RESEARCH ARTICLE

The physiological mechanism of salt-alkali tolerance and cultivation regulation strategies of peonies based on multi-omics technology

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Received: January 6, 2026; **accepted:** May 8, 2026.

To clarify the physiological mechanism of peonies' response to saline-alkali stress and establish an efficient cultivation regulation system, this study systematically analyzed the "Fengdanbai" and "Ziban" peony varieties that exhibited varying degrees of salt tolerance by simulating a stress environment through pot cultivation and integrating multi-omics technologies to elucidate their physiological and molecular response mechanisms. The results showed that salt-alkali stress significantly inhibited growth and caused oxidative damage. Salt-tolerant varieties adapted by enhancing the activity of antioxidant enzymes and accumulating osmotic regulatory substances. Omics analysis revealed a large number of differentially expressed genes, proteins, and metabolites, which were enriched in hormone signaling, mitogen-activated protein kinase (MAPK) pathways, carbon metabolism, and antioxidant processes. Among them, transcription factors of myeloblastosis (MYB) and WRKY played a core role in regulating antioxidant genes and flavonoid synthesis. Cultivation experiments showed that exogenous spraying of a mixture of 50 mg/L salicylic acid and 100 mg/L proline combined with humus-improved substrate and drip irrigation with low-salt water fertilizer management could significantly enhance the survival and growth of peonies in saline-alkali land. This study clarified the mechanism of salt-alkali tolerance of peonies, providing theoretical and technical support for cultivation in saline-alkali land.

Keywords: peony; saline-alkali stress; multi-omics; physiological mechanism; cultivation regulation.

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Introduction

Peony, as an important horticultural plant with ornamental, medicinal, and ecological values, has broad application prospects in the resource utilization of saline-alkali land and the creation of garden landscapes. However, saline-alkali stress has become a key environmental factor restricting its geographical distribution and cultivation promotion.

In a review of the development mechanism of peony flower color and shape, Zuo *et al*. systematically sorted out the molecular regulatory pathways of apparent traits of woody peonies and the application experience in crop breeding, providing a theoretical framework for the quality improvement of peonies and laying the foundation for the collaborative research of stress resistance traits and ornamental traits [1]. Jiang *et al*. revealed the molecular response mechanism of salt-alkali stress tolerance in woody plants through the combined analysis of physiological index detection and transcriptome sequencing and established a "physiological phenotype - molecular regulation" research model, providing an important methodological reference for the stress resistance study of

woody ornamental plants [2]. Zhang *et al*. confirmed the optimization effect of exogenous active substances on the microenvironment of peony growth, opening up a new practical path for improving the salt-alkali tolerance of peonies through exogenous regulation [3]. In a study on the alkali tolerance of Hosta, the strategy of integrating physiology and transcriptome was adopted to analyze the stress resistance mechanism, further verifying the effectiveness of this multi-dimensional research method in the analysis of stress resistance of herbaceous ornamental plants, providing a technical paradigm for the study of salt-alkali tolerance of peonies [4]. Tang *et al*. cloned and functioned the salt tolerance function of the PlWRKY70 transcription factor of herbaceous peony, clarified the core role of this gene in regulating the plant salt stress response, filled the gap in the verification of peony stress resistance function genes, and provided core gene resources for subsequent molecular breeding [5]. The research on improving the growth of herbaceous peonies with compound fertilizers provided a practical solution for the growth regulation of peonies in saline-alkali areas from the perspective of agronomic cultivation, which complemented the research on molecular regulation and improved the system framework of peony stress resistance research [6]. Lv *et al*. reviewed the research progress of plant omics in the *Rosaceae* family, systematically summarizing the application experience of multi-omics technologies such as transcriptomics and proteomics in plant stress resistance and quality formation. Its omics analysis ideas and data mining methods had important reference significance for the multi-omics research of peonies in the *Paeoniaceae* family [7]. Further, Chen *et al*. analyzed the respiratory metabolism regulation mechanism of oat roots in response to salt stress using labeling proteomics technology, providing a technical reference and a comparative basis for molecular pathways for the proteomics study of salt-alkali tolerance in peonies [8]. Gao *et al*. revealed the hormone regulatory network of *Phalaenopsis* flower bud development through the joint analysis of transcriptome and metabolome, further verified the advantages of multi-omics integrated analysis in understanding the regulatory mechanism of complex plant traits and provided methodological support for the construction of the "gene-protein-metabolite" regulatory network of peony salt-alkali tolerance [9].

Although many progresses have been made in the quality improvement of peonies, the verification of individual stress-resistant genes, the optimization of cultivation techniques, the study of stress-resistant omics in other plants, systematic research on the physiological response patterns of peonies to salt-alkali stress, multi-omics integration analysis, and core regulatory networks are still relatively scarce. This study aimed to integrate transcriptomic, proteomic, and metabolomic sequencing technologies to determine physiological parameters such as photosynthetic characteristics, antioxidant systems, and osmoregulatory substances in peonies under salt-alkali stress to systematically analyze the physiological and molecular regulatory mechanisms underlying salt-alkali tolerance in peonies. Through a series of cultivation regulation experiments, efficient stress relief measures were screened out, and a complete research chain of mechanism analysis - technology research and development - application verification was constructed, providing a theoretical basis and technical support for the efficient cultivation of peonies in saline-alkali areas.

Materials and methods

Plant culture and groups

Two-year-old seedlings of peony varieties “Fengdanbai” with strong salt tolerance and “Ziban” with weak salt tolerance were obtained from the Peony Germplasm Resource Garden of Henan Province (Luoyang, Henan, China). The tested seedlings grew vigorously and uniformly with an average plant height of 25 to 30 cm and a ground diameter of 0.8 to 1.0 cm in a mixture

of garden soil and river sand at a ratio of 3:1 (v/v), pH of 6.5 to 7.0, an organic matter content of 18.5 g/kg, and a total salt content of 0.3 g/kg. Saline-alkali stress was carried out using a mixed salt solution prepared with NaCl and Na_2CO_3 in a 1:1 molar ratio. Four concentration gradients were set as 0 mmol/L as the control, 50 mmol/L as mild stress, 100 mmol/L as moderate stress, and 150 mmol/L as severe stress. The experiments were conducted in the greenhouse of Henan Agricultural University (Zhengzhou, Henan, China) from March to October 2023 using the pot method with the pot size of 30 cm in diameter and 25 cm in height. Each pot was filled with 12 kg of cultural substrate, and one seedling was planted. The experiment adopted a completely random design with the combination of each variety and stress concentration being set to 3 replicates and each replicate including 10 pots [10]. The main stress test included a total of 240 plants, while the subsequent cultivation regulation experiment included 270 plants, which made the total number of plants used in the whole study 510.

Plant saline-alkali stress treatments

After the seedlings were transplanted and underwent a two-month acclimatization period, saline-alkali stress treatment was initiated. 200 mL stress solution was irrigated once a week with the control group using the same amount of clear water. Samples were taken on 0, 7, 15, 30, and 45 days after stress treatment to determine the relevant physiological indicators. On 30 days of stress treatment, leaf and root samples were collected for multi-omics analysis [11, 12]. Three treatment groups were set up in the cultivation regulation experiment. The first group was treated with exogenous regulatory substances with salicylic acid at 25 mg/L, 50 mg/L, and 100 mg/L, proline at 50 mg/L, 100 mg/L, and 200 mg/L, and their mixed solutions of 50 mg/L salicylic acid and 100 mg/L proline being sprayed, respectively. The first spray was carried out 3 days before the stress began and then every 10 days for a total of three sprays. The second group consisted of substrate improvement treatments including humus improvement consisting of the mixture of humus, garden soil, river sand at ratios of 1:5:2, 2:5:2, 3:5:2, perlite improvement with perlite, garden soil, river sand at a ratio of 1:5:2, and zeolite improvement with zeolite, garden soil, river sand at a ratio of 1:5:2. The control group adopted a mixture of garden soil and river sand at a ratio of 3:1. The third group was the optimization of water and fertilizer management including drip irrigation and flood irrigation. The amount of drip irrigation was 150 mL each time with an interval of 7 days, while the amount of each flood irrigation was 300 mL with an interval of 10 days. Meanwhile, two fertilization concentrations were applied as the low-salt fertilizer with a nitrogen, phosphorus, potassium ratio of 1:1:1 and a concentration of 0.5 g/L and the conventional fertilizer with a nitrogen, phosphorus, potassium ratio of 2:1:1 and a concentration of 1.0 g/L. The cultivation regulation experiments were all conducted under saline-alkali stress conditions of 100 mmol/L. Each treatment was repeated three times, mainly measuring indicators of plant survival rate, plant height increase, and photosynthetic rate.

Physiological index determination

The plant height and ground diameter were determined by using a ruler and a vernier caliper. The photosynthetic rate (Pn), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci) were determined using LI-COR Li-6400 portable photosynthesis meter (LI-COR, Lincoln, NE, USA). The measurement was conducted on the third fully expanded leaf from the top of the plant between 9:00 and 11:00 on a sunny day with a light intensity of 1,200 μmol/m²·s, a CO_2 concentration of 400 μmol/mol, and a leaf temperature of 25℃. Each treatment was measured on 5 plants with 3 technical replicates per plant. The chlorophyll content was determined by the ethanol extraction method. Briefly, 0.2 g of fresh leaves were cut into pieces, soaked in 25 mL of 95% ethanol, and extracted in the dark at 25℃ for 24 h until the leaf tissue turned completely white. The absorbance of the extract was measured at 665 nm and 649 nm using Shimadzu UV-1800 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan)

to calculate the chlorophyll content. The content of malondialdehyde (MDA) was determined by the thiobarbituric acid (TBA) method. 0.5 g of fresh leaf sample was ground into homogenate with 5 mL of 5% trichloroacetic acid (TCA) and then centrifuged at 4,000 rpm for 10 min. 2 mL of the supernatant was mixed with 2 mL of 0.67% TBA, boiled in a water bath at 100℃ for 30 min, then rapidly cooled down and centrifuged. The absorbances at 450 nm, 532 nm, and 600 nm of the supernatant were measured, and the MDA content was calculated. The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were determined by the azadiazole reduction method, guaiacol method, and potassium permanganate titration method, respectively. For SOD activity determination, 0.5 g of fresh leaf sample was ground with 5 mL of 0.05 mol/L pre-cooled phosphate buffer (pH 7.8) in an ice bath, centrifuged at 10,000 rpm at 4℃ for 20 min, and the supernatant was used as the crude enzyme extract. The SOD activity reaction was conducted by mixing phosphate buffer, methionine, nitroblue tetrazolium (NBT), riboflavin, and enzyme extract, reacting under 4,000 lx light for 20 min, and measuring the absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of the NBT photochemical reduction. POD activity was determined by mixing 0.2% guaiacol, 0.3% H_2O_2, phosphate buffer, and enzyme extract. The change in absorbance at 470 nm was recorded continuously for 3 min. One unit of POD activity was defined as a 0.01 change in absorbance per minute. CAT activity was determined by mixing 0.1 mol/L H_2O_2 and enzyme extract. The remaining H_2O_2 was titrated using 0.1 mol/L potassium permanganate solution at 1 min intervals for 5 min. One unit of CAT activity was defined as the amount of enzyme that decomposed 1 mg of H_2O_2 per minute. All enzyme activity assays were performed at 4℃. The contents of proline (Pro), betaine, and soluble sugar were measured, respectively, by the indole trione colorimetric method, the Ryxell salt colorimetric method, and the anthrone colorimetric method. Pro content was measured by grounding 0.5 g of fresh leaf sample with 5 mL of 3% sulfosalicylic acid, boiling in a water bath for 10 min, centrifuging, followed by mixing 2 mL of glacial acetic acid and 3 mL of 2.5% indole trione with 2 mL of the supernatant, boiled for 40 min, extracted with toluene. The absorbance at 520 nm of the organic phase was measured. For betaine content determination, 0.5 g of dried sample was extracted with 80% methanol. The extract was mixed with Ryxell reagent (Sigma-Aldrich, St. Louis, MO, USA), reacted in the dark for 2 h, and measured the absorbance at 525 nm. The soluble sugar content was determination by extracting 0.2 g of dried sample with 80% ethanol in a water bath at 80℃ for 30 min. The extract was mixed with anthrone reagent (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), boiled for 10 min, and measured the absorbance at 620 nm. Root vitality was determined by the triphenyl tetrazolium chloride (TTC) method. Briefly, 0.5 g of fresh root tip sample was immersed in 10 mL of mixed solution containing 0.4% TTC and 0.1 mol/L phosphate buffer (pH 7.0), incubated in the dark at 37℃ for 3 h, then terminated with 2 mL of 1 mol/L sulfuric acid. The red triphenyl formazan produced was extracted with ethyl acetate, and the absorbance at 485 nm was measured. Root vitality was expressed as the amount of TTC reduced per gram of fresh root weight per hour. Each indicator was measured with 3 biological replicates and 3 technical replicates.

Multi-omics sample preparation and sequencing

Leaf and root samples that had been subjected to saline-alkali stress for 30 days were selected for transcriptome, proteome, and metabolome sequencing analyses with three biological replicates for each sample. Transcriptome sequencing was performed by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) for sample total RNA extraction, NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) for cDNA library construction, and Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) for sequencing following manufacturers' instructions. The original data was filtered, and

impurities were removed to obtain clean reads for differentially expressed genes (DEGs) screening with the criteria of that the absolute value of the expression multiple change was greater than or equal to 1 and the corrected *P* value was less than 0.05. Gene Ontology (GO) (http://geneontology.org/) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/) pathway enrichment analysis were conducted on the differentially expressed genes screened out. Total proteins were extracted by using the SDT protein extraction kit (Beyotime Biotechnology, Shanghai, China) following manufacturer's instructions. The protein sample was added with dithiothreitol (DTT) to a final concentration of 100 mmol/L, denatured in a boiling water bath for 5 min, and cooled down to room temperature. The sample was then mixed with 200 µL of UA buffer (8 mol/L urea and 150 mmol/L Tris-HCl at pH 8.0), transferred to a 30 kD ultrafiltration tube, centrifuged at 12,000 rpm for 15 min, and discarded the filtrate. 100 µL of 50 mmol/L iodoacetamide (IAA) was then added. The reaction was incubated at room temperature in the dark for 30 min before centrifugation. The sample was washed twice with UA buffer and three times with 50 mmol/L tetraethylammonium bromide (TEAB) buffer. 40 µL of trypsin solution (enzyme to protein ratio of 1:50) was added to the ultrafiltration tube, incubated at 37℃ for 16 h, and collected the digested peptides by centrifugation. The peptides were desalted using a C18 solid phase extraction column, dried in Eppendorf vacuum concentrator (Eppendorf, Hamburg, Germany), and then labeled with Tandem Mass Tag (TMT) using the TMT10plex™ Isobaric Label Reagent Set (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The labeled peptides were mixed and fractionated by high pH reversed-phase liquid chromatography and then analyzed by using Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Differentially abundant proteins (DAPs) were screened with the criteria that the absolute value of the expression multiple change was greater than or equal to 1.2 and the *P* value was less than 0.05. GO function and KEGG pathway enrichment analyses were then conducted on differentially abundant proteins [13]. Metabolomics sequencing was performed using a mixed solution of methanol and water in a volume ratio of 1:1. Metabolites were extracted by using a KQ-500DE numerical control ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, Jiangsu, China). The analysis was carried out using an ultra-performance liquid chromatography-tandem mass spectrometry system, in which the liquid chromatography part used Agilent 1290 Infinity II ultra-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) and the mass spectrometry part used AB Sciex QTRAP 6500 plus mass spectrometer (AB Sciex, Framingham, MA, USA). Metabolite identification and differential metabolites (DAMs) screening were conducted using MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca/). The screening criteria were that the projected value of variable importance was greater than or equal to 1 and the *P* value was less than 0.05. Metabolic pathway enrichment analysis was conducted on the identified differential metabolites.

Correlation analysis of multi-omics data

Association analysis was performed on DEGs from the transcriptome, DAPs from the proteome, and DAMs from the metabolome to construct a gene-protein-metabolite regulatory network. The regulatory network was visualized by using Cytoscape 3.9.1 software (https://cytoscape.org/) to screen the core regulatory factors. Through Pearson correlation analysis, the correlation coefficients among genes, proteins, and metabolites were calculated to identify the key regulatory pathways.

Statistical analysis

Data was collated by using Microsoft Excel 2021 (Microsoft, Redmond, WA, USA). SPSS 26.0 software (IBM, Armonk, NY, USA) was employed for two-way ANOVA analysis. Multiple comparisons were conducted using the Duncan method. The Origin 2023 (https://www.originlab.com/) was used to draw

Table 1. Effects of saline-alkali stress on the increment of plant height and ground diameter (mean ± SD).

Variety	Stress concentration (mmol/L)	Coercive time (d)	Plant height (cm)	Ground diameter (mm)
Fengdanbai	0	7	1.2 ± 0.1^{a}	0.3 ± 0.02^{a}
		30	3.5 ± 0.2^{a}	0.8 ± 0.05^{a}
		45	5.2 ± 0.3^{a}	1.3 ± 0.08^{a}
	50	7	1.0 ± 0.1^{ab}	0.25 ± 0.02^{ab}
		30	2.8 ± 0.2^{b}	0.65 ± 0.04^{b}
		45	3.8 ± 0.2^{b}	0.9 ± 0.06^{b}
	100	7	0.7 ± 0.08^{bc}	0.2 ± 0.02^{bc}
		30	1.9 ± 0.1^{c}	0.45 ± 0.03^{c}
		45	2.7 ± 0.2^{c}	0.7 ± 0.05^{c}
	150	7	0.5 ± 0.05^{c}	0.15 ±0.01^{c}
		30	1.2 ± 0.1^{d}	0.3 ± 0.02^{d}
		45	2.2 ± 0.1^{d}	0.7 ± 0.04^{c}
Ziban	0	7	1.1 ± 0.1^{a}	0.28 ± 0.02^{a}
		30	3.2 ± 0.2^{a}	0.75 ± 0.04^{a}
		45	4.8 ± 0.2^{a}	1.2 ± 0.07^{a}
	50	7	0.8 ± 0.07^{b}	0.2 ± 0.02^{b}
		30	2.1 ± 0.1^{c}	0.5 ± 0.03^{b}
		45	2.9 ± 0.2^{c}	0.65 ± 0.04^{b}
	100	7	0.5 ± 0.05^{c}	0.15 ± 0.01^{c}
		30	1.3 ± 0.1^{d}	0.3 ± 0.02^{c}
		45	1.7 ± 0.1^{d}	0.45 ± 0.03^{c}
	150	7	0.3 ± 0.03^{d}	0.1 ± 0.01^{d}
		30	0.7 ± 0.05^{e}	0.2 ± 0.02^{d}
		45	1.3 ± 0.1^{e}	0.4 ± 0.03^{d}

Note: Different lowercase letters after the data in the same column indicated significant differences ($P < 0.05$).

the chart. Multi-omics data analysis was completed by Beijing Novogene Technology Co., LTD. (Beijing, China).

Results

Effects of saline-alkali stress on the growth and physiological characteristics of peonies

1. Impact on growth indicators

Saline-alkali stress significantly inhibited the growth of peony plant height and ground diameter, and the inhibitory effect intensified with the increase of stress concentration and the extension of stress duration. On 45 days of stress, the plant height incretions of “Fengdanbai” and “Ziban” peonies in the 150 mmol/L stress group decreased by 58.3% and 72.5%, respectively, compared to the control group, while the ground diameter incretions decreased by 46.2% and 63.8%, respectively (Table 1). Under the same stress conditions, the increase in plant height and ground diameter of “Fengdanbai” was significantly higher than that of “Ziban”, indicating that “Fengdanbai” had a stronger tolerance to salt and alkali.

2. Effects on photosynthetic characteristics

Saline-alkali stress led to a significant decrease in the photosynthetic rate (Pn) and stomatal conductance (Gs) of peonies with the intercellular CO_2 concentration (Ci) first decreasing and then increasing, while the chlorophyll content continuously decreased. On 30 days of stress, the Pn of “Fengdanbai” and “Ziban” in the 100 mmol/L stress group decreased by 32.6% and 48.9%, respectively, compared to the control group, and the chlorophyll content decreased by 25.3% and 38.7%, respectively (Figure 1). The Pn, Gs, and

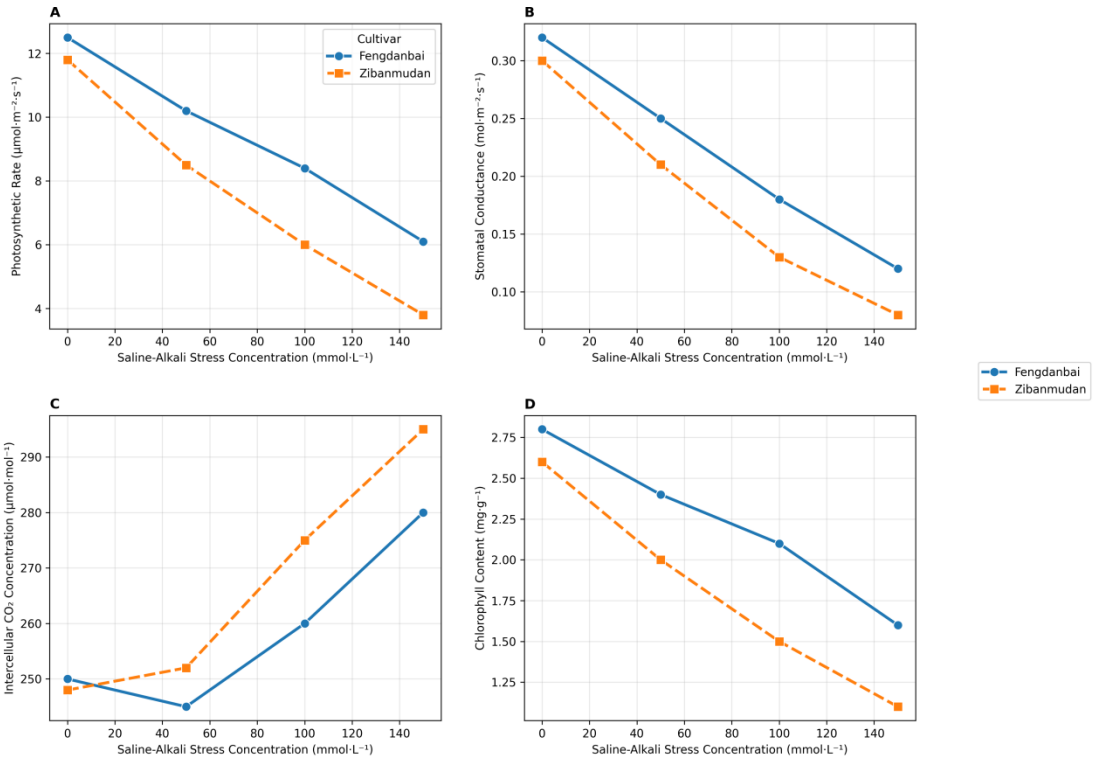

Figure 1. The effect of saline-alkali stresses on the photosynthetic characteristics of peonies. **A.** photosynthetic rate. **B.** stomatal conductance. **C.** intercellular CO_2 concentration. **D.** chlorophyll content.

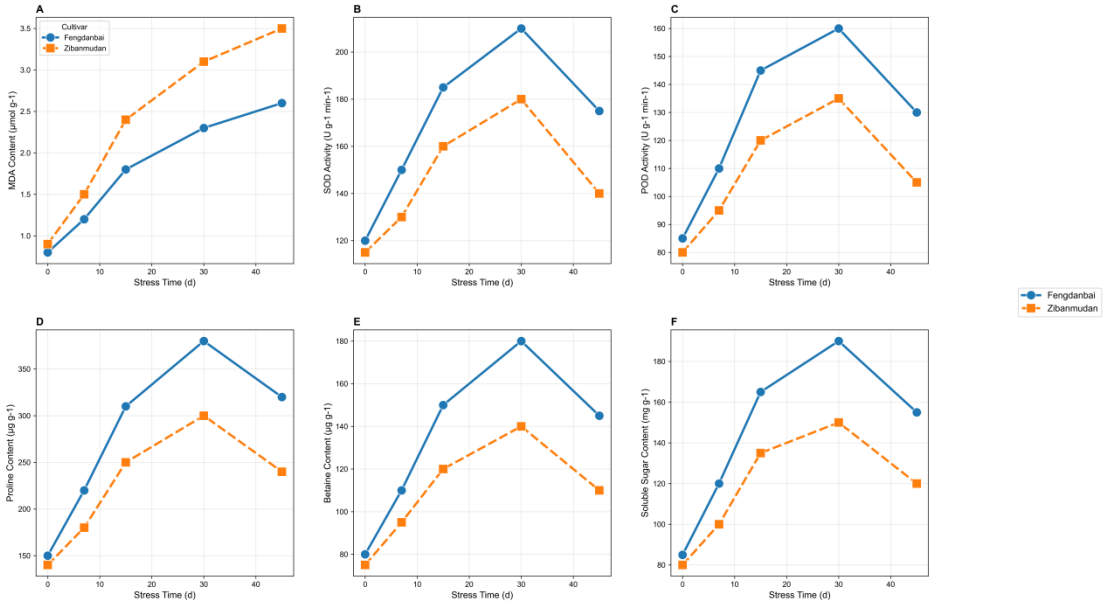

Figure 2. The effects of saline-alkali stress on the antioxidant system and osmotic regulatory substances of peonies. **A.** MDA content. **B.** SOD activity. **C.** POD activity. **D**. Pro content. **E.** betaine content. **F.** soluble sugar content.

chlorophyll contents of "Fengdanbai" under various stress conditions were significantly higher than those of "Ziban", indicating that salt-tolerant varieties could resist salt-alkali stress by

maintaining a relatively high photosynthetic capacity.

3. The influence of antioxidant systems and osmotic regulating substances

Under saline-alkali stress, the MDA content in peony leaves significantly increased, while the activities of SOD, POD, CAT, and the contents of Pro, betaine, and soluble sugar all showed a trend of increasing first and then decreasing (Figure 2). During 15 to 30 days under stress, the activity of antioxidant enzymes and the content of osmotic regulatory substances reached their peaks, and then gradually decreased with the extension of stress time. Under the same stress conditions, the MDA content of “Fengdanbai” was significantly lower than that of “Ziban”, while the activities of SOD, POD, CAT, and the content of osmotic regulatory substances were significantly higher than those of “Ziban”. The results showed that the SOD activity of “Fengdanbai” in the 100 mmol/L stress group was 35.2% higher than that of “Ziban” on 30 days of stress, while the Pro content was 42.8% higher, indicating that “Fengdanbai” could resist oxidative damage caused by salt-alkali stress through stronger antioxidant capacity and osmotic regulation ability.

Transcriptome analysis of salt-alkali tolerance in peony

1. Screening of differentially expressed genes (DEGs)

Transcriptome sequencing results showed that, when both peonies were under 100 mmol/L saline-alkali stress for 30 days, a total of 1,243 DEGs were identified in their leaves and roots, among which 689 genes were up-regulated, and 554 genes were down-regulated (Figure 3A). There were significant differences between varieties with “Fengdanbai” showing 423 unique DEGs and “Ziban” showing 317 unique DEGs. There were 503 common DEGs being demonstrated (Figure 3B).

2. DEGs functional enrichment analysis

GO functional enrichment analysis demonstrated that DEGs were mainly enriched in the reduction-oxidation (REDOX) processes, stress responses, photosynthesis of biological processes (BP), chloroplasts, cell membranes, cytoplasm of cellular components (CC), REDOX enzyme activities, transport protein activities, and oxygen-releasing complex activities of photosynthetic system II of molecular functions (MF) (Figure 3C). KEGG functional enrichment analysis indicated that DEGs were significantly enriched in pathways of plant hormone signal transduction, plant MAPK signaling pathway, flavonoid biosynthesis, phenylpropanin biosynthesis, and carbon metabolism (Figure 3D). In the plant hormone signal transduction pathway, genes related to hormone synthesis and signal transduction such as ABA, JA, and ETH were significantly upregulated, while in the flavonoid biosynthesis pathway, the expression levels of key enzyme genes such as PAL, C4H, and CHS were significantly increased.

3. Proteomic analysis of salt-alkali tolerance in peony

(1) Differential abundance protein (DAPs) screening

A total of 5,823 proteins were identified by proteome sequencing, among which 876 were DAPs (log2FC ≥ 1.2, $P < 0.05$), including 498 up-regulated DAPs and 378 down-regulated DAPs (Figure 4A). The comparative analysis of DAPs between two peonies showed that the number of DAPs upregulated in “Fengdanbai” was significantly higher than that in “Ziban”, especially the abundance of antioxidant enzymes, ion transport proteins, and photosynthetically related proteins increased more significantly.

(2) DAPs functional enrichment analysis

GO functional enrichment analysis indicated that DAPs were mainly enriched in the carbon fixation, antioxidant reaction, ion transport of BP, the thyroids, cytoplasmic matrix, cell membrane of CC, the Rubisco activity, peroxidase activity, and Na^+/H^+ reverse transporter activity of MF (Figure 4B). KEGG functional enrichment analysis revealed that DAPs were significantly enriched in pathways of carbon metabolism,

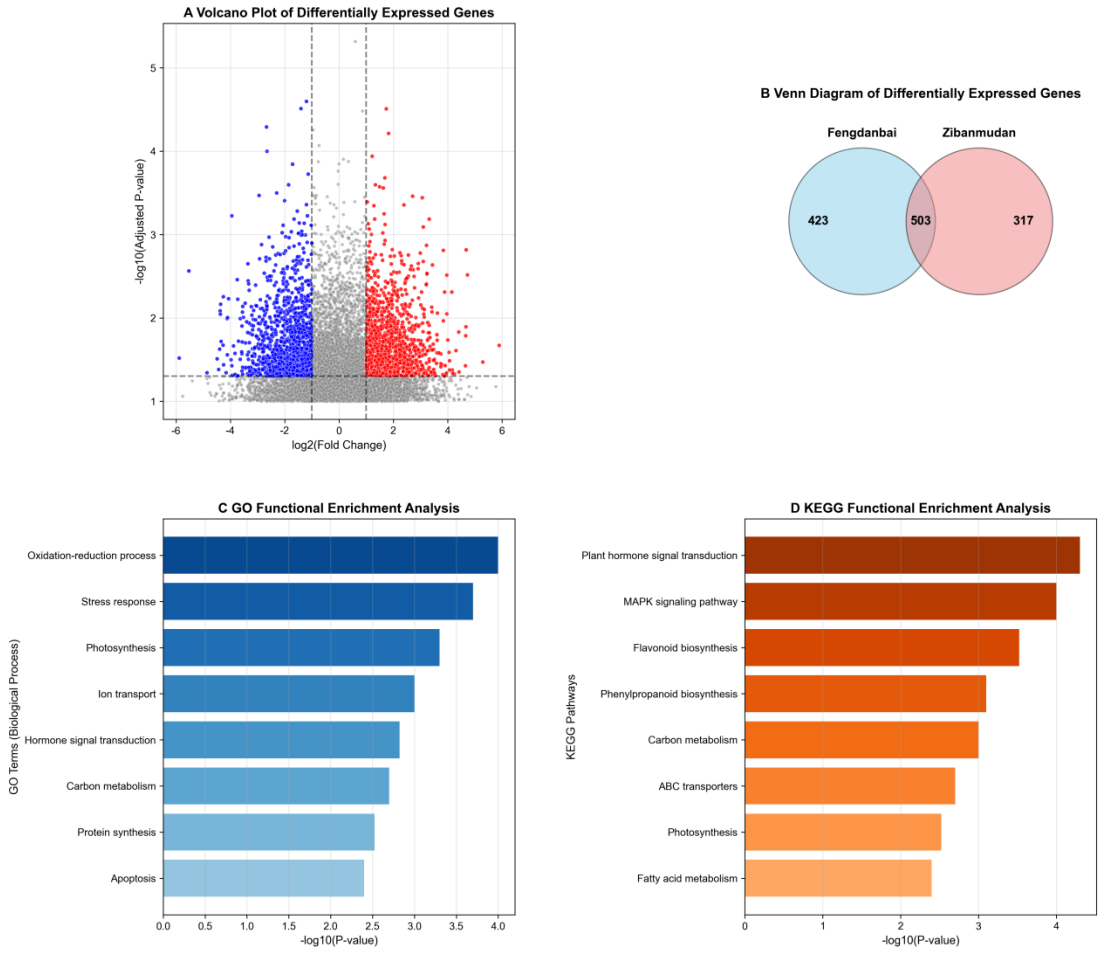

Figure 3. DEGs analysis of peonies under saline-alkali stress.

photosynthetically antenna proteins, antioxidant systems, and ABC transporters (Figure 4C). The down-regulation range of photosynthetic system-related proteins including PsbA, PsbB, Rubisco in "Fengdanbai" was smaller than that in "Ziban". The abundance of antioxidant enzyme proteins such as SOD, POD, and CAT in "Fengdanbai" was significantly higher than that in "Ziban". The abundance of Na^+/H^+ reverse transporter (NHX) and proton pump (H^+-ATPase) plasma transporters was significantly upregulated in "Fengdanbai".

4. Metabolome analysis of salt-alkali tolerance in peony

(1) Differential metabolite (DAMs) screening

A total of 1,845 metabolites were detected, among which 632 were DAMs (VIP ≥ 1, $P < 0.05$), including 386 up-regulated DAMs and 246 down-regulated DAMs (Figure 5A). The DAMs differences between both peonies were significant. The accumulation of metabolites such as flavonoids, phenolic acids, amino acids, and their derivatives in "Fengdanbai" was significantly higher than that in "Ziban".

(2) DAMs functional enrichment analysis

KEGG metabolic pathway enrichment analysis revealed that DAMs were mainly enriched in pathways like flavonoid biosynthesis, phenylalanine biosynthesis, tyrosine metabolism, proline metabolism, and the citric acid cycle (TCA cycle) (Figure 5B). Among them, flavonoid metabolites and phenolic acid metabolites accumulated significantly under saline-alkali stress, and their accumulation in

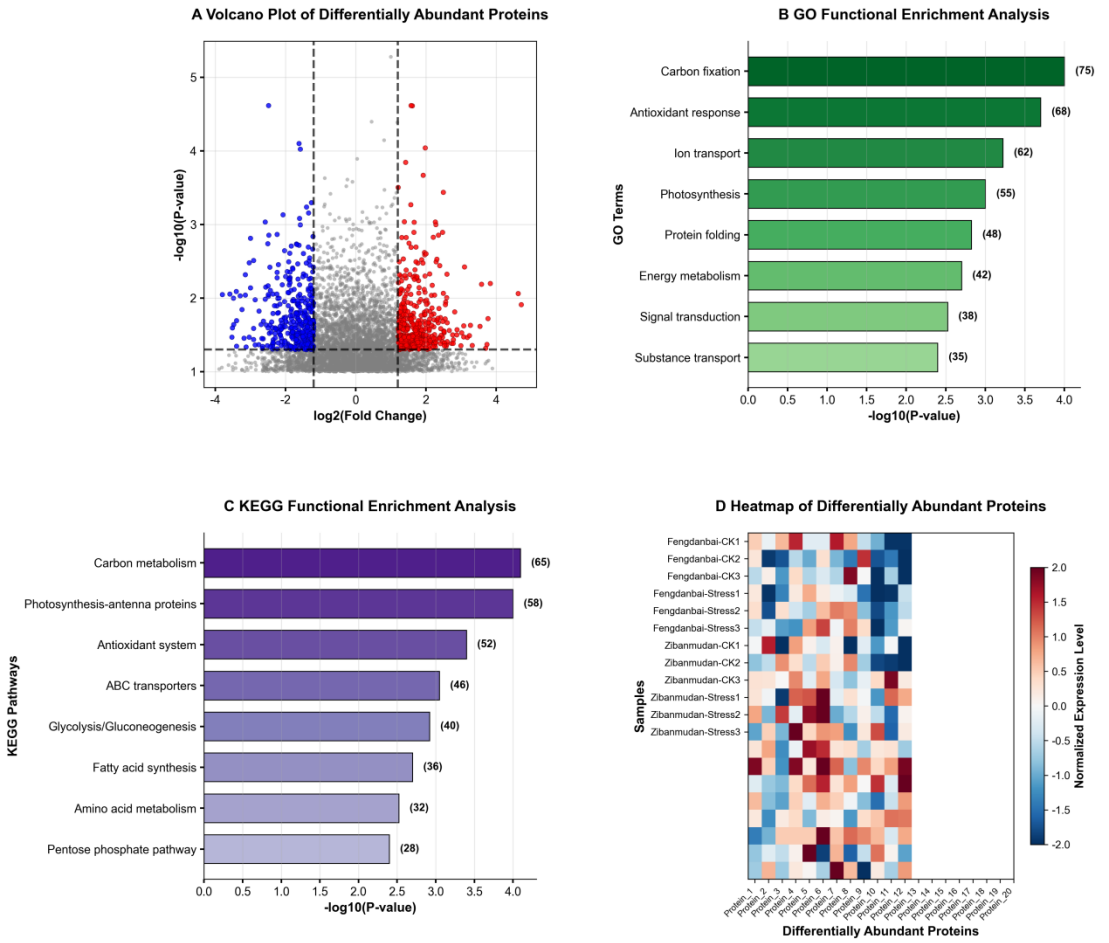

Figure 4. DAPs analysis of peony under saline-alkali stress.

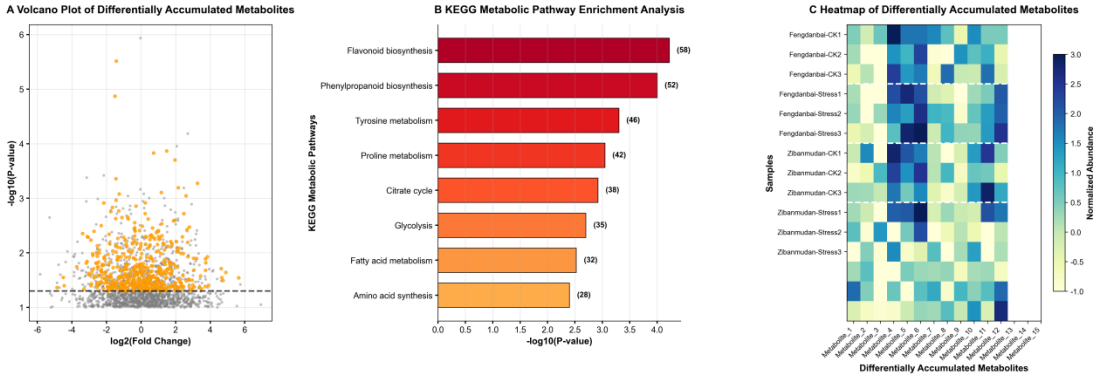

Figure 5. DAMs Analysis of Peony under saline-alkali stress.

"Fengdanbai" was significantly higher than that in "Ziban". The content of amino acids and their derivatives as well as organic acids also demonstrated significant increases, providing a material basis for peonies to resist saline-alkali stress.

5. Correlation analysis of multi-omics data

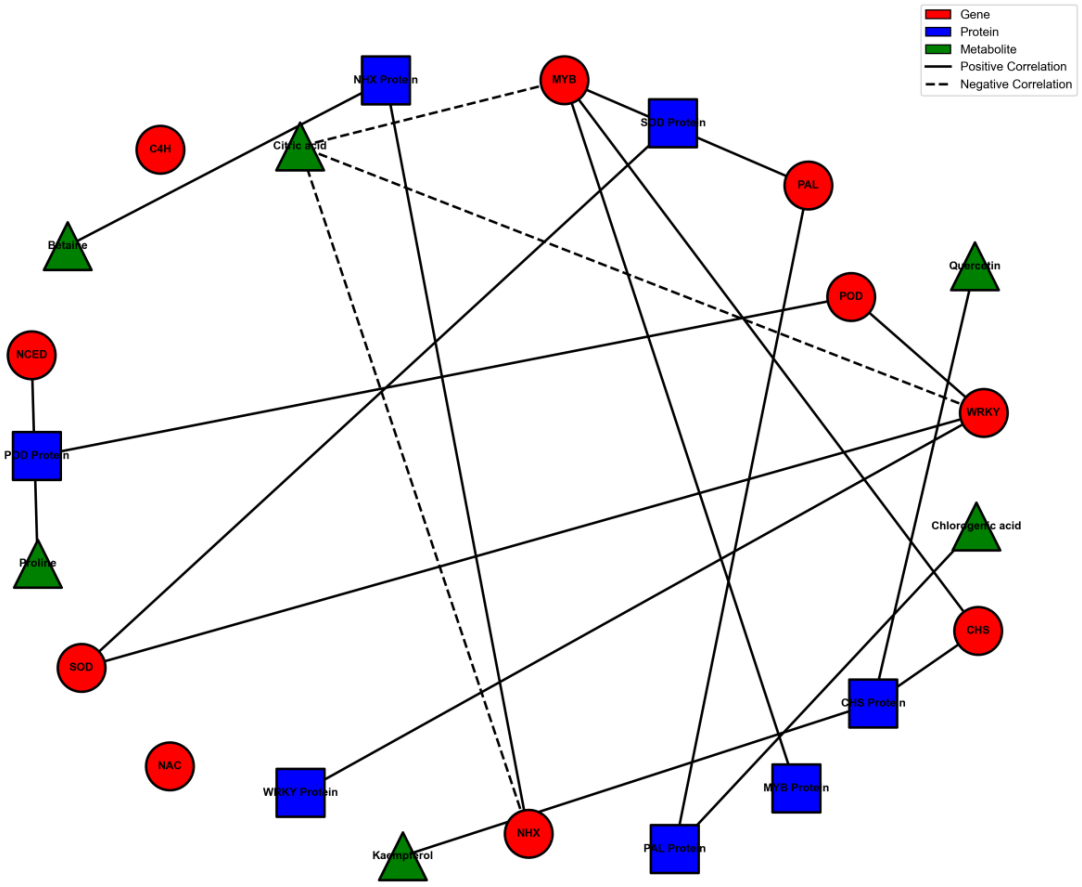

Figure 6. Regulatory network of salt-alkali tolerance genes - proteins - metabolites in peony.

(1) Construction of the gene-protein-metabolite regulatory network

The salt-alkali tolerance regulatory network for peonies demonstrated 286 nodes and 543 edges. The nodes covered 68 genes, 82 proteins, and 136 metabolites. The core nodes in the network mainly included transcription factors such as MYB and WRKY, genes and proteins related to antioxidant enzymes such as superoxide dismutase and peroxidase, genes and proteins related to flavonoid synthesis such as phenylalanine aminase and chalketone synthase, as well as key metabolites such as quercetin and proline (Figure 6).

(2) Analysis of core regulatory pathways

Correlation analysis indicated that MYB transcription factors were significantly positively correlated with flavonoid synthesis-related genes PAL, C4H, CHS and flavonoid metabolites ($r \geq 0.85$, $P < 0.01$). The WRKY transcription factor was significantly positively correlated with antioxidant enzyme genes SOD, POD, CAT and osmotic regulatory substances proline, betaine ($r \geq 0.82$, $P < 0.01$). Combined with the KEGG enrichment results, three core regulatory pathways for salt-alkali tolerance in peonies were identified, which included the transcription factor (MYB/WRKY) - flavonoid synthesis pathway, transcription factor (WRKY/NAC) - antioxidant enzyme system pathway, and ABA signaling pathway - osmotic regulatory substance synthesis pathway. These three pathways worked in synergy to jointly regulate the salt-alkali tolerance response of peonies.

6. Screening of cultivation regulation strategies for peony under saline-alkali stress

(1) Screening of exogenous regulatory substances

Table 2. Effects of exogenous regulatory substances on the growth and photosynthetic characteristics of peonies under saline-alkali stress.

Processing	Variety	Survival rate (%)	Plant height increment (cm)	Photosynthetic rate (μmol/m·s)
Control (clear water)	Fengdanbai	68.3 ± 3.2^{d}	2.7 ± 0.2^{d}	8.5 ± 0.4^{d}
	Ziban	52.5 ± 2.8^{e}	1.7 ± 0.1^{e}	6.2 ± 0.3^{e}
SA 50 (50 mg/L)	Fengdanbai	82.5 ± 3.5^{b}	3.5 ± 0.2^{c}	10.8 ± 0.5^{c}
	Ziban	68.3 ± 3.2^{d}	2.3 ± 0.1^{d}	8.1 ± 0.4^{d}
Pro 100 (100 mg/L)	Fengdanbai	85.0 ± 3.8^{ab}	3.8 ± 0.2^{b}	11.5 ± 0.6^{b}
	Ziban	72.5 ± 3.5^{c}	2.6 ± 0.1^{c}	8.8 ± 0.5^{c}
SA 50 + Pro 100 (mg/L)	Fengdanbai	90.0 ± 4.2^{a}	3.8 ± 0.2^{a}	12.8 ± 0.7^{a}
	Ziban	79.2 ± 3.6^{b}	2.6 ± 0.1^{a}	9.0 ± 0.5^{a}

Note: Different lowercase letters after the data in the same column indicated significant differences ($P < 0.05$).

Table 3. Effects of different matrix improvement treatments on matrix physical and chemical properties and peony growth indicators.

Matrix ratio (volume ratio)	Matrix pH	Total salt (g/kg)	Variety	Survival rate (%)	Plant height increment (cm)
Control (soil:river sand) (3:1)	8.5 ± 0.2^{a}	1.8 ± 0.1^{a}	Fengdanbai	68.3 ± 3.2^{e}	2.7 ± 0.2^{e}
			Ziban	52.5 ± 2.8^{f}	1.7 ± 0.1^{f}
Humus:soil:river sand (1:5:2)	7.9 ± 0.1^{b}	1.3 ± 0.1^{b}	Fengdanbai	77.5 ± 3.5^{d}	3.5 ± 0.2^{d}
			Ziban	65.0 ± 3.1^{e}	2.3 ± 0.1^{e}
Humus:soil:river sand (2:5:2)	7.5 ± 0.1^{c}	1.0 ± 0.1^{c}	Fengdanbai	85.0 ± 3.8^{b}	4.0 ± 0.2^{b}
			Ziban	72.5 ± 3.5^{c}	2.8 ± 0.1^{c}
Humus:soil:river sand (3:5:2)	7.2 ± 0.1^{d}	0.8 ± 0.1^{d}	Fengdanbai	92.5 ± 4.1^{a}	4.2 ± 0.2^{a}
			Ziban	85.0 ± 3.8^{a}	2.8 ± 0.1^{b}
Pumice:garden soil:river sand (1:5:2)	8.2 ± 0.2^{b}	1.5 ± 0.1^{b}	Fengdanbai	75.0 ± 3.4^{d}	3.3 ± 0.2^{d}
			Ziban	62.5 ± 3.0^{e}	2.2 ± 0.1^{e}
Zeolite:garden soil:river sand (1:5:2)	8.1 ± 0.2^{b}	1.4 ± 0.1^{b}	Fengdanbai	72.5 ± 3.5^{d}	3.2 ± 0.2^{d}
			Ziban	60.0 ± 2.9^{f}	2.1 ± 0.1^{f}

Note: Different lowercase letters after the data in the same column indicated significant differences ($P < 0.05$).

Exogenous spraying of different concentrations of SA, Pro, and their mixed solutions all increased the survival rate and growth vigor of peonies under saline-alkali stress to varying degrees. The effects of a mixed solution of 50 mg/L SA and 100 mg/L Pro demonstrated the best results with the survival rates of “Fengdanbai” and “Ziban” being increased by 28.3% and 35.7%, the plant height increments being increased by 42.6% and 51.2%, and the photosynthetic rates being increased by 38.5% and 45.8%, respectively, compared to the control (Table 2). The treatment effect of the mixed liquid was superior to that of a single substance, indicating that SA and Pro had a synergistic effect.

(2) Analysis of substrate improvement effects

All different substrate improvement treatments reduced the salinity and alkalinity of the substrate and enhanced the growth performance of peonies. The improvement effect of humus was the best, which increased with the increase of the humus addition ratio. When the ratio of humus, garden soil, river sand was 3, 5, 2, the pH value of the substrate decreased from 8.5 to 7.2, and the total salt content decreased from 1.8 g/kg to 0.8 g/kg. The survival rates of “Fengdanbai” and “Ziban” reached 92.5% and 85.0%, while the increase in plant height was 55.6% and 64.7% higher than that of the control group, respectively (Table 3). Humus provided favorable environmental conditions for the growth of peonies by improving the physical and chemical properties of the substrate and

Table 4. Effects of different water-fertilizer combinations on the growth, photosynthetic characteristics and soil salinity content of peony under saline-alkali stress.

Water and fertilizer combination	Variety	Survival rate (%)	Plant height increment (cm)	Photosynthetic rate (μmol/m²·s)	Soil salt content (g/kg)
Drip irrigation + low-salt fertilizer (0.5 g/L, N:P:K = 1:1:1)	Fengdanbai	91.7 ± 4.0^{a}	4.0 ± 0.2^{a}	13.2 ± 0.7^{a}	0.9 ± 0.1^{d}
	Ziban	83.3 ± 3.7^{a}	2.8 ± 0.1^{a}	9.5 ± 0.5^{a}	0.9 ± 0.1^{d}
Drip irrigation + conventional fertilizer (1.0 g/L, N:P:K = 2:1:1)	Fengdanbai	80.0 ± 3.6^{b}	3.2 ± 0.2^{b}	11.5 ± 0.6^{b}	1.3 ± 0.1^{b}
	Ziban	68.3 ± 3.2^{c}	2.1 ± 0.1^{c}	8.2 ± 0.4^{c}	1.3 ± 0.1^{b}
Dilute irrigation + low-salt fertilizer (0.5 g/L, N:P:K = 1:1:1)	Fengdanbai	75.0 ± 3.4^{c}	3.0 ± 0.2^{c}	10.8 ± 0.5^{c}	1.2 ± 0.1^{c}
	Ziban	65.0 ± 3.1^{d}	2.0 ± 0.1^{c}	7.8 ± 0.4^{d}	1.2 ± 0.1^{c}
Dilute irrigation + conventional fertilizer (1.0 g/L, N:P:K = 2:1:1)	Fengdanbai	70.0 ± 3.2^{d}	2.8 ± 0.2^{d}	10.2 ± 0.5^{d}	1.5 ± 0.1^{a}
	Ziban	58.3 ± 2.9^{e}	1.8 ± 0.1^{d}	7.2 ± 0.3^{e}	1.5 ± 0.1^{a}

Note: Different lowercase letters after the data in the same column indicated significant differences ($P < 0.05$).

enhancing its water and nutrient retention capacity.

(3) Optimization of water and fertilizer management

The peonies treated with drip irrigation and low-salt fertilizer had the best growth condition, and their survival rate, plant height increase, and photosynthetic rate were significantly higher than those of other water and fertilizer combinations. The survival rates of "Fengdanbai" and "Ziban" were 91.7% and 83.3%, the plant height increments were 4.0 cm and 2.8 cm, and the photosynthetic rates were 13.2 μmol/m²·s and 9.5 μmol/m²·s, respectively (Table 4). Drip irrigation could reduce water evaporation and lower the accumulation of soil salts, while low-salt fertilizers could prevent high-concentration fertilizers from intensifying saline-alkali stress. The combination of the two achieved efficient utilization of water and fertilizer.

(4) Verification of comprehensive cultivation regulation strategies

The optimal schemes of exogenous regulatory substances, substrate improvement, and water and fertilizer management were combined to form a comprehensive regulatory strategy, which was applied to the verification test of severe saline-alkali stress at 150 mmol/L. The exogenous regulatory substances selected were a mixed solution of SA 50 mg/L and Pro 100 mg/L. The substrate improvement adopted a ratio of humus, garden soil, and river sand at 3:5:2. The water and fertilizer management adopted a combination of drip irrigation and low-salt fertilizer. The results showed that, under the treatment of comprehensive regulatory strategies, the survival rates of "Fengdanbai" and "Ziban" reached 87.5% and 76.7%, respectively, both showing significant improvements compared to single regulatory measures. Meanwhile, all physiological indicators including plant height increment, photosynthetic rate, and antioxidant enzyme activity were superior to those of the single treatment group, which indicated that the comprehensive regulatory strategy could effectively enhance the salt-alkali tolerance of peonies through the synergistic effect of multiple measures, thereby alleviating the inhibitory effect of severe salt-alkali stress on their growth.

Discussion

The harm of saline-alkali stress to plants mainly manifests in three aspects including osmotic stress, ionic toxicity, and oxidative damage. Plants resist adverse conditions by initiating physiological mechanisms like osmotic regulation, ionic balance, and antioxidant defense. This study found that the salt-tolerant variety "Fengdanbai" could maintain a high photosynthetic efficiency and root vitality under salt-alkali stress. By accumulating more osmotic

regulatory substances of proline and betaine, it activated stronger antioxidant enzyme activity and reduced MDA accumulation, thereby alleviating stress damage, which was consistent with the salt-alkali tolerance physiological response mechanism of crops such as wheat and cotton. However, “Ziban” with relatively weak salt tolerance suffered severe damage to its photosynthetic system under stress, resulting in insufficient antioxidant capacity and osmotic regulation ability, which significantly inhibited its growth. Multi-omics analysis provided a systematic perspective for understanding the molecular mechanism of salt-alkali tolerance in peonies. Transcriptome analysis indicated that, under saline-alkali stress, peonies responded to adverse conditions by regulating the expression of genes in pathways of plant hormone signal transduction, MAPK signaling pathway, and secondary metabolite synthesis. The upregulation of the expression of NCED, a key gene involved in the synthesis of ABA, the central hormone in stress responses, promoted the accumulation of ABA, thereby activating the expression of downstream stress-tolerance genes. The co-expression of hormone signaling pathway genes of JA and ETH further enhanced the stress tolerance of peonies. Proteomic analysis revealed that the degradation degree of photosynthetically related proteins in the salt-tolerant variety “Fengdanbai” was lower than that in the sensitive variety, while the abundance of antioxidant enzymes and ion transporters significantly increased, indicating that it enhanced salt tolerance by maintaining photosynthetic function, strengthening antioxidant capacity and ion balance capacity. Metabolomics analysis showed that the massive accumulation of flavonoids and phenolic acid metabolites was an important material basis for the salt-alkali tolerance of peonies. These metabolites not only had antioxidant activity but also could participate in processes of cell wall reinforcement and ion chelation, reducing stress damage. The gene-protein-metabolite regulatory network constructed by multi-omics association analysis clarified the core role of transcription factors of MYB and WRKY in the regulation of salt-alkali tolerance in peonies. MYB transcription factors could directly regulate the expression of genes related to flavonoid synthesis and promote the accumulation of flavonoid metabolites. WRKY transcription factor enhanced the antioxidant capacity and osmotic regulatory ability of peonies by activating the expression of antioxidant enzyme genes and the synthesis of osmotic regulatory substances. The discovery of this regulatory network explored the molecular regulatory rules of salt-alkali tolerance in peonies and provided candidate genes for the breeding of salt-tolerant varieties. The research and development of cultivation regulation techniques is the key to the promotion of peony cultivation in saline-alkali areas. The comprehensive regulatory strategies screened out in this study could alleviate saline-alkali stress through multiple pathways in a coordinated manner. Specifically, exogenous regulatory substances could directly enhance the stress-resistant physiological functions of peonies. Humus improvement could enhance the physical and chemical properties of the substrate and reduce the harm of salt. Optimizing water and fertilizer management could enhance the efficiency of water and nutrient utilization and reduce salt accumulation. This strategy had significant effects on severe saline-alkali stress and high practical application value. However, this study had some deficiencies. The multi-omics analysis only focused on the samples under stress for 30 days and failed to reveal the dynamic regulatory process of the salt-alkali tolerance response of peonies. The functional verification of the core regulatory genes had not been carried out, and their regulatory mechanisms still needed to be further clarified. Future research can analyze the dynamic response patterns of salt-alkali tolerance in peonies through time series multi-omics and verify the functions of core genes by combining gene editing, transgenic, and other technologies, which will further improve the molecular regulatory network of salt-alkali tolerance in peonies and provide a more solid theoretical support for the breeding of salt-tolerant varieties and cultivation in saline-alkali land.

Acknowledgements

This study was supported by the second batch of Provincial Science and Technology Research and Development Plan Joint Fund Projects in Henan Province in 2024 (Grant No. 245200810025), the Central Plains Scholars Workstation (Grant No. 244400510006), and the Key Scientific Research Funding Project of Colleges and Universities in Henan Province (Grant No. 25B210018).

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