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Effects of Exogenous Calcium on Adaptive Growth, Photosynthesis, Ion Homeostasis and Phenolics of *Gleditsia sinensis* Lam. Plants under Salt Stress

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Abstract: Salinity is the main environmental factor responsible for limited plant growth in many areas of the world. *Gleditsia sinensis* Lam. is a shelter forest tree species that does not require high-quality soil and can even grow in mild saline soil. This study mainly explored the tolerance of *G. sinensis* to salt and the effect of exogenous calcium addition on the growth of *G. sinensis* in a salinized soil. The concentrations of NaCl were set as 0 mmol/L, 100 mmol/L, and 200 mmol/L. Compared with the control, under the NaCl treatment of 200 mmol/L, it was observed that the leaves of *G. sinensis* turned yellow, the electrical conductivity significantly increased, and the water content and the chlorophyll content significantly decreased, which is probably unfavorable for growth. Our study showed that the addition of 10 mmol/L exogenous calcium chloride under salt stress had a positive effect on the growth and photosynthetic characteristics of *G. sinensis.* Moreover, the addition of exogenous calcium attenuated the cytotoxicity caused by Na^+ under salt stress and promoted the equilibrium of ion homeostasis. More importantly, the addition of exogenous calcium ions was beneficial for the survival of *G. sinensis* plants on salinized land and the increase of effective active ingredient content including phenolic compounds, which is of direct significance for improving environmental problems such as desertification of saline-alkali land. In conclusion, we investigated the effect of salt treatment on *G. sinensis*, as well as the positive effects of exogenous calcium on the survival and growth of *G. sinensis* in salt environment, which provided a scientific basis for the targeted cultivation of *G. sinensis* in salinized land and the effective utilization of salinized and alkaline land.

Keywords: salinized soil; salt stress; *Gleditsia sinensis*; ionic homeostasis; phenolic compounds

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1. Introduction

The soil environment is one of the most important factors affecting the growth of all plants, and soil salinization is becoming an increasingly more serious problem worldwide. At present, about 20% of the world's arable land is affected by salinity [1,2], and the area of saline land in China is more than 9.9×10^7 hm^2 [3]. As an abiotic stress, soil salinity is one of the major environmental factors affecting plant growth, photosynthesis, respiration, nutrient metabolism, hormonal regulation, and osmotic potential [4]. Proper development and utilization of these saline soils could alleviate land resource problems; this alleviation is important, especially with the increasing demands associated with the growing global population. Many countries have carried out research for breeding salt-tolerant varieties and have made some progress in cereal crops such as wheat and rice as well as fruits and vegetables [5,6]. If we wish to keep making advances in the study of plant salt tolerance, we should shift our research focus from crops such as wheat, cotton, barley, oats, and rice

to woody plants, as research into the mechanisms of forest salt tolerance has become an unavoidable trend [7]. The aim is to find more woody plants that can survive on high levels of land salinity, consolidate the land, and reduce further desertification of the saline land.

Growth of many plants in saline soils leads to plant dehydration and yellowing of leaves, mainly because salt stress disrupts ion homeostasis and balance in plant cells [8]. Moreover, increased salinity reduces the water and mineral absorption (osmotic effect) by the plant cells [9], resulting in nutrient deficiency, causing a decrease in chlorophyll content and affecting the function of the pigment–protein complex, thereby reducing the absorption and conversion of chloroplasts to light energy [10].A large number of experimental studies have shown that under NaCl stress, the net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr)of leaves significantly decreased, whereas intercellular carbon dioxide concentration (Ci) increased, indicating that non-stomatal limitation has become the main factor of photosynthetic reduction [11]. Compared with the gas exchange index of plant leaves, the chlorophyll fluorescence parameters of photosystem II reflect the characteristics of plant absorption, transmission, dissipation, and distribution of light energy [12]. In addition, Photosystem II (PSII) has a certain response to salinity compared to Photosystem I (PSI) [13]. However, recent studies have shown that the response of PSII photochemistry to salinity stress remained controversial. Inhibition of PSII activity was observed in *Perennial ryegrass* [14]. On the other hand, there has been no reported influence on PSII in *Suaeda* [15].

Calcium functions as a second messenger, and its overall cellular signaling network is critical for plant response to abiotic stresses [16]. When the plant is subjected to a high level of salinity, an increase in the concentration of Ca^{2+} can decrease the inhibition of growth. Although the underlying mechanisms are still largely unexplained, calcium has a significant potential to maintain membrane stability [17], membrane structure, and functional integrity. Moreover, calcium is involved in regulating plant cross-adaptation, salt ion-selective absorption, and transport to enhance plants stress resistance [18]. Numerous studies have shown that plants can adapt themselves to high-salinity environments by activating signal transduction systems involving Ca^{2+} [19]. Plants that grow in a saline environment are often deficient in calcium, and the application of calcium can impair the severity of specific cytotoxicity, particularly in plants susceptible to sodium and chloride damage [20]. However, the positive effect of Ca^{2+} on the growth of woody plants under salt stress has not been extensively studied.

Gleditsia sinensis Lam. is a deciduous small tree [21]. It is widely distributed in Central and Southeast Asia, as well as in North and South America [22]. *Gleditsia sinensis* has a high economic value. For example, the fruit is a natural raw material for medicine, food, health products, cosmetics, and detergents, and the seeds can be used for digestion and appetite; the Spina Gleditsia (the dry thorn of *G. sinensis*) is a good raw material for Chinese patent medicine [23]. Studies have now found that *G. sinensis* has more than 60 bioactive ingredients with medicinal properties such as triterpenoids, sterols, flavonoids, and alkaloids, the most important of which are phenolic compounds and their derivatives [24]. The plant phenolic compounds, originating from shikimic acid, phenylpropanoid, and acetic acid metabolic pathways, play a crucial role in plant resistance to environmental stresses. Their content is often affected by environmental conditions [25,26]. Numerous studies have shown that the synthesis of phenolic compounds depend mainly on abiotic factors [27,28]. In particular, when the plants are subjected to salt treatment, some significant changes occur in the phenolic compounds [29]. For example, mild salt treatment significantly increases the total polyphenol content in plants such as lettuce [30] and *Amaranthus tricolor* [31]. Studying the changes in plant phenolic compounds after exogenous calcium is added with salt stress is therefore important to understand the mitigation process.

It is worth noting that *G. sinensis* has considerable tolerance to abiotic stresses, such as cold, drought, heat, and salt, as well as resistance to biotic stresses such as pests and pathogens; moreover, it can adapt well to different environments such as plains, hills, and mountainous areas [32]. As a legume, *G. sinensis* can play a role in fixing nitrogen

and improving soil conditions. By analyzing the growth state, water content, electrical conductivity, and degree of membrane peroxidation of *G. sinensis* under different salt concentrations, its tolerance to salt can be effectively determined. Furthermore, through the state of plant photosynthetic system, the composition of Na^+, K^+, and Ca^{2+} homeostasis demonstrates that exogenous calcium can actively alleviate the unfavorable survival conditions of *G. sinensis* under salt stress. Increasing the content of active substances (phenolic compounds, etc.) in *G. sinensis* will have a great influence on its medicinal efficiency. As a result, understanding *G. sinensis*' tolerance to salt stress and decreasing the degree of damage caused by salt stress on it is critical for afforestation and land consolidation in saline areas.

2. Materials and Methods

2.1. Plant Material

Gleditsia sinensis seeds from Northeast Forestry University (Harbin, Heilongjiang, China) were soaked in hot water at 80 °C and stirred until naturally cooled; water was changed every 12 h for 24 h. The water-swelled seeds were selected and planted in a 6:4 matrix of soil and vermiculite. One-week seedlings were transferred to a hydroponic tank using 1/2 Hoagland nutrient solution. The whole Hoagland nutrient solution was used two weeks after the seedlings were adapted to the environment to ensure normal growth.

2.2. Experimental Design

Gleditsia sinensis seedlings after three weeks of hydroponic culture were subjected to NaCl treatment (100 mmol/L(S1) and 200 mmol/L(S2)), and the normal growing plants were used as a control (CK). The above treatments were used to study the tolerance of *G. sinensis* to salt stress. The relief experiment of salt stress by exogenous calcium was carried out on the basis of adding 100mmol/L NaCl to the nutrient solution. The plants treated with 100 mmol/L sodium chloride were simultaneously supplied with 5 mmol/L(S1 + C1), 10 mmol/L(S1 + C2), and 15 mmol/L(S1 + C3) calcium chloride. Calcium chloride is used as an exogenous calcium donor to increase the calcium content in the nutrient solution. After one week of treatment, the phenotype of plants changed significantly. Root, stem, and leaf tissue samples were taken for growth and physiological metabolism analyses. A total of 30 plants were used for each treatment, and three replicates were used for each treatment.

2.3. Plant Growth Parameters

Plant height, root length, fresh weight, and dry weight were measured after one week of NaCl treatment.

2.4. Plant Damage and Lipid Peroxidation

The degree of plant damage was assessed by the relative electrolytic conductivity (REC) and relative water content (RWC) of the leaves. Conductivity experiments were performed using the soaking method, wherein 0.1 g of fully expanded fresh leaves were washed with deionized water and cut into pieces of about 0.5 cm length (avoiding the main vein). Then, the leaves were transferred to a centrifuge tube containing 10 mL of deionized water and shaken at 25 °C for 12 h until the initial conductivity (A1) was measured using a conductivity meter (JENCO-3173, Jenco Instruments, San Diego, CA, USA). After that, leaves were heated in a boiling water bath for 30 min to completely release all electrolytes; then, they were cooled to room temperature and shaken well, and following this, the conductivity of the extract was measured again (A2). Then, REC was calculated as the division of A1 to A2 multiplied by 100 (REC (%) = A1/A2 × 100) [33]. RWC was measured by the method of [34]. The entire leaf was cut, and the fresh weight (FW) was recorded immediately. The leaves were then immersed in distilled water for 4 h at room temperature

to record the swelling weight (TW). The total dry weight (DW) was recorded after drying at 85 °C for 24 h in an oven. RWC was calculated on the basis of the following equation:

$$\text{RWC (\%)} = [(\text{FW-DW})/(\text{TW-DW})] \times 100$$

The content of malondialdehyde (MDA) was determined by an ultraviolet spectrophotometer, and lipid peroxidation was determined on the basis of the thiobarbituric acid (TCA) reaction [35]. The MDA content at 532 nm was calculated by subtracting the absorbance at 600 nm.

2.5. Leaf Photosynthesis, Chlorophyll Fluorescence Parameters, and Chlorophyll Content

The net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) of the leaves were measured by the portable photosynthetic system (li-6400, Li-COR, Lincoln, NE, USA). Leaf photosynthetic parameters were determined at 10 a.m. after the plants were treated with different concentrations of NaCl and treated with different concentrations of calcium chloride for one week. The mature leaves were dark-adapted for 20 min without isolation, and the fluorescence kinetic parameters at room temperature were measured using a portable modulation chlorophyll fluorescence instrument (PAM-2500 Walz, Effeltrich, Germany). For the chlorophyll content, 0.03 g of fresh leaves were extracted in a 10 mL pigment extraction solution containing absolute ethanol and acetone (1:2, *v*/*v*) at 25 °C for 12 h in the dark. The absorbance of the supernatant at 470, 645, and 663 nm was then measured using an ultraviolet spectrophotometer. Chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll content were calculated according to [36].

2.6. Determination of K^+, Na^+, and Ca^{2+}

To determine the K^+, Na^+, and Ca^{2+} ion concentrations, we carefully washed fresh root, stem, and leaf samples with deionized water, placed them in an oven at 105 °C for 20 min, and then kept the temperature constant at 80 °C until the samples were completely dried. The dried plant samples were then grounded in a 5 mL centrifuge tubes using a high-throughput plant tissue ball milling instrument (Scientz-192, Xinzhi Biotechnology Co., Ltd., Ningbo, China). A total of 0.3 g of each sample powder was weighed, and 5 mL of nitric acid and 1 mL of perchloric acid were added for wet digestion. The K^+, Na^+, and Ca^{2+} contents of plant tissue extracts and standard samples (National Institute of Metrology, Beijing, China) were determined by inductively coupled plasma optical emission spectrometer (ICP-OES; PerkinElmer, Optima 8300, Waltham, MA, USA). The concentration of K^+, Na^+, and Ca^{2+} is defined as K^+, Na^+, and Ca^{2+} content (mg) per unit tissue (g) [37].

2.7. Extraction and LC–MS Analysis of Phenolic Compounds

2.7.1. Chemicals and Reagents

UPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other reagents were of analytical purity. Ultrapure water was prepared by a Milli-Q system (Millipore, Bedford, MA, USA) water purification system. The reference compounds required for the experiment were all purchased from ChromaDex Inc. (Santa Ana, CA, USA), including p-hydroxycinnamic acid, p-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, genistein, abscisic acid, petunidin, naringenin, hesperidin, quercetin-3-O-rhamnoside, chlorogenic acid, ferulic acid, myricetin, luteolin, catechin, cinnamic acid, p-coumaric acid, hesperetin, quercetin, caffeic acid, L-phenylalanine, naringin, kaempferol, liquiritigenin, isoliquiritigenin, and vanillic acid. The purities of these standards were higher than 98%.

2.7.2. Preparation of Test Sample Solution

Gleditsia sinensis plant tissues (root, stem, and leaf) treated with different treatments (CK, S1, S2, S1 + C1, S1 + C2, S1 + C3) were grounded and then ultrasonically extracted (100 kHz, 40) for 45 min by adding 10 mL of 70% methanol. After filtration, the residue was further added to 10 mL of the solvent for ultrasonic extraction for 45 min. The two filtrates were combined and concentrated to dryness by a vacuum rotary evaporator, reconstituted with 1 mL of chromatographic grade methanol, and centrifuged at 12,000 r/min for 10 min, and then the supernatant was stored in a refrigerator at −20 until all samples were filtered through micropores filter membrane of 0.22 μm diameters that could be directly injected for LC–MS analysis.

2.7.3. Chromatographic Conditions

Measurement of the major effect compounds was carried with the UPLC–MS system, containing an ultra-performance with an LC-20AD pump, a temperature controller, and a column oven. The chromatographic separation was performed on an Acquity UPLC BEH C18 (1.7 μm, 2.1 mm × 5 mm) column. Analysis was conducted on the gradient elution component using solvent A (0.04% formic acid–water) and solvent B (0.04% formic acid–acetonitrile) as the mobile phase. The gradient elution with the flow rate of 0.5 mL/min was performed as follows: 0.0–20.0 min, 5–95% B; 20.0–22.1 min, 95–5% B; 22.1–28.0 min, 5% B.

2.7.4. Mass Spectrometry Conditions

Mass spectrometry was carried out on a QTRAP 5500 Ion TRAP MASS Spectrometer (AB SCIEX, Boston, MA, USA) equipped with an electrospray ionization source that was operated in positive ion mode. The experimental conditions were as follows: scan time 1.0 s, mass range from *m/z* 50 to 1000, fragmentation voltage 105 V, capillary voltage 3500 V, source temperature set at 350 °C, and curtain gas pressure of 40 psi. In an environment of 25 °C, chromatographic separations were achieved on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 5 mm); the volume injected was 5 μL. The phenolic data were acquired with MassLynx software v 4.1 (Waters, Milford, MA, USA).

2.8. Statistical Analysis

Data were analyzed for variance (ANOVA) using Tukey's multi-range test using the statistical package SPSS (version 20.0; IBM, Armonk, NY, USA), Origin Pro 9.0, and Excel 2010, with a significance level of $p < 0.05$. Results are expressed as mean ± SD, and the letters in the table and histogram show significant differences between treatments of the same category (simultaneous tissue).

3. Results

3.1. Effect of Salt Stress on Growth Parameters of G. sinensis

To understand the direct effect of salt stress on the growth and development of *G. sinensis*, we determined the growth indexes of plant height, root length, fresh weight, and dry weight after one week of salt treatment (Table 1). It was found that the plant height under salt stress was significantly lower than that under normal growth conditions, while the difference in root length was not significant. The fresh weight of plants treated with NaCl was also lower than control plants. According to the comparison, it was found that *G. sinensis* had yellowing of the leaves under salt stress, and the greater the salt concentration, the more the number of yellowing leaves. It indicates that the concentration of 200 mmol/L NaCl has affected the normal growth and development of the *G. sinensis*.

Table 1. Effects of hydroponic cultivation of *G. sinensis* plants on plant height, root length, fresh weight, and dry weight after one week treatment with different concentrations of NaCl and treatment with different concentrations of exogenous calcium and NaCl.

Treatments	Plant Height (cm)	Root Length (cm)	Fresh Weight of Plant (g)	Dry Weight of Plants (g)
CK	15.73 ± 0.31 ^{a}	18.58 ± 0.35 ^{a}	2.15 ± 0.03 ^{a}	0.45 ± 0.05 ^{a}
S1	13.46 ± 0.61 ^{b}	15.58 ± 0.33 ^{b}	1.78 ± 0.11 ^{b}	0.42 ± 0.04 ^{a}
S2	12.79 ± 0.32 ^{c}	16.51 ± 0.21 ^{b}	1.61 ± 0.13 ^{c}	0.38 ± 0.20 ^{b}
S1 + C1	13.94 ± 0.22 ^{ab}	17.24 ± 0.5 ^{a}	1.88 ± 0.09 ^{ab}	0.47 ± 0.12 ^{a}
S1 + C2	14.33 ± 0.10 ^{ab}	15.96 ± 0.36 ^{b}	2.01 ± 0.15 ^{a}	0.47 ± 0.08 ^{a}
S1 + C3	14.55 ± 0.29 ^{a}	16.26 ± 0.24 ^{b}	1.79 ± 0.08 ^{b}	0.45 ± 0.09 ^{b}

Note: Data are the means of three replicates ± standard error. In each column, the mean value represented by the different letters was significantly different in Tukey's test at $p < 0.05$.

3.2. Effect of Salt Stress on Relative Electrical Conductivity (REC) and Relative Water Content (RWC) in G. sinensis Leaves

In order to evaluate the degree of damage caused by salt stress on *G. sinensis*, we determined relative electrical conductivity and relative water content (Figure 1A) of *G. sinensis* leaves under different treatments. Compared to the control, the relative conductivity of the plants treated with salt increased significantly with increasing salt concentration. The relative water content of the leaves treated with salt decreased compared with the control conditions; however, the change of water content was not significant with increasing salt concentration.

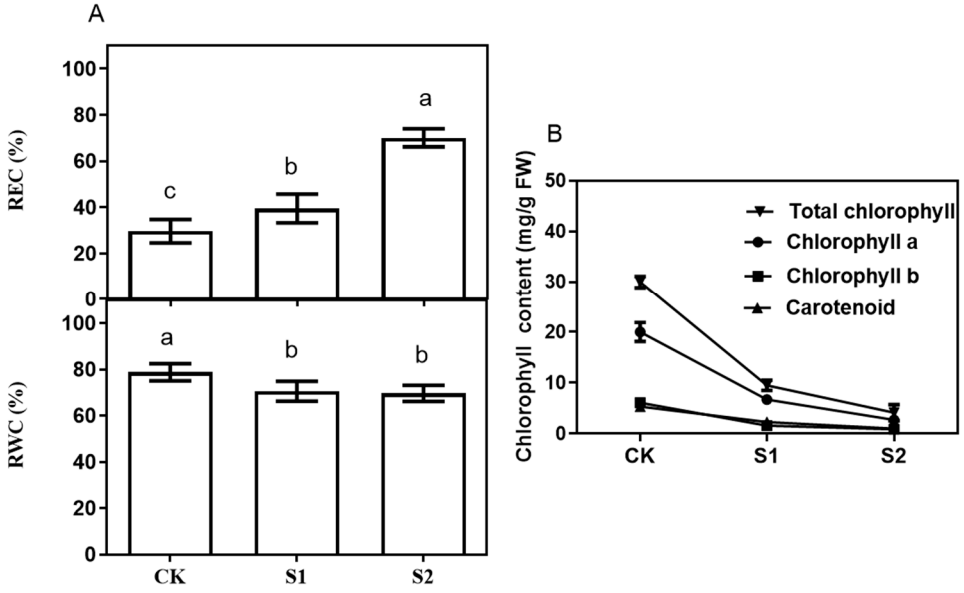

Figure 1. Effect of different concentrations of NaCl on (**A**) relative electrical conductivity (REC) and relative water content (RWC) in leaves of *G. sinensis* seedlings. REC and RWC were analyzed immediately after one week of treatment. (**B**) Photosynthetic pigments in leaves of *G. sinensis*. Photosynthetic pigments were analyzed immediately after treatment of the leaves at the same position in the plant one week later. CK represents *G. sinensis* leaves in normal hydroponic growth in Hogland nutrient solution, and S1 and S2 are *G. sinensis* leaves treated with concentrations of 100 and 200 mmol/L NaCl, respectively. The data are the average SD of three independent replicates. The mean values represented by the different letters were significantly different in Tukey's test at $p < 0.05$.

3.3. Effect of Salt Stress on Photosynthetic Pigments in G. sinensis Leaves

Salt stress significantly reduced the content of the photosynthetic pigments in *G. sinensis* relative to the control and continued to decrease with increasing salt concentration (Figure 1B). The trend of chlorophyll b and carotenoids was the same as that of

chlorophyll a, and the overall trend showed a downward trend. Concretely, *G. sinensis* treated with 100 mmol/L sodium chloride was 68% lower than the control plants in total chlorophyll content, and the 200 mmol/L treatment was reduced by 86%, indicating that the increase in salinity resulted in severe destruction of the chloroplast.

3.4. The Degree of Membrane Peroxidation in G. sinensis Plants under Salt Stress

The malondialdehyde content of various parts of the plants was measured to evaluate the effect of salt stress on membranous peroxidation (Figure 2). It was found that with the increase of salt concentration, the content of malondialdehyde in the plant leaves and roots increased significantly while there was no significant difference in the stems. It indicated that the higher concentration of NaCl caused serious damage to the plant membrane.

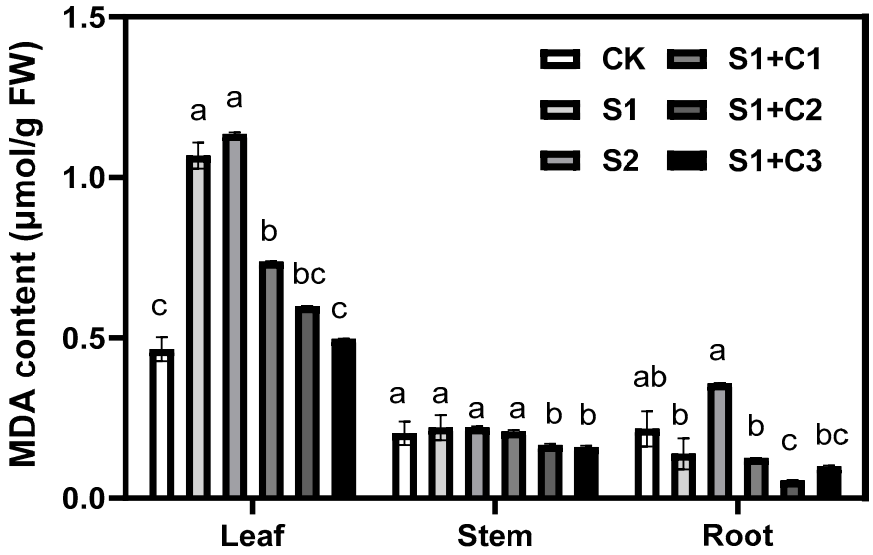

Figure 2. Effect of different salt concentrations and effect of different concentrations of exogenous calcium under salt stress on malondialdehyde content in *G. sinensis* seedlings. CK is a *G. sinensis* plant grown in normal hydroponic culture in Hogland nutrient solution. S1 and S2 are *G. sinensis* plants treated with concentrations of 100 and 200 mmol/L NaCl, respectively; S1 + C1, S1 + C2, and S1 + C3 represent 100 mmol/L NaCl with the addition of 5, 10, and 15 mmol/L$CaCl_2$, respectively. After one week of all plant treatments, the malondialdehyde content was measured and analyzed using a UV spectrophotometer. The data are the average SD of three independent replicates. The mean values represented by the different letters were significantly different in Tukey's test at $p < 0.05$.

3.5. Effect of Adding Exogenous Calcium on Plant Growth Parameters in 100 mmol/L NaCl Treatment

It can be seen from Table 1 that the addition of a certain amount of Ca^{2+} to the nutrient solution treated with salt caused a positive change in the growth of *G. sinensis*. As the concentration of Ca^{2+} increased, the plant height increased. The root length was the longest when the Ca^{2+} concentration reached 5mmol/L, and the root length did not change significantly at higher concentrations. Moreover, the fresh weight and dry weight of the plants were the heaviest when 10 mmol/L $CaCl_2$ was added with salt, compared to the case of adding salt alone. It indicated that the appropriate increase of Ca^{2+} concentration under salt stress can alleviate the inhibition of salt stress on seedling growth to some extent, but excessive Ca^{2+} concentration may further inhibit seedling growth and reduce plant biomass.

3.6. Effects of Different Concentrations of Exogenous Calcium on Lipid Peroxidation of G. sinensis Plants under Salt Stress

We added 5 mmol/L, 10 mmol/L, and 15 mmol/L of calcium chloride on the basis of 100 mmol/L of sodium chloride treatment. The addition of exogenous calcium significantly reduced the malondialdehyde content (Figure 2) caused by salt stress in all parts of tissue,

and 15 mmol/L calcium chloride concentration showed the most significant effect in leaves and stems, while 10 mmol/L calcium chloride concentration was the most significant treatment in roots. It is proved that the addition of exogenous calcium has a certain effect on the degree of membranous peroxidation damage in *G. sinensis* caused by salt stress.

3.7. Effects of Exogenous Calcium on Photosynthetic System Parameters in Leaves of G. sinensis

Salt stress has a harmful effect on the gas exchange of *G. sinensis*, however, the addition of exogenous calcium significantly increased its net photosynthetic rate (Pn) (Figure 3A), which reached the maximum rate with the addition of 10 mmol/L calcium chloride, while there was a decrease in the net photosynthetic rate at 15 mmol/L calcium chloride, but it was still higher than that of the plants treated with 100 mmol/L sodium chloride alone. The change in stomatal conductance (Figure 3B) was not significant, but a slight decrease was observed with the highest concentration of calcium chloride. The intercellular CO_2 concentration (Figure 3C) increased with increasing calcium chloride concentration, and the overall level was higher than that of salt stress alone. Transpiration rate (Figure 3D) showed similar results as the photosynthetic rate. The results demonstrate that the participation of calcium chloride has a certainly positive response to the photosynthesis of *G. sinensis* under salt stress.

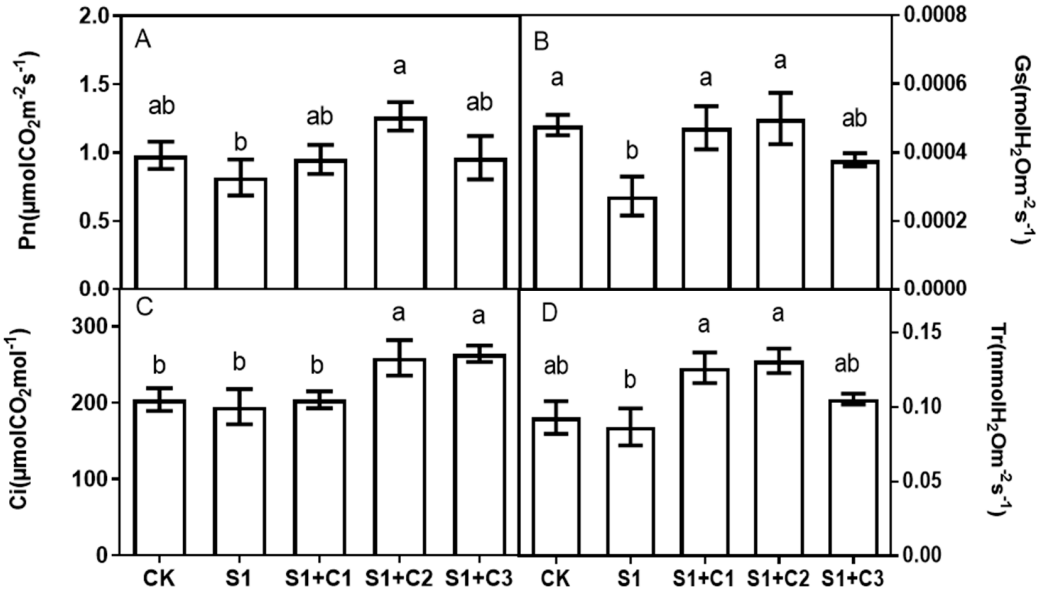

Figure 3. Effect of exogenous calcium on photosynthetic system parameters of *G. sinensis*. Pn, photosynthetic rate (**A**); Gs, leaf stomatal conductance (**B**); Ci, CO_2 concentration between cells (**C**); Tr, transpiration rate (**D**). The hydroponic three-week *G. sinensis* plants grow under normal conditions and were simultaneously treated with 100 mmol/L NaCl and $CaCl_2$ at concentrations of 0, 5, 10, and 15 mmol/L. After one week of treatment, the leaves at the same position were measured using a portable photosynthesis system. The data were obtained on the basis of three independent replicates. The average value was SD. The mean values represented by the different letters were significantly different in Tukey's test at $p < 0.05$.

3.8. Effects of Exogenous Calcium on Chlorophyll Fluorescence Parameters of G. sinensis

The NPQ (non-chemical quenching) of photosystem II in *G. sinensis* leaves was significantly increased as the concentration of exogenous calcium increased compared with salt treatment alone (Figure 4). The maximum photochemical quantum yield (Fv/Fm) reflected the original light energy conversion efficiency of the PSII reaction center, which was also increased with the addition of calcium chloride. Other fluorescence characteristics such as apparent ETR (electron transfer rate) and ΦPSII (actual photochemical quantum efficiency) reached the highest rate at 10 mmol/L calcium chloride, which was significantly

higher than that when no calcium chloride was added. These results indicate that a certain concentration of calcium chloride has a positive effect on maintaining the photosystem II function of the salt-stressed *G. sinensis*.

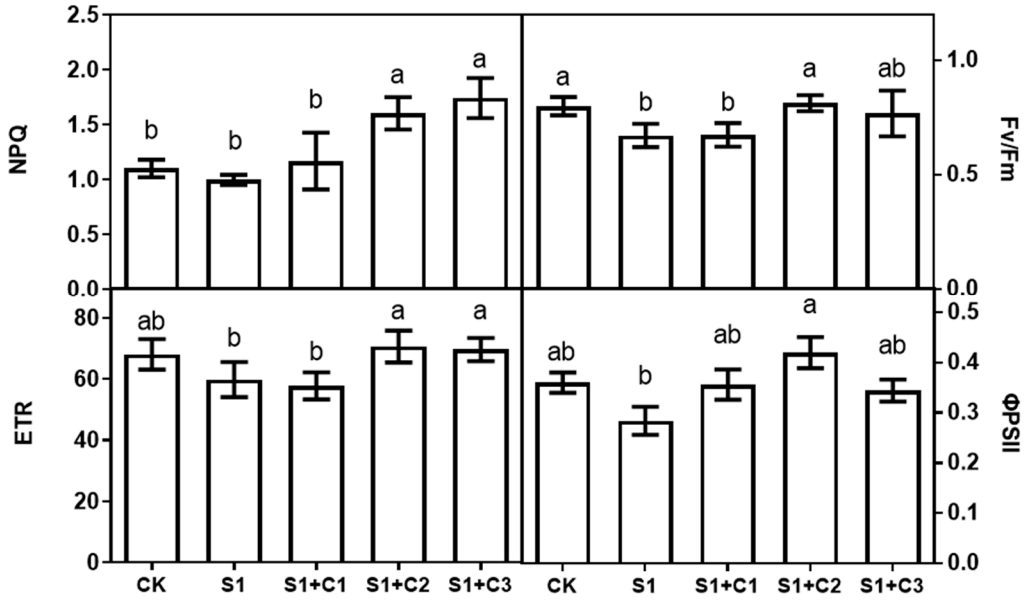

Figure 4. Effect of exogenous calcium on the fluorescence characteristics of leaves of *G. sinensis*. NPQ, non-photochemical quenching coefficient; Fv/Fm, maximum photochemical efficiency; ETR, apparent electron transport rate; ΦPSII, actual photochemical quantum efficiency. The hydroponic three-week *G. sinensis* plants grew under normal conditions and were simultaneously treated with 100 mmol/L NaCl and $CaCl_2$ at concentrations of 0, 5, 10, and 15 mmol/L. After one week of treatment, the leaves were measured using a portable chlorophyll fluorescence instrument (PAM-2500). The data were obtained on the basis of three independent replicates. The average value was SD. The mean values represented by the different letters were significantly different in Tukey's test at $p < 0.05$.

3.9. Content of Na^+, K^+, and Ca^{2+} in G. sinensis under Salt Stress and the Effect of Exogenous Calcium

It was clear that as the salt concentration increased, the Na^+ content increased sharply, but the addition of high concentrations of calcium chloride significantly reduced the Na^+ content in the leaves, stems, and roots of *G. sinensis* (Figure 5A).Conversely, the K^+ content was significantly reduced with increasing Na^+ content in *G. sinensis*, and the addition of exogenous calcium provided some improvement, especially in the roots (Figure 5B). It can be seen from the results that as the salinity increased, the Ca^{2+} in the plant decreased, especially in the leaf and stem, and the addition of exogenous calcium made it reach the normal level (Figure 5C).

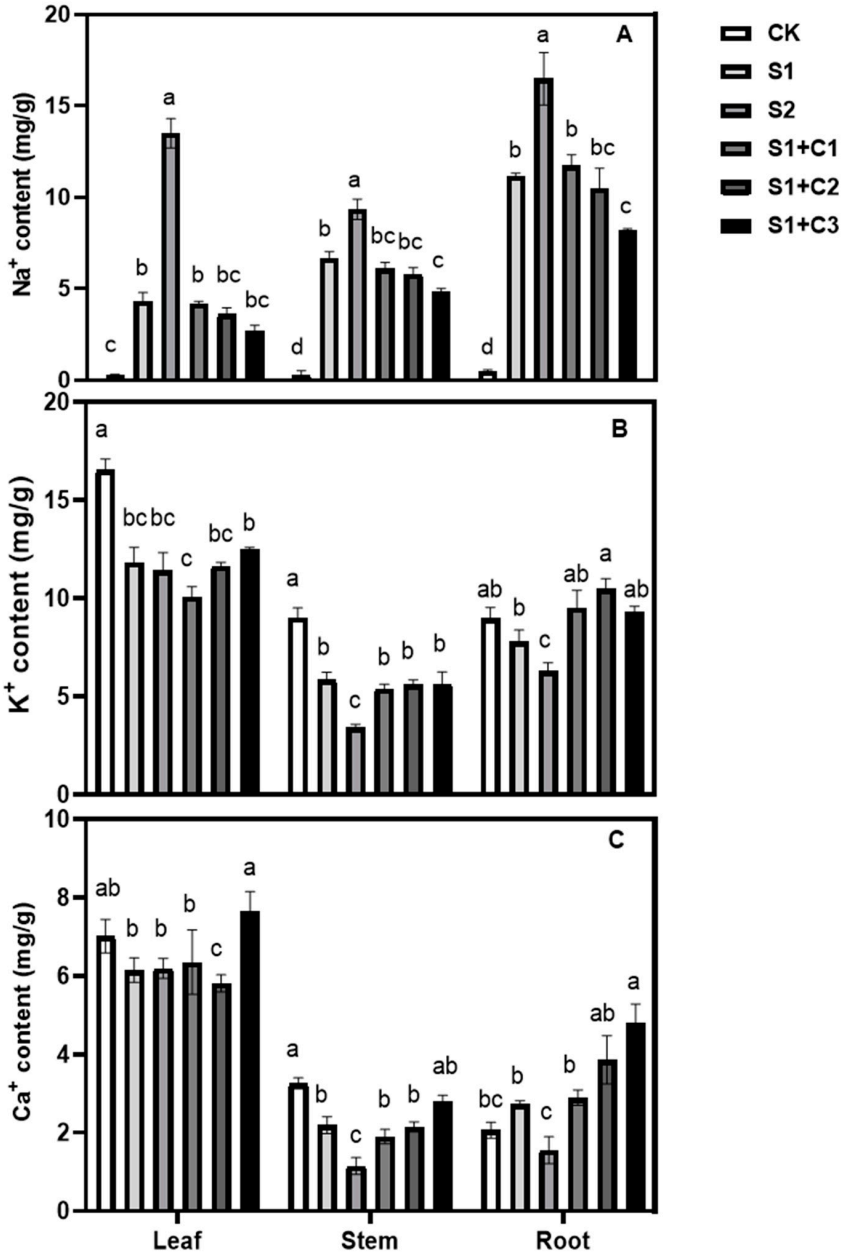

Figure 5. Effects of different salt concentrations and addition of exogenous calcium ions on the contents of Na^+, K^+, and Ca^{2+} in *G. sinensis* seedlings. (**A**–**C**) Na^+, K^+, and Ca^{2+} content in different tissue parts of *G. sinensis* at different salt concentrations and with different concentrations of calcium ions. CK is a *G. sinensis* plant grown in normal hydroponic culture in Hogland nutrient solution. S1 and S2 are *G. sinensis* plants treated with concentration of 100 and 200 mmol/L NaCl, respectively; S1 + C1, S1 + C2, and S1 + C3 represent 100 mmol/L NaCl with the addition of 5, 10, and 15 mmol/L$CaCl_2$, respectively. The ion content was measured and analyzed by inductively coupled plasma optical emission spectrometry after one week of plant treatment. The data are the average SD of three independent replicates. The mean values represented by the different letters were significantly different in Tukey's test at $p < 0.05$.

3.10. Effects of Exogenous Calcium on Plant Phenolic Compounds under Salt Stress

Subsequently, we studied the accumulation of phenolic compounds in different tissues treated with 100 mmol/L NaCl (S1), 100 mmol/L NaCl + 10 mmol/L $CaCl_2$ (S1 + C2), and normal growth state (CK). Visualization of phenolic materials was performed by hierarchical cluster analysis (Figure 6). Twenty-three phenolic compounds and L-phenylalanine were determined by the LC–MS technique in methanol extracts of roots, stems, and leaves

of *G. sinensis*, and the types of compounds measured for the differently treated samples showed no difference. It can be seen from the heat map that these compounds were mainly divided into three groups on the basis of their tissue-specific accumulation patterns and participated in salt stress responses to varying degrees. The C6C3-type compound (p-hydroxycinnamic acid, p-coumaric acid), the C6C3C6-type compound (naringenin, glycyrrhizin, genistein, quercetin), and L-phenylalanine were mainly accumulated in the roots (Figure 7A). There was a slight increase but not significant in the case of salt treatment alone, and a very significant increase in case of exogenous calcium addition. The C6C1-type compound (2,5-dihydroxybenzoic acid) and the C6C3C6 type compound (hesperidin, naringin, luteolin, kaempferol)were similar, showing a high content in the stem and a positive response to the addition of salt to exogenous calcium (Figure 7B). C6C3-type compound (ferulic acid, chlorogenic acid) and C6C3C6-type compound (catechin, petunidin, quercetin-3-O-rhamnoside, myricetin) are highly accumulated phenolic compounds in leaves (Figure 7C). It is worth noting that only the petunidin, quercetin-3-O-rhamnoside, myricetin, and chlorogenic acid in the leaves were specifically responsive to salt stress alone, and the other compounds were added to the salt stress treatment of exogenous calcium. Most phenolic compounds exhibited a positive response to salt stress and the addition of exogenous calcium to salt stress. The most important one was the C6C3C6-type compound, indicating that flavonoids and isoflavones respond more strongly to salt stress.

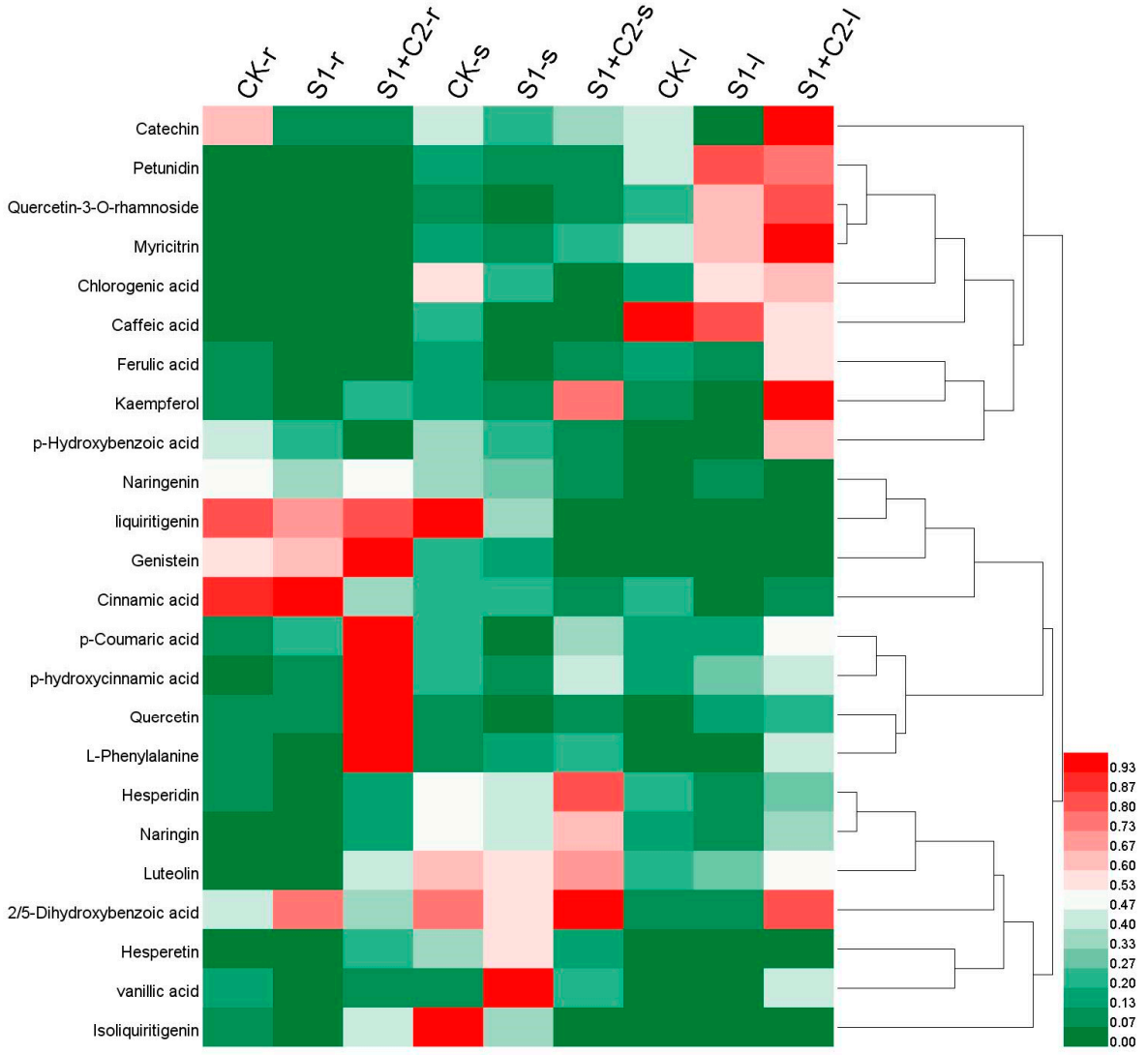

Figure 6. Heat map visualization of phenolics in different treatment of *G. sinensis*. The color range from red to green indicates relative abundance from high to low (color key scale right the heat map). r: root; s: stem; l: leaf.

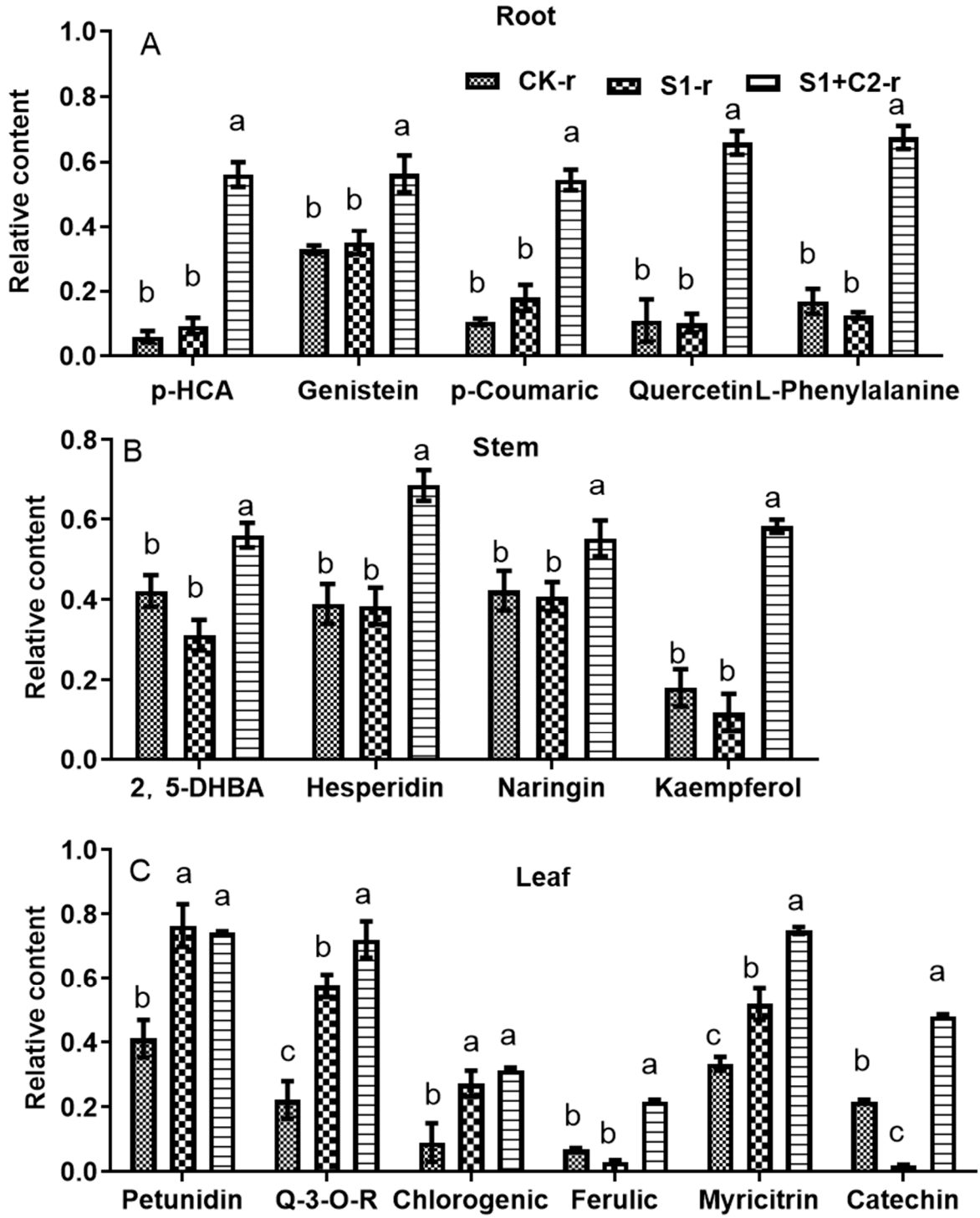

Figure 7. Response of the iconic phenolic compounds in *G. sinensis* to different treatments from root(**A**), stem(**B**) and leaf(**C**), respectively. Normal hydroponic conditions (CK), 100 mmol/L NaCl treatment (S1), 100 mmol/L NaCl + 10 mmol/L $CaCl_2$ (S1 + C2); p-hydroxycinnamic acid (p-HCA), 2,5-dihydroxybenzoic acid (2,5-DHBA), quercetin-3-O-rhamnoside (Q-3-O-R). The data are the average SD of three independent replicates. The mean values represented by the different letters were significantly different in Tukey's test at $p < 0.05$.

4. Discussion

In our results, the adverse effects of salt stress on plant height, root length, fresh weight, and dry weight clearly show that the physiological growth of *G. sinensis* was negatively affected (Table 1). Like many other abiotic stresses, salt stress reduces the growth rate

of plant canopy and biomass and causes yellowing of leaves that become more severe with increasing salt concentration, which is the adaptive characteristic of plants living under stress [38]. Many plants regulate the above-ground and the underground biomass to adapt to different environmental changes [39]. The biomass is reduced to adapt to the stress or due to the downregulation of the metabolism resulted from the stress. However, after different concentrations of $CaCl_2$ are added, the plant height, fresh weight, etc., of *G. sinensis* increased to varying degrees compared with the salt-treated plants (Table 1). The relative water content of plants is a crucial measure of their water status, and water content in plant tissue has a direct impact on plant development and stomatal state, as well as on photosynthetic performance [40]. In this study, 200 mmol/L NaCl (S2) reduced its RWC by 11.69% compared to the control (Figure 1A). This was the case due to the increased osmotic pressure outside the plant tissue from the increasing salinity. Consequently, the relative water content of the leaves was significantly lower than that of normal plants, indicating that the salt-stressed plants were in an extremely water-deficient state.

When a plant is subjected to any kind of stress, the plant cell is easily broken, and the membrane protein is damaged, causing extravasation of the cytosol and increase in the relative conductivity [41]. Hence, relative electrical conductivity is an important physiological and biochemical indicator reflecting the condition of the plant membrane system [42]. Our results clearly show that the surface relative conductivity increased in response to salt stress (Figure 1A). Similar results were obtained by Kaya et al., who reported that high salinity stress increases the membrane permeability in strawberries [43].

Studies usually consider the malonaldehyde (MDA) content as one of the main products of membranous peroxidation; it is generally believed that its accumulation in plants is a manifestation of active oxygen toxicity, and its content is an important indicator to the degree of membrane peroxidation [44]. It can be seen from this study that MDA content increased with increasing salt concentration, especially in the leaves (Figure 2), indicating that salt stress has a certain degree of lipid membrane oxidative damage to *G. sinensis* seedlings mainly reflected in the leaves. It is worth noticing that the addition of calcium ions effectively alleviates this phenomenon (Figure 2). This may be because calcium reduces plasma membrane permeability and maintains the functional and structural integrity of plant cells under salt stress, inconsistent with the conclusion of Khan et al. [45].

Through previous studies, photosynthetic pigment factor is one of the key factors determining photosynthetic efficiency and plant growth [46]. In this study, the content of the photosynthetic pigments such as chlorophyll and carotenoids in the leaves of *G. sinensis* under salt stress decreased significantly, indicating that salt stress negatively affected the synthesis of photosynthetic pigments (Figure 2). These results are consistent with the study of El-Esawi in soybean [47].

Photosynthesis is an extremely important metabolic process in all plants. It has a great influence on plant growth, yield, and resistance. Therefore, photosynthesis can be used as an indicator to the status of plant growth and its stress resistance [48]. Salt stress has multiple effects on plant photosynthesis through affecting photosynthetic electron transport, photosynthetic phosphorylation, and dark reaction-related enzyme activities [49]. Our results showed that salt stress also damaged the photosynthetic system by decreasing the photosynthetic rate (Figure 3A), stomatal conductance (Figure 3B), Co_2 concentration between cells (Figure 3C), and transpiration rate (Figure 3D). These results are expected to be due to the increase of osmotic pressure in plants under salt stress that leads to the loss of water, and hence causes the water potential of plants to decrease, resulting in decreased stomatal conductance and reduced transpiration rate. At the same time, salt stress reduces the rate of photosynthesis and reduces the assimilation and energy supply, thereby limiting the growth and development of plants in consistent with the results of Li et al. [50]. On the other hand, our results showed that the supplement of exogenous calcium significantly enhanced the photosynthesis of *G. sinensis* seedlings under salt stress by alleviating the negative effect on the photosynthetic system parameters (Figure 3). These results are consistent with the results of Wang, which indicated that applying a particular quantity

of calcium to plants under salt stress can improve stomatal sensitivity and regulation, enhance leaf water content, preserve the stability of chloroplast membrane structure, and boost Rubisco enzyme and PEP (phosphoenolpyruvate) levels [42]. Moreover, the results of Zhang et al. showed that the activity of carboxylase increased the carboxylation efficiency of CO_2 and improved the photosynthetic properties of plants [51].

Fv/Fm represents the potential activity and maximum photochemical quantum yield of Photosystem II (PSII) [52]. The Fv/Fm of *G. sinensis* increased to a certain extent when different concentrations of exogenous Ca^{2+} were added to the salt stress system, indicating that the membrane damage of the light energy absorption conversion mechanism of *G. sinensis* alone subjected to salt stress was improved and slowed down. The original light energy conversion efficiency of PSII is adversely affected [53]. The actual photochemical quantum efficiency (ΦPSII) is commonly used to represent the total photochemical quantum yield of PSII under plant photosynthesis, which reflects the actual primary light energy capture efficiency of the PSII reaction center in the partially closed condition [54]. When 10 mmol/L $CaCl_2$ was added under salt stress, the ΦPSII increased, indicating an increase in the photosynthetic performance. ETR and NPQ are also effective parameters for indicating the photosynthetic capacity of plants [54]. ETR increased with the addition of exogenous calcium, indicating an increase in the electron capture efficiency of the ΦPSII reaction center [55]. The NPQ reflects the portion of the light energy that is dissipated in the form of heat by the light energy absorbed by the ΦPSII antenna pigment, cannot be used for photosynthetic electron transport, and is an indicator of the degree of heat dissipation [56]. Plants can avoid excess damage by dissipating excess light energy through heat dissipation. This is a self-protection mechanism of plants and plays a protective role for the photosynthetic apparatus [57]. In this study, the addition of different concentrations of $CaCl_2$ in salt stress led to an upward trend in NPQ, indicating that the ability of salt stress alone to protect the heat dissipation of PSII antennas in plants was restored to some extent.

Ionic conditions are important factor in the salt tolerance of plants. Calcium is one of the main nutrients for plants affected by salt stress. As a component of the cell membrane, Ca^{2+} plays an important role in maintaining the structure and function of the membrane [58]. In our results, the salinity increased the Na^+ concentration in the roots, stems, and leaves of *G. sinensis* (Figure 5A). The concentration of Ca^{2+} and K^+ in the different tissues of the salinized *G. sinensis* plants was downregulated, whereas the reduction of K^+ concentration was more obvious (Figure 5B,C). Na^+ does not function as a macro nutrient, but K^+ and Ca^{2+} play key roles in several physiological processes, and therefore a decrease in the concentration of K^+ and Ca^{2+} caused by a sharp increase in Na^+ may lead to a nutritional imbalance. Addition of calcium reverses the accumulation rate of Na^+, Ca^{2+}, and K^+ (Figure 5). The explanation of this phenomenon is that the root system directly absorbs minerals, and that Ca^{2+} promoted the K^+ channel opening and K^+ uptake of root plasma membrane under salt stress. High Ca^{2+} concentrations reduce the permeability of the plasma membrane to Na^+. The reduced permeability of Ca^{2+} to the Na^+ membrane reduces the accumulation of passive influx of Na^+, as concluded by Cramer et al. [59]. Hence, we can say that the addition of a certain concentration of Ca^{2+} reduces the plant cytotoxicity. Ca^{2+} maintains the ionic homeostasis of cells, which is the most direct factor in alleviating plant loss caused by salt stress.

The physiological and molecular mechanisms of response to salt stress in the process of evolution include the accumulation of antioxidant enzymes and their activity, phytohormone metabolism, signal transduction, and the regulation of halo-tolerant-related genes [60]. The most important point to note is the synthesis of secondary metabolites for osmotic adjustment [61]. Plants have developed their ability to produce large amounts of phenolic secondary metabolites as a response to salt stress, which are not essential in the main processes of growth and development but essential for their interaction with the environment; therefore, their production strategies are crucial [62].

In our study, different phenolic compounds detected in *G. sinensis* showed distinct tissue specificity (Figure 6). In leaves, salt stress increased the accumulation of specific

phenolic compounds such as chlorogenic acid, petunidin, myricetin and quercetin-3-O-rhamnoside(Figure 7C). Chlorogenic acid is an effective phenolic antioxidant, and its antioxidant capacity is stronger than that of caffeic acid, ferulic acid, etc., and it has various pharmacological effects [63]. The other three compounds belonged to the C6C3C6-type compound, indicating that the flavonoids and isoflavones responded more strongly to salt stress. On the other hand, caffeic acid, ferulic acid, kaempferol, and catechin responded negatively to the salt stress, potentially due to decrease in the activity of the enzymes responsible for their accumulation. Phenolic substances such as hesperidin, kaempferol, and naringin in the stem (Figure 7B), and quercetin and L-phenylalanine in the root were also downregulated by salt stress, while coumaric acid was upregulated(Figure 7A). The increase in chlorogenic acid level and the decrease in caffeic acid and ferulic acid in response to stress are consistent with the results of Kısa et al. [64]. Moreover, the effect of stress on myricetin level is similar to the result of Zafari et al. [65], whereas our results showed different effects on ferulic acid, quercetin, kaempferol, and naringin. Further, kaempferol, catechin, and caffeic acid showed different response to stress compared with the results of Poonam et al. [66], indicating that different plants show different mechanisms to respond to different abiotic stresses. However, in general, the mechanism by which salt stress affects the production of phenolic compounds is through activation of the cell signaling process, gene expression, and enzyme activities that would lead to upregulation of phenylpropanoid pathway, which is responsible for the accumulation of phenolic compounds [27].

By adding calcium with salt stress, the specific upregulated compounds showed a stronger accumulation during stress. Moreover, although L-phenylalanine, kaempferol, ferulic acid, and catechin responded negatively to the salt stress, this effect was reversed with the addition of calcium, indicating that calcium not only enhances the accumulation of specific phenolic compounds but also reverses the negative effect of salinity on the production of some specific phenolics. The effect of calcium on L-phenylalanine is essential because it is the precursor to the synthesis of phenolic compounds. Moreover, the increase in kaempferol would raise the medicinal value of *G. sinensis.* This is consistent with the results of Ngadze et al. [67] and Sharma et al. [68], who found that the addition of calcium significantly increased the content of phenolic compounds and the enzymatic activity involved in phenol metabolism (phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidases). In our results, the decrease in cinnamic acid, especially in roots, could refer to downregulation of phenylalanine ammonia lyase activity; however, the accompanied increase in p-coumaric acid indicated that the increase in phenylalanine ammonia lyase activity that is essential for accumulation of phenolic compounds was coupled with increase in cinnamic acid 4-hydroxylase (C4H), which oxidizes cinnamic acid to 4-coumaric acid, consistent with the results of Ma et al. [69] and Castañeda& Pérez [70]. At the same time, the increased levels of p-coumaric acid associated with the decreased levels of caffeic acid indicates the downregulation of p-coumaric acid 3-hydroxylase (C3H), as also concluded by Ma et al. [69].

Hence, we can say that the addition of exogenous calcium alleviated the harmful effect of salt stress on the growth of *G. sinensis* but did not reduce the accumulation of effective phenolic compounds such as L-phenylalanine, chlorogenic acid, and kaempferol, which had a positive effect on the production and the accumulation of effective medicinal and active ingredients within *G. sinensis.* We can target these marker compounds to different tissue sites during culturing and extracting them in order to specifically extract high levels of active ingredients in the future.

5. Conclusions

Salt stress has a harmful effect on the normal growth and development of *G. sinensis*. The plant loses water and brings about certain membrane damage, and the photosynthetic pigment content is also reduced by the increase of salinity. However, the addition of exogenous calcium ions significantly improved the degree of membrane peroxidation disrupted by high salinity, increased the photosynthetic capacity of plants, and impaired

the cytotoxicity due to the sharp increase in Na^+. The addition of Ca^{2+} caused Na^+ and K^+ to balance the steady state, which is the most direct factor that alleviates salt stress. The specific response of phenolic substances in different tissue parts of *G. sinensis* can be used as a chemical signal for calcium to alleviate salt stress. The addition of exogenous calcium ions is beneficial to the survival of *G. sinensis* on salinized land and the increase in effective active ingredient content, which is of direct significance for improving environmental problems such as desertification of saline-alkali land.

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