Research Article

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Comparative Effects of Salt and Alkali Stress on Antioxidant System in Cotton (*Gossypium Hirsutum* L.) Leaves

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Abstract: This pot experiment was to evaluate how salts (NaCl, Na_2SO_4) and alkali (Na_2CO_3+$NaHCO_3$) affect the physiological and biochemical characteristics during the seedling stage of two cotton cultivars (salt-tolerant, L24; salt-sensitive, X45). Salt and alkali stress reduced seedling emergence rate, relative biomass, and chlorophyll content, however, the REC and MDA content increased. Salt and alkali stress increased markedly superoxide dismutase (SOD) activity. Peroxidase (POD) activity increased first and then decreased as the increase of salt and alkali stress. Catalase (CAT) activity initially increased and then decreased as NaCl stress increased. In addition, the SOD activity, REC, and MDA content was markedly higher in salt stress than that in alkali stress. The proline content of L24 was higher than that of X45 under salt and alkali stress. However, glycine betaine and soluble sugar content of L24 was lower than that of X45 under alkali stress. The REC and MDA content of L24 were lower than those of X45, however, the relative biomass, chlorophyll content, SOD, POD, CAT, and Pro were higher than those of X45. In conclusion, salt tolerant cotton cultivars may possess a superior protection effect by increasing antioxidant enzymes activity under salt and alkali stress.

Keywords: Salt stress; Alkali stress; Cotton growth; Antioxidant System; Osmotic adjustment .

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1 Introduction

Salinity is a globally growing problem in agricultural soils, with 6% of the world's land and 20% of irrigated land that were affected by salinization, especially in arid regions. Xinjiang is a region with the widest distribution of saline soil, the heaviest salt accumulation, and the most salinization types in China. In addition to carbonates, chlorides, and sulfates, there are also rare nitrate soils. The pH value of the soil in salinization area of Xinjiang is above 8.5, and the soil alkalinity is an important feature of Xinjiang saline soil as well. Neutral salts (e.g., NaCl and Na_2SO_4) and alkaline salts (e.g., $NaHCO_3$ and Na_2CO_3) are two distinct types of salt stress, therefore, crops can produce different responses and salt tolerance mechanisms [1,2,3]. Currently, some studies have been conducted on the salt tolerance mechanism of crops under NaCl stress, while studies on other salt types have not been profoundly or systematically performed [4, 5, 6]. Salt and alkaline stress not only produces osmotic and ionic stress under neutral salt stress, but also has a negative effect by high pH value. The high pH value of alkaline soils inhibits the absorption of ions by roots, alters the availability of soil nutrients, and leads to an imbalance of crop ions and mineral nutrients. Several studies suggested that alkaline stress is indeed more harmful than salt stress [7,8,9], and some scholars have pointed out that the sensitivity order of cotton is $MgSO_4$ > $MgCl_2$ > Na_2CO_3 > Na_2SO_4 > NaCl > $NaHCO_3$ [10]. However, there are few studies on different types of salt stress, and the understanding of the salt tolerance mechanism of crops under different salt-alkali stresses is seriously insufficient [11,12,13], which is an important basis for proper regulation and improvement of salt tolerance of crops [14].

The most significant effect of salt stress on crops is to inhibit the growth. High salt stress leads to obvious stagnation of plant growth, while low salt stress may cause the decline of plant growth rate. Related studies have shown that with the increase of soil salinity, plant dry matter accumulation decreases [15]. Within the appropriate irrigation, water salinity (EC<4.61dS/m) can promote cotton growth and increase dry matter accumulation and yield of cotton plants [16]; however, excessive salt concentration can significantly inhibit cotton growth, resulting in decrease of biomass [17]. The adverse impact of salt stress on crop growth includes inhibition of physiological and metabolic processes in plant, osmotic stress, ionic toxicity, nutrient and hormonal imbalance, oxidative damage, etc. [18]; besides, it can also destroy the cell membrane structure, inhibit photosynthesis, produce toxic metabolites, and reduce nutrient absorption, leading to obstruction in the growth of plants, decline of productivity, and even death [19, 20]. The relative electrical conductivity (REC) of cells reflected the permeability change and damage degree of cell membrane under adverse conditions, and was an important indicator for reflection of the crop damage. Malonaldehyde (MDA) was a product of lipid peroxidation, which accumulates more under salt stress [21], Therefore, the stability of cell membrane is one of the criteria to evaluate the salt tolerance of crops [22]. In addition to this harm, alkali stress increases the stress of HCO_3^- or CO_3^{2-} and high pH caused by bicarbonate or carbonate. The inhibition effect of salt stress on crop growth is less than that of alkali stress on the same concentration [23, 24]. Salt tolerance of crops is a complicated and comprehensive issue, involving a variety of resistance mechanisms such as ion homeostasis, osmotic equilibrium, and elimination of reactive oxygen species (ROS) [25]. Imbalance of ROS is one of the important causes of oxidative damage and apoptosis in plant cells induced by salt stress [26, 27]. ROS are a by-product of normal cell metabolism of plants, while the abiotic stresses break the balance between the production and elimination of ROS [28]. Salt stress and alkali stress can increase cellular damage due to accumulation of ROS. It has been found that high antioxidant levels could be associated with salt tolerance [29]. When plants are exposed to salt damage, intracellular ROS will dramatically increase, and react with macromolecules, such as lipids, leading to active oxygen imbalance and cell damage [30]. SOD, POD, and CAT are all protective enzymes for the plant's enzymatic defense system against membrane lipid peroxidation, which can remove excessive free radicals under adverse conditions, thus playing a role in protecting the cell membrane structure, and improving plant salt tolerance. The substrate of SOD is superoxide anion radical $O_2{\bullet}^-$, which can be disproportionated into oxygen and hydrogen peroxide. SOD plays an important role in protecting biological cells from superoxide radicals and ROS formed by $O_2{\bullet}^-$ and OH, and then, H_2O_2 is converted into non-toxic H_2O and O_2 by CAT and POD. Under the high salt environment, the crop can deal with nutritional imbalance by regulating ion transport, as well as maintaining ion homeostasis, and producing osmotic regulators (e.g., proline (Pro) and glycine betaine (GB)) to protect against ionic and osmotic stress. In addition, protection against oxidation-reduction equilibrium in the defense of the ROS induced by salt stress includes the involvement of non-enzymatic antioxidants or enzyme antioxidants (e.g., SOD, POD, CAT, etc.) [31]. The mechanism of crop salt tolerance has been a hot topic for researches, and its ultimate goal is to reduce the negative effects of salt and improve the ability of crops to maintain growth and yield in saline soils [32]. It has also been found in recent studies that exogenous protective agents (e.g., osmotic regulators, hormones, signaling molecules, antioxidants, etc.) can effectively alleviate the harms of salt stress, improve the salt tolerance of crops, promote crop growth, and increase yielding [33]. However, the available research is not comprehensive enough in terms of the physiological mechanism of salt tolerance of crops under different salt and alkali stresses.

Cotton is salt tolerant and a major cash crop grown in saline soil. Differences in salt tolerance were found between different cotton cultivars. There are important theoretical and practical significances for study on salt tolerance mechanism of cotton, which can improve cotton salt tolerance and increase cotton yield under salt stress. In this study, we explored the regulation of cotton organic osmosis and enzyme protection mechanisms by observing the effects of different salt and alkali stresses on main salt-tolerant physiological indexes of cotton (cell membrane permeability, MDA, Pro, GB, soluble sugar content, etc.), and biochemical indicators (activities of SOD, POD, CAT, etc.). It will reveal the salt tolerance evaluation of cotton under different salt and alkali stresses, which has important scientific significance in promotion of the salt tolerance, in addition to production and development of cotton in Xinjiang province (China).

2 Methodology

2.1 Materials and Cotton Growth Conditions

This pot experiment was carried out in a greenhouse at Shihezi University, Xinjiang, China. A clay loam soil (grey desert soil) taken from the station field, with depth at the range of 0~30 cm of topsoil soil. Soil physicochemical properties were as follow: soil salinity 0.16 dS/m (1:5 soil:

Table 1: Type and degree of saline and alkaline in soil under different treatments.

Treatment	Saline and alkaline	Add salt content (%)	Electrical conductivity (dS/m)	pH(1: 2.5)
CK	Control- non-salinization (alkalization)	0	0.16	8.38
CS1	NaCl- mild salinization	0.1	0.38	8.41
CS2	NaCl-moderate salinization	0.3	0.85	8.22
CS3	NaCl-severe salinization	0.5	1.4	8.09
SS1	Na_2SO_4- mild salinization	0.2	0.28	8.39
SS2	Na_2SO_4- moderate salinization	0.4	0.9	8.3
SS3	Na_2SO_4- severe salinization	0.6	1.33	8.24
AS1	Na_2CO_3+$NaHCO_3$- mild alkalization	0.1	0.2	8.93
AS2	Na_2CO_3+$NaHCO_3$- moderate alkalization	0.15	0.42	9.83
AS3	Na_2CO_3+$NaHCO_3$- severe alkalization	0.2	0.57	10.21

water extract); pH 8.16; total N 0.57 g/kg; organic matter 6.77 g/kg; available phosphorus 7.21 mg/kg, and available potassium 182 mg/kg.

Three types of saline and alkaline soil including chloride, sulfate and soda basification were used in this experiment, and different soil types and salinity degrees were set by adding NaCl, Na_2SO_4, and Na_2CO_3+$NaHCO_3$ to the sample soil. The cotton cultivars tested were X45 (salt-sensitive) and L24 (salt-tolerant). Each treatment was repeated for 3 times. The specific treatment and soil salinity type and salinization degree are shown in Table 1. As shown in Table 1, the two salts used in salt stress treatment, made the soil conductivity, were basically consistent, and the alkali stress treatment ensured mild salinity degree on the basis of increase of pH value. Before the experiment, the sample soil was air-dried, crushed, and passed through a 2 mm sieve. In addition, NaCl, Na_2SO_4, and Na_2CO_3+$NaHCO_3$ (weight ratio 1:1) were separately prepared into a salt solution, which was then added to the sample soil to a supersaturated state (the same volume of deionized water was added to the control soil), and the soil was placed for 1 month to achieve stability. The treated soil was then air-dried, crushed, and sieved (2 mm sieve). Basins with diameter of 15 cm and height of 20 cm were used to fill the treated soil according to a bulk density of 1.25 g/cm^3, and each pot was filled with 5 kg soil. The irrigation method was drip irrigation with the dripper flow rate of 1.1 L/h and the dripper spacing of 40 cm. The drip irrigation pipe was laid flat on the basin, each basin was supplied with water by one dripper, and the dripper was fixed at the center of the top of the pot. Cotton was sown on May 6, 2016, and 20 seeds were planted in each soil column. After the cotton was emerged, the number was counted and the seedling emergence was accordingly calculated. To ensure emergence of cotton, 1 L of seedling water was dripped from each soil column after sowing. The cotton seedlings were fixed when 2 true leaves were grown, and 4 cotton seedlings with uniform growth were kept in each basin. The water was weighed and regularly replenished during the experiment to ensure the water supply and to keep the soil water content at 60% ~ 80% field capacity. The experiment was completed 45 days after sowing (cotton seedling stage).

2.2 Sampling and measurement methods

2.2.1 Cotton seedling emergence rate

At the 10th day after sowing, the number of treated seedlings were counted, and the seedling emergence = number of emergence / total number of seeds×100%. (1)

45 days after seedling emergence, the cotton samples were taken. When the dry matter of cotton leaves was sampled and measured, 3 representative cottons with each treatment were taken. All leaves were cut and washed with water, then killed out at 105°C for 30 min, and dried to constant weight at 70°C. The dry matter was weighed and calculated for relative dry matter weight of leaves.

2.2.2 Measurement of physiological indexes

The main stem functional leaves were taken for each treatment, all samples were put into the ice box, and returned to the laboratory in-time. Then, the dust and dirt on the surface of the leaves were washed, the surface was dried with blotting paper, and the main veins were removed. Indexes including chlorophyll, relative conductivity (REC), MDA content, SOD, POD, CAT, Pro, soluble sugar content, and GB were measured, respectively.

Chlorophyll, MDA, and REC were measured by the method described by Arnon [34], Zhu [35], Wu et al.'s method [36], respectively. The antioxidant enzymes were extracted according to the Zhou et al.'s method with slight modification [37], SOD, POD, and CAT activities were measured by the method described by Giannoplitis and Ries [38], Kraus and Fletcher [39], Beers and Sizer [40], respectively. Pro, soluble sugar, and GB were measured according to the method of Bates [41], Gao [42], and Greive and Grattan [43], respectively.

2.3 Data analysis

The significance of difference for physiological characteristic was analyzed by analysis of variance (ANOVA) using SPSS software (version SPSS 19.0). Treatment means were separated using least significant difference (LSD) test at 95 or 99 % level of probability.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Seedling emergence and leaf relative biomass

Soil salinity and alkali are major abiotic stresses that limit crop growth and productivity. The seed germination stage is the first and most sensitive period for crops to be affected by salt stress, which is critical for crop growth and yield [44]. In this study, it has been revealed that the increase of salt and alkali stresses reduced the emergence rate of cotton, low concentration of Na_2SO_4 and alkali stress stress has no significant effect on seedling emergence, however, high concentration of Na_2SO_4 & alkali stress significant inhibited seedling emergence, in addition, under NaCl stress, and the cotton emergence rate was minimum (Figure 1A). Yu et al. also found that low concentration of salt and alkali promoted the germination and growth, while the high level (≥150 mmol/L) salt and alkali concentration was toxic to the wheat seedlings and inhibit their growth [45]. The reason for inhibition of salt and alkali stress on cotton emergence may be due to the inhibition of the high concentration of Na^+ ions on cell membrane system of seeds, and the formation of toxins caused the seeds to inhibit germination. Moreover, the osmotic stress of seeds increased with the increase of salt and alkali concentration, which led to insufficient water absorption of cotton seeds with excessive external osmotic pressure, so that the germination rate of cotton seeds decreased with the increase of salt and alkali concentration.The crop growth status is the most direct reflection of the degree of salt and alkali damage. In general, the leaf relative biomass of both cultivars decreased as the increase of salt and alkali stresses, and the relative biomass of L24 leaves was significantly higher than that of X45 (Figure 1B). In addition, the relative biomass reduction of two cultivars under NaCl stress decreased more than that under Na_2SO_4 stress and alkali stress ($NaHCO_3$ and Na_2CO_3).

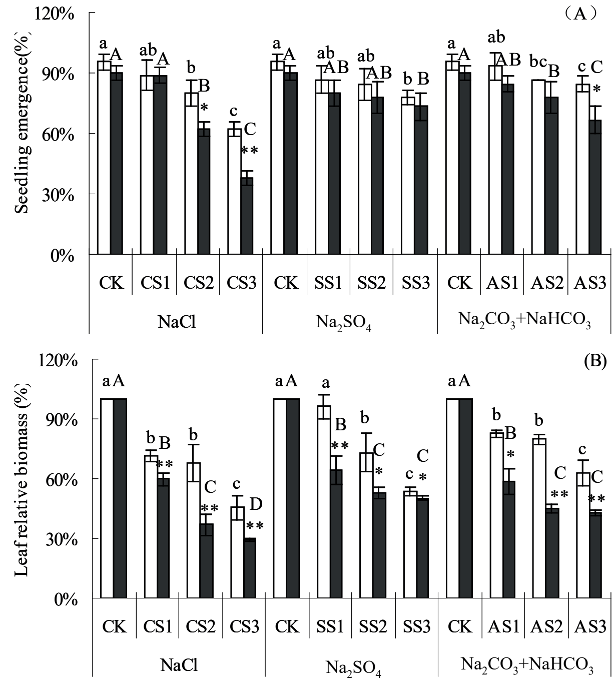

Figure 1: Effect of soil salt and alkali stresses on seedling emergence and leaf relative biomass of cotton. Symbols indicate L24 (salt-tolerant, white bar) and X45 (salt-sensitive, black bar). Error bars represent SDs (n = 3). Different letters represent significant differences (*P<0.05*) between L24 and X45. Asterisks represent a significant difference between L24 and X45 (**P < 0.05; **P < 0.01*).

3.2 Chlorophyll content

The concentration of Chlorophyll in the stressed plants was a key indicator to evaluate plant's health and photosynthesis capacity. Figure 2 shows that there was no significant difference in chlorophyll content between the two cultivars under low salt and alkali stresses, while the chlorophyll content of L24 was significantly higher than that of X45 under medium or severe salt and alkali stresses. NaCl stress has reduced the chlorophyll content of cotton. The reason for chlorophyll content reduction lay in that the saline and alkali soil contained more Na^+ and Cl^-, which caused the cotton leaves to absorb a large amounts of Na^+ and Cl^- to inhibit the absorption of K^+, Ca^{2+}, and Mg^{2+}, and this ion imbalance led to the accelerated chlorophyll degradation and the reduction of chlorophyll content [46]. Xie et al. also found that salt stress in cotton seedling could inhibit the growth of cotton seedlings and reduce the content of chlorophyll [47]. In addition, we have also discovered that the chlorophyll content of L24 was observed in the Na_2SO_4 stress treatment higher than in the CK treatment. Low concentration of Na_2SO_4 stress promoted the chlorophyll content of X45, however, it decreased with the increase of Na_2SO_4 concentration. The chlorophyll content of X45 in the alkali stress treatment is lower than in the CK treatment, however, a high concentration of alkali stress promoted the chlorophyll content of L24. These results indicated that L24 (salt-tolerant) is more resistant to Na_2SO_4 and alkali stress than X45 (salt-sensitive), as a result, the photosynthetic physiological mechanism of salt-sensitive and salt-tolerant cultivars may be different. In summary, the effects of different salt and alkali stresses on the chlorophyll content of cotton leaves were NaCl > Na_2SO_4 > $NaHCO_3$ + Na_2CO_3, indicating that the chlorophyll content significantly decreased under NaCl stress. One explanation is that Na^+ ions concentration in Na_2SO_4 or alkali stress treatments lower than NaCl stress treatment in this study, therefore, there is no dose-dependent effect on chlorophyll content reduction for these groups.

3.3 REC and MDA content

The REC increased with the increase of soil salinity and alkali stresses (Figure 3A). Generally, the REC of L24 leaves was lower than X45, and the effects of different saline and alkali stresses on the REC of leaves were: NaCl > Na_2SO_4 > $NaHCO_3$ + Na_2CO_3. Lin et al. also found that alkaline stress led to an increase in plasma membrane permeability [48], which was consistent with the results of this study. The reason for increase of REC in leaves may be that under salt

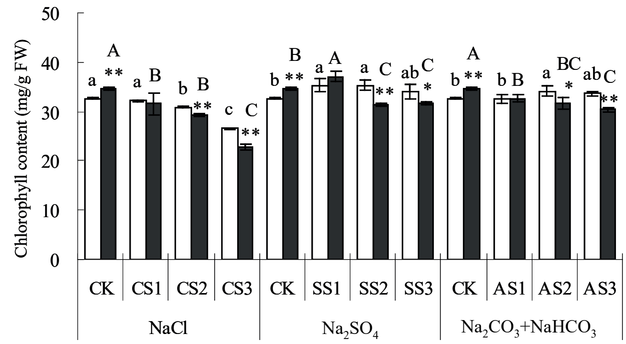

Figure 2: Effect of soil salt and alkali stresses on chlorophyll content of cotton leaves. Symbols indicate L24 (salt-tolerant, white bar) and X45 (salt-sensitive, black bar). Error bars represent SDs (n = 3). Different letters represent significant differences (*$P<0.05$*) between L24 and X45. Asterisks represent a significant difference between L24 and X45 (**$P < 0.05$; **$P < 0.01$*).

stress, the plasma membrane was firstly damaged, and Na^+ replaced with the Ca^{2+} in the plasma membrane, leading to the increase of the permeability of the cell membrane and decrease of selective transmission, and may further cause the occurrence of plasma membrane leakage [49].

The effect of different salt and alkali stresses on MDA content in cotton leaves is shown in Figure 3B. The MDA content in cotton leaves under salt stress treatment was significantly higher than that under alkaline stress, indicating that the physiological responses of different cultivars to salt and alkali stress were different. Saline and alkali stress can cause oxidative damage to the cell membrane, thereby accumulating MDA metabolites. Under salt-alkali stress, crops with weak salt-tolerant ability were more susceptible to accumulate a relatively higher MDA. In general, the MDA content of L24 leaves was lower than that of X45, indicating that the damage of salt-tolerant cotton cultivar under salt and alkali stresses was less than that of salt-sensitive cultivar, and the effect of alkaline stress on MDA content of cotton leaves was less than salt stress. One explanation is that may be because the salt-tolerant cultivar has a strong antioxidant enzyme system, and reduces intracellular superoxide radicals and alleviates membrane lipid peroxidation, thereby reducing the MDA content.

3.4 Antioxidant enzymes activities

SOD was one of the essential enzymes for cellular against ROS in aerobic organisms. Figure 4A shows that the SOD activity of L24 and X45 leaves increased as the soil salt and alkali stress increased. Changes in salt-induced

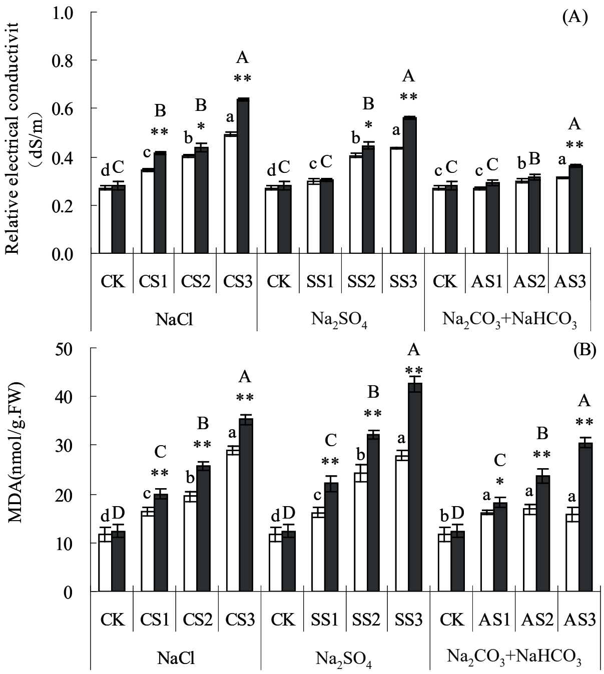

Figure 3: Effect of soil salt and alkali stresses on REC and MDA content of cotton leaves. Symbols indicate L24 (alt-tolerant, white bar) and X45 (salt-sensitive, black bar). Error bars represent SDs (n = 3). Different letters represent significant differences (*P<0.05*) between L24 and X45. Asterisks represent a significant difference between L24 and X45 (**P < 0.05; **P < 0.01*). The symbols REC and MDA represent the relative electrical conductivity and malondialdehyde treatments, respectively.

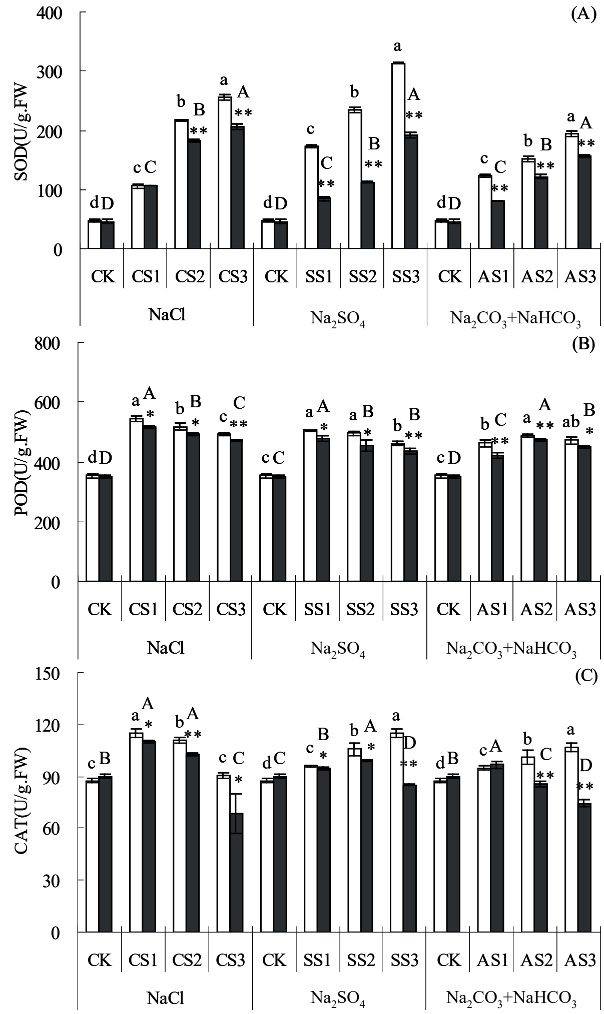

Figure 4: Effect of soil salt and alkali on antioxidant enzyme activities of cotton leaves. Symbols indicate L24 (salt-tolerant, white bar) and X45 (salt-sensitive, black bar). Error bars represent SDs (n = 3). Different letters represent significant differences (*P<0.05*) between L24 and X45. Asterisks represent a significant difference between L24 and X45 (**P < 0.05; **P < 0.01*). The symbols SOD, POD and CAT represent the superoxide dismutase, peroxidase and catalase treatments, respectively.

SOD activity were more conspicuous in L24 than that in X45 as well, suggesting that the scavenging ability of the salt-tolerant cultivar was superior than the salt-sensitive cultivar. POD and CAT activities in salt-tolerance plants were higher, thus protecting plants against oxidative stress; however, POD and CAT activities were not observed in salt-sensitive plants [50]. Figures 4B and 4C showed that the activities of CAT and POD in cotton leaves were significantly controlled under saline and alkali stresses, and the activities of CAT and POD of L24 leaves was higher than that of X45, indicating that salt-tolerant cultivar had higher antioxidase activity than salt-sensitive cultivar under salt and alkali stresses, which assisted to scavenge ROS. Additionally, it has also been discovered that the CAT and POD activities of the two cotton cultivars decreased as the increase of NaCl stress; similarly, POD and CAT activities were reduced in responding to excess salinity in the leaves and roots of various plant species [51]. Hence, it can be concluded that under salt and alkali stress, the activity of endogenous protective enzymes in plants accordingly changed, however, the changes often varied with different plant materials and different stress intensities.

3.5 Osmotic adjust material

The effects of different salt and alkali stress on the contents of Proline (Pro), soluble sugar, and glycine betaine (GB) in cotton leaves are shown in Figure 5. Figure 5A shows Pro content increased significantly as the increase of salt and alkali stress, and the increase of Pro content under

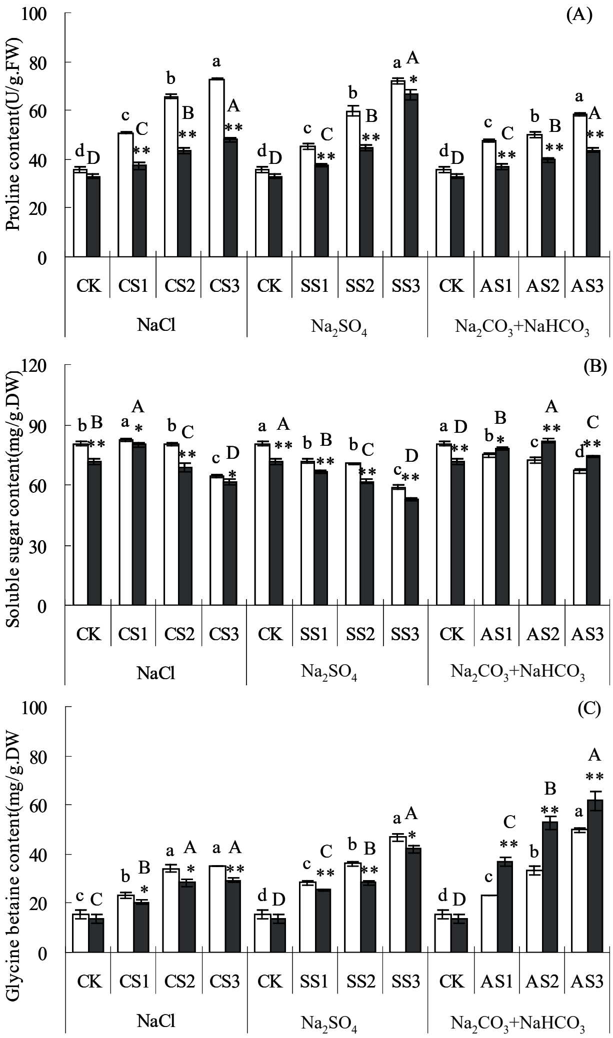

Figure 5: Effect of soil salt and alkali on contents of proline, soluble sugar, and glycine betaine of cotton leaves. Symbols indicate L24 (salt-tolerant, white bar) and X45 (salt-sensitive, black bar). Error bars represent SDs (n = 3). Different letters represent significant differences (*P<0.05*) between L24 and X45. Asterisks represent a significant difference between L24 and X45 (**P < 0.05; **P < 0.01*).

salt stress was higher than that under alkaline stress, and the Pro content of L24 was significantly higher than X45. Some reports also showed that the concentration of Pro increased with an increase in the salinity level [52-56]. In addition, salt stress affected sugar metabolism process of cotton. Figure 5B shows the increase of salt stress reduced the soluble sugar content of cotton leaves, the soluble sugar content of X45 decreased more than that of L24, and the alkali stress significantly decreased the soluble content of L24, however, the soluble sugar content of X45 initially increased and then decreased with the increase of alkali stress, besides, the soluble sugar content of X45 was significantly higher than that of L24. The accumulation of GB in plants under salt stress was an important physiological phenomenon that was beneficial to the growth of plants under stress. Figure 5C shows that the GB content of L24 and X45 leaves increased as the soil salt and alkali rate increased, and the GB content of L24 was significantly higher than that of X45 under salt stress, however, the trend was opposite under alkaline stress, suggesting that X45 mainly relied on GB and soluble sugars to resist against alkaline stress.

4 Conclusions

Saline and alkali stress reduced the relative biomass, chlorophyll content, increased REC, MDA content, SOD activity, POD activity, and Pro and GB contents of cotton leaves. The CAT activity and soluble sugar content varied remarkedly under different saline and alkali stresses. Under salt stress, the increase of REC, MDA content, and SOD activity of leaves was more than that of alkali stress, however, the GB content showed an opposite trend. L24 was higher than that of X45 in seedling emergence rate, leaf biomass, antibody oxidase activity, and Pro content, while it was lower than X45 in leaf REC and MDA content. The content of soluble sugar and GB in L24 was higher than that in X45 under salt stress, but was lower than that in X45 under alkaline stress. In summary, this can be attributed to different mechanisms of oxidative stress damage because of the difference of MDA content, SOD, POD, and CAT activities, Pro, soluble sugar, and GB contents of salt-tolerant and salt-sensitive cotton under salt and alkali stresses. L24 mainly removed ROS through antioxidant enzyme activity and protected cell membrane from damage. X45 may adapt to alkaline stress through the contents of soluble sugar and GB in the body. The damage degree of cotton leaves by different saline and alkali stresses was NaCl > Na_2SO_4 > $NaHCO_3$ + Na_2CO_3, indicating that the adverse effect of alkali stress alone, as a result, the adverse effect of pH on cotton was less than that of salt stress. The results of this study are of great significance for a more comprehensive understanding of the nutrient growth behavior of cotton in saline and alkali soils.

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