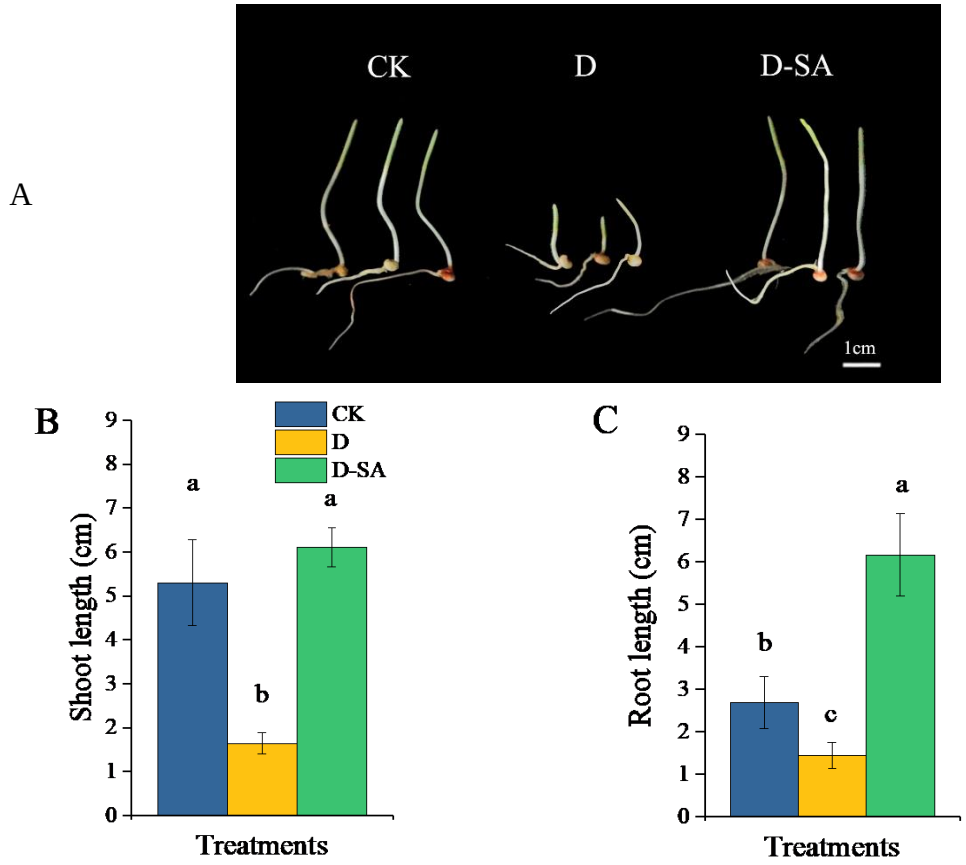

Figure 1. *Germination characteristics of 'JZ22' sorghum under normal and drought-stressed conditions. (A): Germination phenotype of 'JZ22' sorghum under different treatments for 72 h of germination. (B): Differences in shoot length of 'JZ22' sorghum under different treatments. (C): Differences in root length of 'JZ22' sorghum under different treatments. CK stands for normal germination, D stands for unprimed seeds germinated under drought stress, and D-SA stands for 0.1 mM SA-primed seeds germinated under drought stress. Columns with different letters were significantly different at the $p < 0.05$ level*

Multivariate analysis of primary metabolites

Multivariate statistical analysis can reduce and simplify high-dimensional and complex data while retaining the original data to the utmost extent. Here, PCA was performed with several principal components (PCs) to reflect the multidimensional metabolomics data. Overall, the first PC explained 57.73% of the variation, while the

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second PC explained 20.41% (*Fig. 3A*). In the PCA, duplicate quality control (QC) samples were projected to the same area, indicating that they had similar metabolic profiles and that the analysis was stable and repeatable. In order to further illustrate the differential distribution of metabolites across samples, a clustering heat map was created (*Fig. 3B*). Drought stress (D) resulted in decreased accumulation of metabolites, while seed priming with SA (D-SA) increased the accumulation of metabolites. Both the PCA and HCA demonstrated a clear separation of the treatments into three different groups. These results illustrate that each treatment group was characterized by a distinct metabolite profile, which was consistent with the phenotypic analysis.

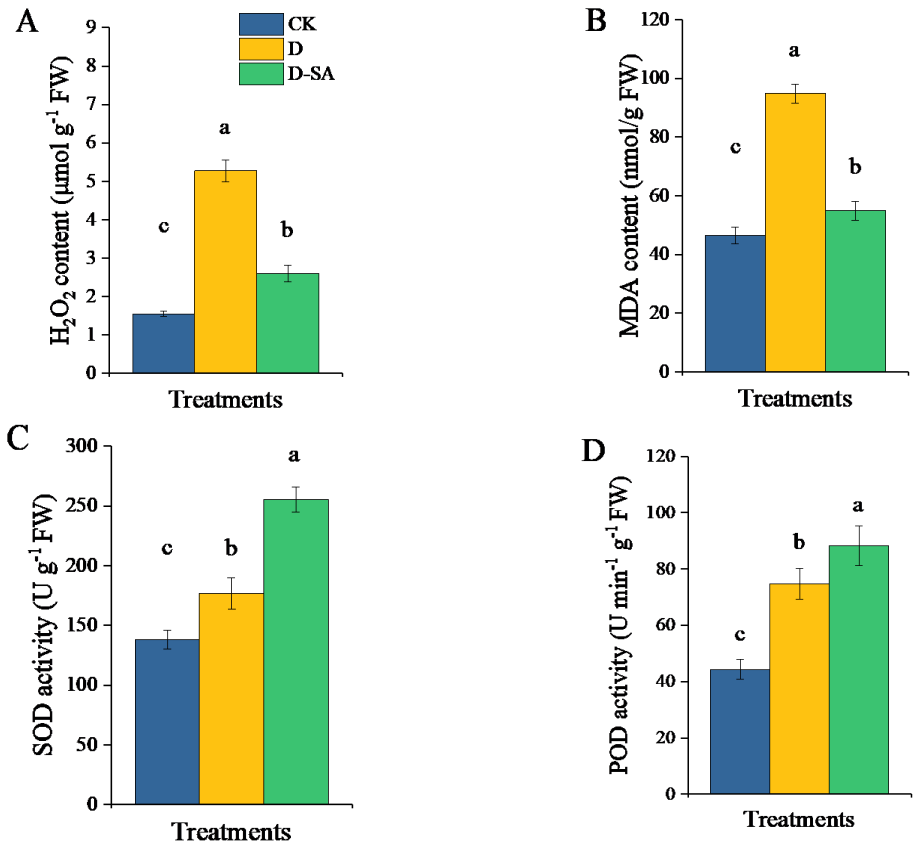

Figure 2. *Effects of SA priming on the contents of H_2O_2 (A) and MDA (B), and the enzymatic activities of SOD (C) and POD (D), in sorghum seeding after 72 h of germination under different treatments. CK stands for normal germination, D stands for unprimed seeds germinated under drought stress, and D-SA stands for 0.1 mM SA-primed seeds germinated under drought stress. Columns with different letters were significantly different at the $p < 0.05$ level*

Differential metabolite analysis

OPLS-DA is a multivariate statistical analysis method with supervised pattern recognition which can effectively eliminate irrelevant influences and screen differential metabolites. According to the OPLS-DA model, the metabolite profiles of the CK vs D ($Q^2 = 0.995$, $P < 0.005$) and D vs D-SA ($Q^2 = 0.979$, $P < 0.005$) groups were significantly different (*Fig. 4A, B*). We further performed differential metabolite screening among all 518 metabolites based on the FC (≥2 or ≤ 0.5) and VIP (>1) scores,

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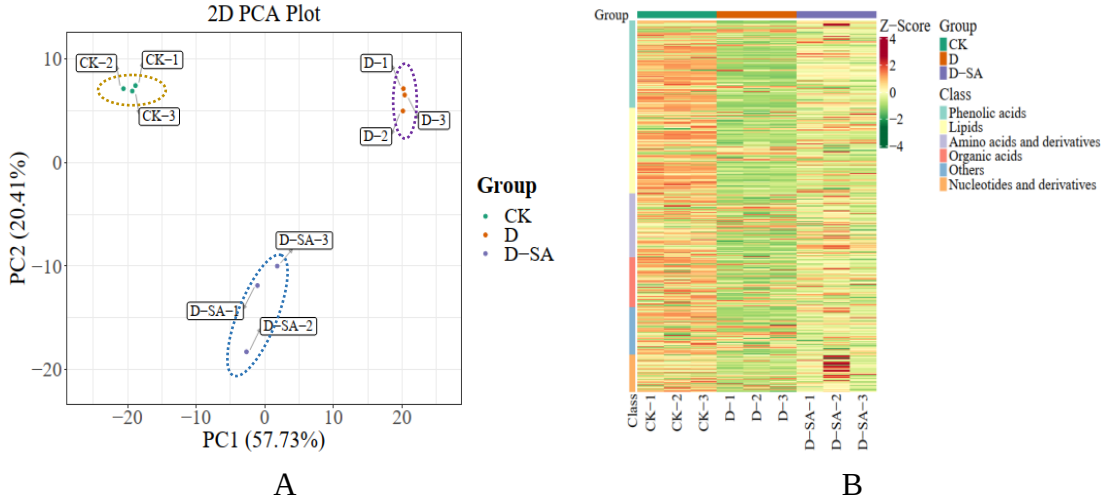
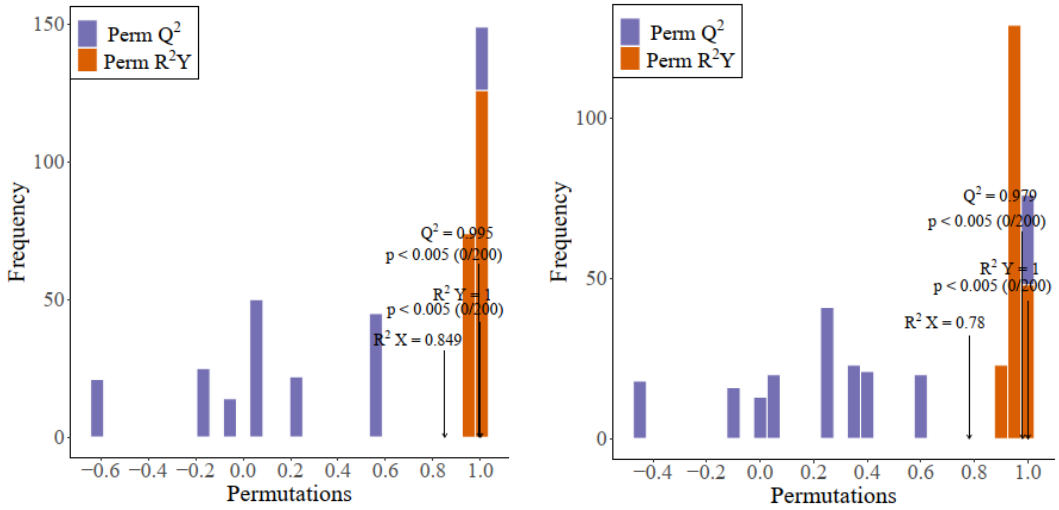
and presented the results as volcano plots (*Fig. 4C, D*). A total of 158 differential metabolites were identified between CK vs D, including 22 which were significantly upregulated and 136 which were significantly downregulated. A total of 75 differential metabolites were identified between D vs D-SA, including 64 which were significantly upregulated and 11 which were significantly downregulated. The top 20 FC metabolites are shown in *Figure 4E* and *F*. Among these, the content of isoascorbic acid changed the most in the CK vs D and D vs D-SA groups.



A B

Figure 3. *Multivariate analysis of primary metabolites of sorghum seedlings under different treatments. (A): PCA. (B): Heatmap visualization of metabolites. CK stands for normal germination, D stands for unprimed seeds germinated under drought stress, and D-SA stands for 0.1 mM SA-primed seeds germinated under drought stress. The content of each metabolite was normalized to complete linkage hierarchical clustering. Each example is visualized in a single column and each metabolite is represented by a single row. Red indicates high abundance, whereas green indicates low abundance (color key scale shown at right of the heat map)*



CK VS D D VS D-SA

A **B**

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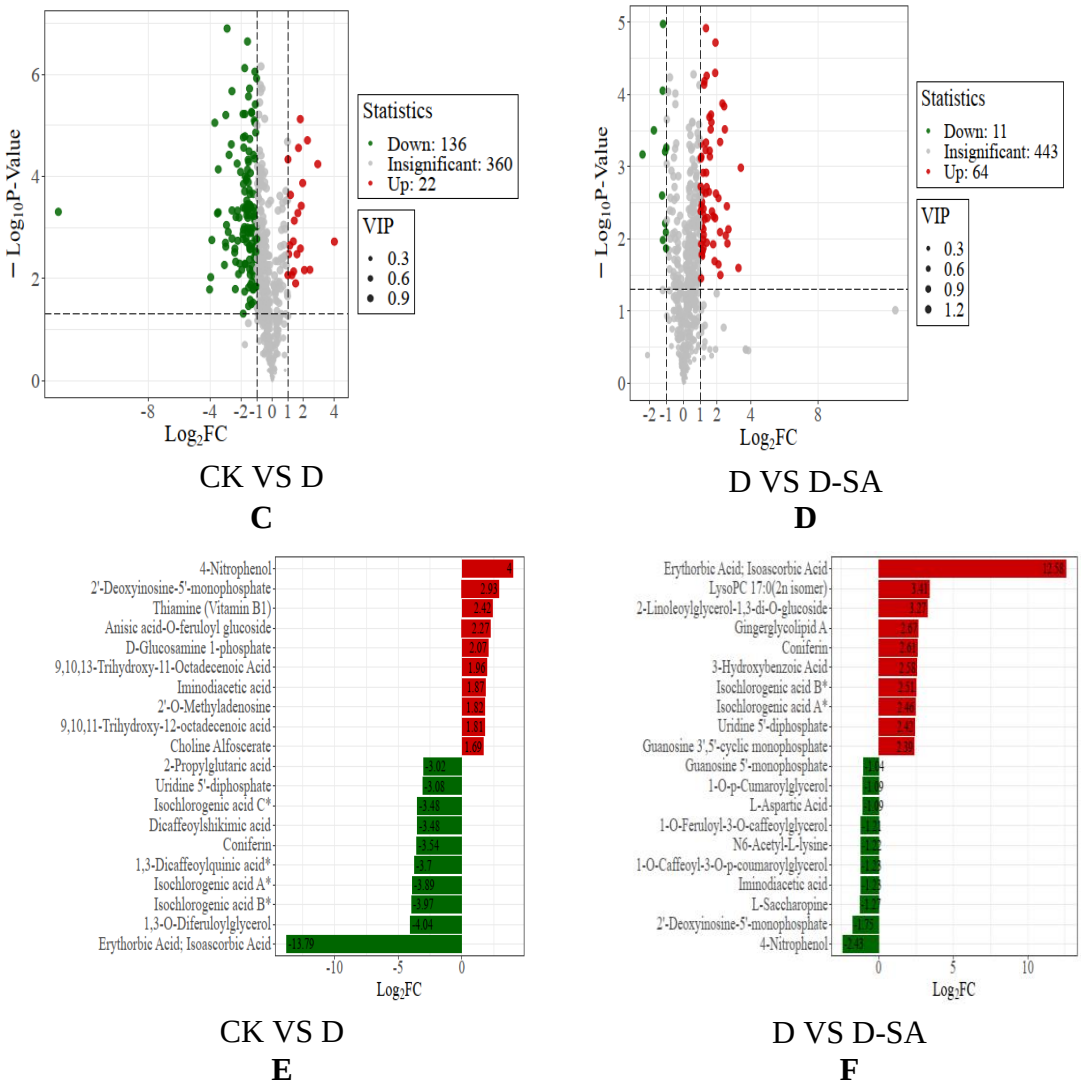

Figure 4. *Analysis of differential metabolites of sorghum seedlings under different treatments. (A, B): OPLS-DA. Note: The model is best when $p < 0.05$. (C, D): Differential metabolite volcano plots. Note: CK stands for normal germination, D stands for unprimed seeds germinated under drought stress, and D-SA stands for 0.1 mM SA-primed seeds germinated under drought stress. Each point in the volcano plot represents a metabolite. Upregulated metabolites are shown in green, downregulated metabolites are shown in red, and metabolites with no change are shown in grey. The abscissa represents a certain metabolite. In the logarithm of the relative content difference (log_2FC) of the metabolite between the two groups, the larger the absolute value of the abscissa, the greater the relative content difference of the metabolite between the two groups. The ordinate represents the significance level of the difference ($-log_{10}$ p-value), and the size of the dot represents the VIP value. The larger the value, the more significant the difference, and the more reliable the result. (E, F): Top 20 FC metabolites. Note: the ordinate represents the metabolite, with downregulated metabolites shown in green and upregulated metabolites shown in red*

Differential metabolic pathway analysis

In the CK vs D group, a total of 159 metabolites were annotated to 67 metabolic pathways according to KEGG analysis (*Fig. 5A*), of which the categories of metabolic pathways, secondary metabolite synthesis, and cofactor synthesis accounted for 74.24%,

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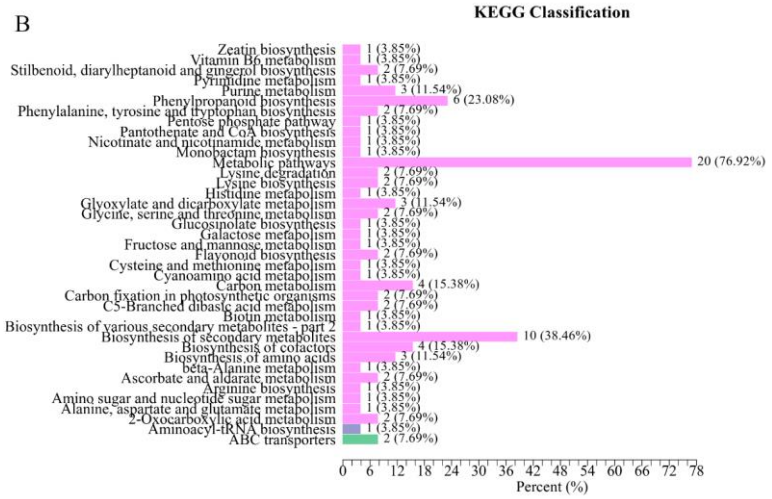
37.88%, and 21.21%, respectively. In the D vs D-SA group, a total of 82 metabolites were annotated to 39 metabolic pathways (*Fig. 5B*), of which the categories of metabolic pathways, secondary metabolite synthesis, and phenylpropane synthesis accounted for 76.92%, 38.46%, and 23.08%, respectively. In both the CK vs D (*Fig. 5C*) and D vs D-SA (*Fig. 5D*) groups, the metabolic pathways were found to be primarily enriched in stilbenoid, diarylheptanoid, and gingerol biosynthesis and flavonoid biosynthesis. Therefore, it is likely that these are the primary metabolic pathways altered by drought stress in sorghum seedlings.

CK vs D



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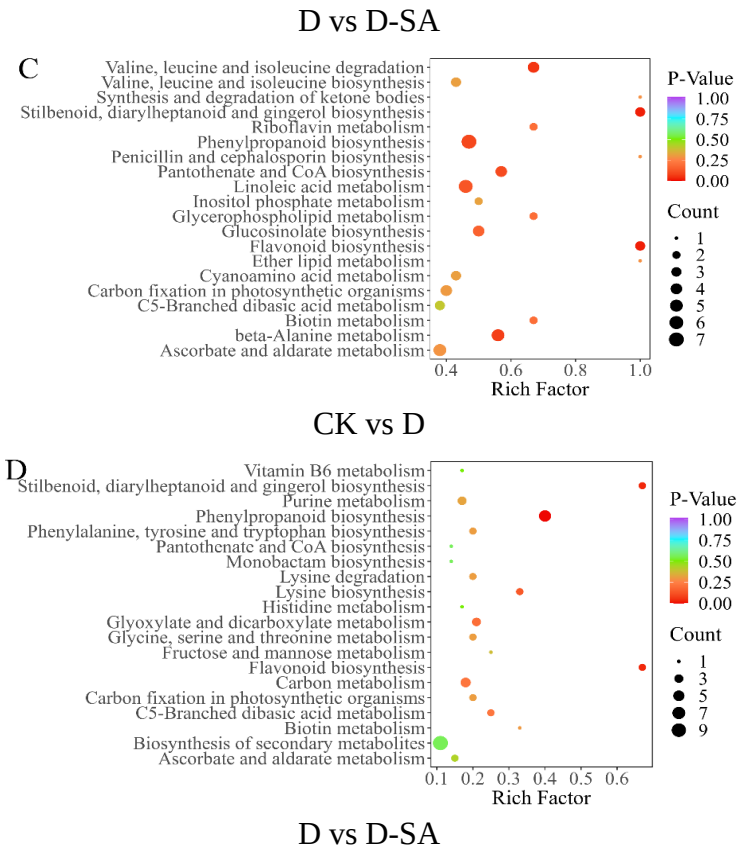

Figure 5. *Analysis of differential metabolic pathways of sorghum seedlings under different treatments. (A, B): Differential KEGG pathway classification map. Note: The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the number of metabolites annotated to this pathway and the ratio of that number to the total number of annotated metabolites. (C, D): Differential KEGG metabolic pathway enrichment map. Note: CK stands for normal germination, D stands for unprimed seeds germinated under drought stress, and D-SA stands for 0.1 mM SA-primed seeds germinated under drought stress. The abscissa represents the Rich Factor corresponding to each pathway and the ordinate is the name of the pathway. The color of the point reflects the p-value, with redder values indicating greater significance. The size of the dots represents the number of enriched differential metabolites*

Core differential metabolite analysis

To better understand the mechanism by which seed priming with SA affects the drought resistance of sorghum seedlings, we identified the core differential metabolites between the CK vs D and D vs D-SA groups (*Fig. 6*). In these pairwise comparisons, 68 overlapping differential core metabolites were identified, including 6 amino acids and derivatives, 42 phenolic acids, 4 nucleotides and derivatives, 8 organic acids, 4 lipids, and 6 others (*Table 2*). Phenolic acids accounted for the largest proportion of differential metabolites, and these compounds decreased under drought stress. However, seed priming with SA significantly increased phenolic acid accumulation, suggesting that seed priming may improve drought resistance by upregulating the biosynthesis of phenolic acids. *Figure 7* shows how metabolites within the phenylpropane biosynthesis pathway change under drought stress and seed priming, including substances such as coniferin, chlorogenic acid, and sinapinaldehyde, among others.

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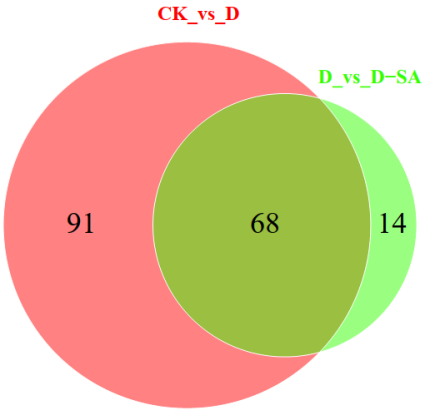

Figure 6. *Venn diagram depicting the shared and specific metabolites between the two treatment groups. CK stands for normal germination, D stands for unprimed seeds germinated under drought stress, and D-SA stands for 0.1 mM SA-primed seeds germinated under drought stress*

Table 2. *Core metabolite profiles of sorghum seedlings*

Metabolite category	CK vs D	CK vs D ∩ D vs D-SA	D vs D-SA
Amino acids and derivatives	11	4	6
Phenolic acids	69	42	48
Nucleotides and derivatives	12	4	5
Organic acids	22	8	8
Others	19	6	7
Lipids	26	4	8

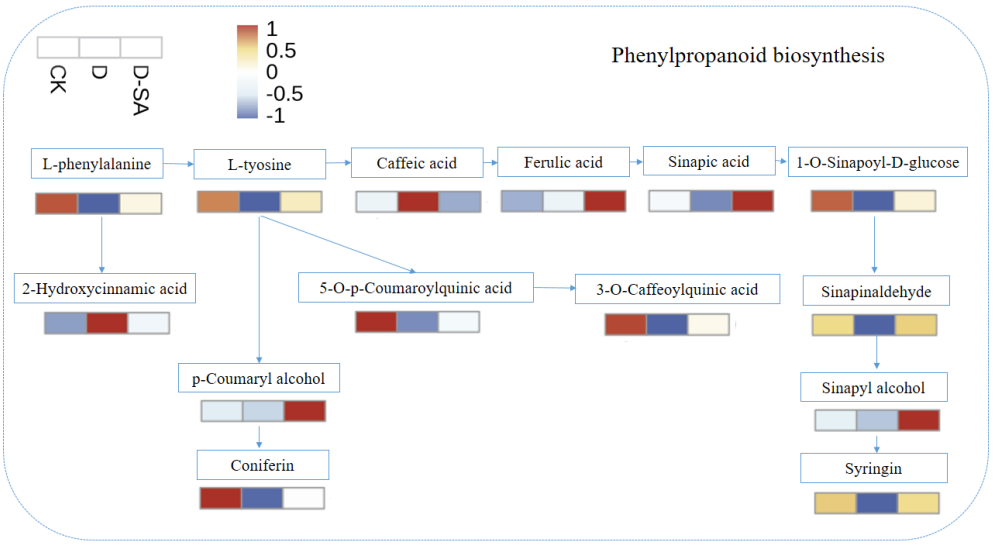

Figure 7. *The responses of differential metabolites related to phenylpropane biosynthesis to drought stress. Changes in differential metabolites are represented by the log_2 ratio. Blue represents a decrease in content and red represents an increase in content. CK stands for normal germination, D stands for unprimed seeds germinated under drought stress, and D-SA stands for 0.1 mM SA-primed seeds germinated under drought stress*

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Discussion

In general, sorghum seeds are sown within the top 3-5 cm of the soil, which is prone to drought compared to deeper soil layers. Although sorghum is generally considered drought tolerant, drought stress often negatively influences seedling germination and emergence. Certain cultivation practices, such as seed priming, can significantly improve the ability of sorghum to emerge under drought stress (Dembele et al., 2021; Patane et al., 2016). In this study, we found that seed priming with SA increased the shoot length under drought stress compared to unprimed seeds. Increased shoot length can improve the rate and uniformity of seed germination, as has been reported in basil (*Ocimum basilicum* L.) (Kulak et al., 2021). In addition, we also found that seed priming with SA increased the root length. Longer roots facilitate water uptake, even under drought, thus improving seedling emergence. Overall, our results suggest that seed priming with SA can improve the drought resistance of sorghum seedlings.

In order to ascertain the mechanism by which seed priming with SA improves the drought resistance of sorghum seedlings, we examined the oxidative status of drought-stressed sorghum seedlings. Drought stress often induces oxidative stress in plants, resulting in reactive oxygen species (ROS) accumulation, lipid peroxidation, enzyme inactivation, DNA damage, metabolic disorder, and apoptosis (Zhang et al., 2019). To protect themselves from oxidative stress and maintain normal cellular functions, plant utilize both enzymatic and non-enzymatic antioxidants, including ascorbic acid and glutathione, to scavenge excess ROS and H_2O_2. Sun et al. found that exogenous fulvic acid (0.1 g L^{-1}) significantly enhanced the drought tolerance of tea plants (*C. sinensis*) by improving ascorbic acid metabolism, glutathione metabolism, and flavonoid biosynthesis (Sun et al., 2020). In this study, we found that isoascorbic acid was the most variable metabolite in the CK vs D and D vs D-SA groups (*Fig. 3E, F*), suggesting that isoascorbic plays an important role in improving drought resistance of sorghum primed with SA. Nonetheless, whether isoascorbic acid has a similar effect to ascorbic acid remains to be further verified.

The KEGG database allows the analysis of genes, expression profiles, and metabolite content as a whole network in terms of metabolic pathways, such as those of carbohydrates, nucleosides, and amino acids, as well as the enzymatic biodegradation of organic compounds. As such, it is a powerful tool for metabolic analysis and metabolic network studies. Here, KEGG metabolic pathway enrichment analysis showed that stilbene, diarylheptane, and gingerol biosynthesis and flavonoid biosynthesis were the key metabolic pathways affected by drought stress in sorghum seedlings. Flavonoids are a group of antioxidant compounds consisting of a 15-carbon skeleton that are widely distributed in plants, and numerous studies have shown that flavonoid biosynthesis is closely related to stress. For example, drought stress induced the expression of flavonoid biosynthesis genes in hybrid popular (*Populus tremula* × *P. alba*), and the accumulation of phenolic and flavonoid compounds improved the antioxidant status of these trees (Ahmed et al., 2021). In Tibetan hulless barley (*Hordeum vulgare* L. var. *nudum*), drought stress induced the biosynthesis of flavonoids and anthocyanins, and flavonoid-enriched Tibetan hulless barley is more tolerant to drought stress (Xu et al., 2021). We theorize that one mechanism by which seed priming with SA improves the drought tolerance of sorghum may be increased flavonoid biosynthesis.

Metabolites in the phenylpropane metabolic pathway have numerous biological functions, including regulating plant growth (Xu et al., 2020), participating in plant stress resistance, and scavenging free radicals, among others. Furthermore,

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phenylpropanoid metabolism has been shown to be involved in plant stress resistance (Nakabayashi et al., 2014). For example, Savoi et al. (2016) compared the secondary metabolism of white grapes (*Vitis vinifera* L.) under normal and drought-stressed conditions using a combined metabolomic and transcriptomic approach and found that the phenylpropanoid metabolic pathway could improve grapevine tolerance to drought (Savoi et al., 2016). In this study, we found that drought stress significantly reduced the accumulation of substances in the phenylpropane metabolic pathway, while seed priming with SA significantly increased the accumulation of these substances. In the phenylpropane metabolic pathway, p-coumarol, caffeic acid, ferulic acid, sinapaldehyde, sinapiol, sinapinic acid, trans-5-O-shikimic acid, 5-Op-coumaroylquinic acid, conifer chlorogenic acid, syringin, and 1-O-spinacyl-D-glucose all play important roles in drought resistance. These results suggest that exogenous SA may increase the drought tolerance of sorghum seedlings by interacting with the phenylpropane metabolic pathway.

The results of this study present an important step toward understanding the mechanism by which seed priming with SA increases the drought resistance of sorghum seedlings. However, seed germination, under both normal and drought-stressed conditions, is a complex physiological process regulated by numerous genes and proteins. To fully characterize this process, more work needs to be done, such as the identification of key responsive genes and proteins. Through these, we will further reveal the molecular mechanism by which seed priming increases drought stress.

Conclusion

Here, we studied the comprehensive metabolite profiles of SA-primed sorghum seedlings under normal and drought-stressed conditions. Our results confirmed that drought stress severely restrains sorghum seed germination. However, seed priming with SA mitigated the effects of drought stress. The metabolite analyses revealed that both the stilbene, diarylheptane, and gingerol biosynthesis and the flavonoid biosynthesis pathways are involved in the drought resistance conferred by seed priming with SA. Further analyses of core metabolites indicated that phenolic acids and other substances within the phenylpropane biosynthesis pathway are enriched by seed priming with SA, including coniferin, chlorogenic acid, and sinapinaldehyde, among others. The phenolic acids may be the core metabolites influenced by exogenous SA, and may be at least partly responsible for the increased drought resistance exhibited by primed sorghum seedlings.

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