

Physiological and Molecular Mechanisms of *Medicago ruthenica* in Response to Different Saline-Alkali Stresses

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Research Article

Keywords: *Medicago ruthenica*, saline-alkali stress, physiological and biochemical, transcriptomic and metabolomic, joint analysis

Posted Date: January 6th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8386389/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Plant Biology on March 3rd, 2026.
See the published version at <https://doi.org/10.1186/s12870-026-08474-1>.

Abstract

Soil salinization is a global issue that constrains agricultural production and ecological restoration. *Melissitus ruthenica*, a stress-tolerant leguminous forage, holds significant potential for the rehabilitation of salinized grasslands. This study systematically compared the effects of three single salts (NaCl, Na₂SO₄, NaHCO₃) and their mixed saline-alkali solutions at varying concentrations on *M. ruthenica* seedlings. Through integrated physiological-biochemical assays, as well as transcriptomic and metabolomic analyses, we elucidated the physiological and molecular mechanisms underlying the response of *M. ruthenica* to saline-alkali stress. The results indicated that alkaline salt (NaHCO₃) stress caused significantly greater damage to plants compared to neutral salt, with *M. ruthenica* being unable to survive under 1.2% NaHCO₃ stress. Osmotic adjustment substances increased significantly with rising stress concentrations and were notably higher under alkaline salt treatment than in other treatments ($P < 0.05$). Transcriptome analysis revealed that the number of upregulated genes (4,835) and downregulated genes (7,286) in the NaHCO₃ versus CK groups was over 3.4 times higher than in other groups. The four core pathways identified were the biosynthesis of secondary metabolites, motor proteins, plant hormone signal transduction, and the MAPK signaling pathway in plants. Transcriptomic results demonstrated that amino acid metabolism plays a central role in the stress response, with 26 common differential metabolites identified as amino acids and their derivatives. L-arginine and L-ornithine exhibited significant accumulation under alkaline stress. Two pathways, D-amino acid metabolism and lysine degradation, were identified through conjoint analysis, with D-amino acid metabolism showing significantly greater enrichment under alkali stress compared to other treatments. This study systematically elucidates the multi-level regulatory mechanisms of *M. ruthenica* in response to saline-alkali stress, providing both theoretical foundations and candidate gene resources for the genetic improvement of saline-alkali tolerant forage varieties.

1. Introduction

Soil salinization represents a significant global environmental challenge that severely limits agricultural productivity and ecosystem stability. It is estimated that over 1 billion hectares of land worldwide are affected by salinization, and this figure continues to rise(1). Salt-alkali stress primarily consists of neutral salt stress (e.g., NaCl and Na₂SO₄) and alkaline salt stress (e.g., NaHCO₃ and Na₂CO₃). These two forms of stress collectively influence plant growth through osmotic stress, ion toxicity, and oxidative stress. Furthermore, alkaline salt stress contributes to elevated soil pH, which exacerbates deficiencies of essential nutrients such as iron and phosphorus, leading to more complex and severe damage to plants(2, 3).

Leguminous forage plays a crucial role in agricultural systems and ecological restoration in arid and semi-arid regions. It not only provides high-quality feed but also enhances soil fertility through biological nitrogen fixation(4). *Medicago ruthenica*, an exceptional perennial leguminous forage, has garnered significant attention due to its strong stress resistance, which includes drought tolerance, cold hardiness, and certain saline-alkali tolerance, positioning it as a promising species for the improvement

and utilization of saline-alkali grasslands(5, 6). Currently, while research has been conducted on the salt tolerance of *M. ruthenica*, most studies focus exclusively on single NaCl stress(7). However, in natural environments, saline-alkali stress typically involves a complex interplay of multiple salts, and its physiological and molecular mechanisms are considerably more intricate than those associated with single-salt stress(8). Therefore, systematically comparing the response differences of *M. ruthenica* to various types of single salts (NaCl, Na₂SO₄, NaHCO₃) and their mixed salts is essential for comprehensively revealing its true salt-alkali tolerance mechanisms.

The response of plants to saline-alkali stress constitutes a complex regulatory network involving multiple levels. At the physiological level, plants mitigate stress damage by regulating the accumulation of osmotic substances, activating antioxidant enzyme systems to scavenge reactive oxygen species, and maintaining ion homeostasis(9, 10). These physiological responses are driven by the coordinated regulation of a series of key genes and metabolites. In recent years, the rapid advancement of omics technologies, particularly the integrated analysis of transcriptomics and metabolomics, has provided powerful tools for systematically deciphering the mechanisms of plant stress resistance(11). The analysis of salt stress responses in alfalfa (*Medicago sativa*) demonstrated a significant upregulation of various genes associated with proline synthesis, ion transport, and reactive oxygen species (ROS) scavenging, including P5CS, NHX, and members of the SOD gene family. This genetic response was accompanied by the accumulation of osmoregulatory substances such as proline and betaine, which collectively contribute to the maintenance of cellular homeostasis(12). In contrast, research on yellow-flowered alfalfa (*Medicago falcata*) utilized transcriptomic and metabolomic association analyses to elucidate the critical role of the flavonoid metabolic pathway in the response to salt stress(13). Additionally, studies on chickpea (*Cicer arietinum*)(14) and soybean (*Glycine max*)(15) have identified a range of genes and metabolites related to salt tolerance, thereby preliminarily establishing their regulatory networks for salt tolerance. Through this integrated analysis, regulatory pathways from gene expression to metabolite changes can be constructed, thereby identifying core genes and key metabolites involved in stress responses(16).

This study aims to comprehensively investigate the physiological and molecular response mechanisms of *M. ruthenica* seedlings to various types of saline-alkali stress. We designed stress experiments using three single salts (NaCl, Na₂SO₄, NaHCO₃) and their compound salts at different concentrations. Integrated approaches were employed, including phenotypic observation, measurement of physiological and biochemical parameters, and transcriptomic and metabolomic analyses. The research outcomes will provide a solid theoretical foundation for elucidating the saline-alkali tolerance mechanisms of *M. ruthenica*, as well as valuable genetic resources and a theoretical basis for breeding novel stress-resistant legume forage varieties through genetic engineering approaches.

2. Materials and methods

2.1 Experimental design.

The test species, *M. ruthenica*, was provided by the College of Animal Science and Veterinary Medicine at Qinghai University. The experiment was conducted indoors using potted plants, specifically selected disposable plastic flowerpots measuring 10 cm × 10 cm. Initially, the flat clover seeds were germinated in a Petri dish. Once the seedlings reached the two-leaf stage, they were transplanted into the pots, with five plants per pot, and thoroughly watered for the first time. The seedlings were then placed in an artificial climate chamber for soil cultivation, utilizing a mixture of nutrient soil, perlite, and vermiculite in a 2:1:1 ratio. The cultivation conditions maintained a temperature of 25±1°C (day/night), a photoperiod of 16/8 hours (day/night), and a relative humidity of 60±5%. During the cultivation period, a nutrient solution with a pH of 7 was applied weekly, while distilled water was administered every three days. After 45 days of pot cultivation, stress treatment commenced. Distilled water was used to prepare three single-salt solutions (NaCl, Na₂SO₄, NaHCO₃) and a mixed salt solution with a 2:1:1 ratio of the three salts, designated as treatment solutions A, B, C, and D, respectively. Each group was established with three concentration gradients: 0.3%, 0.6%, and 1.2%, referred to as 1, 2, and 3, with distilled water serving as the control group. Following 72 hours of stress treatment, physiological, biochemical, and transcriptional metabolic indicators were measured, with each treatment replicated three times.

2.2 Sample collection.

For each treatment, the third fully expanded leaves from the top were collected as fresh samples. A total of 0.3 g of leaf tissue was utilized for metabolomic analysis, while 1.0 g was allocated for RNA sequencing. The remaining samples were reserved for physiological index measurements, with three biological replicates for metabolomics, transcriptomics, and physiological indices. All samples were stored at -80°C.

2.3 Physiological index measurement.

Soluble sugars(SS) were quantified using the phenol-sulfuric acid colorimetric method(17). Soluble proteins(SP) were assessed via the Bradford method, while proline(Pro) levels were determined using acidic ninhydrin colorimetry(18). Malondialdehyde(MDA) concentrations were measured through the thiobarbituric acid (TBA) colorimetric method(19). Catalase(CAT) activity was analyzed using ultraviolet spectrophotometry(20), and superoxide dismutase(SOD) was evaluated by the nitroblue tetrazolium(NBT) photoreduction inhibition method(18). Gibberellin(GA) and abscisic acid(ABA) were quantified through isotope dilution-UPLC-ESI-MS/MS (21, 22), and total flavonoids(TF) were measured using the NaNO₂-Al(NO₃)₃ colorimetric method(23).

2.4 Transcriptome analysis.

2.4.1 RNA extraction, library construction and sequencing.

Total RNA was extracted using the TRIzol kit, and the integrity and quality of the RNA were assessed using an Agilent 2100 Bioanalyzer and agarose gel electrophoresis. mRNA was enriched with Oligo(dT) magnetic beads, followed by fragmentation and first-strand cDNA synthesis utilizing random primers.

Subsequently, second-strand cDNA was synthesized using DNA polymerase I, RNase H, and dNTPs. The resulting products were purified and underwent end repair, A-tailing, and ligation of Illumina adapters. Fragment size selection was executed via agarose gel electrophoresis, followed by PCR amplification and sequencing on the Illumina NovaSeq platform.

2.4.2 Data analysis and functional annotation.

The raw sequencing data underwent quality control filtering to eliminate reads containing adapters, those with an N ratio exceeding 10%, or reads of low quality, defined as those where bases with a quality score (Q) ≤ 20 comprised more than 50% of the entire read. This process resulted in clean reads. Following this, the clean reads were aligned to the reference genome using HISAT2. Transcripts were reconstructed using StringTie, and gene expression levels were quantified as FPKM using RSEM. Differential expression analysis was conducted with DESeq2, identifying significantly differentially expressed genes based on an FDR threshold of 1. Subsequently, KEGG enrichment analysis was performed on the differentially expressed genes using the hypergeometric test, with pathways deemed significantly enriched if they had a Q value ≤ 0.05 after FDR correction.

2.5 Metabolomics analysis.

2.5.1 Metabolite extraction and LC-MS analysis.

A suitable amount of tissue sample should be taken, followed by the addition of an extraction solvent consisting of methanol, water, and formic acid in a ratio of 15:4:1, which contains 0.5% BHT. The mixture is vortex mixed and subjected to ultrasonication. After standing at -40°C , the mixture is centrifuged to collect the supernatant. Subsequently, purification is carried out using a solid-phase extraction column, which involves activation, adsorption, washing, and elution. The eluate is then concentrated to dryness and reconstituted with an 80% methanol-water solution. Following this, centrifugation is performed, and the supernatant is collected for LC-MS/MS analysis. The analysis utilizes a Waters Acquity UPLC system equipped with an Acquity UPLC BEH C18 column ($1.7\text{ }\mu\text{m}$, $2.1 \times 100\text{ mm}$). The column temperature is maintained at 35°C , and the flow rate is set at 0.30 mL/min . The mobile phase comprises water (containing 10 mM ammonium formate) and methanol, with gradient elution over a duration of 8 minutes, and an injection volume of $6\text{ }\mu\text{L}$. Mass spectrometric detection is conducted using an AB SCIEX 5500 QQQ-MS with an ESI ion source, where the ion spray voltage is established at 4500 V and the ion source temperature at 450°C . The curtain gas and collision gas are set at 35 arb and 7 arb , respectively. Data acquisition is performed in multiple reaction monitoring (MRM) mode, and relative quantification is achieved using the internal standard method. Metabolite levels are calculated based on the peak area ratio of the internal standard to the target compound through integration using MultiQuant software. Quality control samples are prepared by pooling equal amounts of each test sample to monitor instrument stability and systematic errors.

2.5.2 Data analysis and multivariate statistics.

Following the quality control of raw data, the relative standard deviation (RSD) of metabolites in quality control (QC) samples was utilized to assess data quality, with an RSD threshold of less than 10% deemed acceptable. Multivariate statistical analyses were conducted using the R programming language, incorporating unsupervised principal component analysis (PCA), and orthogonal partial least squares-discriminant analysis (OPLS-DA). The reliability of the OPLS-DA model was confirmed through cross-validation and permutation tests, while the contribution of metabolites to intergroup differences was evaluated based on variable importance in projection (VIP) values.

2.5.3 Differential metabolite screening and pathway analysis.

Differential metabolites were screened based on VIP values derived from OPLS-DA, combined with t-tests ($P < 0.05$), and their fold changes (FC) were calculated. The identified differential metabolites underwent KEGG pathway annotation and enrichment analysis. Pathway significance was assessed using hypergeometric tests, with a $Q \text{ value} \leq 0.05$ after FDR correction considered indicative of significantly enriched pathways. Additionally, metabolite set enrichment analysis (MSEA) was utilized to further identify key metabolic pathways.

2.6 Statistical analysis.

Analysis of variance (ANOVA) was conducted using the IBM SPSS statistical software package. Treatment means were separated using the least significant difference (LSD) method at the significance level of $P = 0.05$. Bar graphs were generated with GraphPad Prism version 10.1.2. Heatmaps and Venn diagrams were created using the online drawing tool available at <https://www.omicshare.com>. The network regulation diagram was constructed with the Cytoscape software.

3. Results

3.1 Phenotypic differences of *M. ruthenica* plants under different treatments.

This study investigates the phenotype and plant height of *M. ruthenica* under various treatments (Figure_1), revealing that the growth inhibition of *M. ruthenica* seedlings intensifies with increasing saline-alkali concentrations across different saline-alkali stresses. Notably, under NaCl stress, the 0.3% concentration exhibited no inhibitory effect and instead promoted plant growth compared to the control (CK). In contrast, other treatments consistently inhibited plant growth. Under NaHCO_3 stress, at a concentration of 0.6%, numerous dry and yellow leaves appeared at the base of the plants. At 1.2% concentration, all leaves drooped and exhibited wilting with necrotic symptoms, indicating that *M. ruthenica* cannot survive when NaHCO_3 concentration reaches 1.2%. Under mixed salt-alkali stress, at 0.6% concentration, the plants displayed relatively sparser leaves compared to 0.3%, although their height remained unaffected. However, at 1.2% concentration, the lower leaves of the plants drooped, with some leaves drying out and falling off.

3.2 Physiological indices of *M. ruthenica* under different treatments.

Under saline-alkali stress, SS, SP, and Pro play crucial roles in physiological regulation. As osmoregulatory substances, these compounds increase intracellular solute concentration, reduce cellular osmotic potential, and alleviate osmotic stress induced by saline-alkali conditions. As illustrated in Figure 2(a, b, c), when *M. ruthenica* is subjected to saline-alkali stress, the levels of osmoregulatory substances significantly increase with rising stress concentrations. Notably, SS content surges under NaHCO_3 stress, exhibiting levels significantly higher than those of the control group (CK) ($P<0.05$), while under NaCl and mixed saline-alkali stress, it remains lower than CK. The trends for SP and Pro content were consistent across different saline-alkali stresses: both were significantly higher under NaHCO_3 stress compared to Na_2SO_4 stress, higher under Na_2SO_4 stress than NaCl stress, and higher under NaCl stress than mixed saline-alkali stress ($P<0.05$). However, the control group (CK) demonstrated significantly greater SP content but significantly lower Pro content compared to all stress treatments ($P<0.05$).

Under salt-alkali stress, MDA, CAT, and SOD collectively contribute to the oxidative stress response in plants. Analysis of antioxidant indices in *M. ruthenica* revealed (Fig_2d, 2e, 2f) that the levels of antioxidant-related indicators significantly increased with rising stress concentrations ($P<0.05$). The malondialdehyde content peaked under NaHCO_3 stress, significantly surpassing that of other salt-alkali stresses, followed by mixed salt-alkali stress, indicating the most severe cell membrane damage in *M. ruthenica* under NaHCO_3 stress. At 0.3% and 0.6% Na_2SO_4 concentrations, malondialdehyde content was lower than that of the control (CK), whereas all other stress treatments exhibited significantly higher malondialdehyde levels compared to CK. The CAT content was highest under mixed saline-alkali stress, followed by NaHCO_3 stress, with all stress treatments demonstrating significantly elevated CAT levels compared to CK. The SOD content was highest under NaCl stress, followed by Na_2SO_4 stress, and all stress treatments showed significantly increased SOD levels compared to CK ($P<0.05$).

Under saline-alkali stress, GA primarily mitigates the inhibitory effects on plant growth by promoting development. ABA reduces water loss through transpiration by regulating stomatal closure. Total flavonoids, as secondary metabolites of plants, exhibit antioxidant functions, scavenging reactive oxygen species and alleviating oxidative damage to plant cells. As illustrated in Figure 2 (j, h, i), the concentrations of both GA and ABA significantly increased with rising stress levels. Except under NaCl stress, total flavonoid content under other stress treatments also showed a significant increase with higher concentrations ($P<0.05$). The GA content peaked under NaHCO_3 stress, significantly surpassing that of the control (CK) ($P<0.05$). The ABA content reached its maximum under 1.2% NaHCO_3 stress, which was significantly higher than that of other stress treatments ($P<0.05$). Under mixed saline-alkali stress, the ABA content consistently exceeded that of the control (CK), while other saline-alkali stresses only surpassed CK at high or medium-high concentrations. The total flavonoid content was highest under NaHCO_3 stress, followed by mixed saline-alkali stress. Notably, the total flavonoid content under all saline-alkali stress treatments was significantly greater than that of CK ($P<0.05$).

3.3 Screening and identification of differentially expressed genes (DEGs).

3.3.1 Differentially expressed genes.

This study conducted transcriptomic and metabolomic analyses on four distinct saline-alkali stress treatments at a concentration of 1.2%, alongside a control group. Genes meeting the criteria of $FDR < 0.05$ and $\log_2FC > 2$ were considered as significantly differentially expressed between groups. The number of differentially expressed genes (DEGs) exhibited substantial variations among the different saline-alkali stress treatments when compared to the control group (CK), as illustrated in Supplementary Figure 1. In the comparison between CK vs A, there were 1,404 upregulated DEGs and 1,649 downregulated DEGs. For the CK vs B, 1,316 genes were upregulated while 1,403 were downregulated. In the CK vs C group, 4,835 genes displayed upregulated expression, whereas 7,286 genes showed downregulated expression. In the CK vs D group, there were 1,370 upregulated DEGs and 1,899 downregulated DEGs. Notably, across the various treatment comparisons, the number of downregulated DEGs consistently exceeded that of upregulated DEGs, with this discrepancy being particularly pronounced in the CK vs D group, where downregulated genes significantly outnumbered upregulated ones. It is evident that under NaHCO_3 stress, the total number of DEGs (both upregulated and downregulated) compared to CK was the highest, markedly surpassing those in the other treatment groups. The abundance of upregulated DEGs was at least 3.4 times greater than in the other groups, while the downregulated DEGs exceeded those in the other groups by at least 3.8 times.

3.3.2 KEGG pathway enrichment analysis of differentially expressed genes.

To elucidate the functions of differentially expressed genes, we conducted KEGG pathway enrichment analysis using an adjusted p-value cutoff of 0.05 (FDR). Only the top 15 pathways are presented in Supplementary Figure 2. The results of the KEGG enrichment analysis, which compared various saline-alkali treatments with the control group, revealed four significantly enriched pathways characterized by a higher number of differentially expressed genes. These pathways include: biosynthesis of secondary metabolites, motor proteins, plant hormone signal transduction, and the MAPK signaling pathway in plants.

Secondary metabolites are a class of non-essential small-molecule compounds that are formed during the long-term evolution of plants. This pathway synergistically enhances plant tolerance to salt and alkali at physiological, biochemical, and molecular levels by synthesizing antioxidants, osmoregulatory substances, structural reinforcement compounds, and signaling molecules. Venn diagram analysis of genes involved in this pathway across the four groups revealed 195 common differentially expressed genes. Among these, the 7OMT and PRP1 genes exhibited high expression levels in all four treatment groups, indicating their regulatory roles under saline-alkali stress. In contrast, the CAB13, CAB3, ACO1, Redox2, and RZPF34 genes displayed high expression under salt stress and mixed stress conditions but low expression under alkali stress, making them potential key markers for distinguishing between responses to saline and alkali stress.

Motor proteins are a class of molecular machines that utilize ATP hydrolysis for energy to transport cargo directionally along the cytoskeleton. This pathway serves as the core molecular machinery for

plants to cope with saline-alkali stress. Through three key mechanisms—regulation of microtubule dynamics, organelle transport, and signal transduction—this pathway achieves a coordinated optimization of ion homeostasis, energy metabolism, and antioxidant defense. The motor proteins pathway was significantly enriched across different saline-alkali treatments compared to the control group. Genes such as K1F11, K1F10, K1F19, K1F22, K1F15, K1FC1, K1FC2/3, and ACTF were all annotated in this pathway. With the exception of the CK vs A group, these genes were downregulated in all other treatment groups. Most of these genes are classified as kinesins, indicating that saline-alkali stress inhibits the synthesis of kinesins within plant motor proteins.

The plant hormone signal transduction pathway mitigates the effects of ion toxicity, osmotic stress, and oxidative damage on plants under saline-alkali stress through the integration and balance of multiple hormones. This pathway was significantly enriched in four comparison groups. Genes ABI1/2, DELLA, PP2C, SnRK2, and ABF were upregulated in both the salt stress and mixed saline-alkali stress groups compared to the CK group, while genes PYR/PYL, ERF1/2, and YUCCA were downregulated. Notably, JAZ was upregulated in all four comparison groups, whereas MYC2 was downregulated. These findings indicate that the aforementioned genes play crucial roles in plant responses to saline-alkali stress.

The MAPK signaling pathway in plants enhances tolerance to saline-alkali stress through a cascade mechanism. By perceiving stress signals and sequentially activating the MAPKKK-MAPKK-MAPK cascade, this pathway performs dual regulatory functions. First, it amplifies the activity of downstream transcription factors to modulate the expression of genes involved in the synthesis of osmoprotectants and antioxidant enzymes. Second, it interacts with phytohormone signaling pathways to coordinately regulate physiological processes such as stomatal closure and root growth adaptation. This integrated response mitigates cellular damage from ion toxicity and oxidative stress. The pathway exhibited significant enrichment across four control groups, with the genes PP2C and SnRK2 being upregulated under both salt stress and mixed saline-alkali stress, while the genes WRKY33 and PYR/PYL were downregulated. Notably, the genes MEKK1, MKS1, MKK9, MYC2, and SPCH were consistently annotated as downregulated across all four groups, resulting in a substantial number of shared genes being annotated as downregulated within this pathway.

3.3.3 qRT-PCR validation of RNA-seq data.

We selected 10 shared pathways from the KEGG database and subsequently identified 10 highly expressed genes (HEGs) that were present in all four control groups. The accuracy of the transcriptome data for these 10 HEGs was validated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). The relative expression profile analysis presented in supplementary Figure 3 demonstrated that these 10 HEGs exhibited similar expression trends, with a high correlation between the qRT-PCR and RNA-seq data, indicating that the transcriptome data obtained in this experiment are relatively accurate.

3.4 Screening and identification of differentially accumulated metabolites (DAMs).

3.4.1 Metabolite profile.

We identified a total of 1,230 metabolites across five distinct treatment groups, comprising 217 amino acids and their derivatives, 207 flavonoids, 114 lipids, and 110 carbohydrates along with their derivatives (Figure_3a). To further explore the intrinsic differences in the metabolites of *M. ruthenica* under various saline-alkali stress treatments, we conducted principal component analysis (PCA) on the metabolite data (Figure_3b). The PCA results facilitate the visualization of overall metabolic differences between groups and the variability among samples within the same group. The analysis indicates that the first principal component accounts for 29.8% of the total variability in the dataset, while the second principal component explains 18.9%, demonstrating a clear separation of the samples. Notably, different saline-alkali treatments are distinctly separated along the first principal component, with treatment C positioned on the right side of PC1 and the other treatments on the left. This distribution correlates with the intensity of stress, as the NaHCO_3 treatment induces a stronger stress response compared to the other treatments.

3.4.2 Screening of identified metabolites.

To optimize intergroup separation, this experiment utilized Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) for further analysis following the removal of quality control samples. In the model, R^2X (cum) and R^2Y (cum) represent the explanatory rates of the constructed model for the X and Y matrices, respectively, while Q^2 indicates the model's predictive ability. The results revealed a clear separation between the different treatments and the control group (Supplementary Figure_4a-d). Comparisons of CK vs A ($R^2X=0.908$, $R^2Y=1$, $Q^2=0.998$), CK vs B ($R^2X=0.928$, $R^2Y=0.999$, $Q^2=0.995$), CK vs C ($R^2X=0.991$, $R^2Y=1$, $Q^2=0.999$), and CK vs D ($R^2X=0.954$, $R^2Y=1$, $Q^2=0.999$) all yielded high values for R^2X , R^2Y , and Q^2 , indicating that these analyses are reproducible, reliable, and suitable for screening differential metabolites. To prevent overfitting during the modeling process, permutation tests were employed to validate the model and ensure its effectiveness. The gradual decrease of R^2 and Q^2 in the stochastic model indicated that there was no overfitting in the original model (Supplementary Figure 4a1-d1), demonstrating that the separation of intergroup metabolites was statistically significant.

In this study, differential metabolite screening was based on the criteria of $p\text{-value}<0.05$ and $\text{VIP}>1$. The results were visualized using scatter plots (Figure_3c), revealing a total of 124 significantly differential metabolites in the comparison of CK vs A (78 up-regulated and 46 down-regulated), 100 in CK vs B (49 up-regulated and 51 down-regulated), 102 in CK vs C (36 up-regulated and 66 down-regulated), and 102 in CK vs D (61 up-regulated and 41 down-regulated). We performed a comparative analysis to identify similarities and differences among the various treatments relative to the control, generating a Venn diagram (Figure_3d). A total of 46 common differential metabolites were identified, which could serve as potential biomarkers for distinguishing different saline-alkali stress conditions. Notably, 26 of these 46 differential metabolites were amino acids and their derivatives (Supplementary Table_1). Among these 26 amino acids, 4-Aminobutyric acid, isoleucine, L-Leucine, N-Methyl- α -aminoisobutyric acid, and beta-Alanine methyl ester demonstrated higher concentrations under salt stress and mixed stress compared to the control (CK), while exhibiting lower levels under alkali stress in relation to CK. These metabolites

should be prioritized in future research focusing on the differentiation between salt stress and alkali stress.

3.4.3 KEGG pathway enrichment analysis of DAMs

KEGG pathway enrichment analysis was conducted on metabolites across various control groups to elucidate their biological functions, identifying significantly enriched pathways ($p\text{-value} \leq 0.05$). We annotated the differential metabolites in each group and categorized them into distinct pathways. The differential metabolites observed between CK vs A, CK vs B, CK vs C, and CK vs D were associated with 83, 76, 76, and 76 pathways, respectively, with the major pathways illustrated in Figures 4a-d. Notably, the top three pathways under salt stress and mixed stress are identical to those in the control group: Biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid; Biosynthesis of alkaloids derived from terpenoid and polyketide; and 2-Oxocarboxylic acid metabolism. In contrast, the D-Amino acid metabolism pathway exhibits more significant enrichment under alkali stress. Additionally, amino acid-related pathways such as Lysine degradation, Biosynthesis of alkaloids derived from histidine and purine, Biosynthesis of amino acids, Alanine, aspartate, and glutamate metabolism, and Aminoacyl-tRNA biosynthesis were all enriched in Group 4, indicating that these pathways are regulated when plants are subjected to saline-alkali stress.

3.4.5 Targeted metabolomics analysis.

Through analyses of physiological and biochemical indicators, transcriptomics, and broad-target metabolomics, we found that saline-alkali stress significantly affects the amino acid content in plants. Consequently, we conducted targeted amino acid metabolomics research on *M. ruthenica* to further validate the response of amino acid metabolites under saline-alkali stress. We identified and quantified 36 amino acid metabolites. By intersecting the common amino acid pathways enriched in the four broad-target metabolomics groups with these 36 amino acids, we identified six differential metabolites (Supplementary Figure_5a-f). The results indicate that the content of these six metabolites was highest under NaHCO_3 stress, significantly exceeding that of the control group and other stress treatments ($P < 0.05$).

These amino acids work synergistically through multiple pathways, including osmotic regulation, antioxidant defense, signal transduction, ion homeostasis, hormone synthesis, and energy supply, to help plants alleviate salt stress damage. Among them, L-arginine (polyamine/NO pathway) and L-ornithine (polyamine synthesis) play particularly crucial roles. As shown in Supplementary Figure 5 (d, f), these two amino acids exhibit consistent responses to various saline-alkali stresses, with the response pattern being $C > A > D > CK > B$.

3.5 Conjoint analysis.

3.5.1 Integrated analysis of physiological biochemistry and transcriptomics.

To investigate the relationships among the aforementioned research contents, we conducted a correlation analysis between physiological and biochemical indicators and all genes. According to the expression profiles, biological replicates demonstrated good clustering within groups, while samples from different treatments exhibited significant separation trends (Supplementary Figure_6a). To assess whether genes share similar expression patterns, we selected scale independence in the power plot, with the soft threshold as the x-axis and the R^2 value of correlation coefficients as the y-axis. This demonstrated that the network topology conforms to a scale-free distribution, ensuring both robustness and biological significance of the network (Supplementary Figure_6b). To avoid selecting an optimal soft threshold that is either too sparse or too dense during the construction of the gene co-expression network, we directly observed changes in network topological properties under different power values through the average connectivity of power values, confirming that gene connections follow a scale-free network distribution (Supplementary Figure_6c).

A clustering tree was constructed using the dynamic tree-cutting method based on the correlation of gene expression levels in WGCNA (Figure_5a). This process led to the identification of 23 gene co-expression modules (Figure_5b). After filtering for genes with expression levels ≥ 2 , we identified a total of 23,929 correlated genes, which were subsequently merged into 23 modules by combining similar correlated modules. Four modules—dark turquoise, turquoise, dark red, and light cyan—were identified as exhibiting significant correlations with physiological and biochemical traits, using thresholds of $R \geq 0.8$ and $P < 0.05$, with a preference for positive correlations. As illustrated in Figure 10b, the genes within the dark turquoise module showed significant positive correlations with CAT, SOD, MDA, GA, and TF; the turquoise module's genes exhibited significant positive correlations with SS, Pro, GA, ABA, and TF; the dark red module's genes demonstrated significant positive correlations with Pro, SOD, ABA, and TF; while the light cyan module's genes displayed significant positive correlations with Pro, CAT, SOD, and TF.

3.5.2 Integrated analysis of physiology, biochemistry and metabolomics.

We employed a consistent approach to conduct a correlation analysis between physiological and biochemical indicators and all metabolites. The results demonstrated strong clustering among biological replicate groups, with the network topology conforming to a scale-free distribution. The connections between metabolites also adhered to a scale-free network distribution, rendering them suitable for subsequent division into metabolite modules.

A clustering tree was constructed based on the correlation of metabolite expression levels using Weighted Gene Co-expression Network Analysis (WGCNA) (Figure_5c), which identified eight metabolite clustering modules (Figure_5d). The 1,230 correlated metabolites detected were grouped into eight modules, each containing between 67 and 493 metabolites, after merging related similar modules. Three modules (turquoise, brown, and red) exhibiting significant correlations with physiological and biochemical characteristics were identified at $R \geq 0.8$ and $P < 0.05$. The metabolites in the turquoise module displayed significant positive correlations with SS, Pro, GA, ABA, and TF, while those in the brown module showed significant negative correlations with Pro, CAT, SOD, MDA, GA, ABA, and TF. The

metabolites in the red module demonstrated significant negative correlations with SP, CAT, SOD, MDA, and TF.

3.5.3 Network regulation graph based on pearson correlation coefficient model.

Based on the results of the WGCNA analysis, we selected 21 genes from four relevant modules and 30 metabolites from three relevant modules. Following the correlation analysis between these genes and metabolites, Figure 6 was generated, which illustrates a network diagram depicting relationship pairs with absolute correlation coefficients exceeding 0.8. In this figure, blue dots represent genes, pink dots represent metabolites, solid lines indicate positive correlations, and dashed lines indicate negative correlations. The data indicate that the genes Os06g0486800, At5g07050, TDT, FAB1D, DTX27, TDT, CCL4, UGE5, and MUCNAIN negatively regulate the metabolites 4'-Hydroxyacetophenone and L-arginine while positively regulating other metabolites. Additionally, the gene At3g50520 negatively regulates 4'-Hydroxyacetophenone, L-arginine, and Cocamidopropyl betaine, while positively regulating other associated metabolites. The gene dnaJ1 negatively regulates Cocamidopropyl betaine, whereas AS and GALM positively regulate it. Furthermore, the gene ENODL2 negatively regulates the associated metabolite, while the genes SNL4 and At5g64700 positively regulate it.

3.5.4 Integrated analysis of transcriptomics and metabolomics.

Through integrated transcriptomic and metabolomic analyses, this study identified two significantly enriched pathways: D-Amino acid metabolism and Lysine degradation. Among these, D-Amino acid metabolism exhibited more pronounced enrichment under alkali stress compared to other treatments, while Lysine degradation was identified as a common pathway. As illustrated in Figure 7a, the comparison between the CK and C groups revealed the highest number of enriched genes and metabolites, with Figure 7b further demonstrating that this group contained the most enriched genes. Additionally, we have created simplified schematic diagrams of the two pathways (Figure_7c). The figure indicates that metabolites such as L-lysine, L-Arginine, L-Proline, D-Proline, L-Ornithine, L-Histidine, and D-Histidine are annotated within the D-Amino acid metabolism pathway. Notably, D-Proline was found to be enriched under salt stress but decreased under alkali stress and mixed saline-alkali stress. With the exception of D-Proline, all other metabolites exhibited downregulation following saline-alkali stress, with significant downregulation observed after alkali stress. The LYSA1 gene, annotated within this pathway, demonstrated downregulation under alkali stress while remaining unchanged or upregulated under salt stress and mixed stress. Metabolites such as L-lysine, L-Saccharopine, 2-Aminoadipic acid, Nepsilon-Acetyl-L-lysine, L-Pipecolate, Succinic acid, and Trimethyl-lysine were annotated within the lysine degradation pathway. L-Saccharopine, 2-Aminoadipic acid, and Succinic acid were consistently upregulated under all stress conditions, while L-lysine, L-Pipecolate, and Trimethyl-lysine were generally downregulated. Nepsilon-Acetyl-L-lysine exhibited upregulation following NaCl and combined stress, but downregulation under alkaline stress. Genes annotated in this pathway include LKR/SDH, ALDH7B4, At2g24580, ACCT1, LPD2, EZA1, ALDH3H1, and CaMKMT, among others. Notably, At2g24580 was downregulated after Na₂SO₄ stress but upregulated under other treatments, whereas LPD2

demonstrated a downward trend following all stress treatments. The remaining genes were all upregulated after stress treatments, with the most significant upregulation observed under alkaline stress.

4. Discussion

This study systematically elucidates the complex regulatory network of *M. ruthenica* in response to varying saline-alkali stresses through integrated physiological-biochemical, transcriptomic, and metabolomic analyses. Our findings not only confirm the fundamental differences in plant damage caused by different types of saline-alkali stress but also, from a systems biology perspective, elucidate the core mechanisms by which *M. ruthenica* enhances stress tolerance through the coordinated regulation of signal transduction, hormone homeostasis, secondary metabolism, and amino acid metabolism.

4.1 Unique toxic effects of alkaline salt stress and the extreme response of *M. ruthenica*.

Phenotypic and physiological indicators consistently demonstrated that alkaline salt (NaHCO_3) stress exerted significantly greater toxicity on *M. ruthenica* compared to neutral salts (NaCl , Na_2SO_4) and mixed salts. The severe wilting and necrosis of plants under NaHCO_3 treatment, along with a marked increase in MDA content, collectively indicated the most severe oxidative damage to their cellular membrane systems(24). This finding aligns with research conclusions reported in *Medicago sativa*(25) and *Cicer arietinum*(26). This commonality arises from the dual mechanisms of damage induced by alkaline stress: on one hand, the ionic toxicity and osmotic stress caused by Na^+ , and on the other hand, the disruption of ion absorption balance and induction of deficiency symptoms in essential elements such as iron and phosphorus due to high pH environments(27). Consequently, the significant accumulation of osmoregulatory substances and antioxidant enzymes under NaHCO_3 stress reflects the considerable physiological cost that plants incur to maintain survival. This response pattern has also been observed in salt-alkali tolerant *Medicago falcata*(13). The transcriptomic data provide compelling molecular evidence that NaHCO_3 treatment triggered a significantly higher number of differentially expressed genes (4,835 upregulated) compared to other treatments. This indicates that *M. ruthenica* must activate an unprecedented and highly specific gene regulatory program to cope with this extreme environment, which explains why alkaline soils generally present greater challenges to plants under natural conditions. Notably, the 1.2% NaHCO_3 stress in this study pushed *M. ruthenica* to the brink of death, while some alkali-tolerant alfalfa varieties were able to survive despite experiencing growth inhibition under similar conditions(13). This suggests that *M. ruthenica* may be more sensitive to nutrient deficiencies induced by high pH.

4.2 Construction and synergistic effects of multi-level regulatory networks.

Our transcriptome analysis identified four core pathways: the biosynthesis of secondary metabolites, motor proteins, plant hormone signal transduction, and the MAPK signaling pathway in plants. These pathways collectively form a well-structured and functionally complementary synergistic response

network. Signal perception and transduction involve the rapid activation of the MAPK signaling pathway and various plant hormone signal transduction pathways as upstream signaling modules. The MAPK cascade functions as a conserved amplifier of stress signals(28), while hormone pathways—particularly those involving abscisic acid (ABA) and jasmonic acid (JA)—play a crucial role in integrating stress signals (29). In this study, the significantly differential expression of core ABA signaling components, such as ABI1/2, SnRK2, and ABF, alongside key JA signaling genes, including JAZ and MYC2, underscores the central role of these two hormones in coordinating stomatal closure, osmotic regulation, and the expression of stress-responsive proteins(30). This mechanism has also been documented in studies on salt tolerance in alfalfa(31) and soybean(32). A key finding is the widespread downregulation of motor protein pathways. Motor proteins are essential for energy-intensive intracellular cargo transport and organelle positioning(33). Their suppressed expression may signify a strategic contraction, during energy crises, plants reallocate limited ATP resources from fundamental infrastructure to more urgent wartime tasks, such as synthesizing defensive compounds. This reflects the energy optimization strategy of plants under stress. This phenomenon has been observed in the stress responses of both yeast and Arabidopsis(34, 35), indicating an ancient and conserved survival mechanism. Under the regulation of signaling pathways and the facilitation of energy redistribution, the biosynthetic pathway of secondary metabolites is robustly activated as an effector module. This pathway extensively synthesizes antioxidant compounds, including flavonoids and alkaloids, which directly counteract oxidative stress(36, 37). This response closely aligns with alfalfa's adaptation to drought and salt stress(38). Our metabolomic data confirm the significant accumulation of flavonoids and various amino acid derivatives under stress.

These four pathways create an efficient closed loop of signal perception (MAPK/hormones) → energy optimization (motor proteins) → defense execution (secondary metabolism), collectively enhancing the salt-alkali tolerance of *M. ruthenica*.

4.3 Amino acid metabolic reprogramming.

One of the most significant findings of this study is the pivotal role of amino acid metabolism in the saline-alkali response of *M. ruthenica*, with 26 out of 46 common differential metabolites identified. This indicates that the reprogramming of amino acid metabolism serves as a fundamental metabolic strategy for *M. ruthenica* in adapting to saline-alkali stress. Amino acids function not only as building blocks for protein synthesis but also play diverse roles as osmoregulators (e.g., proline), antioxidant precursors (e.g., tyrosine-derived flavonoids), and signaling molecules(39, 40).

The levels of specific amino acids, including 4-aminobutyric acid and isoleucine, were found to increase under both salt stress and combined stress, while they decreased under alkali stress. This variation indicates their potential as biomarkers for differentiating between various types of stress. Targeted metabolomics further clarified the critical roles of L-arginine and L-ornithine in response to severe alkali stress. These two amino acids act as direct precursors for polyamine synthesis, which is vital for maintaining ion homeostasis, scavenging reactive oxygen species, and stabilizing membrane

structures(41, 42). In salt-tolerant wild soybean, the arginine biosynthesis pathway was also significantly enriched(43). Our findings suggest that the arginine-polyamine metabolic axis may be a crucial pathway for *M. ruthenica* to manage high-pH stress. Furthermore, the differential response patterns of certain branched-chain amino acids and GABA under salt and alkali stress enhance their potential as biomarkers for distinguishing between stress types, thereby offering new insights for the future development of rapid diagnostic technologies.

4.4 Integrated analysis reveals gene-metabolite interaction network.

Through Weighted Gene Co-expression Network Analysis (WGCNA) and correlation network analysis, we successfully established a visualized regulatory network that links physiological phenotypes, gene expression, and metabolite accumulation. For example, several genes exhibited significant co-expression relationships with 4'-Hydroxyacetophenone, a phenolic antioxidant, and L-arginine. These modules included a variety of functional genes, such as transporters and enzymes involved in cell wall modification. This characteristic of complex network interactions mirrors the stress response networks documented in poplar(44) and rice(45), highlighting the systemic and intricate nature of plant stress resistance. The network clearly indicates that the salt-alkali tolerance of *M. ruthenica* is not governed by a single 'star gene', rather, it is supported by a robust network comprising numerous functional proteins and metabolites. The combined analysis identified the D-amino acid metabolism and lysine degradation pathways, particularly their specific responses to alkaline stress, providing precise entry points for future in-depth research into the differential regulatory mechanisms of various salt-alkali stresses.

5. Conclusion

In summary, this study systematically elucidates that *M. ruthenica* responds to saline-alkali stress through a multi-level synergistic network. This network, centered around MAPK and plant hormone signaling, regulates energy allocation by inhibiting motor proteins, ultimately facilitating the substantial synthesis of secondary metabolites, particularly amino acids and flavonoids. This process establishes an effective defense system at both physiological and biochemical levels. Notably, alkaline salt stress induces more intense and specific transcriptional and metabolic reprogramming, with the upregulation of the arginine-polyamine metabolic pathway potentially serving as a key response to high-pH stress. These findings not only enhance our understanding of the mechanisms underlying saline-alkali tolerance in leguminous forage but also offer valuable candidate genes and metabolic markers for improving crop stress resistance through molecular breeding approaches.

Declarations

Acknowledgments

Thanks to the support of project funding project: Qinghai Provincial Major Science and Technology Special Project(2023-NK-A3) and Processes, Mechanisms, and Research Methods of Rhizosphere

Synthetic Microbial Communities Promoting Vegetation Restoration in Saline-Alkali Lands of the Qaidam Basin(U23A2043).

Authors' contributions

Xiaoli Wei: Writing-original draft, Resources, Investigation. Xiaojian Pu: Visualization, Formal analysis. Wei Wang: Project administration, Conceptualization. Yuanyuan Zhao: Funding acquisition. Chuyu Tang: Supervision. Guangxin Lu: Supervision. Chengti Xu: Writing-review & editing, Funding acquisition, Conceptualization. All authors contributed critically to the drafts and gave final approval for publication.

Funding

Qinghai Provincial Major Science and Technology Special Project(2023-NK-A3) and Processes, Mechanisms, and Research Methods of Rhizosphere Synthetic Microbial Communities Promoting Vegetation Restoration in Saline-Alkali Lands of the Qaidam Basin (U23A2043) .

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the SRA repository, <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1395193>.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare no conflicts of interest.

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Figures

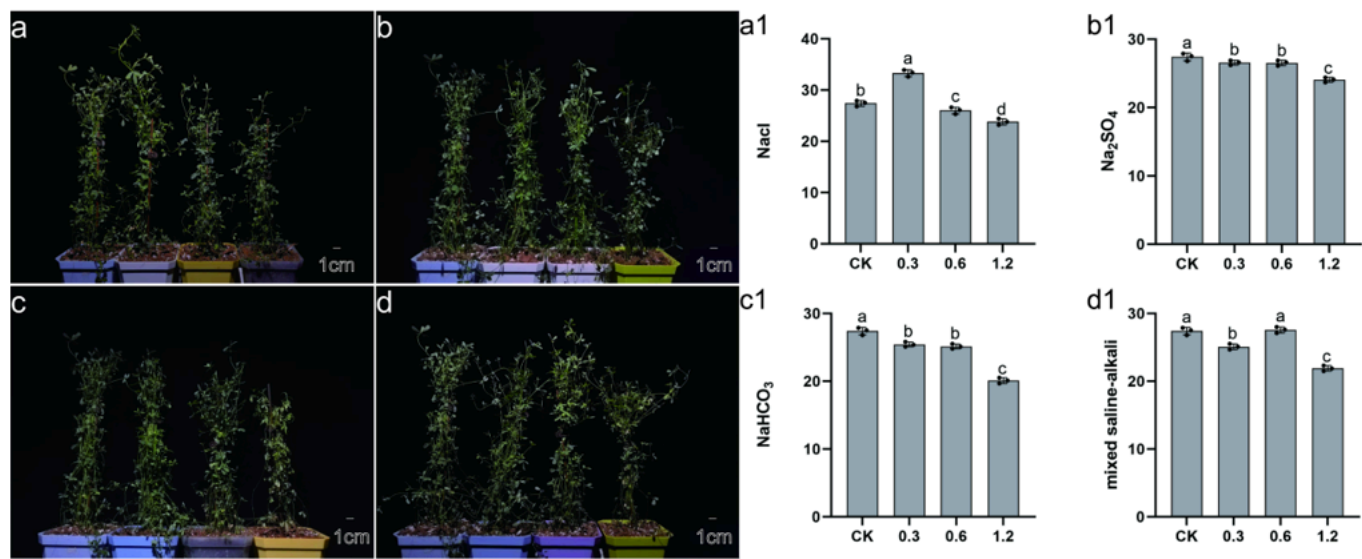


Figure 1

The phenotypic differences and plant heights of *M. ruthenica* plants after 72 hours of different treatments. Notes: (a, a1) NaCl stress; (b, b1) Na_2SO_4 stress; (c, c1) NaHCO_3 stress; (d, d1) mixed saline-alkali stress. Each image, arranged from left to right, represents control (CK), and treatment concentrations of 0.3%, 0.6%, and 1.2%, respectively.

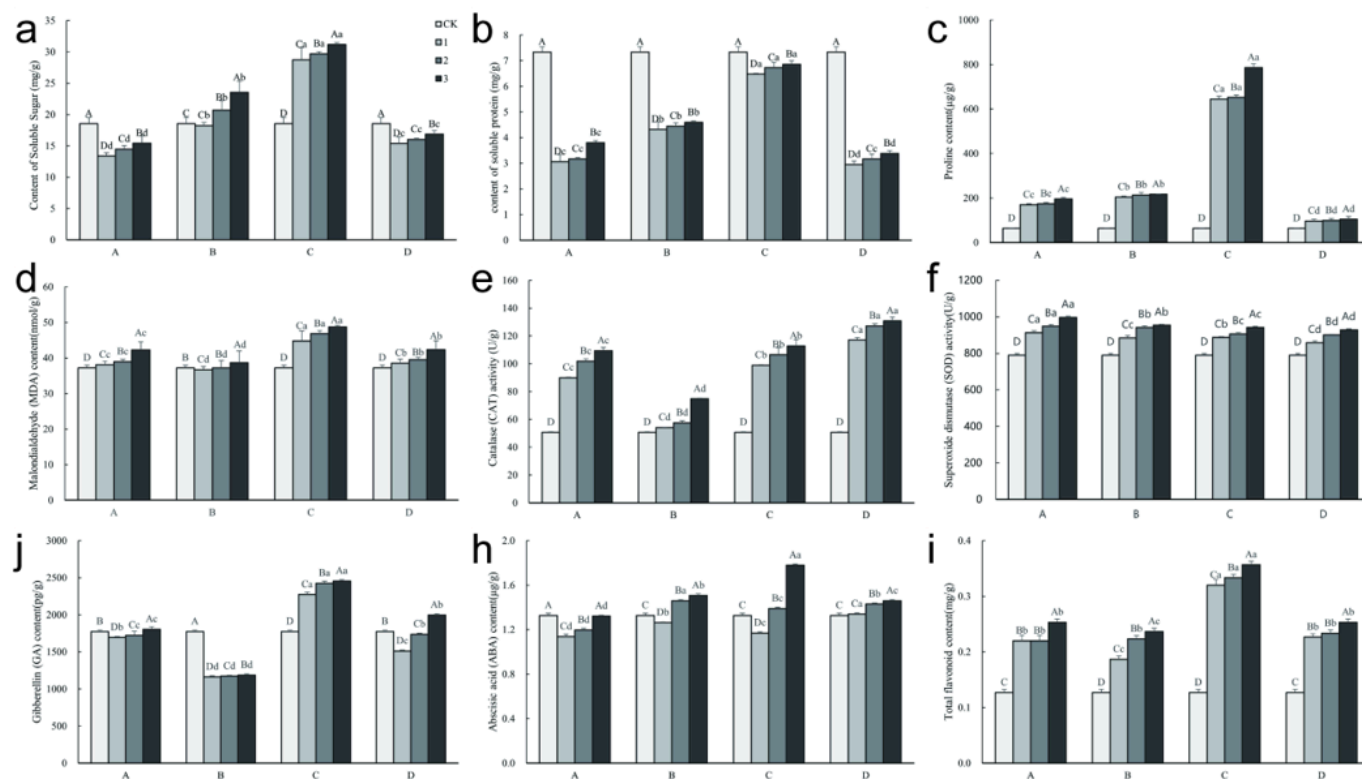


Figure 2

Physiological index results. Note: The horizontal coordinates A, B, C, and D represent NaCl, Na₂SO₄, NaHCO₃, and mixed saline-alkali stress, respectively. The numbers 1, 2, and 3 denote concentration gradients of 0.3%, 0.6%, and 1.2%, respectively. Different uppercase letters indicate significant differences ($P < 0.05$) among various concentrations under the same saline-alkali stress, while different lowercase letters indicate significant differences ($P < 0.05$) among various saline-alkali stresses at the same concentration.

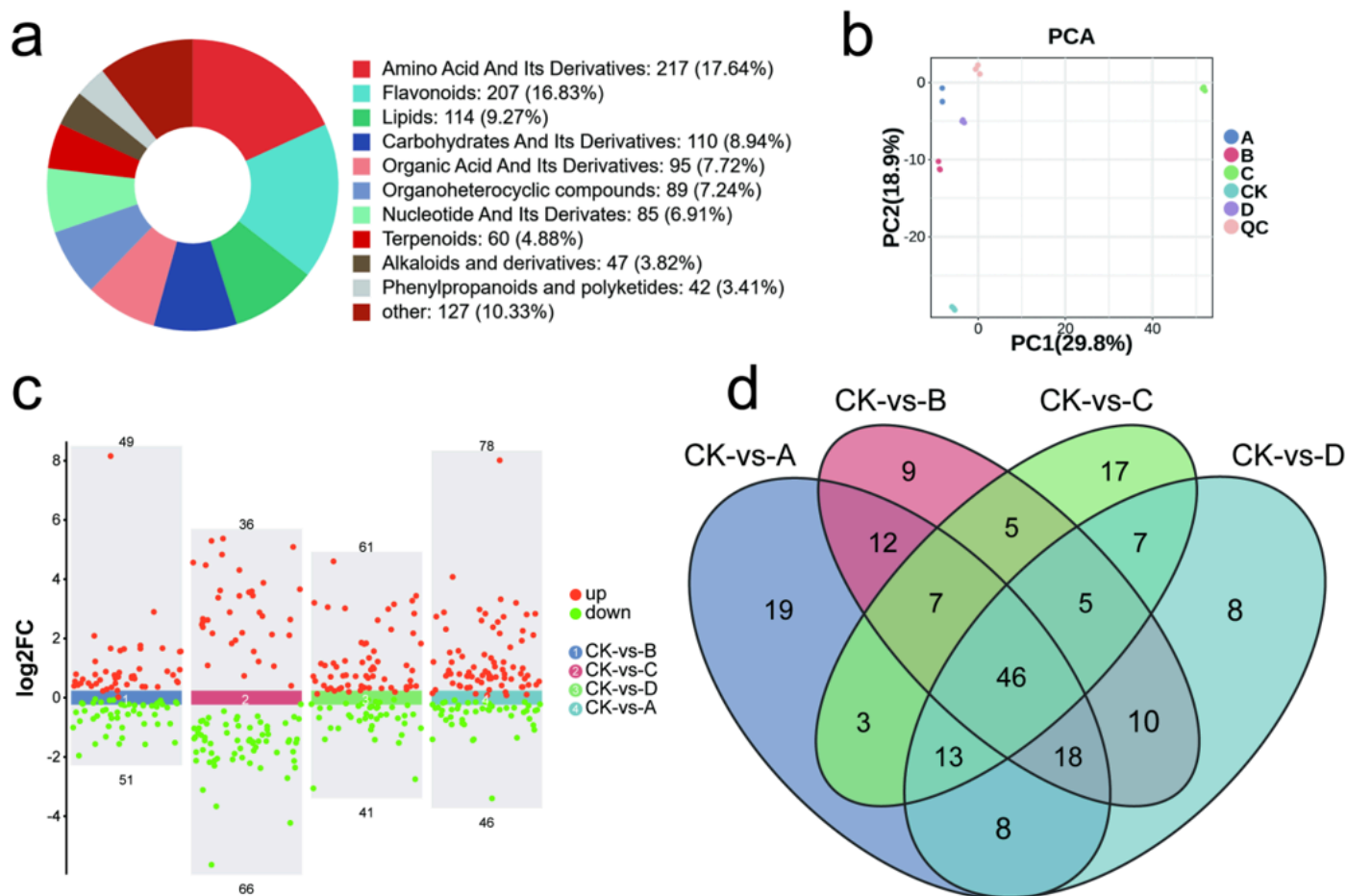


Figure 3

Metabolomic analysis of *M. ruthenica* seedlings under different saline-alkali stress conditions. (a) Classification of total metabolites. The top three categories are amino acids and their derivatives, flavonoids, and lipids. (b) Principal component analysis (PCA) of metabolic differences in *M. ruthenica* seedlings under different saline-alkali stress conditions. (c) Scatter plot of differential metabolites between different treatments. The scatter plot primarily displays the screened differential metabolites. This study identified differential metabolites based on the criterion of $p\text{-value} < 0.05$. (d) Venn diagram of differential metabolites between different treatments.

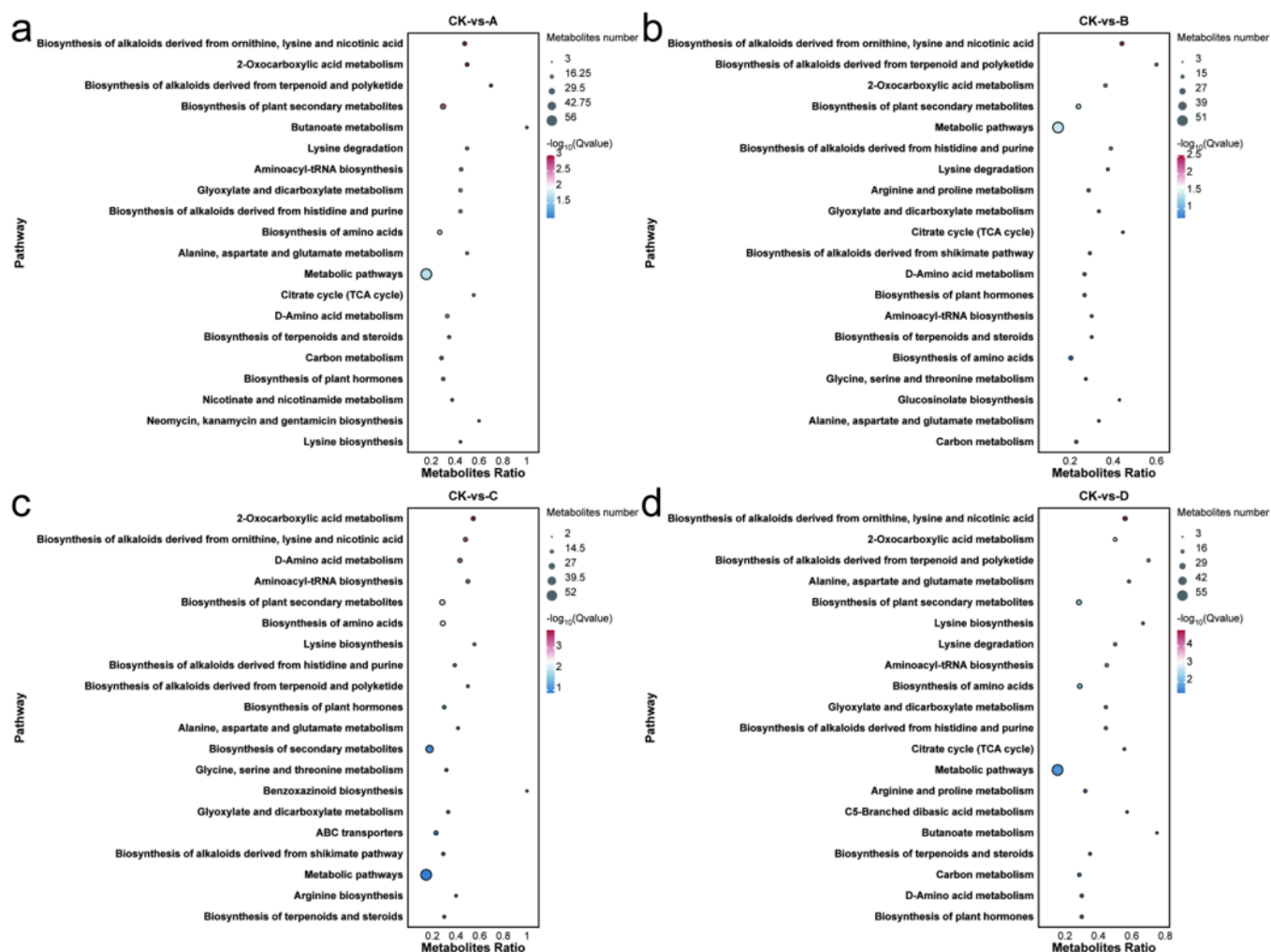


Figure 4

KEGG enrichment analysis of metabolites in *M. ruthenica* seedlings under different saline-alkali stress conditions. The x-axis represents the enrichment score, while the y-axis displays the top 20 pathway entries. Larger bubble size indicates a greater number of differential metabolites. The bubble color gradient from blue to red denotes decreasing enrichment p-values, reflecting increasing statistical significance.

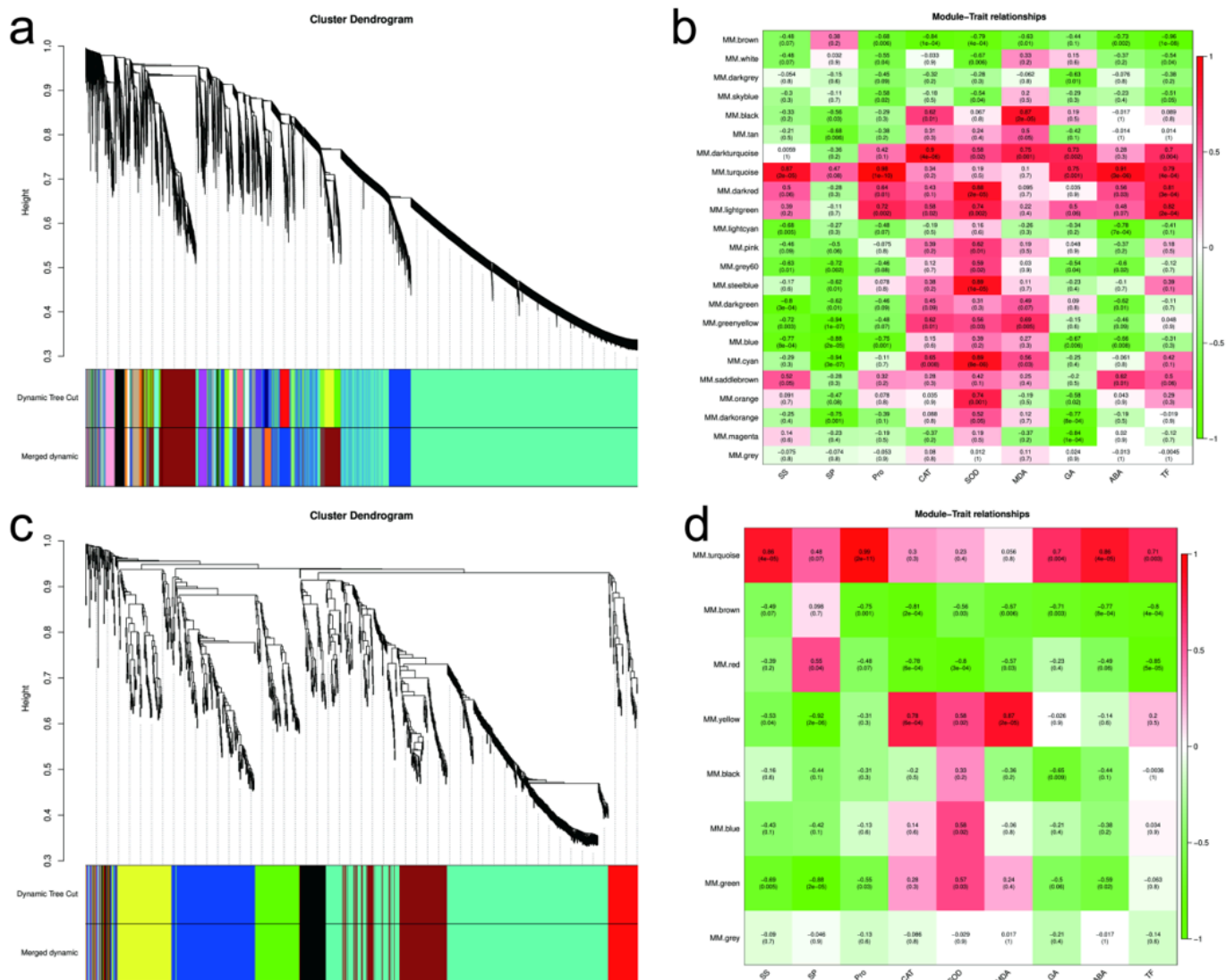


Figure 5

Correlation Analysis of Transcriptome, Metabolome, and Physiological-Biochemical Characteristics. Each row and column corresponds to a specific module. The numerical value within each square represents the Pearson correlation coefficient between two modules, with the corresponding P-value provided in parentheses. Darker-colored squares (either redder or greener) signify stronger correlations, whereas lighter-colored squares denote weaker correlations.

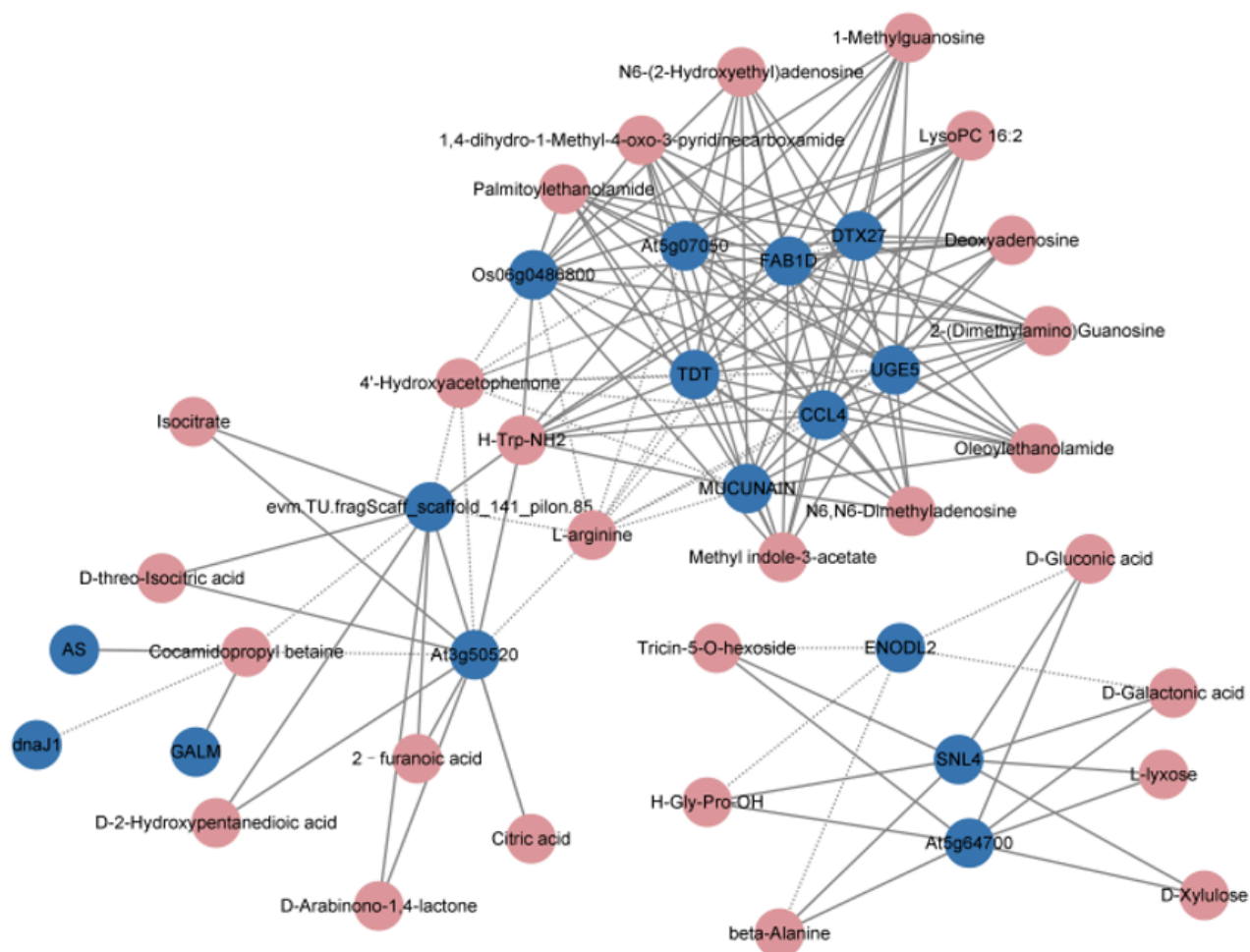


Figure 6

Association network between genes and metabolites. Blue dots represent genes, pink dots represent metabolites, solid lines indicate positive correlations, and dashed lines indicate negative correlations.

