

Article

The Difference in the Mechanisms of the TCA Cycle, Organic Acid Metabolism and Secretion of Rapeseed Roots Responding to Saline and Alkaline Stresses

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Abstract

Currently, the differences in the responses of the organic acid metabolism in rapeseed (*Brassica napus* L.) roots to saline and alkaline stresses are still unknown. To clarify the differences, different saline (100 (LS) and 200 (HS) mmol/L NaCl) and alkaline (20 (LA) and 40 (HA) mmol/L Na₂CO₃) treatments were applied to rapeseed. Then, targeted metabolomics was used to quantitatively analyze the changes in organic acid metabolism in the root system. The results showed that compared with the control group without stress (CK), 21, 18, 27, and 20 differentially accumulated organic acid metabolites were detected in the rapeseed roots under LS, HS, LA, and HA, respectively. In addition, 26, 6, 34, and 14 differentially accumulated organic acids were detected in the rapeseed root exudates under LS, HS, LA, and HA, respectively. Based on the activities of key enzymes related to the tricarboxylic acid cycle (TCA), antioxidant enzyme activities, organic acid metabolism, and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis in rapeseed roots, rapeseed mainly resisted saline and alkaline stresses by increasing organic acid synthesis and scavenging reactive oxygen species. Specifically, rapeseed resisted saline stress mainly by increasing the secretion of TCA cycle-related organic acids such as succinic acid, L-malic acid, fumaric acid, and cis-aconitic acid. In addition to secreting organic acids, rapeseed also resisted alkaline stress by increasing the secretion of phenolic acids such as 4-hydroxybenzoic acid, ferulic acid, and 4-coumaric acid. Notably, the number of secreted organic acid types and the increase in organic acid content under alkaline stress were higher than those under saline stress. The results of this study provide an important basis for the breeding of saline and alkaline stress-tolerant rapeseed varieties.

Keywords: rapeseed; saline stress; alkaline stress; targeted metabolomics; organic acid metabolism



Received: 28 November 2025

Revised: 8 January 2026

Accepted: 9 January 2026

Published: 13 January 2026

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1. Introduction

Soil salinization is one of the major abiotic stresses leading to reduced crop yields. China has about 99.13 million ha salinized land, accounting for 9% of its total land area [1]. Soil salinization is mostly a mixture of salinization and basification, with complex components and varying degrees. According to the soil and salt characteristics in China, salt stress can be divided into saline (NaCl or Na₂SO₄) and alkaline (NaHCO₃ or Na₂CO₃) stresses [2]. Saline stress mainly affects the distribution of various ions in plant cells and

destroys the intracellular ion homeostasis. Alkaline stress causes significant pH-induced damage, osmotic stress, and ion toxicity [3,4]. Clarifying the response of plants to saline and alkaline stresses and the stress tolerance mechanism is of great significance to salinized soil remediation and sustainable agricultural development.

Plants resist saline and alkaline stresses by synthesizing small-molecule soluble substances such as betaine, proline, polyamine, and soluble sugars [5]. In addition, plants also secrete a large amount of organic acids (i.e., citric acid, malic acid, and oxalic acid) to alleviate the pH-induced damage and ionic toxicity. The changes in the content of organic acids are closely related to organic acid metabolism pathways such as the tricarboxylic acid (TCA) and glyoxylic acid cycles [6]. For example, saline stress induced a decrease in the content of 2-ketoglutaric acid, malic acid, and fumaric acid in cotton leaves, which in turn led to TCA cycle inhibition [7]. Saline stress also induced an increase in the content of organic acids such as citric acid, succinic acid, malic acid, and oxalic acid in tomatoes [8,9]. Therefore, organic acid metabolism plays an important role in the saline stress resistance of plants. Organic acid metabolism also plays an important role in resisting alkaline stress. For example, under alkaline stress, the tomato root systems secreted large amounts of organic acids such as citric acid, succinic acid, and malic acid to chelate exchange bases (Na^+ , Mg^{2+}) in the soil, reducing ion toxicity and changing the soil pH of the root zone [10,11]. Most studies have shown that the TCA cycle is a hub for the mutual conversion of various organic matters, where changes in intermediate products and key enzyme activity are crucial for plants to resist saline and alkaline stresses. For example, Xu et al. [12] showed that the activity of pyruvate dehydrogenase and citrate synthase showed an increasing trend under saline stress, resulting in an increase in pyruvate and citric acid content. Li et al. [13] found that the intermediate products (oxaloacetic acid, fumaric acid) of the TCA cycle in *Nitraria sibirica* Pall. accumulated significantly under saline stress, and the expression of key enzyme genes controlling the TCA cycle was up-regulated compared with that of the control. These studies indicate that the activities of TCA cycle-related enzymes and TCA cycle intermediates have a certain regulatory effect on plant responses to saline and alkaline stresses.

Brassica napus L. is the largest oil crop in China, with a planting area of 7.8 million hectares. In addition to traditional oil use, it can also be used as a green manure crop for salinized soil remediation. Currently, it is widely planted in coastal (mainly NaCl), inland (mainly Na_2SO_4 and Na_2CO_3), and soda-type (mainly NaHCO_3 and Na_2CO_3) salinized lands [14,15]. Although a series of progress has been made on the mechanisms of saline and alkaline stress tolerance in various plants, there is little research on the differences in the response of organic acid metabolism in rapeseed roots to saline and alkaline stresses. Therefore, this study quantitatively analyzed the organic acids secreted *in vivo* and *in vitro* by the root systems of salt-tolerant rapeseed varieties under simulated saline and alkaline stresses through targeted metabolomics. Then, by combining with plant physiological parameters and the activities of key enzymes related to the TCA cycle, the differences in organic acid metabolism of rapeseed roots under saline and alkaline stresses were explored. This study will provide a scientific basis for the breeding of salt-tolerant rapeseed varieties.

2. Materials and Methods

The rapeseed variety S03 provided by Huazhong Agricultural University, China was used in this experiment. A hydroponic experiment was conducted on 15 June 2023 in the phytotron of the Agricultural College of Shihezi University, China. The day and night temperatures were 28 and 23 °C, respectively, the humidity was 70%, the photoperiod was 14 h per day, and the light intensity was 400–450 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Rapeseed seeds were sown in trays (32 holes per tray, 4 cm in diameter), using vermiculite as the substrate.

Rapeseed seedlings were transplanted in hydroponic boxes containing 1/4 Hoagland nutrient solution (pH was adjusted with $\text{H}_2\text{SO}_4/\text{KOH}$). The hydroponic box was a five-hole black cuboid ($38 \times 28 \times 14$ cm). One hole was used to connect an air pump for oxygen supply, and the other four holes were used for hydroponics. The nutrient solution was updated every two days. The nutrient solution was updated every two days: the old solution was discarded, and 1000 mL of freshly prepared nutrient solution was added to each hydroponic box. When the seedlings had five leaves, the 1/4 Hoagland nutrient solution was replaced with the 1/2 Hoagland solution. After three days of cultivation, different concentrations of saline (NaCl) and alkaline (Na_2CO_3) solutions were added to the hydroponic boxes [15]. A pretest was conducted before the experiment to determine the critical NaCl and Na_2CO_3 concentrations of the rapeseed variety. This experiment had five treatments: CK (1/2 Hoagland nutrient solution), 100 mmol/L NaCl treatment (LS), 200 mmol/L NaCl treatment (HS), 20 mmol/L Na_2CO_3 treatment (LA), and 40 mmol/L Na_2CO_3 treatment (HA). Each treatment had six replicates. NaCl solution was added at a rate of 50 mmol/L every 12 h (2.925 g of NaCl dissolved in 1000 mL of half-strength Hoagland nutrient solution), and the old solution was discarded prior to each addition, and the rate for Na_2CO_3 solution was 10 mmol/L every 12 h (1.06 g of Na_2CO_3 dissolved in 1000 mL of half-strength Hoagland nutrient solution), and the old solution was discarded prior to each addition. Target salt concentrations were simultaneously reached for all treatments. Samples were taken for analysis after 7 days of saline and alkaline treatments.

2.1. Determination of Growth Parameters for Rapeseed Seedlings

After 7 days of saline and alkaline treatments, the plant height of fifteen rapeseed seedlings was determined using a ruler and averaged [16]. Then, the root length from the cotyledon node to the main root tip was determined using a ruler [17].

2.2. Determination of Root Vitality

Root viability was determined using the method of Zhou et al. [18]. In short, fresh root samples (0.3 g) were rinsed with deionized water. Then, the samples were immersed in a mixture (10 mL) containing 4 g/L TTC (2,3,5-tribenzotetrazolium chloride) solution and 66 mmol/L phosphate-buffer solution (pH = 7.0) (V:V = 1:1), and placed in the dark at 37 °C for 2–3 h. When the tips of the root systems turned red, 2 mL of 1 mol/L sulfuric acid solution was added to terminate the reaction. After drying the root systems with filter paper, the root samples were ground with 3–5 mL of ethyl acetate. After filtration, the extracts were transferred to 10 mL volumetric flasks, and the volume was made to 10 mL with ethyl acetate. Finally, the extracts were subjected to colorimetry at 485 nm using a spectrophotometer (UV-5100, METASH, Shanghai, China). The amount of TTC reduction was determined based on a standard curve, followed by the calculation of the root vitality [18].

$$TTC = \frac{TTC \text{ reduction volume(g)}}{Root \text{ weight(g)} \times Time(h)} \quad (1)$$

2.3. Determination of Soluble Sugar and Soluble Protein Contents

The content of soluble sugars and soluble proteins was determined using the methods of He et al. [19]. Fresh root samples (0.5 g) were ground, followed by repeated extraction with 80% anhydrous ethanol. After adding 5 mL of anthraone solution, the absorbance was measured at 625 nm. Finally, the soluble sugar content was determined using the standard curve.

Fresh root samples (0.5 g) were ground and centrifuged, followed by supernatant collection. After repeated operation, 5 mL of Coomassie Brilliant Blue G-250 solution was

added. The absorbance was measured at 595 nm. Finally, the soluble protein content was determined using the standard curve.

2.4. Determination of Proline Content

The content of proline (Pro, $\mu\text{g}\cdot\text{g}^{-1}$ FW) in the roots was determined using a kit (Suzhou Grace Biotechnology Co., Ltd., Suzhou, China). The formula for calculating proline content based on sample mass was as follows:

$$\text{Pro} = [(\Delta A + 0.0071) \div 0.1304] \div (W \times V1 \div V) \times D = 25.6 \times (A + 0.0071) \div W \times D \quad (2)$$

$$\Delta A = A \text{ (measured value)} - A \text{ (value of the control group)} \quad (3)$$

where V is the volume of the extraction solution (1 mL), V1 is the volume of the extraction solution added to the reaction system (0.3 mL), V2 is the volume of the liquid sample (0.1 mL), W is the sample mass (g), D is the dilution factor (1 for undilution), and A is the absorbance value at 520 nm measured by a visible spectrophotometer (UV-5100, METASH, Shanghai, China).

2.5. Determination of Antioxidant Enzyme Activity and Malondialdehyde (MDA) Content

The activities of antioxidant enzymes were determined using the methods of Li et al. [20]. Potassium phosphate buffer (5 mL, 50 mM, pH: 7.8) and 0.5 g of fresh root samples were ground in an ice bath, and centrifuged at 4 °C for 20 min. The supernatant was spectrophotometrically determined to obtain the activities of SOD (superoxide dismutase), catalase (CAT), and peroxidase (POD).

The MDA content ($\mu\text{mol}\cdot\text{g}^{-1}$ FW) was determined using the method of Heath and Packer [21]. Fresh roots were homogenized in 0.1% trichloroacetic acid, centrifuged ($10,000\times g$), and cooled. The supernatant was added to the reaction mixture containing 0.5% thiobarbituric acid (TBA) and 20% trichloroacetic acid, followed by a water bath at 95 °C for 30 min. After cooling to room temperature and centrifugation, colorimetry was carried out at 532 and 600 nm.

2.6. Determination of the Activity of Key Enzymes in the TCA Cycle

The activities of A-ketoglutaric dehydrogenase, succinate dehydrogenase, succinyl CoA synthetase, citrate synthase, citrate lyase, isocitric dehydrogenase, fumarase, cis-aconitase, and malate dehydrogenase were determined using the kits produced by Suzhou Grace Biotechnology Co., Ltd., Suzhou, China.

2.7. Determination of Organic Acids and Metabolites

Organic acids and their metabolites were determined by the MetWare (<http://www.metware.cn/> accessed on 12 January 2024) platform. After 7 days of stress treatments, fresh plants were collected, and the roots were washed 2–3 times with deionized water. Then, 0.5 g of the root samples was stored in liquid nitrogen at $-80\text{ }^{\circ}\text{C}$ in a refrigerator. The MetWare (<http://www.metware.cn/> accessed on 12 January 2024) platform was used to determine the content of organic acids and their metabolites. Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using the platform to draw an OPLS-DA scoring map. A Venn diagram was drawn to compare the number of organic acid metabolites among different treatments. K-means clustering analysis was performed on the relative content of metabolites in different treatments.

2.8. Collection of Root Exudates and Determination of Organic Acid Type and Content

Root exudates were collected using the method described by Wang et al. [22]. After 7 days of saline and alkaline treatments, the rapeseed plants were pulled out and rinsed

2–3 times with deionized water. Then, each plant (six replicates per treatment, four plants per replicate) was transferred into a centrifuge tube containing 50 mL of deionized water. The centrifuge tubes were wrapped with tin foil to prevent the root systems from being exposed to light. After cultivation under natural light for 24 h, the solution in each tube was filtered and collected. After mixing the solutions of the four replicates, the mixture was stored at -80°C refrigerator for the determination of organic acids secreted by roots. Organic acids and their metabolites were determined using the MetWare (<http://www.metware.cn/> accessed on 12 January 2024) platform.

2.9. Statistical Analysis

At least three biological replicates for each treatment were used for each measurement. Data sorting, mean and standard deviation calculations, and outlier filtering were completed in Microsoft Excel 2021. One-way ANOVA and Duncan's multiple range test were done in SPSS 26 software ($p < 0.05$). Plotting was completed in Origin 2021.

3. Results

3.1. Effects of Saline and Alkaline Stresses on the Growth and Physiological Parameters of Rapeseed Seedlings

3.1.1. Effects of Saline and Alkaline Stresses on the Plant Height and Root Length

The plant height under saline and alkaline stresses was lower than that of CK, and the inhibitory effect increased with the increase in salt concentration. The plant height of the LS, LA, HS, and HA treatments was 10.73%, 9.07%, 40.11%, and 43.85% lower than that of CK, respectively ($p < 0.05$). Similarly, the root length gradually decreased with the increase in salt concentration. The root length of the LS, LA, HS, and HA treatments was 10.15%, 21.07%, 13.86%, and 11.46% lower than that of CK, respectively ($p < 0.05$, Figure 1).

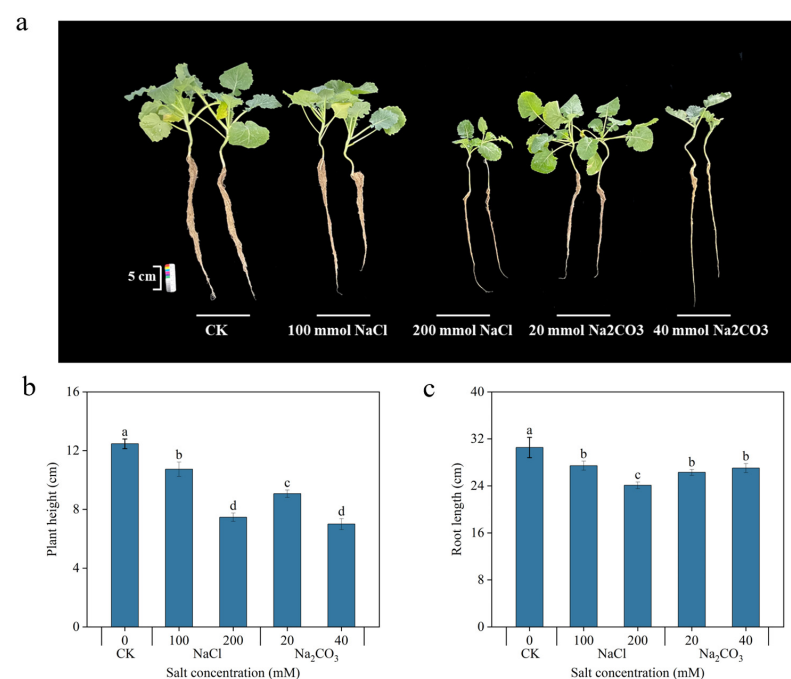


Figure 1. Effects of saline and alkaline treatments on the growth parameters of rapeseed plants. (a) Plant phenotype; (b) Plant height; (c) Root length. Different lowercase letters indicate significant differences among various treatments ($p < 0.05$).

3.1.2. Effects of Saline and Alkaline Stresses on Root MDA Content and Vitality

The content of MDA in the rapeseed roots under saline and alkaline stresses was higher than that of CK, and increased with the increase in salt concentration (Figure 2a).

The content of MDA of the LS, LA, HS, and HA treatments was 32.2%, 23.6%, 48.9%, and 41.3% higher than that of CK, respectively ($p < 0.05$). The root vitality under saline and alkaline stresses was significantly lower than that of CK, and gradually decreased with the increase in salt concentration (Figure 2b). The root vitality of the LS, LA, HS, and HA treatments was 40.9%, 35.8%, 70.6%, and 61.9% lower than that of CK, respectively ($p < 0.05$).

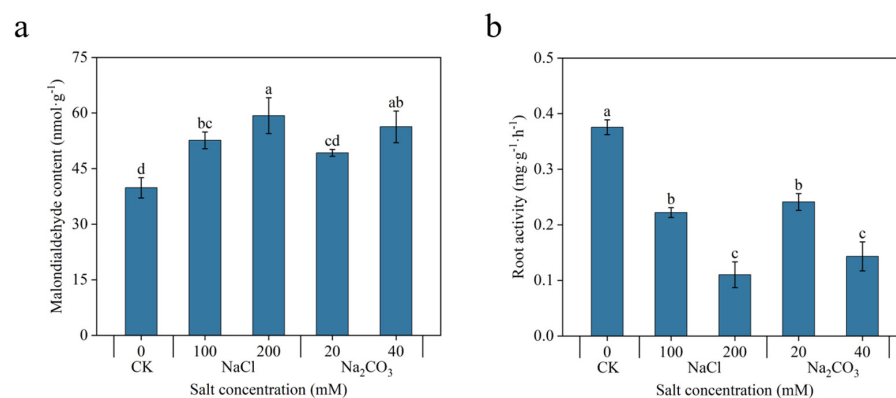


Figure 2. Effects of saline and alkaline stresses on rapeseed root malondialdehyde content (a) and vitality (b). Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

3.1.3. Effect of Saline and Alkaline Stresses on the Content of Osmolytes in Rapeseed Roots

The changes in the content of soluble sugars, soluble proteins, and proline in rapeseed roots under saline and alkaline stresses are shown in Figure 3. The content of soluble sugars, soluble proteins, and proline in rapeseed roots under saline and alkaline stresses was significantly higher than that of CK, and increased with the increase in salt concentration. The content of soluble sugars, soluble proteins, and proline of the LS treatment was 36.8%, 27.5%, and 52.8% higher than that of CK, respectively ($p < 0.05$). Similar results were also obtained in HS vs. CK (62.2%, 37.6%, and 81.0%, respectively), LA vs. CK (38.9%, 25.3%, and 50.4%, respectively), and HA vs. CK (62.6%, 42.1%, and 93.9%, respectively) ($p < 0.05$).

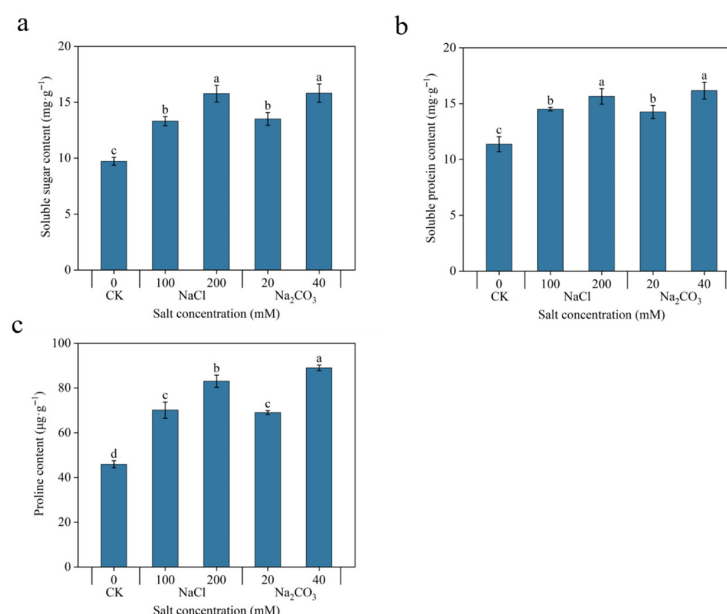


Figure 3. The content of osmolytes in rapeseed roots under saline and alkaline stresses. (a) Soluble sugar content; (b) Soluble protein content; (c) Proline content. Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

3.1.4. Effects of Saline and Alkaline Stresses on the Activities of Antioxidant Enzymes in Rapeseed Roots

Under saline and alkaline stresses, the activities of CAT and POD in rapeseed roots were significantly higher than those of CK, and showed a gradually increasing trend with the increase in salt concentration (Figure 4a,c). The activity of CAT of the LS, HS, LA, and HA treatments was 18.9%, 26.4%, 21.4%, and 31.1% higher than that of CK, respectively ($p < 0.05$), and the activity of POD were 24.7%, 43.3%, 22.6%, and 38.9% higher than that of CK, respectively ($p < 0.05$).

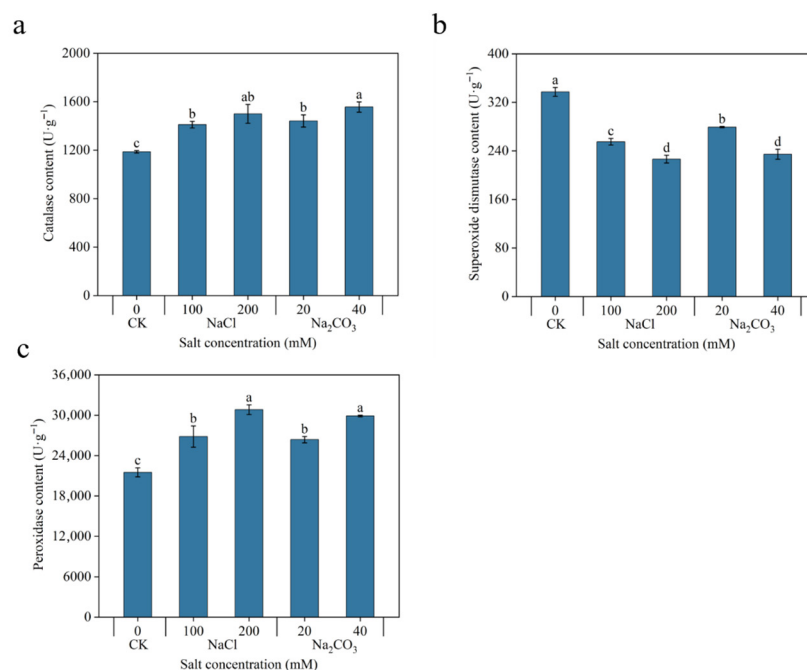


Figure 4. Changes in antioxidant enzyme activities in rapeseed roots under saline and alkaline stresses. (a) Catalase activity; (b) Superoxide dismutase activity; (c) Peroxidase activity. Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

The activity of SOD under saline and alkaline stresses was significantly lower than that of CK, and gradually decreased with the increase in salt concentration (Figure 4b). The activity of SOD of the HS and HA treatments was 32.8% and 30.5% lower than that of CK, respectively ($p < 0.05$).

3.2. Metabolomics Analysis of Organic Acids in Rapeseed Roots Under Saline and Alkaline Stresses

3.2.1. Metabolomics Analysis of Organic Acids in Rapeseed Roots Under Saline Stress

According to the OPLS-DA, the LS and HS treatments were completely isolated from CK (Figure 5a,b). The total variation rate between LS and CK was 72.1%, of which 42.2% was related to the separation between LS and CK, and 29.9% was related to the intra-group variation. The permutation test of the OPLS-DA of CK and LS treatment showed that the predictive squared correlation coefficient (Q^2) was 0.852, and the explained sum of squares (R^2) was 0.994, indicating high stability and reliability of the model. The total variation rate between HS and CK was 55.9%, of which 26% was related to the separation between HS and CK, and 29.9% was related to the intra-group variation. The permutation test of CK and HS treatment showed that the Q^2 was 0.779, and the R^2 was 0.998, indicating high stability and reliability of the model (Figure 5c,d). The top 20 organic acids with higher variable importance in projection (VIP) scores were displayed. The differentially accumulated in vivo organic acids for LS vs. CK mainly included indole-3-acetic acid, cinnamic acid, citraconic acid, suberic acid, and cis-Aconitic acid (Figure 5e), and those for HS vs. CK

mainly included hippuric acid, maleic acid, 2-hydroxyisovaleric acid, 3-hydroxyisovaleric acid, and benzoic acid (Figure 5f).

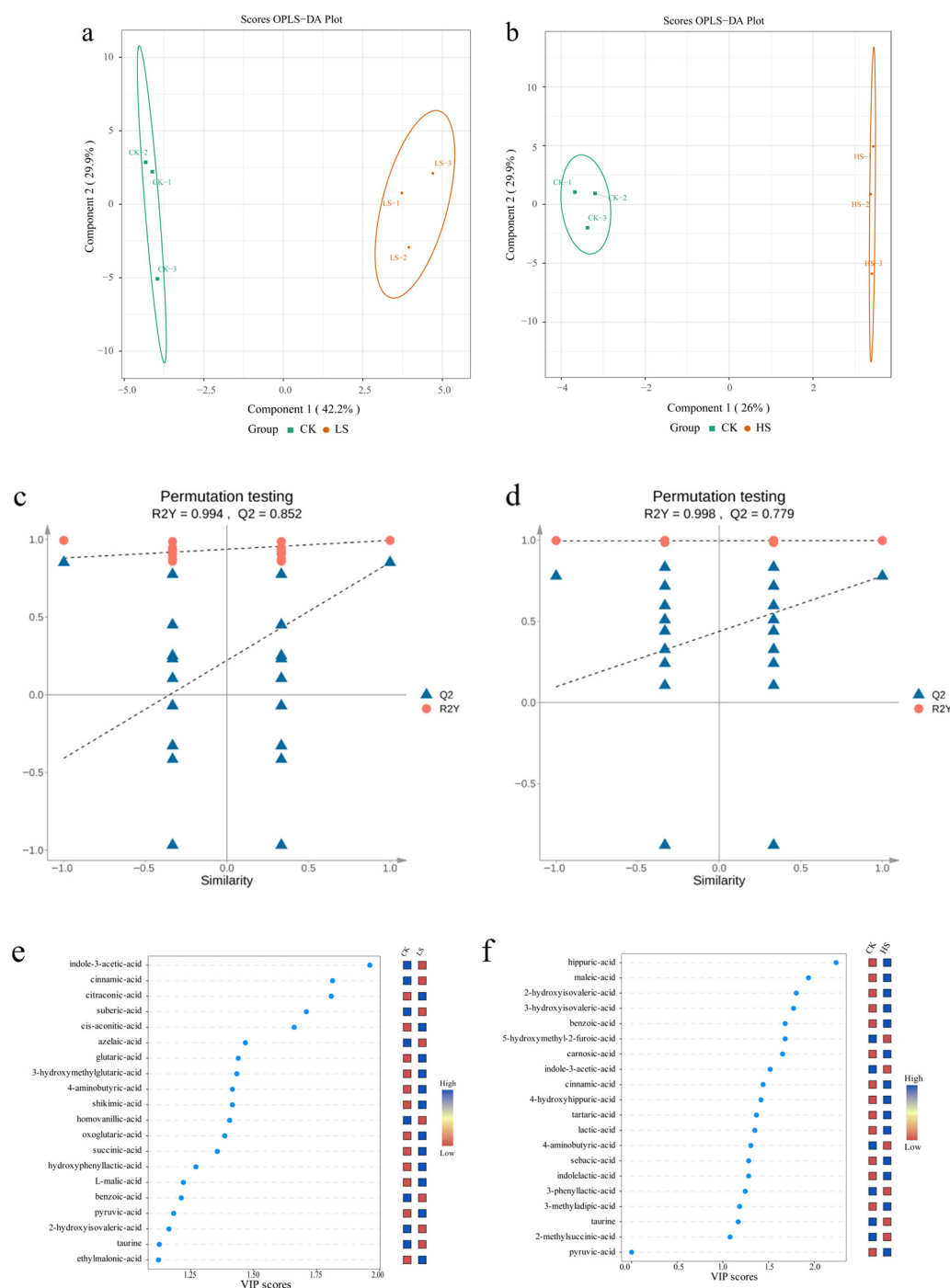


Figure 5. OPLS-DA score (a,b), permutation test (c,d), and variable importance in projection (VIP) score (e,f) of the top 20 organic acids in the root system of rapeseed seedlings under different levels of saline treatments.

According to the Venn diagram, eight differentially accumulated metabolites were detected in both LS vs. CK and HS vs. CK. Thirteen and ten unique differential metabolites were detected in LS vs. CK and HS vs. CK, respectively (Figure 6c). The cluster analysis results showed that differential metabolites under different saline stresses were divided into 8 subclasses (Figure 6b). Subclasses 1, 5, and 7 showed similar metabolite changes, i.e., the content of the differential metabolites under saline and alkaline stresses was lower

than that of CK. Among them, the content of the differential metabolites of subclasses 1 and 5 was the lowest under LS, and that of subclass 7 decreased with the increase in salt concentration. The content of differential metabolites in subclasses 2, 3, and 8 under LS was higher than that of CK, but the opposite phenomenon was observed in HS vs. CK. The content of differential metabolites in subclass 4 under HS and subclass 6 under LS and HS was higher than that of CK. In addition, cluster analysis of organic acids under saline stress showed that the organic acids significantly accumulated in the rapeseed root system under LS were cinnamic acid, 3-methyladipic acid, pyruvic acid, 2-hydroxyisovaleric acid, and fumaric acid, up-regulating by 2.27, 1.55, 1.38, 1.27, and 1.06 times, respectively, compared with those of CK. The organic acids significantly accumulated in the root system under HS were 3-hydroxymethylglutaric acid, pyruvic acid, hydroxyphenyllactic acid, oxoglutaric acid, and L-malic acid, up-regulating by 1.73, 1.7, 1.5, 1.44, and 1.44 times, respectively, compared with those of CK (Figures 6a and S1, Table S1).

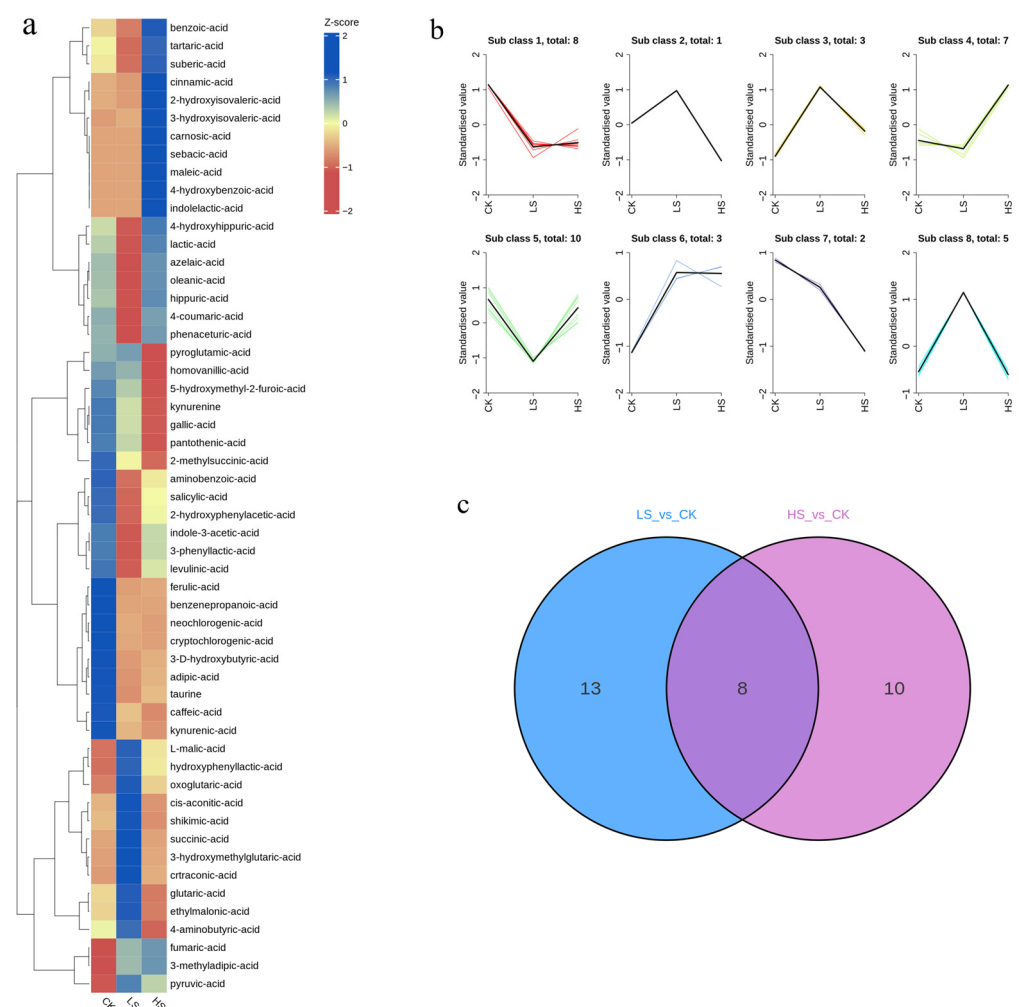


Figure 6. Cluster heat map (a), analysis of K-means (b), and Venn diagram (c) of differentially accumulated metabolites (organic acids) in rapeseed roots under different levels of saline stress. CK, Control group; LS, 100 mmol/L NaCl treatment; HS, 200 mmol/L NaCl treatment.

KEGG enrichment analysis of differentially accumulated metabolites in rapeseed roots under saline stress revealed that under LS, the significantly enriched metabolic pathways in rapeseed roots mainly included Glyoxylate and dicarboxylate metabolism, TCA cycle, Carbon metabolism, and Biosynthesis of secondary metabolism (Figure 7a). Under HS, the significantly enriched metabolic pathways included Nicotinate and nicotinamide metabolism, Butanoate metabolism, Arginine and proline metabolism, Tyrosine metabolism, Alanine,

aspartate and glutamate metabolism, and Phenylalanine metabolism (Figure 7b). Alanine, aspartate and glutamate metabolism, Tyrosine metabolism, Phenylalanine metabolism, and Butanoate metabolism were the significantly enriched metabolic pathways under both LS and HS.

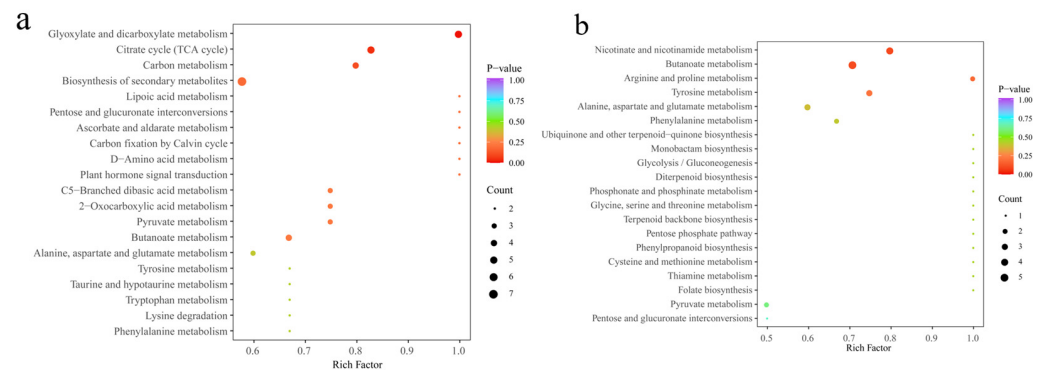


Figure 7. KEGG enrichment analysis of differentially accumulated metabolites (organic acids) in rapeseed roots under different levels of saline stress. (a) 100 mmol/L NaCl; (b) 200 mmol/L NaCl.

3.2.2. Metabolomics Analysis of Organic Acids in Rapeseed Roots Under Alkaline Stress

According to the OPLS-DA (Figure 8a,b), the LA and HA treatments were completely separated from CK. The total variation rate between LA and CK was 62.2%, of which 39.4% was related to the separation between LA and CK, and 22.8% was related to the intra-group variation. The permutation test of LA and CK showed that the Q^2 was 0.888, and the R^2 was 0.997. The total variation rate between HA and CK was 82.7%, of which 57.6% was related to the separation between HA and CK, and 25.1% was related to the intra-group variation. The permutation test of HA and CK showed that the Q^2 was 0.979, and the R^2 was 0.997 (Figure 8c,d). The top 20 organic acids by VIP score in rapeseed roots under different levels of alkaline stress were displayed. The differentially accumulated *in vivo* organic acids for LA vs. CK mainly included indolelactic acid and cis-aconitic acid (Figure 8e), and those for HA vs. CK mainly included hydroxyphenyllactic acid, L-malic acid, succinic acid, cis-Aconitic acid, indolelactic acid, and glutaric acid (Figure 8f).

According to the Venn diagram (Figure 9c), there were a total of 15 differentially accumulated metabolites in LA vs. CK and HA vs. CK, of which 5 and 12 were unique to LA vs. CK and HA vs. CK, respectively. The K-means cluster analysis after normalization and centralization of the relative content of differential metabolites under different levels of alkaline stresses showed that differential metabolites under alkaline stresses were divided into 10 subclasses (Figure 9b). Subclasses 1 and 6 showed similar change trends, i.e., the content of differential metabolites under alkaline stresses, particularly LA, was lower than that of CK. The content of differential metabolites in subclasses 2, 4, 9, and 10 decreased with the increase in alkaline salt concentration. The content of differential metabolites in subclasses 3 and 7 was higher than that of CK under LA, but lower than that of CK under HA. The content of differential metabolites in subclasses 5 and 8 under LA and HA was higher than that of CK. In addition, cluster analysis of organic acids in the rapeseed root system under alkaline stresses showed that under LA, organic acids accumulated in the rapeseed root system were mainly cinnamic acid, fumaric acid, pyruvic acid, benzenepropanoic acid, and taurine, up-regulating by 5.22, 1.63, 1.26, 1.18, and 1.12 times, respectively, compared with those of CK (Figures 9a and S3, Table S2). Under HA, organic acids accumulated in the root system were mainly cinnamic acid, suberic acid, and fumaric acid, up-regulating by 2.97, 1.75, and 1.52, respectively, compared with those of CK (Figures 9a and S3, Table S2).

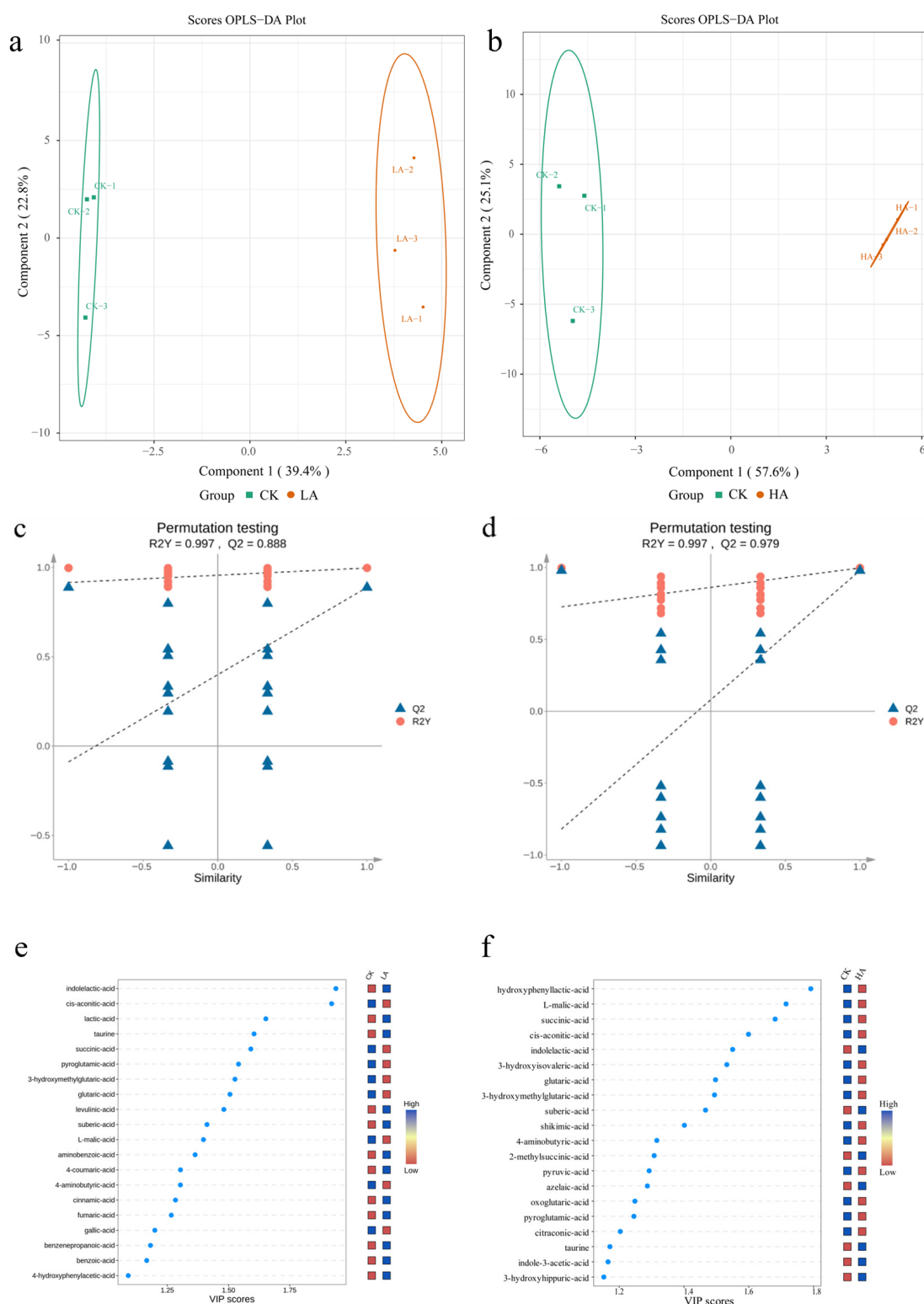


Figure 8. OPLS-DA score (a,b), permutation test (c,d), and VIP score of the top 20 organic acids in the root system of rapeseed seedlings under different levels of alkaline stresses (e,f).

KEGG enrichment analysis of differentially accumulated metabolites in rapeseed roots under alkaline stresses revealed that under LA, the significantly enriched metabolic pathways in rapeseed roots included Arginine and proline metabolism, beta-Alanine metabolism, Pantothenate and CoA biosynthesis, and Nicotinate and nicotinamide metabolism (Figure 10a). Under HA, the significantly enriched metabolic pathways in the root system included beta-Alanine metabolism, Phenylpropanoid biosynthesis, Fatty acid

degradation, Ubiquinone and other terpenoid-quinone biosynthesis, and Folate biosynthesis (Figure 10b). Arginine and proline metabolism, beta-Alanine metabolism, Pantothenate and CoA biosynthesis, Nicotinate and nicotinamide metabolism, Butanoate metabolism, Biosynthesis of cofactors, Phenylpropanoid biosynthesis, Ubiquinone and other terpenoid-quinone biosynthesis, and Folate biosynthesis were the significantly enriched metabolic pathways under both LA and HA.

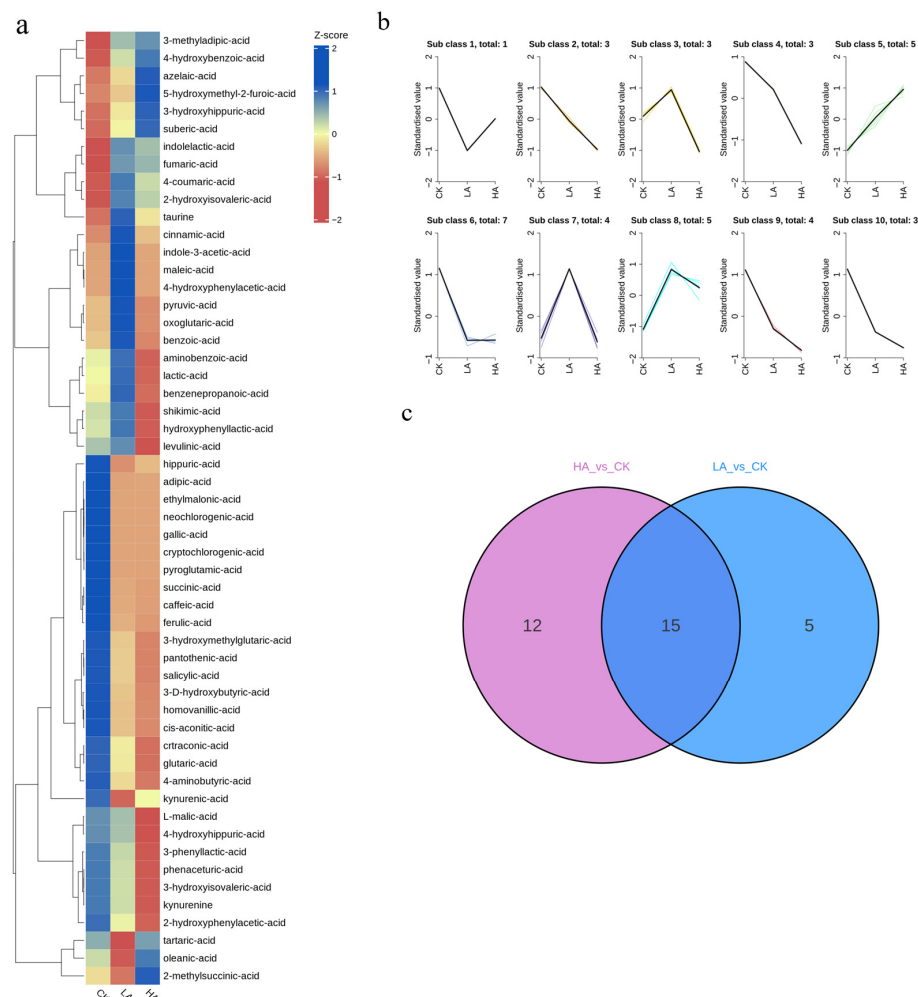


Figure 9. Cluster heat map (a), analysis of K-means (b), and Venn diagram (c) of differentially accumulated metabolites (organic acids) in rapeseed roots under different levels of alkaline stresses. CK, Control group; LA, 20 mmol/L Na₂CO₃ treatment; HA, 40 mmol/L Na₂CO₃ treatment.

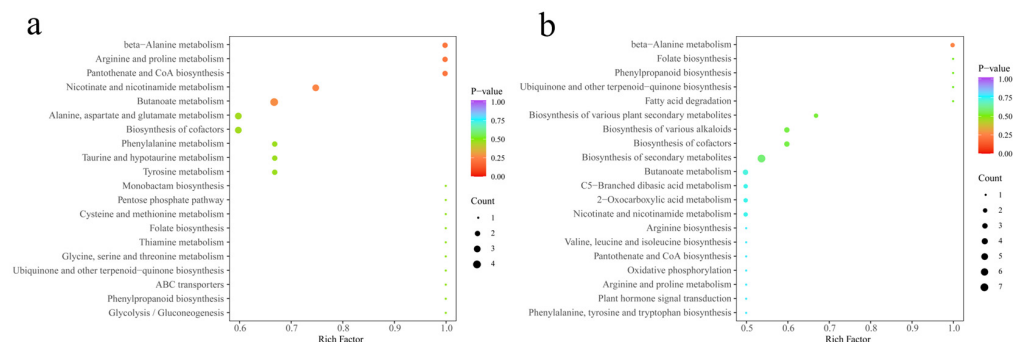


Figure 10. KEGG enrichment analysis of differentially accumulated metabolites (organic acids) in rapeseed roots after 7 days of alkaline treatments. (a) 20 mmol/L Na₂CO₃ treatment; (b) 40 mmol/L Na₂CO₃ treatment.

3.3. Activities of Key Enzymes in the TCA Cycle in Rapeseed Roots Under Saline and Alkaline Stresses

Further analysis of citrate synthase, succinyl-CoA synthetase, cis-aconitase, succinate dehydrogenase, fumarase, and isocitrate dehydrogenase showed that under saline stress, the activities of citrate lyase, cis-aconitase, isocitrate dehydrogenase, α -ketoglutaric dehydrogenase, and fumarase in rapeseed roots were significantly lower than those of CK, and gradually increased with the increase in salt concentration (Figure 11b–e,h). The activity of citrate synthase was significantly lower than that of CK and gradually decreased with the increase in salt concentration (Figure 11a). However, the activity of succinyl-CoA synthetase was significantly higher than that of CK and gradually increased with the increase in salt concentration (Figure 11f). In addition, the activity of succinate dehydrogenase was significantly higher than that of CK, and gradually decreased with the increase in salt concentration (Figure 11g).

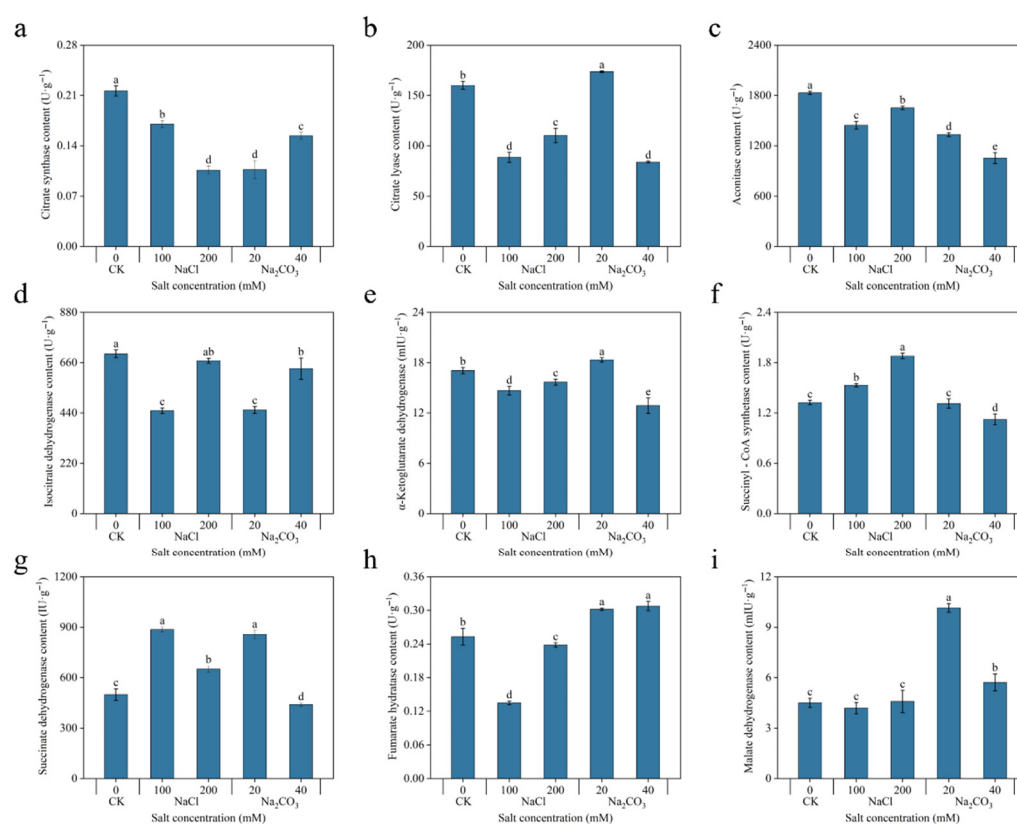


Figure 11. Effects of saline and alkaline stresses on the activities of key enzymes involved in the tricarboxylic acid cycle in rapeseed roots. (a) Citrate synthase; (b) Citrate lyase; (c) Cis-aconitase; (d) Isocitrate dehydrogenase; (e) α -ketoglutaric dehydrogenase; (f) Succinyl-CoA synthetase; (g) Succinate dehydrogenase; (h) Fumarase; (i) Malate dehydrogenase. Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

Under alkaline stress, the activities of citrate synthase and isocitrate dehydrogenase were significantly lower than those of CK, and gradually increased with the increase in salt concentration (Figure 11a,d). The activities of citrate lyase, α -ketoglutaric dehydrogenase, and succinate dehydrogenase of the LA treatment were significantly higher than that of CK, while it was opposite in HA vs. CK (Figure 11b,e,g). Under alkaline stress, the activity of cis-aconitase was significantly lower than that of CK, and gradually decreased with the increase in salt concentration (Figure 11c). The activities of fumarase and malate dehydrogenase were significantly higher than that of CK (Figure 11h,i), among which the

activity of malate dehydrogenase of the LA and HA treatments was 125.2% and 27.0% higher than that of CK, respectively (Figure 11i).

3.4. Metabolomics Analysis of In Vitro Organic Acids Secreted by Rapeseed Roots Under Saline and Alkaline Stresses

3.4.1. Metabolomics Analysis of In Vitro Organic Acids Secreted by Rapeseed Roots Under Saline Stress

The OPLS-DA scoring map showed that both LS and HS treatments were completely separated from CK (Figure 12a). The total variation rate between LS and CK was 57.3%, among which 18.4% was related to the separation between LS and CK, and 38.9% was related to the intra-group variation. The total variation rate between HS and CK was 64.2%, among which 34.5% was related to the separation between HS and CK, and 29.7% was related to the intra-group variation. Under LS, the contents of organic acids such as pyroglutamic acid, glutaric acid, aminobenzoic acid, 3-methyladipic acid, L-malic acid, and pantothenic acid were down-regulated compared with those of CK. Under HS, the contents of organic acids tartaric acid, 4-coumaric acid, indole-3-acetic acid, 3-D-hydroxybutyric acid, glutaric acid, and 2-hydroxyisovaleric acid were up-regulated, while the content of 4-aminobutyric acid was down-regulated, compared with those of CK (Figure 12c,d).

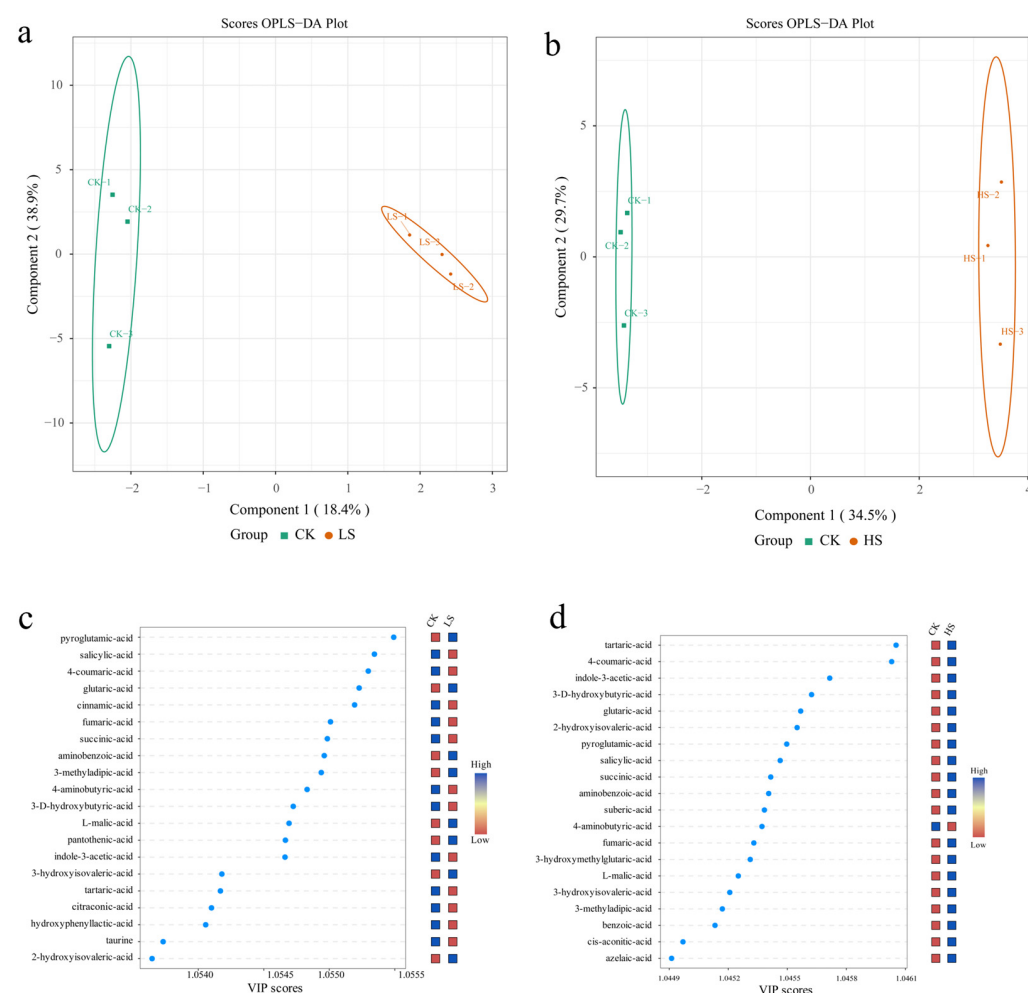


Figure 12. OPLS-DA scoring map (a,b) and VIP score (c,d) of the top 20 in vitro organic acids secreted by the root system of rapeseed seedlings under different levels of saline treatments.

The Venn diagram showed that there were 4 common in vitro differentially accumulated metabolites for LS vs. CK and HS vs. CK. In addition, there were 2 and 22 differential metabolites unique to LS vs. CK and HS vs. CK, respectively (Figure 13c). The in vitro

differential metabolites secreted by rapeseed roots were divided into 6 subclasses under saline stress (Figure 13b), among which subclasses 1, 2, and 6 showed similar changes in differential metabolite content, i.e., the differential metabolite content of the three classes increased with the increase in salt concentration. Under LS, subclass 3 had higher differential metabolite contents compared with CK, but there was no difference in HS vs. CK. Subclass 4 had lower differential metabolite content under LS but had higher content under HS, compared with CK. The differential metabolite content of subclass 5 decreased with the increase in salt concentration. In addition, cluster analysis of in vitro organic acids secreted by rapeseed roots under saline stresses showed that the in vitro organic acids secreted by rapeseed roots under LS were mainly 3-methyladipic acid, 4-hydroxybenzoic acid, 3-hydroxyhippuric acid, and pyroglutamic acid, up-regulating by 2.37, 1.9, 1.68, and 1.44 times, respectively, compared with those of CK (Figures 13a and S5, Table S3). The in vitro organic acids secreted under HS were mainly 4-coumaric acid, L-malic acid, fumaric acid, pyroglutamic acid, and ferulic acid, up-regulating by 6.03, 5.59, 5.07, 4.71, and 4.67 times, respectively, compared with those of CK (Figures 13a and S5, Table S3).

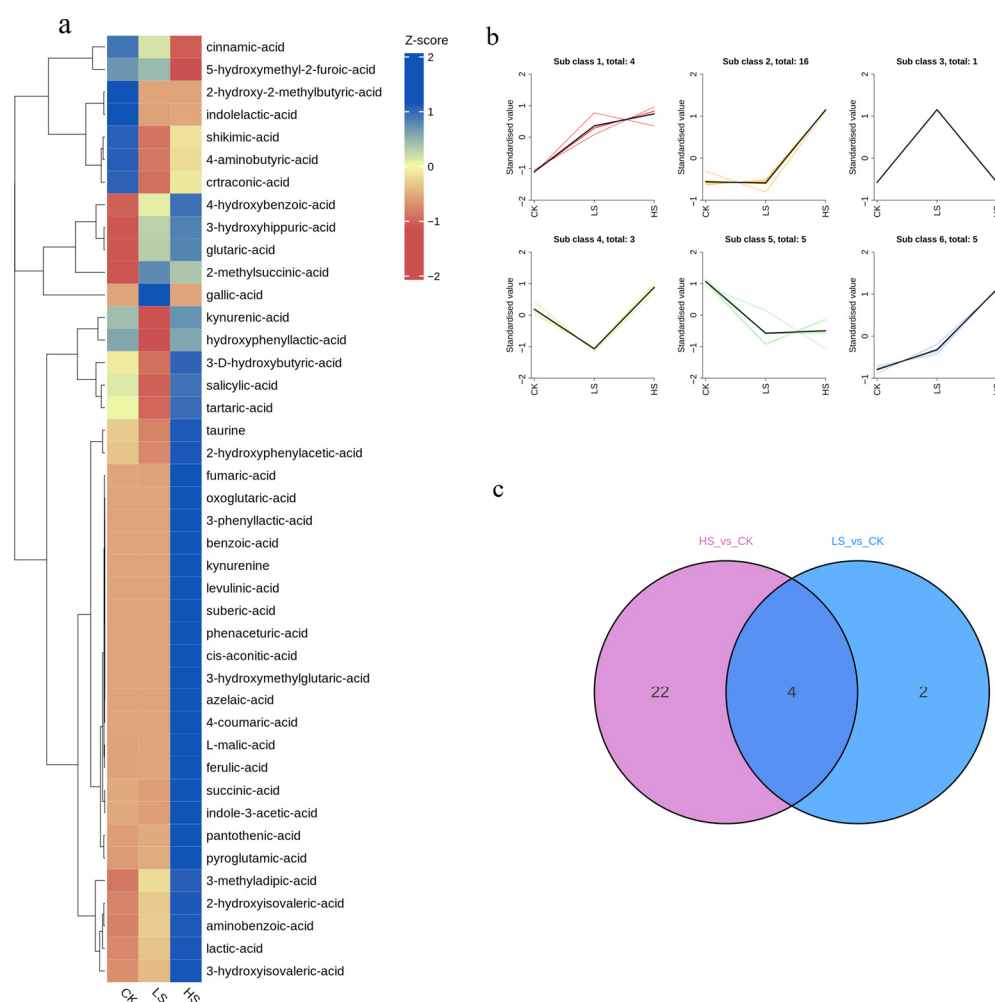


Figure 13. Cluster heat map (a), analysis of K-means (b), and Venn diagram (c) of in vitro differentially accumulated metabolites (organic acids) secreted by rapeseed roots under saline stresses. CK, Control group; LS, 100 mmol/L NaCl treatment; HS, 200 mmol/L NaCl treatment.

3.4.2. Metabolomic Analysis of In Vitro Organic Acids Secreted by Rapeseed Roots Under Alkaline Stress

The OPLS-DA scoring map showed that LA and HA treatments were completely separated from CK (Figure 14a,b). The total variation rate between LA and HA was 42%,

of which 15.6% was related to the separation between LA and HA, and 26.4% was related to intra-group variation. The total variation rate between HA and CK was 71.5%, of which 55% was related to the separation between HA and CK, and 16.5% was related to intra-group variation. The top 20 in vitro organic acids ($p < 0.05$) by VIP score were displayed (Figure 14c,d). Under LA, the contents of indole-3-acetic acid, cis-aconitic acid, 3-hydroxymethylglutaric acid, pyroglutamic acid, and 4-coumaric acid were up-regulated compared with those of CK. The contents of cinnamic acid, salicylic acid, 3-D-hydroxybutyric acid, citraconic acid, 4-aminobutyric acid, and hydroxyphenyllactic acid were down-regulated. Under HA, the contents of indole-3-acetic acid, kynurenic acid, 4-coumaric acid, 4-hydroxybenzoic acid, salicylic acid, and ferulic acid were up-regulated compared with those of CK.

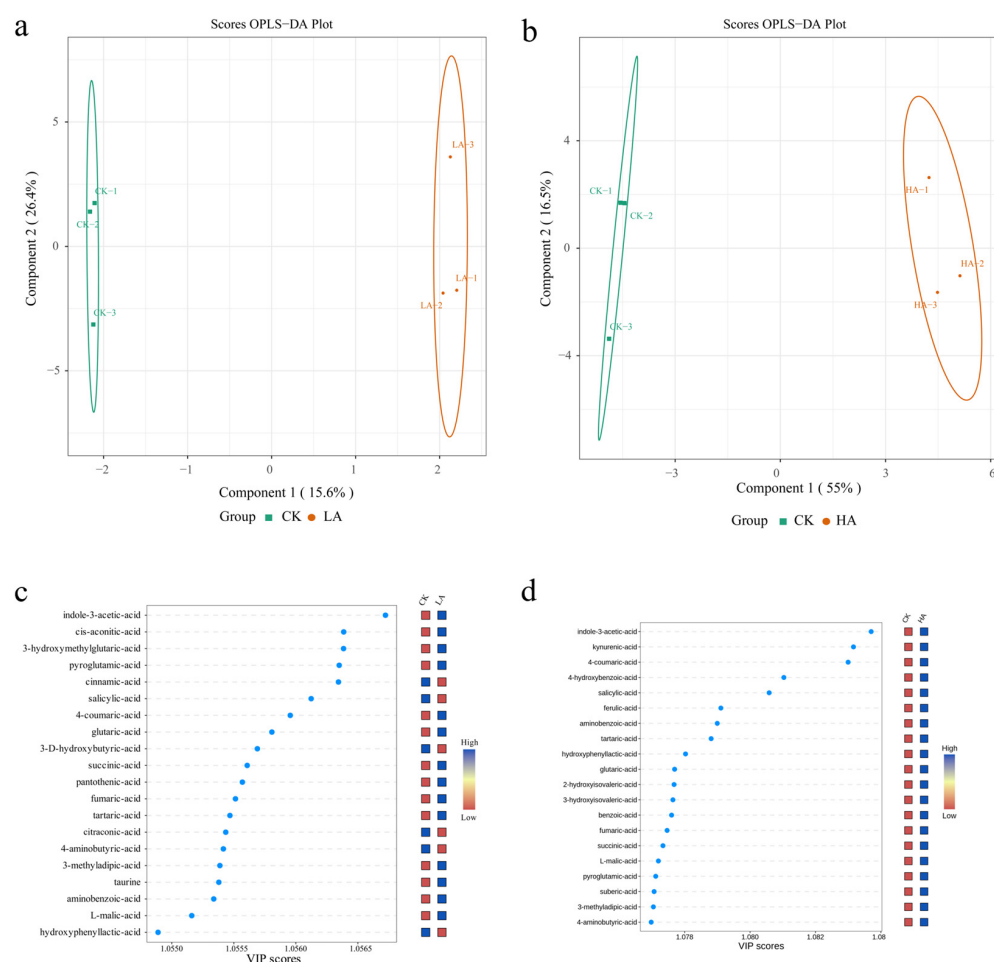


Figure 14. OPLS-DA scoring maps (a,b) and VIP score (c,d) of the top 20 in vitro organic acids secreted by rapeseed roots under different levels of alkaline treatments.

The Venn diagram showed that there were 3 common differentially accumulated metabolites for LA vs. CK and HA vs. CK. In addition, there was 1 differential metabolite unique to LA vs. CK, and 21 differential metabolites unique to HA vs. CK (Figure 15c). The K-means cluster analysis showed that the in vitro differential metabolites secreted by rapeseed roots under alkaline stresses were divided into 8 subclasses (Figure 15b). Among them, the contents of in vitro differential metabolites in subclass 1 were all lower than those of CK under alkaline stresses, particularly under LA. The contents of in vitro differential metabolites in subclasses 2 and 6 were lower than those of CK under LA but higher than those of CK under HA. The content of in vitro differential metabolites in subclasses 3, 4, 5, and 7 showed a similar changing trend, i.e., the contents of in vitro differential metabolites

increased with the increase in salt concentration under alkaline stresses. The contents of in vitro differentially metabolites in subclass 8 were higher than that of CK under LA but lower than that of CK under HA. Furthermore, cluster analysis of the in vitro organic acids under alkaline stresses indicated that under LA, the main in vitro organic acids secreted by rapeseed roots were L-malic acid, fumaric acid, 4-coumaric acid, pantothenic acid, and succinic acid, up-regulating by 5.01, 4.86, 2.77, 2.47, and 1.98 times, respectively, compared with those of CK (Figures 14a and S7, Table S4). Under HA, the main in vitro organic acids secreted by the root system were 4-coumaric acid, L-malic acid, fumaric acid, 2-hydroxyphenylacetic acid, and ferulic acid, up-regulating by 9.07, 8.27, 8, 6.88, and 6.63 times, respectively, compared with those of CK (Figures 15a and S7, Table S4).

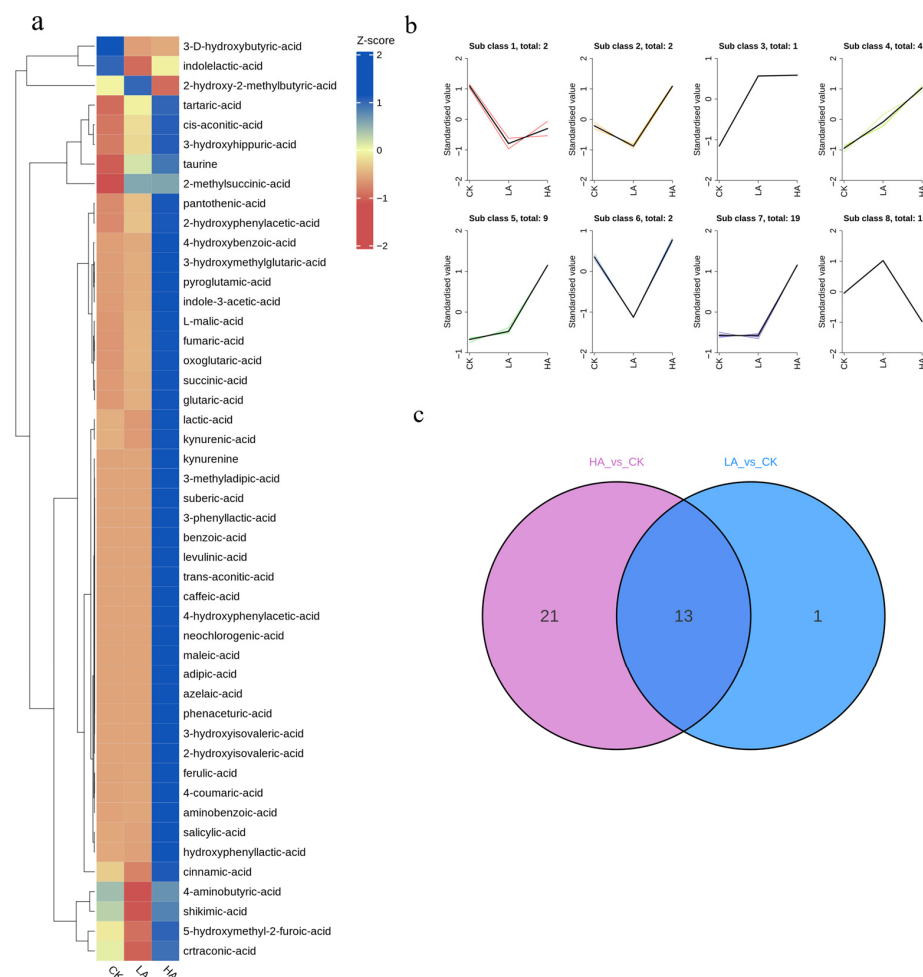


Figure 15. Cluster heat map (a), analysis of K-means (b), and Venn diagram (c) of in vitro differentially accumulated metabolites (organic acids) secreted by rapeseed roots under alkaline stress. CK, Control group; LA, 20 mmol/L Na₂CO₃ treatment; HA, 40 mmol/L Na₂CO₃ treatment.

4. Discussion

4.1. Saline and Alkaline Stresses Inhibited Rapeseed Growth and Triggered an Increase in Antioxidant Enzyme Activities and Osmolyte Contents in the Root System

Under saline and alkaline stresses, excessive sodium ions enter crop cells, causing an ion imbalance and growth inhibition [4]. This study found that saline and alkaline stresses significantly impacted the root vitality, MDA content, antioxidant enzyme activities, and osmolyte contents of rapeseed. However, there were no significant differences between LS and LA, nor between HS and HA. This is consistent with the results of research on *Carum copticum*, *Oryza sativa* L., and *Leymus chinensis* [23–25]. This study also found that

both saline and alkaline stresses led to significantly higher MDA and osmolyte contents compared with CK. However, most previous studies suggest that alkaline stress has a more significant effect on crop growth than saline stress [26,27]. This may be due to that researchers keep the same Na^+ concentration in exploring the effects of different levels of saline and alkaline stresses on plants, leading to higher pH damage under alkaline stress. For example, in the study of the organic acid metabolism mechanism of *Chloris virgata* Sw. under saline and alkaline stresses [26], under the consistent Na^+ concentrations, the pH was 6.70–6.72 for saline stress and 10.46–10.62 for alkaline stress. However, in this study, saline and alkaline treatments were carried out at different Na^+ concentrations, but both were the salt-tolerance critical concentrations of rapeseed seedlings.

The MDA content and root vitality can reflect the response of plants to adversity [28]. For example, Wang et al. [29] found that MDA played a major role under salt stress, and the MDA content in rapeseed leaves increased with the increase in salt concentration and stress duration. Zhang et al. [30] found that severe salt stress significantly impacted the membrane structure and function of buckwheat root cells, inducing low root vitality. This study found that both low- and high-level saline and alkaline stresses significantly inhibited the root vitality of rapeseed. As the concentration of saline and alkaline salts increased, the MDA content in rapeseed roots showed an upward trend, and the root vitality gradually decreased. This is consistent with the research results of Sun et al. [31], i.e., saline and alkaline stresses lead to lipid peroxidation of cell membranes, severe oxidative damage, and low root vitality.

Under salt stress, plants mainly clear in vivo harmful substances through enzymatic reactions in the reactive oxygen species (ROS) scavenging system. Enzymatic reactions regulate the balance of ROS in plants through the coordination of related enzymes such as SOD, POD, and CAT [32]. Farghaly et al. [33] found that both saline and alkaline stresses significantly increased the activities of SOD, CAT, and POD compared with the control group, and the effect of alkaline stress on antioxidant enzyme activities was stronger than that of saline stress. Garg and Chandel [34] found that salt stress increased the activities of CAT and POD in maize and sorghum roots compared with the control group. Besides, it enhanced the SOD activity in the short term but reduced the SOD activity in the long term. Kumar et al. [35] found that long-term salt stress led to a decrease in SOD activity in non-salt-tolerant varieties, but a significant increase in CAT and POD activities compared with the control group. The results of this study showed that both saline and alkaline stresses significantly increased the activities of CAT and POD, and the effect on antioxidant enzyme activities gradually increased with the increase in salt concentrations. This is consistent with the research results of Dai et al. [36]. However, in this study, the SOD activity of rapeseed roots showed a decreasing trend with the increase in salt concentrations. This may be related to the stress duration, crop type and variety.

Proline, soluble sugars, and soluble proteins are important components of the non-enzymatic redox system in plants, playing a crucial role in coping with stresses such as salinity, drought, and high temperature, as well as maintaining normal cellular physiological functions [37]. Isin, Y et al. [38] found that the production of proline in crops could regulate the water balance inside and outside cells, which was the main way for plants to resist salt stress. Saline and alkaline stresses led to a significant increase in proline content, but the effect of alkaline stress was significantly stronger than that of saline stress. Lv et al. [24] also reported that the proline content in rice roots increased with the increase in salt concentration. This study showed that saline and alkaline stresses significantly increased the proline content in rapeseed roots, and the effect of the 40 mM Na_2CO_3 treatment was significantly stronger than that of other treatments. Under adversity, plant cells also regulate osmotic pressure by synthesizing other osmolytes, such as soluble sugars and

soluble proteins. Li et al. [39] reported that the accumulation of soluble sugars could enhance the tolerance of flax to saline stress, but the synthesis of soluble sugars was inhibited under severe alkaline stress. There is also a study indicating that saline stress promotes the accumulation of soluble sugars in plant leaves, but the content of soluble sugars shows a decreasing trend under severe saline stress [40]. In this study, saline and alkaline stresses significantly increased the soluble sugar and soluble protein contents of rapeseed roots, and the soluble sugar and soluble protein contents increased significantly with the increase in salt concentrations. Previous studies have shown that the decrease in soluble sugar content in leaves under severe saline stress may be due to the salt concentration exceeding the crop tolerance threshold, which disrupts leaf osmoregulation and metabolism, making the plants unable to resist osmotic stress caused by high salinity [41].

4.2. Both Saline and Alkaline Stresses Stimulated the Synthesis and Secretion of Organic Acids in Rapeseed Seedlings, and This Was More Pronounced Under Alkaline Stress

Organic acids are small-molecule compounds with carboxyl groups produced through basal metabolism and secondary metabolic pathways in plants. The synthesis, accumulation, and secretion of organic acids play an important role in plants adapting to various adversities [42]. Research has found that organic acids participate in plants' salt resistance through multiple mechanisms such as ion chelation, pH regulation, energy metabolism, and microbial interaction [43]. This study showed that there were significant differences in the content and types of organic acids in the root system of rapeseed under saline (LS, HS) and alkaline (LA, HA) stresses. For example, the contents of the intermediate products of the TCA cycle under saline stress, such as pyruvic acid, fumaric acid, malic acid, and ketoglutaric acid, were higher than those of CK, but there were no differences between alkaline stress treatments and CK. The up-regulation of organic acids such as cinnamic acid, taurine, and succinic acid was much higher under alkaline stress than under saline stress. The accumulation of these intermediate products in the TCA cycle may provide precursors for the synthesis of osmolytes such as proline and soluble proteins [15]. Unlike the mechanism under saline stress, rapeseed seedlings under alkaline stress reduce oxidative damage by accumulating sulfur-containing organic acids such as taurine to chelate excess metal ions in alkaline environments and maintain cell pH homeostasis, thus alleviating high pH stress [44,45]. These findings indicate that the root system of rapeseed seedlings focuses on providing antioxidant capacity and promoting the synthesis of osmolytes to alleviate saline stress. However, the root system focuses more on regulating pH to adapt to adverse environments under alkaline stress.

The TCA cycle is a common metabolic pathway in aerobic organisms, playing a critical role in response to saline and alkaline stresses. Changes in the activities of key enzymes involved in the TCA cycle directly affect crops' ability to resist saline and alkaline stresses [46]. Zhong et al. [47] showed that saline stress significantly reduced the activities of isocitrate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase compared with the control, disrupting the TCA cycle and leading to a decrease in organic acid secretion. This study found that under saline stress, the activities of citrate lyase, cis-aconitase, isocitrate dehydrogenase, α -ketoglutaric dehydrogenase, and fumarase increased as the salt concentration increased, while the activities of citrate synthase and succinate dehydrogenase showed an opposite trend. Under alkaline stress, the activities of citrate synthase and isocitrate dehydrogenase increased with the increase in salt concentration, while the activities of citrate lyase, cis-aconitase, α -ketoglutaric dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, and malate dehydrogenase decreased with the increase in salt concentration. It can be seen that there were differences in the responses of key enzymes in the TCA cycle of rapeseed roots to saline and alkaline stresses. Saline stress led to an increase in the activity of succinate-CoA synthase, which further led to a

decrease in root succinic acid content. However, alkaline stress led to an opposite change. Under saline stress, there was no significant change in the activity of malate dehydrogenase, but the activity of fumarase decreased, resulting in a significant conversion of fumarate to malic acid in the root system of rapeseed seedlings. Under alkaline stress, both the activities of fumarase and succinate dehydrogenase increased, leading to an increase in root fumaric acid and malic acid. This indicates that saline and alkaline stresses have different impacts on the TCA cycle and organic acid metabolism in the roots of rapeseed seedlings (Figure 16). This is consistent with the study results of Li et al. [48]. Wang et al. [49] found that under alkaline stress, the content of citric acid and malic acid as well as the activity of TCA cycle-related enzymes in the halophilic plant *Chloris virgata* were higher than those of the control, while there was no significant difference under saline stress [50]. The results of this study are not completely consistent with previous research results, which may be related to differences in the saline and alkaline stress tolerance of different rapeseed varieties [46] or the different metabolic regulation mechanisms of different crops under saline and alkaline stresses.

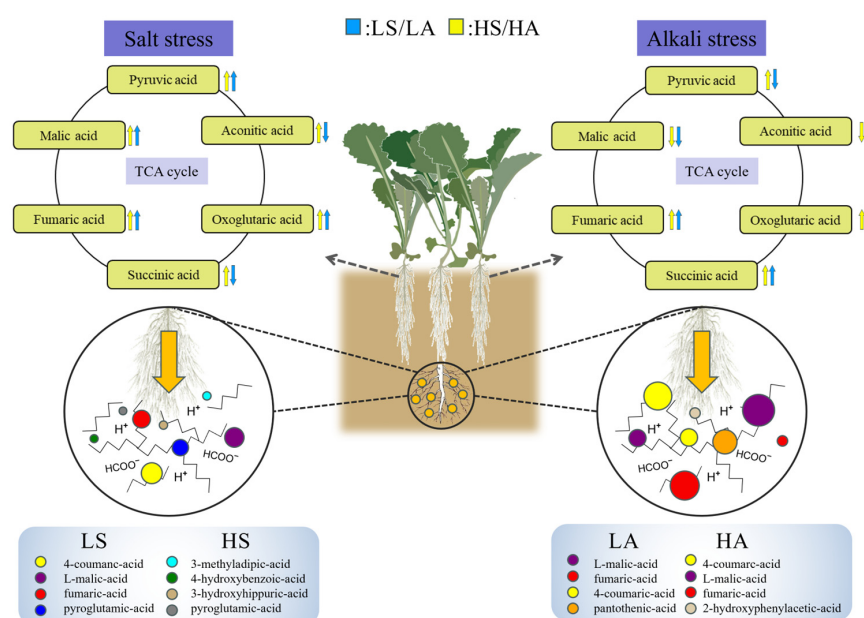


Figure 16. Differential response model of organic acid metabolism and secretion in the root system of rapeseed seedlings to saline and alkaline stresses. Under stress conditions, the synthesis and secretion of organic acids related to the TCA cycle in the root system increased compared with those of CK. The yellow arrows represent the changes in organic acid content in the root system under low levels of saline and alkaline stresses, while the blue arrows represent those under high levels of saline and alkaline stresses. Different circle sizes indicate different up-regulation factors of organic acids secreted by the root system, and different circle colors indicate different types of organic acids secreted by the root system.

The root system is the part of plants in direct contact with the soil. Changes in organic acids secreted by plants play a key role in resisting saline and alkaline stresses [51]. Research [52] has found that when rapeseed is subjected to alkaline stress, its root system regulates the pH of the root-zone soil by increasing the secretion of organic acids such as malic acid, citric acid, and succinic acid. Musazade et al. [53] also found that saline and alkaline stresses induced the secretion of TCA-cycle metabolites by rice roots, such as succinic acid, malic acid, and aconic acid. The results of this study showed that there were no differences in the contents of TCA cycle-related organic acids secreted by rapeseed (i.e., succinic acid, L-malic acid, fumaric acid, and cis-aconitic acid) between LS and CK. However, under HS, the contents of these TCA cycle-related organic acids were significantly

higher than those of CK. Similar result were also observed under alkaline stress, that is, under HA, the contents of TCA cycle-related organic acids (succinic acid, L-malic acid, fumaric acid, and cis-aconitic acid) were significantly higher than those of CK. This may be due to the fact that under severe saline and alkaline stresses with higher salt concentrations, the soil environment has a greater impact on plants. Plants secrete organic acids into the soil environment to form chelates with metal ions [54]. Notably, different from the results obtained under saline stress, under HA, the contents of many stress resistance-related organic acids (i.e., 4-coumaric-acid, cinnaic-acid, pantothenic-acid, taurine, ferulic-acid, and 4-hydroxybenzoic-acid) were higher than those of CK. These secreted organic acids can dissociate H^+ in the soil environment, effectively reducing the pH of the rhizosphere environment [55].

Some studies also suggest that phenylpropane metabolites (coumaric acid, cinnamic acid, and ferulic acid) under alkaline stress can not only scavenge ROS, but also act as precursors for the synthesis of large molecules such as lignin and suberin. That is, rapeseed roots may physically restrict the entry of saline and alkaline ions into cells, thereby alleviating stress [56]. Therefore, both saline and alkaline stresses can stimulate the secretion of organic acids from rapeseed roots to the rhizosphere environment. The secreted organic acids can reduce rhizosphere soil pH and alleviate the damage caused by saline and alkaline stresses. Notably, this phenomenon is more pronounced under alkaline stress [57].

Cinnamic acid and pyruvate are antioxidants produced by plants under stress [58]. Cinnamic acid contains unsaturated double bonds that can bind to free radicals, thereby scavenging superoxide anions and free radicals and reducing their damage to cell membranes. Pyruvate can indirectly enhance the free radical scavenging ability in plants through electron transfer [59]. Hydroxybenzoic acid and coumaric acid are hydroxy derivatives of cinnamic acid. The phenolic hydroxyl groups derived from them have significant reducibility. They can react directly with ROS and regulate antioxidant capacity, reducing oxidative stress [60,61]. This study detected cinnamic acid, pyruvate, and suberic acid in the root system of rapeseed seedlings under saline and alkaline stresses. In addition, this study also detected phenolic acids such as coumaric acid, pantothenic acid, hydroxybenzoic acid, hydroxyphenylacetic acid, and ferulic acid in root exudates. Notably, the up-regulation factors of *in vivo* and *in vitro* organic acids of rapeseed roots under saline stress relative to CK were smaller than those of alkaline stress.

5. Conclusions

Low-level saline (100 mmol/L NaCl) and alkaline (20 mmol/L Na_2CO_3) stresses had similar effects on the physiological parameters of rapeseed seedlings as high-level saline (200 mmol/L NaCl) and alkaline (40 mmol/L Na_2CO_3) stresses. Both saline and alkaline stresses caused consistent changing trends in malondialdehyde content, root vitality, and organic solutes (soluble sugar, soluble protein) in the roots of rapeseed seedlings, but had inconsistent and even opposite effects on the activity of key TCA cycle-related enzymes. Metabolomic analysis showed that the levels of synthesis and secretion of TCA cycle-related organic acids (cis-Aconitic acid, succinic acid, fumaric acid, L-malic acid) under saline stress were higher than those of the control. Although the secretion of TCA cycle-related organic acids under alkaline stress was also higher than that of the control, the synthesis of some organic acids was lower. Under alkaline stress, the roots of rapeseed seedlings can alleviate pH toxicity by increasing the secretion of phenolic acids such as 4-hydroxybenzoic acid, ferulic acid, and 4-coumaric acid. Moreover, the number of types and up-regulation factors of organic acids secreted by the root system of rapeseed seedlings under alkaline stress were significantly higher than those of saline stress. Therefore, there are differences in the metabolic responses of

rapeseed to saline and alkaline stresses, but the specific mechanism of action is still unclear. Further in-depth research is needed to determine the key genes that regulate saline and alkaline stress tolerance in rapeseed. This study provides a scientific basis for the genetic improvement of salt stress tolerance in rapeseed and the breeding of salt-tolerant varieties.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16020189/s1>, Figure S1: Bar chart of fold changes in differential root-borne organic acid metabolites under low and high salt stress; Figure S2: Volcano plot of differential root-borne organic acid metabolites under low and high salt stress; Figure S3: Bar chart of fold changes in differential root-borne organic acid metabolites under low and high alkali stress; Figure S4: Volcano plot of differential root-borne organic acid metabolites under low and high alkali stress; Figure S5: Bar chart of fold changes in differential root-secreted organic acid metabolites under low and high salt stress; Figure S6: Volcano plot of differential root-secreted organic acid metabolites under low and high salt stress; Figure S7: Bar chart of fold changes in differential root-secreted organic acid metabolites under low and high alkali stress; Figure S8: Volcano plot of differential root-secreted organic acid metabolites under low and high alkali stress; Table S1: Mean values of root-borne organic acid metabolites in *Brassica napus* under salt stress; Table S2: Mean values of root-borne organic acid metabolites in *Brassica napus* under alkali stress; Table S3: Mean values of root-secreted organic acid metabolites in *Brassica napus* under salt stress; Table S4: Mean values of root-secreted organic acid metabolites in *Brassica napus* under alkali stress.

Author Contributions: C.Z.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing—original draft, Writing—review & editing. L.S.: Investigation, Data curation, Methodology, Writing—review & editing. D.C.: Investigation, Methodology, Writing—review & editing. X.Z.: Investigation, Methodology, Writing—review & editing. F.Z.: Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Tianshan Talent & Leading Talent in Science and Technology Innovation Program of Xinjiang, China (Grant Number: 2022 TSY CLJ 0051) and Xinjiang Agriculture Research System (Grant number: XJARS-05).

Data Availability Statement: The data that support this study are available in the article and accompanying online Supplementary Material.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

CK	control treatment
LS	low concentration salt stress
HS	high concentration salt stress
LA	low concentration alkali stress
HA	high concentration alkali stress
TCA	Tricarboxylic acid (circulating)
TTC	triphenyltetrazolium chloride
MDA	malondialdehyde
SOD	superoxide dismutase activity
POD	peroxidase activity
CAT	catalase activity
PCA	principal component analysis
OPLS-DA	orthogonal partial least squares-discriminant analysis
VIP	variable importance in the projection
KEGG	kyoto encyclopedia of genes and genomes

References

- Wang, W.; Pang, J.; Zhang, F.; Sun, L.; Yang, L.; Siddique, K.H.M. Transcriptomic and metabolomics-based analysis of key biological pathways reveals the role of lipid metabolism in response to salt stress in the root system of *Brassica napus*. *Plant Growth Regul.* **2022**, *97*, 127–141. [\[CrossRef\]](#)
- Lei, S.; Jia, X.; Zhao, C.; Shao, M. A review of saline-alkali soil improvements in China: Efforts and their impacts on soil properties. *Agric. Water Manag.* **2025**, *317*, 109617. [\[CrossRef\]](#)
- Zhang, J.; Flowers, T.J.; Wang, S. Mechanisms of sodium uptake by roots of higher plants. *Plant Soil* **2010**, *326*, 45–60. [\[CrossRef\]](#)
- Liu, C.; Mao, B.; Yuan, D.; Chu, C.; Duan, M. Salt tolerance in rice: Physiological responses and molecular mechanisms. *Crop J.* **2022**, *10*, 13–25. [\[CrossRef\]](#)
- Sun, J.; He, L.; Li, T. Response of seedling growth and physiology of *Sorghum bicolor* (L.) Moench to saline-alkali stress. *PLoS ONE* **2019**, *14*, 0220340. [\[CrossRef\]](#)
- Guo, R.; Yang, Z.; Li, F.; Yan, C.; Zhong, X.; Liu, Q.; Xia, X.; Li, H.; Zhao, L. Comparative metabolic responses and adaptive strategies of wheat (*Triticum aestivum*) to salt and alkali stress. *BMC Plant Biol.* **2015**, *15*, 170. [\[CrossRef\]](#) [\[PubMed\]](#)
- Guo, J.; Lu, X.; Tao, Y.; Guo, H.; Min, W. Comparative Ionomics and Metabolic Responses and Adaptive Strategies of Cotton to Salt and Alkali Stress. *Front. Plant Sci.* **2022**, *13*, 871387. [\[CrossRef\]](#) [\[PubMed\]](#)
- Timpa, J.D.; Burke, J.J.; Quisenberry, J.E.; Wendt, C.W. Effects of water stress on the organic Acid and carbohydrate compositions of cotton plants. *Plant Physiol.* **1986**, *82*, 724–728. [\[CrossRef\]](#)
- Fougere, F.; Le Rudulier, D.; Streeter, J.G. Effects of Salt Stress on Amino Acid, Organic Acid, and Carbohydrate Composition of Roots, Bacteroids, and Cytosol of Alfalfa (*Medicago sativa* L.). *Plant Physiol.* **1991**, *96*, 1228–1236. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wang, X.; Geng, S.; Ri, Y.; Cao, D.; Liu, J.; Shi, D.; Yang, C. Physiological responses and adaptive strategies of tomato plants to salt and alkali stresses. *Sci. Hortic.* **2011**, *130*, 248–255. [\[CrossRef\]](#)
- Zhang, J.; Yang, D.; Li, M.; Shi, L. Metabolic Profiles Reveal Changes in Wild and Cultivated Soybean Seedling Leaves under Salt Stress. *PLoS ONE* **2016**, *11*, 0159622. [\[CrossRef\]](#)
- Xu, N.; Lu, B.; Wang, Y.; Yu, X.; Yao, N.; Lin, Q.; Xu, X.; Lu, B. Effects of salt stress on seed germination and respiratory metabolism in different *Flueggea suffruticosa* genotypes. *PeerJ* **2023**, *11*, 15668. [\[CrossRef\]](#)
- Li, H.; Tang, X.; Yang, X.; Zhang, H. Comprehensive transcriptome and metabolome profiling reveal metabolic mechanisms of *Nitraria sibirica* Pall. to salt stress. *Sci. Rep.* **2021**, *11*, 12878. [\[CrossRef\]](#)
- Yang, S.; Xu, Y.; Tang, Z.; Jin, S.; Yang, S. The Impact of Alkaline Stress on Plant Growth and Its Alkaline Resistance Mechanisms. *Int. J. Mol. Sci.* **2024**, *25*, 13719. [\[CrossRef\]](#)
- Wang, W.; Pang, J.; Zhang, F.; Sun, L.; Yang, L.; Zhao, Y.; Yang, Y.; Wang, Y.; Siddique, K.H.M. Integrated transcriptomics and metabolomics analysis to characterize alkali stress responses in canola (*Brassica napus* L.). *Plant Physiol. Biochem.* **2021**, *166*, 605–620. [\[CrossRef\]](#)
- Zhao, H.; Zheng, D.; Feng, N.; Zhou, G.; Khan, A.; Lu, X.; Deng, P.; Zhou, H.; Du, Y. Regulatory effects of Hemin on prevention and rescue of salt stress in rapeseed (*Brassica napus* L.) seedlings. *BMC Plant Biol.* **2023**, *23*, 558. [\[CrossRef\]](#)
- Zeng, L.; Cai, J.; Li, J.; Lu, G.; Li, C.; Fu, G.; Zhang, X.; Ma, H.; Liu, Q.; Zou, X.; et al. Exogenous application of a low concentration of melatonin enhances salt tolerance in rapeseed (*Brassica napus* L.) seedlings. *J. Integr. Agric.* **2018**, *17*, 328–335. [\[CrossRef\]](#)
- Zhou, J.; Yuan, W.; Di, B.; Zhang, G.; Zhu, J.; Zhou, P.; Ding, T.; Qian, J. Relationship among Electrical Signals, Chlorophyll Fluorescence, and Root Vitality of Strawberry Seedlings under Drought Stress. *Agronomy* **2022**, *12*, 1428. [\[CrossRef\]](#)
- He, M.; Mei, S.; Zhai, Y.; Geng, G.; Yu, L.; Wang, Y. Effects of Melatonin on the Growth of Sugar Beet (*Beta vulgaris* L.) Seedlings Under Drought Stress. *J. Plant Growth Regul.* **2023**, *42*, 5116–5130. [\[CrossRef\]](#)
- Li, B.; Feng, Y.; Zong, Y.; Zhang, D.; Hao, X.; Li, P. Elevated CO₂-induced changes in photosynthesis, antioxidant enzymes and signal transduction enzyme of soybean under drought stress. *Plant Physiol. Biochem.* **2020**, *154*, 105–114. [\[CrossRef\]](#)
- Heath, R.L.; Packer, L. Reprint of: Photoperoxidation in Isolated Chloroplasts I. Kinetics and Stoichiometry of Fatty Acid Peroxidation. *Arch. Biochem. Biophys.* **2022**, *726*, 109248. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wang, P.; Ma, L.; Ge, J.; Feng, F.; Wan, Q.; Zeng, D.; Yu, X. Colonization Mechanism of Endophytic *Enterobacter cloacae* TMX-6 on Rice Seedlings Mediated by Organic Acids Exudated from Roots. *J. Agric. Food Chem.* **2023**, *71*, 4802–4809. [\[CrossRef\]](#)
- Dehghan-Harati, Z.; Mahdavi, B.; Hashemi, S.E. Ion contents, physiological characteristics and growth of *Carum copticum* as influenced by salinity and alkalinity stresses. *Biol. Futur.* **2022**, *73*, 301–308. [\[CrossRef\]](#)
- Lv, B.S.; Li, X.W.; Ma, H.Y.; Sun, Y.; Wei, L.X.; Jiang, C.J.; Liang, Z.W. Differences in Growth and Physiology of Rice in Response to Different Saline-Alkaline Stress Factors. *Agron. J.* **2013**, *105*, 1119–1128. [\[CrossRef\]](#)
- Yan, G.; Shi, Y.; Mu, C.; Wang, J. Differences in Organic Solute and Metabolites of *Leymus chinensis* in Response to Different Intensities of Salt and Alkali Stress. *Plants* **2023**, *12*, 1916. [\[CrossRef\]](#)
- Wang, H.; Bai, B.; Bai, Z.Y.; Shi, L.J.; Ye, J.L.; Fan, S.W.; Xu, H.H.; Shi, D.C. Enzymatic regulation of organic acid metabolism in an alkali-tolerant halophyte *Chloris virgata* during response to salt and alkali stresses. *Afr. J. Biotechnol.* **2016**, *15*, 2243–2250. [\[CrossRef\]](#)

27. Zhang, H.; Liu, X.; Zhang, R.; Yuan, H.; Wang, M.; Yang, H.; Ma, H.; Liu, D.; Jiang, C.; Liang, Z. Root Damage under Alkaline Stress Is Associated with Reactive Oxygen Species Accumulation in Rice (*Oryza sativa* L.). *Front. Plant Sci.* **2017**, *8*, 1580. [\[CrossRef\]](#)
28. Sreenivasulu, N.; Grimm, B.; Wobus, U.; Weschke, W. Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica*). *Physiol. Plant.* **2000**, *109*, 435–442. [\[CrossRef\]](#)
29. Wang, D.; Gao, Y.; Sun, S.; Lu, X.; Li, Q.; Li, L.; Wang, K.; Liu, J. Effects of Salt Stress on the Antioxidant Activity and Malondialdehyde, Soluble Protein, Proline, and Chlorophyll Contents of Three Malus Species. *Life* **2022**, *12*, 1929. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Zhang, K.; Tang, J.; Wang, Y.; Kang, H.; Zeng, J. The tolerance to saline–alkaline stress was dependent on the roots in wheat. *Physiol. Mol. Biol. Plants* **2020**, *26*, 947–954. [\[CrossRef\]](#)
31. Sun, L.; Cai, X.; Chen, D.; Cai, Y.; Zhang, F. Physiological and biochemical responses of cotton (*Gossypium hirsutum*) seedlings to NaCl stress and analysis of salt tolerance thresholds. *Funct. Plant Biol.* **2025**, *52*, 24204. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Shao, J.; Huang, K.; Batool, M.; Idrees, F.; Afzal, R.; Haroon, M.; Noushahi, H.A.; Wu, W.; Hu, Q.; Lu, X.; et al. Versatile roles of polyamines in improving abiotic stress tolerance of plants. *Front. Plant Sci.* **2022**, *13*, 1003155. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Farghaly, F.A.; Radi, A.A.; Abdel-Wahab, D.A.; Hamada, A.M. Effect of salinity and sodicity stresses on physiological response and productivity in *Helianthus annuus*. *Acta Biol. Hung.* **2016**, *67*, 184–194. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Garg, N.; Chandel, S. Role of arbuscular mycorrhiza in arresting reactive oxygen species (ROS) and strengthening antioxidant defense in *Cajanus cajan* (L.) Millsp. nodules under salinity (NaCl) and cadmium (Cd) stress. *Plant Growth Regul.* **2015**, *75*, 521–534. [\[CrossRef\]](#)
35. Kumar, V.; Shriram, V.; Nikam, T.D.; Jawali, N.; Shitole, M.G. Antioxidant enzyme activities and protein profiling under salt stress in indica rice genotypes differing in salt tolerance. *Arch. Agron. Soil Sci.* **2009**, *55*, 379–394. [\[CrossRef\]](#)
36. Dai, R.; Zhan, N.; Geng, R.; Xu, K.; Zhou, X.; Li, L.; Yan, G.; Zhou, F.; Cai, G. Progress on Salt Tolerance in *Brassica napus*. *Plants* **2024**, *13*, 1990. [\[CrossRef\]](#)
37. Che-Othman, M.H.; Jacoby, R.P.; Millar, A.H.; Taylor, N.L. Wheat mitochondrial respiration shifts from the tricarboxylic acid cycle to the GABA shunt under salt stress. *New Phytol.* **2020**, *225*, 1166–1180. [\[CrossRef\]](#)
38. Isin, Y.; Türkan, I.; Sekmen, A.H.; Demiral, T. Salinity tolerance of purslane (*Portulaca oleracea* L.) is achieved by enhanced antioxidative system, lower level of lipid peroxidation and proline accumulation. *Environ. Exp. Bot.* **2007**, *1*, 49–57. [\[CrossRef\]](#)
39. Li, W.; Meng, R.; Liu, Y.; Chen, S.; Jiang, J.; Wang, L.; Zhao, S.; Wang, Z.; Fang, W.; Chen, F.; et al. Heterografted chrysanthemums enhance salt stress tolerance by integrating reactive oxygen species, soluble sugar, and proline. *Hortic. Res.* **2022**, *9*, uhac073. [\[CrossRef\]](#)
40. Guo, R.; Zhou, Z.; Cai, R.; Liu, L.; Wang, R.; Sun, Y.; Wang, D.; Yan, Z.; Guo, C. Metabolomic and physiological analysis of alfalfa (*Medicago sativa* L.) in response to saline and alkaline stress. *Plant Physiol. Biochem.* **2024**, *207*, 108338. [\[CrossRef\]](#)
41. Hare, P.D.; Cress, W.A.; Staden, J.V. Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environ.* **1998**, *21*, 535–553. [\[CrossRef\]](#)
42. Xiang, G.; Ma, W.; Gao, S.; Jin, Z.; Yue, Q.; Yao, Y. Transcriptomic and phosphoproteomic profiling and metabolite analyses reveal the mechanism of NaHCO₃-induced organic acid secretion in grapevine roots. *BMC Plant Biol.* **2019**, *19*, 383. [\[CrossRef\]](#)
43. Fang, S.; Hou, X.; Liang, X. Response Mechanisms of Plants Under Saline-Alkali Stress. *Front. Plant Sci.* **2021**, *12*, 667458. [\[CrossRef\]](#)
44. Farooq, U.; Rehman, A.; Ashraf, M.A.; Rasheed, R.; Shahid, M.; Ali, S.; Sarker, P.K. Taurine priming improves redox balance, osmotic adjustment, and nutrient acquisition to lessen phytotoxic effects of neutral and alkaline salts on pea (*Pisum sativum* L.). *Plant Signal. Behav.* **2025**, *20*, 2480224. [\[CrossRef\]](#)
45. Hafeez, A.; Rasheed, R.; Rizwan, M.; Habib, N.; Zafar, S.; Shad, M.I.; Ali, S.; Ashraf, M.A. Taurine Enervates Nickel Stress in Lagenaria siceraria by regulating Redox Balance, Methylglyoxal Detoxification, H₂S Production, and Nickel Uptake. *J. Soil Sci. Plant Nutr.* **2024**, *24*, 2334–2353. [\[CrossRef\]](#)
46. Zhang, Y.; Fernie, A.R. The Role of TCA Cycle Enzymes in Plants. *Adv. Biol.* **2023**, *7*, 2200238. [\[CrossRef\]](#)
47. Zhong, M.; Yuan, Y.; Shu, S.; Sun, J.; Guo, S.; Yuan, R.; Tang, Y. Effects of exogenous putrescine on glycolysis and Krebs cycle metabolism in cucumber leaves subjected to salt stress. *Plant Growth Regul.* **2016**, *79*, 319–330. [\[CrossRef\]](#)
48. Li, H.; Xu, C.; Han, L.; Li, C.; Xiao, B.; Wang, H.; Yang, C. Extensive secretion of phenolic acids and fatty acids facilitates rhizosphere pH regulation in halophyte *Puccinellia tenuiflora* under alkali stress. *Physiol. Plant.* **2022**, *174*, 13678. [\[CrossRef\]](#)
49. Wang, J.; Zhang, Y.; Yan, X.; Guo, J. Physiological and transcriptomic analyses of yellow horn (*Xanthoceras sorbifolia*) provide important insights into salt and saline-alkali stress tolerance. *PLoS ONE* **2020**, *15*, 0244365.
50. Yang, G.; Liang, K.; Zhou, Z.; Wang, X.; Huang, G. UPLC-ESI-MS/MS-Based Widely Targeted Metabolomics Analysis of Wood Metabolites in Teak (*Tectona grandis*). *Molecules* **2020**, *25*, 2189. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Duan, X.; Jin, K.; Mao, Z.; Liu, L.; He, Y.; Xia, S.; Hammond, J.P.; White, P.J.; Xu, F.; Shi, L. Compacted soil adaptability of Brassica napus driven by root mechanical traits. *Soil Tillage Res.* **2023**, *233*, 105785. [\[CrossRef\]](#)

52. Wang, H.; Zhao, S.; Sun, B.; Osman, F.M.; Qi, Z.; Ding, D.; Liu, X.; Ding, J.; Zhang, Z. Carboxylic acid accumulation and secretion contribute to the alkali-stress tolerance of halophyte *Leymus chinensis*. *Front. Plant Sci.* **2024**, *15*, 1366108. [\[CrossRef\]](#)
53. Musazade, E.; Zhao, Z.; Shang, Y.; He, J.; Wang, Z.; Wu, M.; Xu, M.; Guo, L.; Feng, X. Differential Responses of Rice Seedlings to Salt and Alkaline Stresses: Focus on Antioxidant Defense, Organic Acid Accumulation, and Hormonal Regulation. *J. Soil Sci. Plant Nutr.* **2025**, *25*, 4323–4340. [\[CrossRef\]](#)
54. Pohlman, A.A.; Mc Coll, J.G. Kinetics of Metal Dissolution from Forest Soils by Soluble Organic Acids. *J. Environ. Qual.* **1986**, *15*, 86–92. [\[CrossRef\]](#)
55. Jones, D.L.; Dennis, P.G.; Owen, A.G.; Van Hees, P.A.W. Organic acid behavior in soils—Misconceptions and knowledge gaps. *Plant Soil* **2003**, *248*, 31–41.
56. Dixon, R.A.; Achnine, L.; Kota, P.; Liu, C.; Reddy, M.S.S.; Wang, L. The phenylpropanoid pathway and plant defence—a genomics perspective. *Mol. Plant Pathol.* **2002**, *3*, 371–390. [\[PubMed\]](#)
57. Lu, X.; Ma, L.; Zhang, C.; Yan, H.; Bao, J.; Gong, M.; Wang, W.; Li, S.; Ma, S.; Chen, B. Grapevine (*Vitis vinifera*) responses to salt stress and alkali stress: Transcriptional and metabolic profiling. *BMC Plant Biol.* **2022**, *22*, 528. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Singh, P.K.; Singh, R.; Singh, S. Cinnamic acid induced changes in reactive oxygen species scavenging enzymes and protein profile in maize (*Zea mays* L.) plants grown under salt stress. *Physiol. Mol. Biol. Plants* **2013**, *19*, 53–59.
59. Lin, C.Y.; Chung, H.H.; Kuo, C.T.; Yiu, J.C. Exogenous cinnamic acid alleviates salinity-induced stress in sweet pepper (*Capsicum annuum* L.) seedlings. *N. Z. J. Crop Hortic. Sci.* **2020**, *48*, 164–182. [\[CrossRef\]](#)
60. Mattila, P.; Pihlava, J.; Hellström, J. Contents of Phenolic Acids, Alkyl- and Alkenylresorcinols, and Avenanthramides in Commercial Grain Products. *J. Agric. Food Chem.* **2005**, *53*, 8290–8295. [\[CrossRef\]](#)
61. Boz, H. *p*-Coumaric acid in cereals: Presence, antioxidant and antimicrobial effects. *Int. J. Food Sci. Technol.* **2015**, *50*, 2323–2328. [\[CrossRef\]](#)

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