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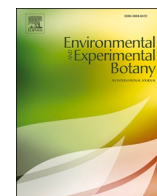
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## Research paper

Stomatal and non-stomatal regulations of photosynthesis in response to salinity, and K and Ca fertigation in cotton (*Gossypium hirsutum* L cv.)Yingying Ma<sup>a,b</sup>, Zuoqiang Yuan<sup>b,g,\*\*</sup>, Zhenhua Wei<sup>d</sup>, Fei Yan<sup>h,\*</sup>, Xuezhi Liu<sup>e</sup>, Xiangnan Li<sup>f</sup>, Jingxiang Hou<sup>i</sup>, Zhanqing Hao<sup>b,g</sup>, Fulai Liu<sup>c,\*</sup><sup>a</sup> Xi'an FanYi University, Xi'an, Shaanxi 710105, China<sup>b</sup> School of Ecology and Environment, Northwestern Polytechnical University, Xi'an, Shaanxi 710072, China<sup>c</sup> Department of Plant and Environmental Science, Faculty of Science, University of Copenhagen, Højbakkegaard Alle 13, Taastrup 2630, Denmark<sup>d</sup> College of Water Resources and Architectural Engineering, Northwest A&F University, Weihui Road 23, Yangling, Shaanxi 712100, China<sup>e</sup> School of Civil and Hydraulic Engineering, Ningxia University, Yinchuan 750021, China<sup>f</sup> Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Science, Changchun 130102, China<sup>g</sup> Laboratory of Qinling Ecological Intelligent Monitoring and Protection, Northwestern Polytechnical University, Xi'an, Shaanxi 710129, China<sup>h</sup> Biological and Chemical Engineering College, Panzhihua University, Panzhihua 617000, China<sup>i</sup> Center for Agricultural Water Research in China, China Agricultural University, Beijing 100083, China

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## ABSTRACT

To study the response of photosynthesis of cotton plants to salinity, and K and Ca fertigation, leaf gas exchange, light and CO<sub>2</sub> response curves, chlorophyll fluorescence parameters, leaf N, P, Mg, K, Na and Ca concentrations, and antioxidant enzyme activities were measured. Cotton plants were grown at either 0 or 150 mM NaCl salinity (C and S treatment, respectively), and fertigated with or without K and Ca addition (O and K+Ca treatment, respectively). The results showed that 150 mM NaCl salinity decreased stomatal conductance ( $g_s$ ), transpiration rate ( $T_r$ ) and chloroplast CO<sub>2</sub> concentration ( $C_c$ ). Yet it increased light saturated photosynthetic rate ( $A_L$ ), CO<sub>2</sub> saturated photosynthetic rate ( $A_C$ ), mesophyll conductance ( $g_m$ ), maximum electron transport rate ( $J_{max}$ ), maximum Rubisco carboxylation rate ( $V_{cmax}$ ) and triose phosphates use rate (TPU), resulting in a similar net photosynthesis rate ( $A_n$ ) to that of C plants. K+Ca treatment enhanced  $A_n$ ,  $g_s$ ,  $T_r$  and  $C_c$ , particular under S condition, while it had no significant effects on  $g_m$ ,  $J_{max}$ ,  $V_{cmax}$  and TPU. Salt-induced increases in  $J_{max}$ ,  $V_{cmax}$  and TPU were associated with higher  $[N]_{leaf}$  and  $[P]_{leaf}$ , whereas the  $J_{max}/V_{cmax}$  ratio decreased with increasing  $[N]_{leaf}$  and  $[P]_{leaf}$ , and  $C_c$  decreased with increasing  $[N]_{leaf}$ . Salt-induced improvement in  $A_C$  was linked to lower  $J_{max}/V_{cmax}$  ratio and  $C_c$ , while the increase in  $A_L$  could be ascribed to the lower non-photochemical quenching and higher photosystem II efficiency, which could be partially attributed to the salt-caused improvement in leaf superoxide dismutase, peroxidase and catalase activities. In conclusion, 150 mM NaCl salinity increased stomatal limitation but decreased non-stomatal limitation on  $A_n$ , resulting in sustained  $A_n$  and significantly lowered  $g_s$  and  $T_r$  and hence an improved water use efficiency. K and Ca addition could alleviate the salinity-induced decrease in  $g_s$  and increase in stomatal limitation.

## 1. Introduction

Photosynthesis is one of the most salt-sensitive processes, and reduced plants growth under higher soil salinity could be closely linked to the limitation of photosynthesis. Stomatal and non-stomatal limitation are two major constraints to photosynthesis under salinity stress (Shabala et al., 1998). The former is mainly caused by the

salinity-induced decrease in stomatal conductance ( $g_s$ ), while the non-stomatal limitation is mainly related to the reduced mesophyll conductance ( $g_m$ ). Moreover, the  $g_m$  determines chloroplast CO<sub>2</sub> concentration ( $C_c$ ). The photosynthetic capacity can be assessed by the maximum rate of carboxylation ( $V_{cmax}$ ) and maximum electron transport rate ( $J_{max}$ ) (Quebbeman and Ramirez, 2016). Soil salinity normally inhibited the  $V_{cmax}$  and  $J_{max}$ , though such effect is species dependent

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(Aspinwall et al., 2020; Ge et al., 2014; Suárez and Medina, 2006). Salt-tolerant species like barley grown with lower Na concentration (25 mM NaCl) showed 25 % higher photosynthetic capacity than those with 0 mM NaCl, but further increase of Na concentration significantly decreased photosynthetic capacity (James et al., 2006).

Plants grown under higher NaCl levels with restricted growth typically accompanied with enhanced Na absorption but declined K and Ca, K/Na and Ca/Na ratio (Kaya et al., 2007; Maswada and Abd El-Kader, 2016). Thus, supplying exogenous K and Ca has been advocated to be an efficient strategy to improve plant salt-tolerance by maintaining higher K, Ca, K/Na and Ca/Na ratio (Cramer et al., 1987; Lahaye and Epstein, 1969; Ju et al., 2021; Yurtseven et al., 2005). Although the enhanced photosynthetic capacity has been largely found when salt-stressed plants were supplied with K and Ca (Shabala et al., 2003; Shabala and Cuin, 2007; Jan et al., 2017; Ebrahimi et al., 2023; Vafadar and Ehsanzadeh, 2023), the mechanisms behind such alleviation effect of K and Ca on  $A_n$  in salt-stressed plants still need further studies.

The importance of leaf N and P status in influencing photosynthetic capacity has also been demonstrated in many high plants (Domingues et al., 2010; Walker et al., 2014). It was found that an enhanced  $J_{max}$  and  $V_{cmax}$  but decreased  $J_{max}/V_{cmax}$  ratio often associated with increased leaf N, while increasing leaf P sometimes could improve the sensitivity of  $V_{cmax}$  to leaf N. The chloroplast  $CO_2$  concentration ( $C_c$ ) also increased with increased leaf N in rice (Li et al., 2009), but tended to be lower in wheat (Caemmerer and Evans, 1991). Soil salinity could alter root structures and activities, thereby disturbing plant N and P acquisition and metabolism (Gimeno et al., 2009; Robin et al., 2016; Abbras et al., 2018; Tang et al., 2019). While applying K and Ca could promote plant N and P absorption as K often coupled with  $NO_3^-$  for plants uptake (Dong et al., 2004), and Ca combined with carbon could improve soil texture (Nishimura and Matsubara, 2021). Thus, it is essential to investigate the effects of soil salinity, and K and Ca application on leaf N and P status, and photosynthetic capacity.

Additionally, photosynthetic  $CO_2$  starvation caused by stomatal closure under salinity condition may lead to over-accumulation of reactive oxygen species (ROS, such as  $\cdot O_2^-$  and  $H_2O_2$ ) (Begum et al., 2020; Khan et al., 2021). Meanwhile, antioxidant defense systems could be activated by ROS via Ca or abscisic acid (ABA) (Zhang et al., 2006), thus sustain leaf photosynthesis from salinity-induced oxidative damage. The ROS-scavenging capacity is closely related to the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). For instance, SOD can metabolize  $\cdot O_2^-$  to  $H_2O_2$  (Farhangi-Abriz and Torabian, 2017), and then  $H_2O_2$  can be furtherly abolished by CAT and POD into  $H_2O$  and  $O_2$  (Begum et al., 2020). Salinity-caused depression of chlorophylls,  $F_v/F_m$ , and the maximal efficiency of photosystem II (PSII) could be alleviated by enhanced activities of SOD, CAT and POD (Yan et al., 2011; Nounjan et al., 2012). However, whether K and Ca addition affects plant growth by regulating photosynthetic capacity through altering antioxidants system under salinity stress remains largely unclear.

Cotton contributes 80 % of the global natural fiber production (Townsend, 2020). However, soil salinity is currently the major limiting factor for cotton cultivation (Li et al., 2023). Therefore, the objective of this study was to investigate the mechanisms of photosynthesis regulation of cotton (*Gossypium hirsutum*) plants as affected by K and Ca fertigation under NaCl salinity. We exposed the plants to two different salt stress levels (0 and 150 mM NaCl) in combination with two different K and Ca fertigation regimes (O and K+Ca treatment). The leaf gas exchange rates, photosynthetic light and  $CO_2$  response curves, chlorophyll fluorescence parameters, leaf N, P, Mg, K, Na and Ca concentrations, antioxidant enzyme activities and plant growth characteristics were determined. It was hypothesized that: 1) both stomatal and non-stomatal limitations are involved in photosynthesis response of plants to salinity stress; 2) an increased  $g_m$  thus a reduced non-stomatal limitation, together with enhanced  $V_{cmax}$ ,  $J_{max}$ , and TPU would cause a less reduction in  $A_n$  of plants grown under salinity stress; 3) K and Ca

addition could alleviate the negative effects of salt stress on  $g_s$  hence reduce the stomatal limitation on  $A_n$ .

## 2. Materials and methods

### 2.1. Plant material and experiment setup

The experiment was performed from May to October 2020 in a natural sunlight greenhouse at Northwest A&F University (N 34° 15', E 108° 04'), Yangling, Shaanxi, China, by using plastic pots with a volume of 9 L each pot (26 cm in diameter at the top edge, 24 cm in height and 22 cm in diameter at the bottom). Each pot was filled with 10 kg air dried soil, which was sieved through a 0.5 cm mesh, with a bulk density of 1.28 g/cm<sup>3</sup>. The soil was collected from nearby field at Yangling, Shaanxi, China, in a depth of 30–40 cm. The soil physicochemical properties are shown in Supplementary Table 2. Cotton seeds (*Xinluzhong No. 46*, *Gossypium hirsutum* L.) were sown on 16th May 2020 and transplanted at four-leaf stage (5th June 2020) with one seedling per pot. 4 days after transplanting, 3.2 g urea (N: 46.67 %) and 3.5 g  $KH_2PO_4$  (K: 28.70 %; P: 22.80 %) were applied to every pot. To minimize soil evaporation, the soil surface was covered by a 2 cm layer perlite. From 18th to 28th June 2020, 100 mL full strength Hoagland solution (Supplementary Table 3) was added every two days, in total 600 mL for every pot. All of the pots were irrigated daily to 90 % SWHC<sub>mass</sub> throughout the experiment. The temperature (T), relative humidity (RH) and vapor pressure deficit (VPD) in the greenhouse were monitored every 1 hours by using a Humidity & Temperature meter (TH-Logger, China). Evolution of daily T, RH and VPD in cell are show in Supplementary Fig. 1.

### 2.2. Treatments

The pot experiment was a complete factorial randomized design including 4 treatments: two salt stress levels (0 mM NaCl and 150 mM NaCl) and two fertilization regimes (without K and Ca addition, and with 0.6 mM K and 3.25 mM Ca addition). Each treatment included four replications. Both salt stress and fertilizer treatment were established at 30 days after transplanting (4th July 2020). Half of the plants were subjected to 150 mM NaCl salinity (2 g NaCl was applied every two days until NaCl concentration rose to 150 mM on 21th July, 2020, total 19 g NaCl was added per pot) assigned as salt stress treatment (S). While another half of the plants were exposed to 0 mM NaCl as control (C). Simultaneously, half of the C and S plants were irrigated daily with modified Hoagland solution without K and Ca set as O treatment, and another half were fertigated daily with modified Hoagland solution contain 0.6 mM K and 3.25 mM Ca (Supplementary Table 3) considered as K+Ca treatment. During the treatment, soil water content was measured daily between 16:00–18:00 by an analytical balance.

### 2.3. Leaf gas exchange measurement

During the treatments, leaf net photosynthetic rate ( $A_n$ ), stomatal conductance ( $g_s$ ) and transpiration rate ( $T_r$ ) were measured 5 times at budding and flowering stages (8th, 15th and 21th August, 4th and 11th September 2020, respectively) with a portable photosynthetic system (LI-6800, Li-Cor, USA) on the upper fully expanded leaves. The measurements were performed between 9:00–11:00 h at 25°C chamber temperature, 1500  $\mu mol\ m^{-2}s^{-1}$  photosynthetic active radiation and 400 ppm  $CO_2$  concentration. Intrinsic water use efficiency ( $WUE_i$ ) and instantaneous water use efficiency ( $WUE_{leaf}$ ) were computed as the ratio of  $A_n/g_s$  and  $A_n/T_r$ , respectively.

Photosynthesis-light responses curves (LRC) and  $CO_2$  response curves ( $A_n-C_i$ ) were measured before the final harvest on the same leaf for  $A_n/g_s/T_r$  measurements between 8:00–17:00 h. LRC was measured under conditions of 25°C chamber temperature and 400 ppm  $CO_2$  concentration at 1500, 1300, 1100, 900, 800, 700, 500, 200, 150, 100, 50

and  $0 \mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photo flux density (PPFD) levels.  $A_n$ - $C_i$  curves was measured under conditions of  $25^\circ\text{C}$  chamber temperature and  $1500 \mu\text{mol m}^{-2}\text{s}^{-1}$  PPFD at 400, 300, 200, 100, 50, 0, 400, 400, 600, 800, 1000, 1200, and  $1500 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  concentrations.

The LRC were fitted according to the method described by Richardson et al. (2002):

$$A_n(I) = \frac{\alpha I + A_L - \sqrt{(\alpha I + A_L)^2 - 4\theta\alpha I A_L}}{2\theta} - R_D$$

where  $A_n(I)$  is the net photosynthetic rate [ $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ ],  $\alpha$  is the initial quantum yield,  $I$  is the photosynthetic photo flux density (PPFD) [ $\mu\text{mol m}^{-2}\text{s}^{-1}$ ],  $A_L$  is the light saturated photosynthetic rate [ $\mu\text{mol (CO}_2\text{) m}^{-2}\text{s}^{-1}$ ],  $\theta$  is the convexity factor ( $0 < \theta \leq 1$ ),  $R_D$  is the dark respiration rate [ $\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$ ].

The  $A_n$ - $C_i$  curves were fitted with a Microsoft Excel estimating utility “A/Ci curve fitting utility version 1.1” based on Sharkey et al. (2007).  $\text{CO}_2$  saturated photosynthetic rate ( $A_C$ ,  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), maximum rate of carboxylation by Rubisco ( $V_{\text{cmax}}$ ,  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), maximum rate of electron transport ( $J_{\text{max}}$ ,  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), rate of triose phosphates use (TPU,  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), respiration rate ( $R_d$ ,  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) and mesophyll conductance ( $g_m$ ,  $\text{mmol m}^{-2}\text{s}^{-1}\text{Pa}^{-1}$ ) were estimated.

The chloroplast  $\text{CO}_2$  concentration ( $C_c$ ,  $\mu\text{mol mol}^{-1}$ ) was calculated as (Yamori et al., 2011):

$$C_c = C_i - \frac{A_n}{g_m}$$

where  $C_i$  is the intercellular  $\text{CO}_2$  concentration ( $\mu\text{mol mol}^{-1}$ ),  $A_n$  is the net photosynthetic rate ( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), and  $g_m$  is the mesophyll conductance ( $\text{mmol m}^{-2}\text{s}^{-1}\text{Pa}^{-1}$ ).

Stomatal limitation ( $l_s$ ) was calculated as (Duan et al., 2014):

$$l_s = 1 - \frac{C_i}{C_a}$$

where  $C_i$  is the intercellular  $\text{CO}_2$  concentration ( $\mu\text{mol mol}^{-1}$ ),  $C_a$  is the ambient  $\text{CO}_2$  concentration ( $\mu\text{mol mol}^{-1}$ ). Non-stomatal limitation was calculated as  $1-l_s$ .

## 2.4. Determination of chlorophyll content and chlorophyll fluorescence characteristics

At the same time during the leaf gas exchange measurement, the chlorophyll content, and the photosystem II (PSII) efficiency were determined by using a portable, battery-powered fluorometer (PAR-FluorPen FP 110, PSI). Minimum fluorescence ( $F_0$ ) and maximum fluorescence in light-adapted state ( $F_m'$ ) were determined at 33 % Super Pulse and 20 % Flash Pulse. 30 min after dark adaption, minimum fluorescence ( $F_0$ ) and maximum fluorescence in dark-adapted state ( $F_m$ ) were determined at 33 % Super Pulse and 20 % Flash Pulse. Quantum Yield of PSII (QY) was calculated as the ratio of  $(F_m - F_0)/F_m$ . Non-Photochemical Quenching (NPQ) was computed as the ratio of  $(F_m - F_m')/F_m'$ .

## 2.5. Activity of antioxidant enzymes

At final harvest (12th September 2020), leaf samples were collected, frozen in liquid nitrogen and stored in  $-80^\circ\text{C}$ . Finely grounded fresh leaf samples were homogenized with 5 mL extraction buffer (50 mM of pre-chilled phosphate buffer pH 7.0 and 0.4 % polyvinylpyrrolidone) in a chilled mortar. After centrifuging extracted homogenate, supernatant was used to superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities analysis. SOD, POD and CAT activities were determined according to the methods reported by Li et al. (2020). SOD activity was measured by tracking the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) and its unit was the enzyme amount causing

half-maximal suppression of NBT reduction at 560 nm absorbance. POD activity was measured by using oxidizing guaiacol to produce 4-o-methoxyphenyl and its unit was identified as the enzyme amount needed for an increase in absorbance of 0.01 per min at 470 nm. CAT activity was measured by observing the decrease of absorbance at 240 nm through decomposing  $\text{H}_2\text{O}_2$  and its unit was the enzyme amount that decrease by 0.1 absorbance per min.

## 2.6. Growth traits, water consumption, leaf N, P, Mg, K, Na and Ca concentrations, and plant water use efficiency

At the end of the treatments (12th September 2020), plant height (H), stem diameter (D) and leaf area (LA) were determined with a ruler, a vernier caliper and a portable leaf area meter (LI-3100, LI-COR, USA), respectively. Leaf biomass ( $\text{DM}_{\text{leaf}}$ ), stem biomass ( $\text{DM}_{\text{stem}}$ ), boll biomass ( $\text{DM}_{\text{boll}}$ ) and root biomass ( $\text{DM}_{\text{root}}$ ) were determined respectively, after drying at  $75^\circ\text{C}$  in an oven to constant weight. Shoot biomass ( $\text{DM}_{\text{shoot}}$ ) was the sum of  $\text{DM}_{\text{leaf}}$ ,  $\text{DM}_{\text{stem}}$  and  $\text{DM}_{\text{boll}}$ . Specific leaf area (SLA) was calculated as the ratio of  $\text{LA}/\text{DM}_{\text{leaf}}$ . Plant water consumption (WU) was calculated from the initiation of salt stress and fertilizer treatments by using gravimetric method. Water use efficiency of plant biomass ( $\text{WUE}_p$ ) and water use efficiency of boll biomass ( $\text{WUE}_y$ ) were calculated as the ratio of  $\text{DM}_{\text{shoot}}/\text{WU}$  and  $\text{DM}_{\text{boll}}/\text{WU}$ , respectively. Oven-dried leaf samples were finely grounded into powder, and passed through a 0.25 mm sieve used for leaf N ( $[\text{N}]_{\text{leaf}}$ ), P ( $[\text{P}]_{\text{leaf}}$ ), Mg ( $[\text{Mg}]_{\text{leaf}}$ ), K ( $[\text{K}]_{\text{leaf}}$ ), Na ( $[\text{Na}]_{\text{leaf}}$ ) and Ca ( $[\text{Ca}]_{\text{leaf}}$ ) concentrations measurement.  $[\text{N}]_{\text{leaf}}$ ,  $[\text{P}]_{\text{leaf}}$  and  $[\text{K}]_{\text{leaf}}$  were determined using an Automatic Flow Elemental Analyzer (AA3). After extraction with 1 N  $\text{NH}_4\text{OAC}$ ,  $[\text{Mg}]_{\text{leaf}}$ ,  $[\text{Na}]_{\text{leaf}}$  and  $[\text{Ca}]_{\text{leaf}}$  were measured using an Atomic Absorption Spectroscopy (PE-pinAAcle, 900 F).

## 2.7. Statistical analysis

Two-way ANOVA was used to detect the effects of salt stress, K and Ca fertigation, and the combination of salt stress and fertigation on soil and plant variables using SPSS version 23.0 (IBM, Corporation, New York, USA), distinguishing the differences where significant ( $p < 0.05$ ). Pearson correlations between the different photosynthetic indicators were performed using the “ggcorrplot” package in the R software (R version 4.4.1).

## 3. Results

### 3.1. Soil water content

Mean of gravimetric soil water content ( $\text{SWC}_{\text{mean}}$ ) is shown in Supplementary Fig. 2. The  $\text{SWC}_{\text{mean}}$  in S treatment was 3 % higher than those in C treatment. Both under C and S conditions, there was no significant differences in  $\text{SWC}_{\text{mean}}$  between O and K+Ca treatments.

### 3.2. Leaf gas exchange

Salt stress, and K and Ca addition had significant effects on stomatal conductance ( $g_s$ ) and transpiration rate ( $T_r$ ) (Fig. 1b, c), while there was no significant effect on net photosynthetic rate ( $A_n$ ) (Fig. 1a).  $g_s$  and  $T_r$  in plants grown under S with O treatment were 42 % and 41 % lower than those grown under C with O treatment, respectively. However, both under C and S conditions,  $g_s$  in plants grown with K+Ca treatment were 6 % and 26 % higher than those with O treatment, respectively, and  $T_r$  in plants grown with K+Ca treatment were 8 % and 28 % higher than those with O treatment, respectively. Under S condition,  $A_n$  in plants grown with K+Ca treatment was slightly higher (8 %) than those with O treatment.

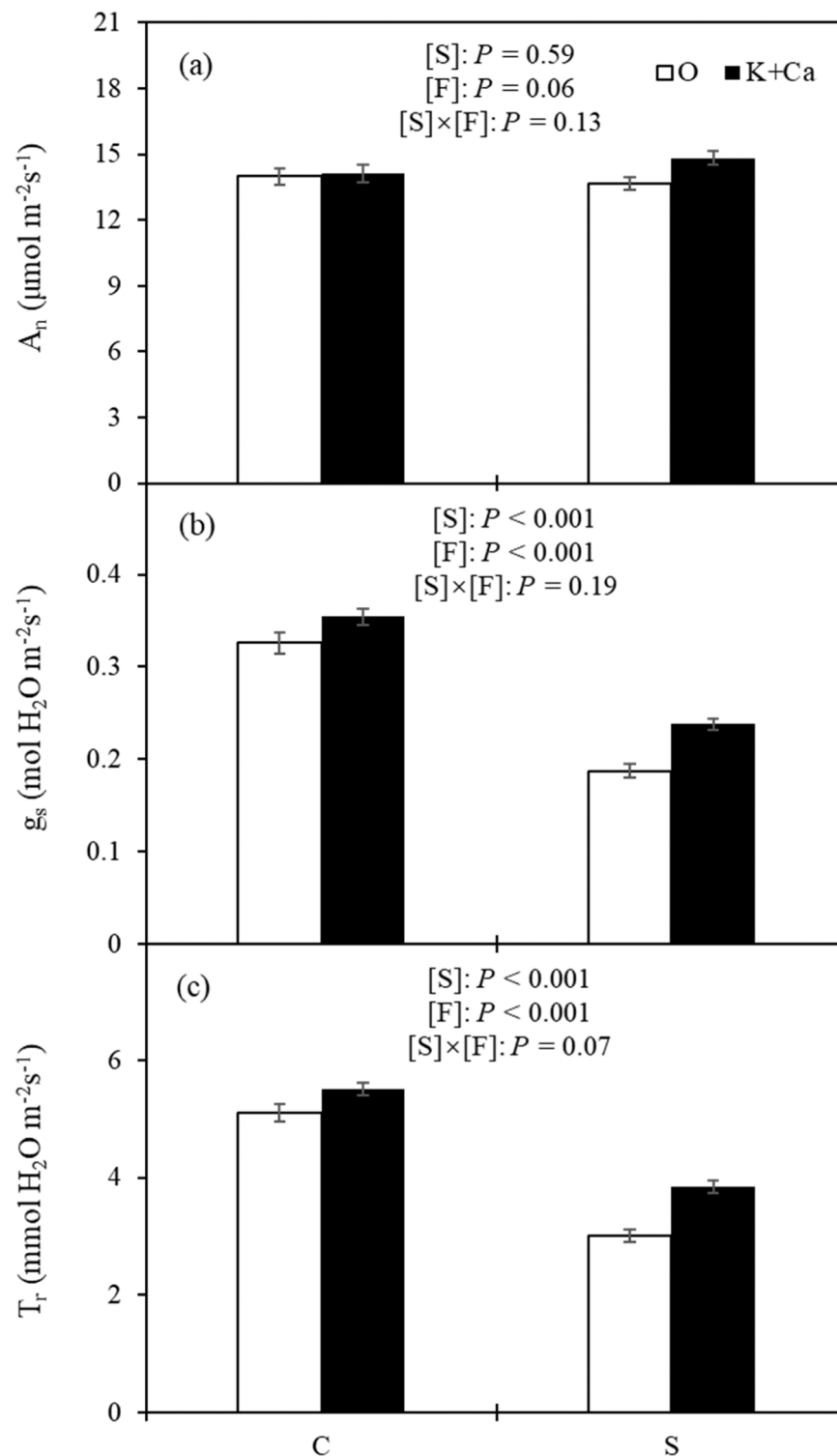


**Fig. 1.** Average of net photosynthetic rate ( $A_n$ ) (a), stomatal conductance ( $g_s$ ) (b) and transpiration rate ( $T_r$ ) (c) of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), and different K and Ca fertilization treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Bars are means  $\pm$  SE ( $n = 60$ ).

### 3.3. Light response curves

Obvious differences in light response curves occurred between control and salt-stressed plants (Fig. 2a, b). With regard to photosynthetic parameters fitted from light responses curves, initial quantum yield ( $\alpha$ ), convexity factor ( $\theta$ ) and dark respiration rate ( $R_D$ ) were not significantly affected by salt stress, K and Ca addition, and the combination of salt

stress and K and Ca addition (Table 1). However, salt stress, and the combination of salt stress and K and Ca addition had significant effects on light-saturated photosynthetic rate ( $A_L$ ) (Table 1).  $A_L$  in plants grown under S with O treatment was 25 % higher than those grown under C with O treatment. Besides,  $A_L$  in plants grown under S with K+Ca treatment was 2 % higher than those grown under C with O treatment.

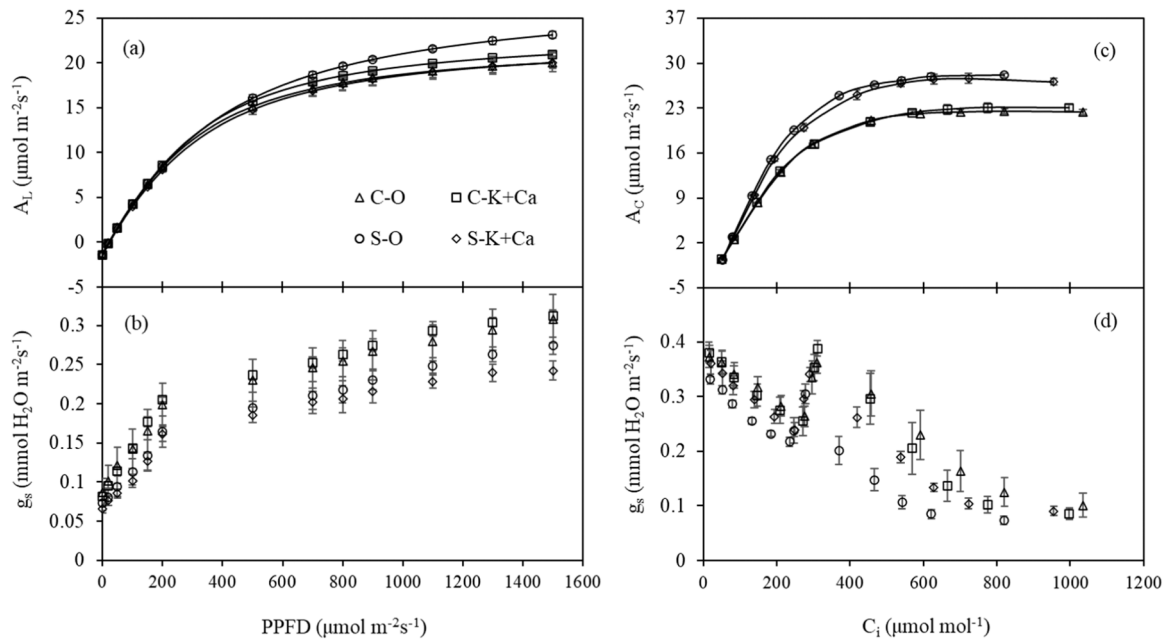


**Fig. 2.** Responses of net photosynthetic rate ( $A_n$ ) (a, c) and stomatal conductance ( $g_s$ ) (b, d) to photosynthetic photon flux density (PPFD) (a, b) and ambient  $CO_2$  concentration (c, d) of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), and different K and Ca fertigation treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Points are means  $\pm$  SE ( $n = 4$ ).

**Table 1**

Initial quantum yield ( $\alpha$ ), light saturated photosynthetic rate ( $A_L$ ), convexity factor ( $\theta$ ) and dark respiration rate ( $R_D$ ) under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), and different K and Ca fertigation treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Values are Means  $\pm$  SE ( $n = 4$ ).

| Salt                       | Fertilizer | $\alpha$             | $A_L$<br>( $\mu\text{mol CO}_2$<br>$\text{m}^{-2}\text{s}^{-1}$ ) | $\theta$           | $R_D$<br>( $\mu\text{mol CO}_2$<br>$\text{m}^{-2}\text{s}^{-1}$ ) |
|----------------------------|------------|----------------------|-------------------------------------------------------------------|--------------------|-------------------------------------------------------------------|
| C                          | O          | 0.061<br>$\pm 0.001$ | 23.83 $\pm$ 1.38 <sup>b</sup>                                     | 0.61<br>$\pm 0.04$ | 1.22 $\pm$ 0.10                                                   |
|                            | K+Ca       | 0.063<br>$\pm 0.001$ | 25.00 $\pm$ 0.44 <sup>b</sup>                                     | 0.62<br>$\pm 0.03$ | 1.39 $\pm$ 0.02                                                   |
| S                          | O          | 0.065<br>$\pm 0.003$ | 29.83 $\pm$ 1.86 <sup>a</sup>                                     | 0.36<br>$\pm 0.19$ | 1.38 $\pm$ 0.07                                                   |
|                            | K+Ca       | 0.060<br>$\pm 0.002$ | 24.30 $\pm$ 0.72 <sup>b</sup>                                     | 0.56<br>$\pm 0.06$ | 1.33 $\pm$ 0.02                                                   |
| P value from two-way ANOVA |            |                      |                                                                   |                    |                                                                   |
| S                          |            | 0.81                 | 0.05                                                              | 0.15               | 0.39                                                              |
| F                          |            | 0.49                 | 0.10                                                              | 0.31               | 0.36                                                              |
| S×F                        |            | 0.18                 | 0.02                                                              | 0.38               | 0.11                                                              |

#### 3.4. $A_n$ - $C_i$ curves

Significant difference was also found in  $A_n$ - $C_i$  curves between the control and salt-stressed plants (Fig. 2c, d). Salt stress significantly increased stomatal limitation ( $l_s$ ) (Table 2). Compared to the plants grown under C with O treatment, those grown under S with O treatment showed 42 % higher  $l_s$  but 17 % lower  $1-l_s$ . In terms of other photosynthetic characteristics derived from the  $A_n$ - $C_i$  curves,  $CO_2$  saturated photosynthetic rate ( $A_C$ ), maximum velocity of Rubisco for carboxylation ( $V_{cmax}$ ), RuBP-regeneration maximum electron transport rate ( $J_{max}$ ), rate of use of triose phosphates (TPU) and respiration rate ( $R_d$ ) of plants grown under S with O treatment were 26 %, 48 %, 33 %, 29 % and 78 %, respectively, higher than those grown under C with O treatment (Table 2). Salt-stressed plants had 38 % higher mesophyll conductance ( $g_m$ ) in comparison to the control, though the difference was not evident statistically. The chloroplast  $CO_2$  concentration ( $C_c$ ) in plants grown under S with O treatment was 17 % lower than those grown under C with O treatment (Table 2). While under C and S conditions,  $C_c$  in plants grown with K+Ca treatment were 7 % and 8 % higher, respectively, than those grown with O treatment (Table 2).

#### 3.5. Leaf N, P, Mg, Na, K and Ca concentrations

Compared to the plants grown under C with O treatment, those

**Table 2**

Stomatal limitation ( $l_s$ ), non-stomatal limitation ( $1-l_s$ ), chlorophyll content, initial chlorophyll fluorescence yield ( $F_0$ ), photosystem II (PSII) maximum efficiency under light ( $F_v'/F_m'$ ) and under dark-adapted ( $F_v/F_m$ ), and Non-Photochemical Quenching (NPQ) of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), and different K and Ca fertigation treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Values are Means  $\pm$  SE ( $n = 4$ ).

| Salt                       | Fertilizer | $l_s$           | $1-l_s$         | Chlorophyll content | $F_0$           | $F_v'/F_m'$     | $F_v/F_m$       | NPQ             |
|----------------------------|------------|-----------------|-----------------|---------------------|-----------------|-----------------|-----------------|-----------------|
| C                          | O          | 0.26 $\pm$ 0.02 | 0.74 $\pm$ 0.02 | 22.97 $\pm$ 0.27    | 2497 $\pm$ 38.6 | 0.78 $\pm$ 0.00 | 0.83 $\pm$ 0.00 | 0.37 $\pm$ 0.03 |
|                            | K+Ca       | 0.26 $\pm$ 0.02 | 0.74 $\pm$ 0.02 | 21.94 $\pm$ 0.33    | 2532 $\pm$ 50.5 | 0.79 $\pm$ 0.00 | 0.84 $\pm$ 0.00 | 0.24 $\pm$ 0.04 |
| S                          | O          | 0.37 $\pm$ 0.03 | 0.63 $\pm$ 0.03 | 26.60 $\pm$ 0.35    | 2520 $\pm$ 52.2 | 0.79 $\pm$ 0.00 | 0.84 $\pm$ 0.00 | 0.27 $\pm$ 0.02 |
|                            | K+Ca       | 0.32 $\pm$ 0.02 | 0.68 $\pm$ 0.23 | 26.14 $\pm$ 0.21    | 2340 $\pm$ 35.4 | 0.80 $\pm$ 0.00 | 0.84 $\pm$ 0.00 | 0.21 $\pm$ 0.03 |
| P value from two-way ANOVA |            |                 |                 |                     |                 |                 |                 |                 |
| S                          |            | 0.001           | 0.001           | 0.001               | 0.08            | 0.01            | 0.03            | 0.07            |
| F                          |            | 0.18            | 0.18            | 0.001               | 0.13            | 0.003           | 0.008           | 0.01            |
| S×F                        |            | 0.22            | 0.22            | 0.17                | 0.03            | 0.22            | 0.66            | 0.23            |

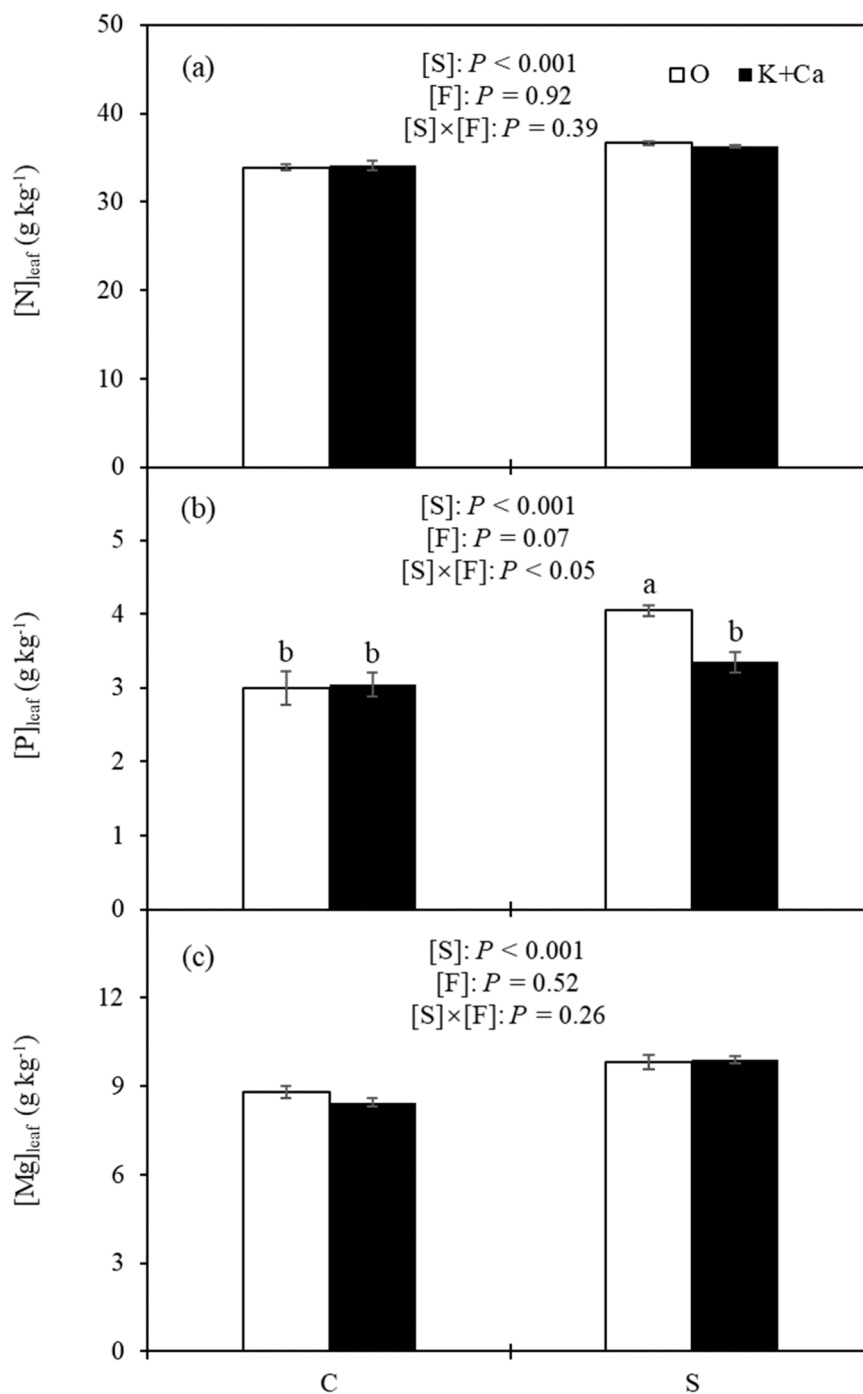


**Fig. 3.** Leaf nitrogen concentration ( $[N]_{\text{leaf}}$ ) (a), leaf phosphorus concentration ( $[P]_{\text{leaf}}$ ) (b) and leaf magnesium concentration ( $[Mg]_{\text{leaf}}$ ) (c) of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), and different K and Ca fertilization treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Bars are means  $\pm$  SE ( $n = 4$ ).

grown under S with O treatment had 8 % higher  $[N]_{\text{leaf}}$  (Fig. 3a).  $[P]_{\text{leaf}}$  in plants grown under S with O treatment was 35 % higher than those grown under C with O treatment (Fig. 3b). However, under S condition,  $[P]_{\text{leaf}}$  was 17 % lower in plants grown with K+Ca treatment than those with O treatment (Fig. 3b). Compared to the plants grown under C with O treatment, those grown under S with O treatment had 11 % higher  $[Mg]_{\text{leaf}}$  (Fig. 3c). Compared to the plants grown under C with O treatment, those grown under S with O treatment had 39 % higher leaf Na concentration ( $[Na]_{\text{leaf}}$ ) (Fig. 4b) but 10 %, 6 %, 38 %, 35 % and 35 % lower leaf K  $[K]_{\text{leaf}}$ , Ca concentration  $[Ca]_{\text{leaf}}$ , and the ratio of K/Na ( $[K/Na]_{\text{leaf}}$ ), Ca/Na ( $[Ca/Na]_{\text{leaf}}$ ) and (K+Ca)/Na ( $[(K+Ca)/Na]_{\text{leaf}}$ ),

respectively (Fig. 4a, c, d, e, f). Besides, both under C and S conditions, of plants grown with K+Ca treatment showed 22 % and 12 % lower  $[Na]_{\text{leaf}}$ , 10 % and 19 % higher  $[K]_{\text{leaf}}$ , 43 % and 35 % higher  $[K/Na]_{\text{leaf}}$ , and 34 % and 20 % higher  $[(K+Ca)/Na]_{\text{leaf}}$ , respectively, relative to those grown with O treatment (Fig. 4a, b, d, f).

### 3.6. Chlorophyll content and chlorophyll fluorescence parameters

Compared to the plants grown under C with O treatment, those grown under S with O treatment had 16 % higher leaf chlorophyll content, 1 % higher light-adapted PSII efficiency ( $F_v'/F_m'$ ) and 1 %

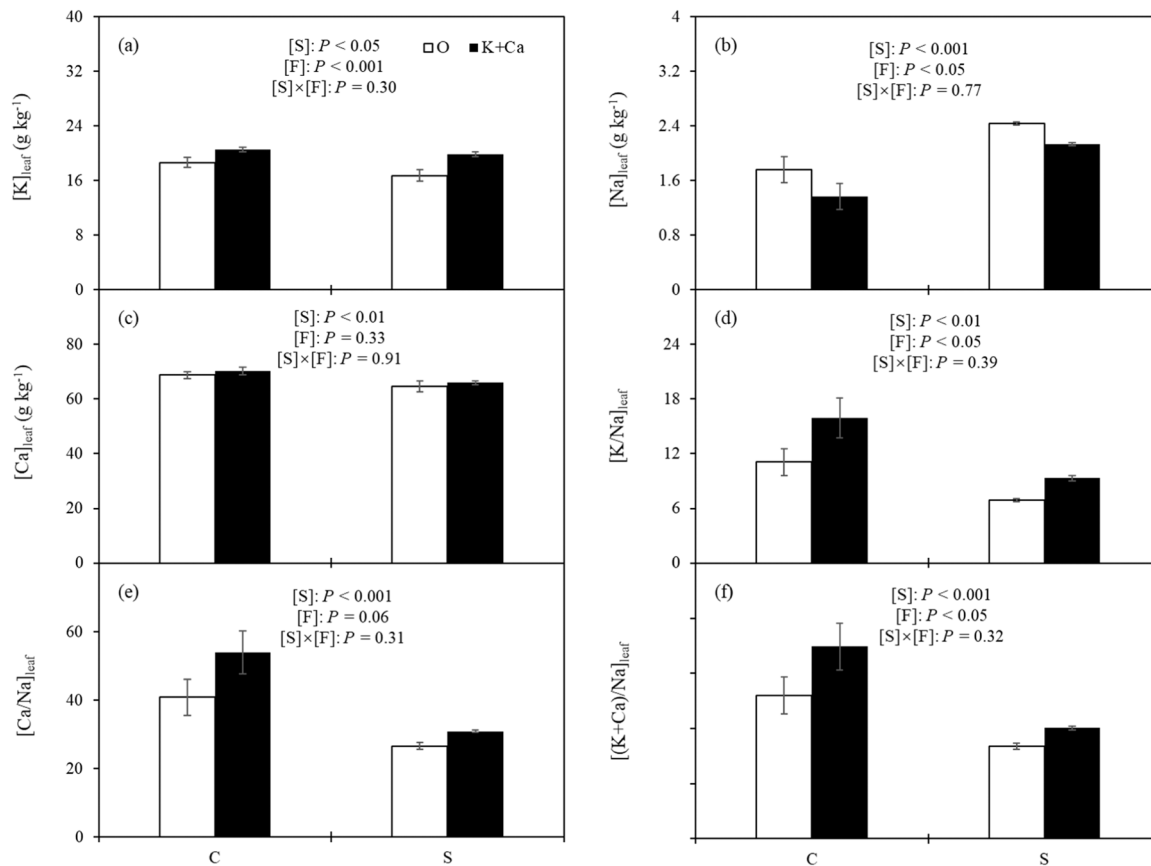


**Fig. 4.** leaf K concentration ( $[K]_{\text{leaf}}$ ), leaf Na concentration ( $[Na]_{\text{leaf}}$ ), leaf Ca concentration ( $[Ca]_{\text{leaf}}$ ), ratio of  $[K]_{\text{leaf}}$  to  $[Na]_{\text{leaf}}$ , ratio of  $[Ca]_{\text{leaf}}$  to  $[Na]_{\text{leaf}}$  and ratio of  $[(K+Ca)/Na]_{\text{leaf}}$  of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl) and different K and Ca fertigation treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca), respectively. Bars are means  $\pm$  SE of 4 replicate plants. Letters of mean discrimination are placed above the bars when there are significant ( $P < 0.05$ ) treatment differences.

higher dark-adapted PSII efficiency ( $F_v/F_m$ ), respectively (Table 2). However, both under C and S conditions, of plants grown with K+Ca treatment showed 4 % and 2 % lower chlorophyll content, respectively, relative to those grown with O treatment. Despite the values of  $F_v/F_m$  and  $F_v/F_m$  were slightly higher in plants with K+Ca treatment than O treatment under these conditions. The Non-Photochemical Quenching (NPQ) in plants grown under S with O treatment was 27 % lower than those grown under C with O treatment (Table 2). Both under C and S conditions, NPQ in plants grown with K+Ca treatment were 35 % and 22 % lower than those grown with O treatment (Table 2).

### 3.7. Antioxidant enzymes

The leaf superoxide dismutase activity ( $[SOD]_{\text{leaf}}$ ) in salt-stressed plants was slightly higher than the control plants, but  $[SOD]_{\text{leaf}}$  in plants with K+Ca treatment was slightly lower than those with O treatment both under C and S conditions (Fig. 5a). The leaf peroxidase activity ( $[POD]_{\text{leaf}}$ ) and leaf catalase activity ( $[CAT]_{\text{leaf}}$ ) in plants grown under S with O treatment were 151 % and 59 %, respectively, higher than those grown under S with O treatment (Fig. 5b, c). Both under C and S conditions,  $[POD]_{\text{leaf}}$  in plants with K+Ca treatment were 54 % and 29 %, respectively, higher than those with O treatment, and  $[CAT]_{\text{leaf}}$  in plants with K+Ca treatment were 20 % and 26 %, respectively, higher than those with O treatment, though there was no statistical difference between these values.

### 3.8. Relationships between leaf photosynthetic capacity and related biochemical attributes

Pearson correlation coefficient analysis (Fig. 6) showed that stomatal limitation ( $l_s$ ) on  $A_n$  had significant positive correlation with  $g_s$  but negative correlations with  $g_m$  and  $C_c$ . By contrast, non-stomatal limitation ( $1-l_s$ ) on  $A_n$  had significant negative correlation with  $g_s$  but positive correlations with  $g_m$  and  $C_c$ .  $g_s$  had significant negative correlation with  $[Na]_{\text{leaf}}$  but significant positive correlations with  $[K]_{\text{leaf}}$ ,  $[Ca]_{\text{leaf}}$ ,  $[K/Na]_{\text{leaf}}$ ,  $[Ca/Na]_{\text{leaf}}$  and  $[(K+Ca)/Na]_{\text{leaf}}$ .  $A_c$ ,  $V_{cmax}$ ,  $J_{max}$  and TPU had significant positive correlations with  $F_v/F_m$ ,  $C_c$ ,  $[N]_{\text{leaf}}$ ,  $[P]_{\text{leaf}}$ ,  $[Mg]_{\text{leaf}}$ ,  $[SOD]_{\text{leaf}}$  and  $[CAT]_{\text{leaf}}$ , while  $J_{max}/V_{cmax}$  had significant negative correlations with  $F_v/F_m$ ,  $[N]_{\text{leaf}}$ ,  $[P]_{\text{leaf}}$  and  $[POD]_{\text{leaf}}$ . Besides,  $C_c$  had significant positive correlations with  $[N]_{\text{leaf}}$ ,  $[P]_{\text{leaf}}$ ,  $[Mg]_{\text{leaf}}$ ,  $[SOD]_{\text{leaf}}$ ,  $[POD]_{\text{leaf}}$  and  $[CAT]_{\text{leaf}}$ . Moreover,  $F_o$  and NPQ negatively correlated with  $[POD]_{\text{leaf}}$  and  $[CAT]_{\text{leaf}}$ , while  $F_v/F_m$  and  $F_v/F_m$  positively correlated with  $[POD]_{\text{leaf}}$  and  $[CAT]_{\text{leaf}}$ .

### 3.9. Plant growth, water consumption and water use efficiency

The plant height (H), stem diameter (D), leaf area (LA), specific leaf area (SLA), shoot biomass accumulation ( $DM_{\text{shoot}}$ ), root biomass accumulation ( $DM_{\text{root}}$ ) and plant water use (WU) in plants grown under S with O treatment were 33 %, 27 %, 44 %, 4 %, 41 %, 25 %, 42 %, respectively, lower than those grown under C with O treatment (Table 4). Both under C and S conditions, H in plants grown with K+Ca treatment were 9 % and 22 %, respectively, higher than those with O treatment, and  $DM_{\text{shoot}}$  in plants grown with K+Ca treatment were 9 % and 17 %, respectively, higher than those with O treatment (Table 4).

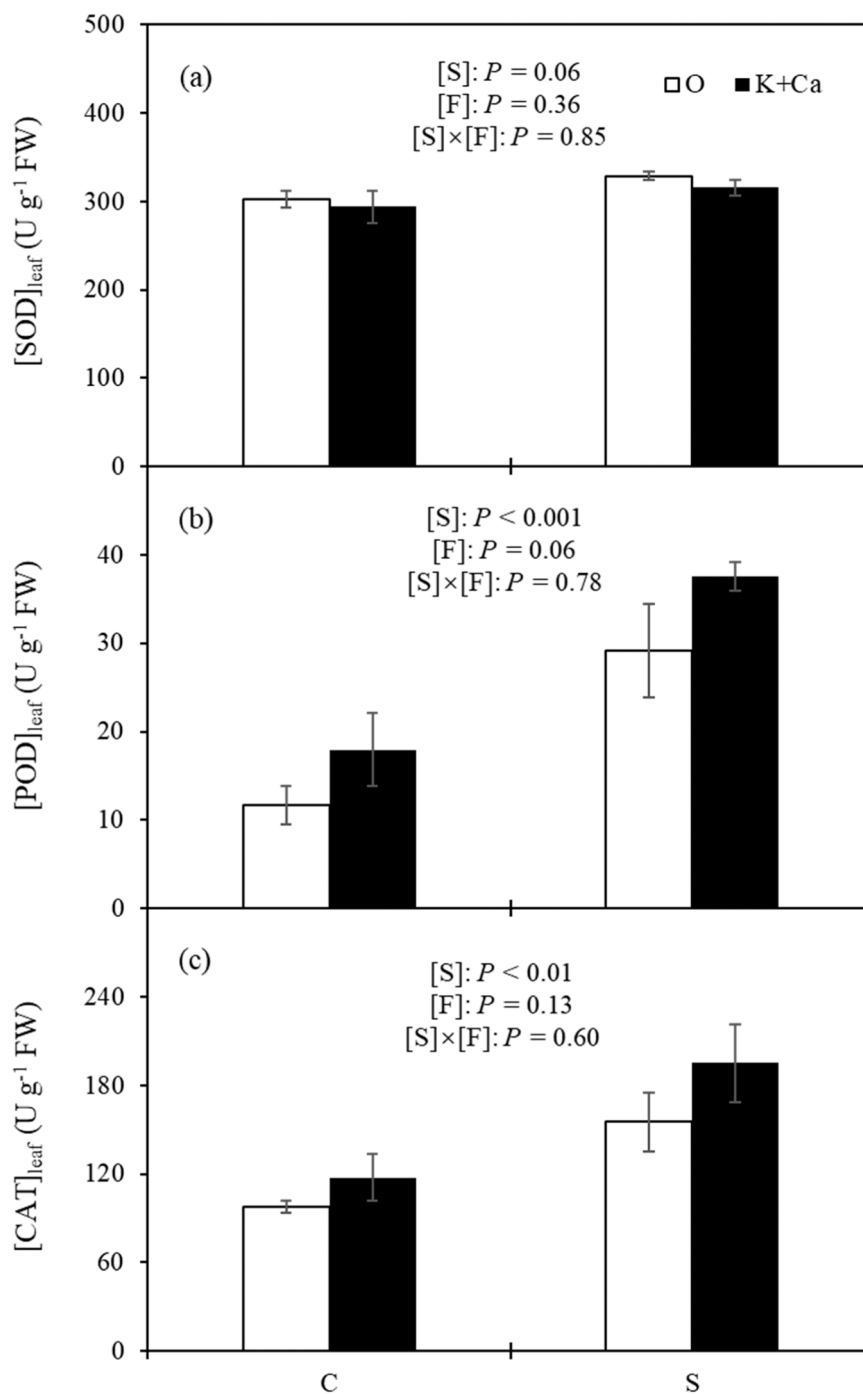


**Fig. 5.** Leaf superoxide dismutase content ( $[SOD]_{leaf}$ ) (a), leaf peroxidase content ( $[POD]_{leaf}$ ) (b) and leaf catalase content ( $[CAT]_{leaf}$ ) (c) of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), different K and Ca fertilization treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Bars are means  $\pm$  SE ( $n = 4$ ).

The intrinsic water use efficiency ( $WUE_i$ ) and instantaneous water use efficiency ( $WUE_{leaf}$ ) in plants grown under S with O treatment were 73 % and 70 %, respectively, higher than those grown under C with O treatment (Fig. 7a). However, compared to those grown under S with O treatment, those grown under S with K+Ca treatment had 18 % lower  $WUE_i$  and 18 % lower  $WUE_{leaf}$ , respectively (Fig. 7b).  $WUE_p$  in plants grown with K+Ca treatment were 3 % and 4 % higher than those grown with O treatment both under C and S conditions, respectively (Fig. 7c).

#### 4. Discussion

Decreases in photosynthesis, stomatal conductance and plants growth as affected by soil salinity could be alleviated by applying K or Ca, such findings have been reported in several crop species (Shabala et al., 2003; Ju et al., 2021). Similarly, our recent study in pot-grown cotton plants exposed to 150 mM NaCl salinity also found that supplying K and Ca (with 0.6 mM K and 3.25 mM Ca in irrigation solution) could significantly alleviate the negative effect of salt on photosynthesis ( $A_n$ ), stomatal conductance ( $g_s$ ) and transpiration rate ( $T_r$ ) (Ma et al., 2021b). Besides, we also found that K and Ca fertilization had a more

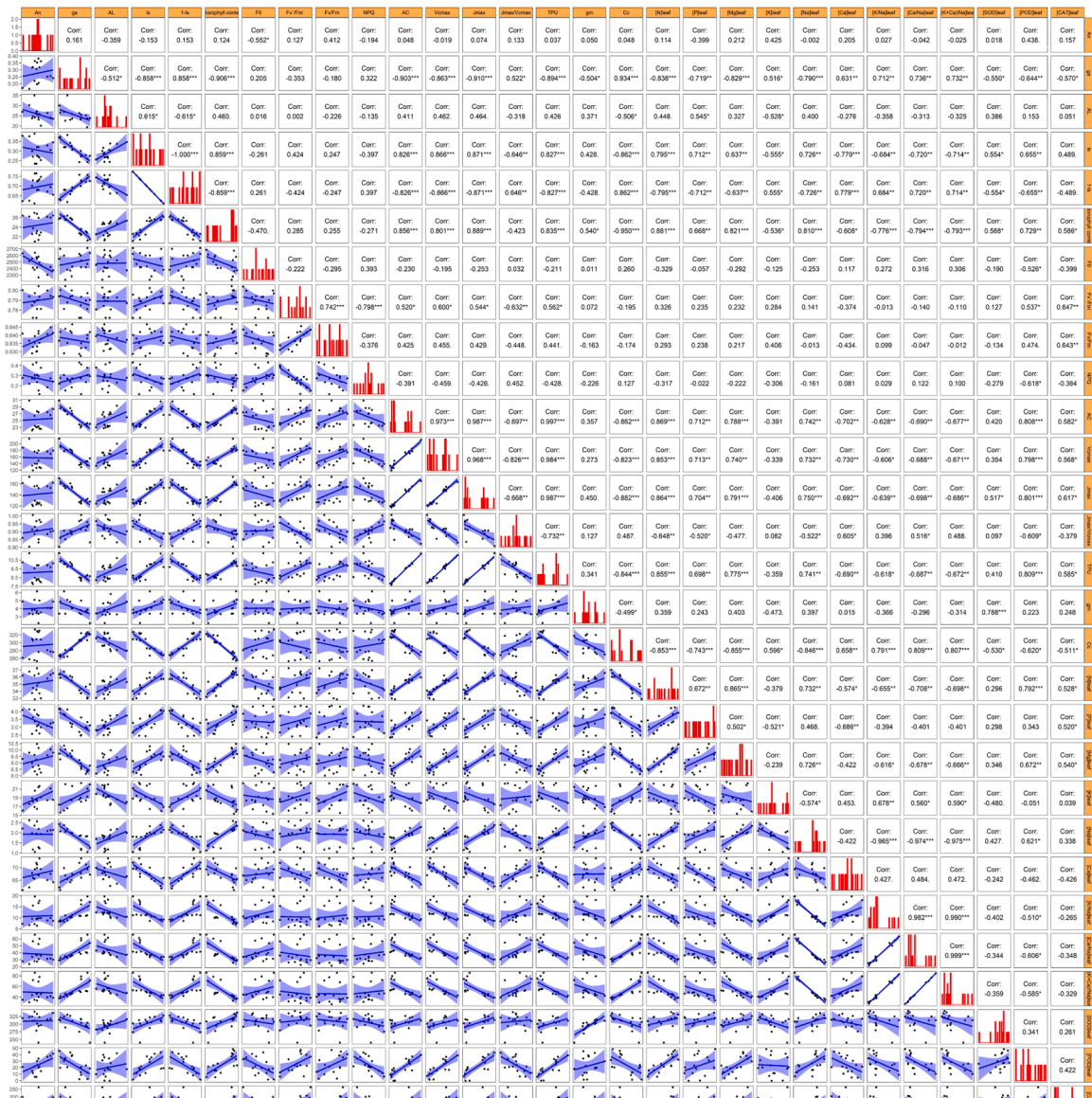


Fig. 6. Pearson correlation coefficients between various indicators across salt stress, and K and Ca fertigation treatments. \*, \*\* and \*\*\* indicates statistically significance at  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ , respectively.

pronounced effects on  $g_s$  than on  $A_n$  (Ma et al., 2021a, b), probably because non-stomatal processes participated in photosynthesis regulation under these conditions. Similar results were also found in the present study (Fig. 1).

For salt-sensitive plants such as tomato, 140 mM NaCl salinity tended to decrease net photosynthetic rate at high PPFD ( $>1000 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) (Zhang et al., 2018). However, in the present study, 150 mM NaCl salinity increased the light-saturated net photosynthetic rate ( $A_L$ ) of cotton plants (Fig. 2a; Table 1), despite it suppressed stomatal conductance at higher PPFD (Fig. 2b). The different response might be ascribed to the higher tolerance of cotton to soil salinity than tomato plants (Maas, 1990). Besides, salt-stressed cotton plants showed higher maximum rate of carboxylation by Rubisco ( $V_{\text{max}}$ ), maximum rate of electron transport ( $J_{\text{max}}$ ), rate of triose phosphates use (TPU) and mesophyll conductance ( $g_m$ ) (Table 3) than control plants, supporting the hypothesis that salinity-caused improvements in these variables sustained the net photosynthetic rate under high light (PPFD  $> 1000 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) conditions.

Higher photosynthesis is associated with the maintenance of higher electron transport rate of plants under stress conditions (Kitao et al.,

2003; Flexas et al., 2006). The regulation of electron transport rate would affect the change of PSII efficiency. As earlier study demonstrated in banana, salinity aggravated photoinhibition of the electron transport system, resulting in a decreased PSII efficiency and suppressed  $F_v/F_m$  (Thomas and Turner, 2001). Recent study by Ebrahimi et al (2023) also supported that the presumed decrease in photosynthesis was confirmed by the observation of suppression of  $F_v/F_m$  of the salt-exposed plants. By contrast, in the present study, the higher  $A_L$  was accompanied with lower NPQ but higher initial chlorophyll fluorescence yield ( $F_0$ ), light ( $F_v/F_m'$ ) and dark-adapted ( $F_v/F_m$ ) PSII efficiency in salt-stressed plants than control plants (Table 2). This indicate that moderate salinity could reduce excess energy dissipation of higher salt tolerant cotton plants, thus providing more energy for electron transport. The enhanced PSII efficiency under salt stress might be related to the enhanced chlorophyll content (Table 2; Ebrahimi et al., 2023), which could be ascribed to the improved leaf N and Mg concentration in the salt-stressed plants (Akram, 2014; Shao et al., 2020; Fig. 3a, c). It was also reported that amending the salt-stressed plants with Ca improved chlorophylls and  $F_v/F_m$  (Ebrahimi et al., 2023; Vafadar and Ehsanzadeh, 2023). However, in the present study, applying K and Ca decreased

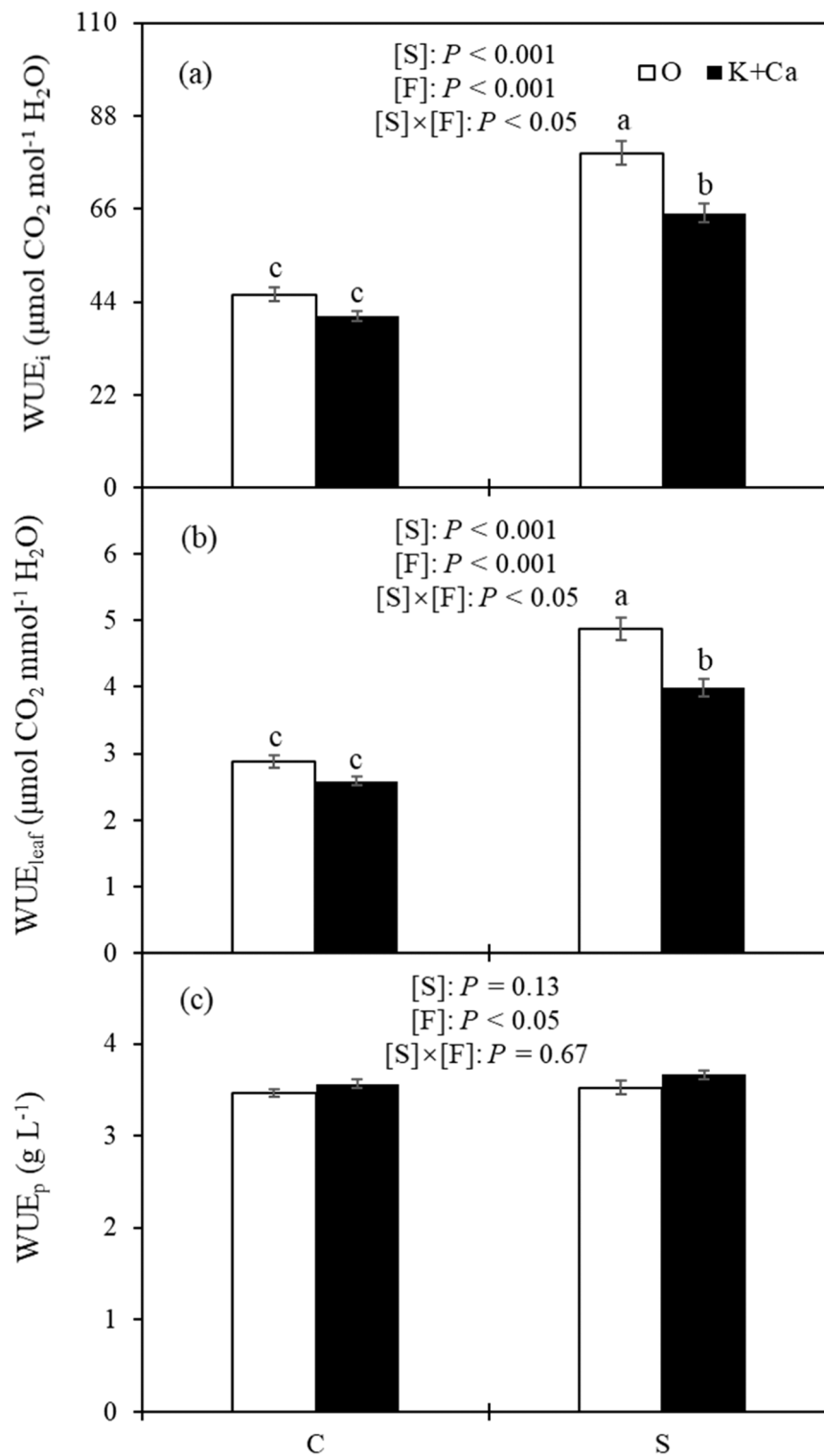


**Fig. 7.** Intrinsic water use efficiency ( $\text{WUE}_i$ ) (a), instantaneous water use efficiency ( $\text{WUE}_{\text{leaf}}$ ) (b) and water use efficiency of plant biomass ( $\text{WUE}_p$ ) of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), and different K and Ca fertilization treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Values are means  $\pm$  SE ( $n = 60$  for  $\text{WUE}_i$  and  $\text{WUE}_{\text{leaf}}$ ,  $n = 4$  for  $\text{WUE}_p$ ).

chlorophyll content of the salt-exposed cotton plants (Table 4) thus with thinner leaf.

150 mM NaCl salinity significantly increased leaf peroxidase ( $[\text{POD}]_{\text{leaf}}$ ) and catalase ( $[\text{CAT}]_{\text{leaf}}$ ) contents (Fig. 5b, c), which positively correlated with  $F_v/F_m$ ,  $F_v/F_m$ ,  $V_{\text{cmax}}$ ,  $J_{\text{max}}$ , TPU, and negatively correlated with  $J_{\text{max}}/V_{\text{cmax}}$  (Fig. 6). This suggests that salinity-induced increase in chlorophyll content,  $F_v/F_m$ ,  $F_v/F_m$ ,  $J_{\text{max}}$ ,  $V_{\text{cmax}}$ , and

decrease in  $J_{\text{max}}/V_{\text{cmax}}$  could be partially ascribed to the salinity-caused improvement in  $[\text{POD}]_{\text{leaf}}$  and  $[\text{CAT}]_{\text{leaf}}$  (Ge et al., 2014; Lu and Zhang, 1999). Earlier studies have reported that chloroplasts are the prevailing sites for producing reactive oxygen species (ROS) (Foyer et al., 1994), which play major role in inhibiting PSII efficiency (Nishiyama et al., 2001). However, an up-regulation of antioxidant enzyme activity could effectively scavenge these ROS such as  $\text{O}_2$  and  $\text{H}_2\text{O}_2$  (Sakihama and

**Table 3**

Average of CO<sub>2</sub> saturated photosynthetic rate ( $A_c$ ), maximum rate of carboxylation by Rubisco ( $V_{cmax}$ ), maximum rate of electron transport ( $J_{max}$ ), ratio of  $J_{max}$  to  $V_{cmax}$  ( $J_{max}/V_{cmax}$ ), rate of triose phosphates use (TPU), respiration rate ( $R_d$ ), mesophyll conductance ( $g_m$ ) and chloroplast CO<sub>2</sub> concentration ( $C_c$ ) under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), different irrigation levels (FI, 90 % of SWC; DI, 70 % of SWC), and different K and Ca fertigation treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Values are means  $\pm$  SE (n = 4).

| Salt                       | Fertilizer | $A_c$<br>( $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) | $V_{cmax}$<br>( $\text{mmol m}^{-2}\text{s}^{-1}$ ) | $J_{max}$<br>( $\text{mmol m}^{-2}\text{s}^{-1}$ ) | $J_{max}/V_{cmax}$ | TPU<br>( $\text{mmol m}^{-2}\text{s}^{-1}$ ) | $R_d$<br>( $\text{mmol m}^{-2}\text{s}^{-1}$ ) | $g_m$<br>( $\text{mmol m}^{-2}\text{s}^{-1} \text{Pa}^{-1}$ ) | $C_c$<br>( $\mu\text{mol mol}^{-1}$ ) |
|----------------------------|------------|--------------------------------------------------|-----------------------------------------------------|----------------------------------------------------|--------------------|----------------------------------------------|------------------------------------------------|---------------------------------------------------------------|---------------------------------------|
| C                          | O          | 22.51 $\pm$ 0.27                                 | 126.47 $\pm$ 4.26                                   | 121.54 $\pm$ 0.91                                  | 0.96 $\pm$ 0.02    | 7.96 $\pm$ 0.12                              | 1.37 $\pm$ 0.25                                | 3.53 $\pm$ 0.15                                               | 308.0 $\pm$ 0.9                       |
|                            | K+Ca       | 23.11 $\pm$ 0.24                                 | 139.00 $\pm$ 5.79                                   | 126.33 $\pm$ 1.68                                  | 0.91 $\pm$ 0.03    | 8.31 $\pm$ 0.10                              | 1.83 $\pm$ 0.07                                | 3.69 $\pm$ 0.81                                               | 330.1 $\pm$ 1.4                       |
| S                          | O          | 28.28 $\pm$ 1.32                                 | 187.74 $\pm$ 13.61                                  | 161.50 $\pm$ 5.59                                  | 0.86 $\pm$ 0.02    | 10.24 $\pm$ 0.53                             | 2.44 $\pm$ 0.31                                | 4.87 $\pm$ 0.80                                               | 256.7 $\pm$ 1.9                       |
|                            | K+Ca       | 27.44 $\pm$ 0.50                                 | 175.89 $\pm$ 3.10                                   | 155.64 $\pm$ 3.16                                  | 0.89 $\pm$ 0.01    | 9.90 $\pm$ 0.13                              | 2.27 $\pm$ 0.12                                | 4.09 $\pm$ 0.43                                               | 277.6 $\pm$ 1.1                       |
| P value from two-way ANOVA |            |                                                  |                                                     |                                                    |                    |                                              |                                                |                                                               |                                       |
| S                          |            | < 0.001                                          | < 0.001                                             | < 0.001                                            | 0.01               | < 0.001                                      | 0.007                                          | 0.19                                                          | < 0.001                               |
| F                          |            | 0.87                                             | 0.16                                                | 0.88                                               | 0.51               | 0.98                                         | 0.52                                           | 0.63                                                          | < 0.001                               |
| S×F                        |            | 0.35                                             | 0.97                                                | 0.15                                               | 0.12               | 0.26                                         | 0.18                                           | 0.46                                                          | 0.67                                  |

**Table 4**

Plant height (H), stem diameter (D), leaf area (LA), specific leaf area (SLA), biomass accumulation in shoot ( $DM_{shoot}$ ), biomass accumulation in root ( $DM_{root}$ ) and plant water consumption (WU) of cotton plants grown under different salt stress levels (C, 0 mM NaCl; S, 150 mM NaCl), and different K and Ca fertigation treatments (O, modified Hoagland solution without K and Ca; K+Ca, modified Hoagland solution with 0.6 mM K and 3.25 mM Ca). Values are Means  $\pm$  SE (n = 4).

| Salt                       | Fertilizer | H (cm)            | D (mm)          | LA (cm <sup>2</sup> ) | SLA (cm <sup>2</sup> /g)        | $DM_{shoot}$ (g) | $DM_{root}$ (g) | WU (L)           |
|----------------------------|------------|-------------------|-----------------|-----------------------|---------------------------------|------------------|-----------------|------------------|
| C                          | O          | 118.75 $\pm$ 3.74 | 8.50 $\pm$ 0.34 | 3273.56 $\pm$ 191.97  | 290.03 $\pm$ 1.36 <sup>a</sup>  | 35.69 $\pm$ 2.17 | 1.53            | 10.28 $\pm$ 0.62 |
|                            | K+Ca       | 130.00 $\pm$ 5.80 | 8.43 $\pm$ 0.35 | 3484.81 $\pm$ 236.67  | 285.51 $\pm$ 2.47 <sup>ab</sup> | 38.94 $\pm$ 1.63 | 1.56            | 10.92 $\pm$ 0.49 |
| S                          | O          | 80.00 $\pm$ 1.08  | 6.18 $\pm$ 0.12 | 1847.33 $\pm$ 131.67  | 277.59 $\pm$ 1.54 <sup>c</sup>  | 21.05 $\pm$ 1.46 | 1.15            | 5.96 $\pm$ 0.38  |
|                            | K+Ca       | 97.50 $\pm$ 1.50  | 6.75 $\pm$ 0.23 | 2101.47 $\pm$ 069.32  | 281.73 $\pm$ 2.39 <sup>bc</sup> | 24.64 $\pm$ 0.89 | 1.35            | 6.73 $\pm$ 0.38  |
| P value from two-way ANOVA |            |                   |                 |                       |                                 |                  |                 |                  |
| S                          |            | < 0.001           | < 0.001         | < 0.001               | 0.002                           | < 0.001          | 0.06            | < 0.001          |
| F                          |            | 0.002             | 0.38            | 0.20                  | 0.92                            | 0.05             | 0.41            | 0.11             |
| S×F                        |            | 0.40              | 0.27            | 0.90                  | 0.05                            | 0.92             | 0.56            | 0.87             |

Yamasaki, 2002), sustaining the chlorophyll content (Jan et al., 2017) and protecting PSII from the salinity-induced oxidative damage (Nounjan et al., 2012).

Enhanced antioxidant enzymatic activity by exogenous application of K and Ca has also been demonstrated in wheat and grasses plants (Jan et al., 2017; Jiang and Huang, 2001). In the present study, there was no significant differences in SOD, POD and CAT between O and K+Ca treatment both under C and S conditions (Fig. 5). Correspondingly, K and Ca fertigation had no significant effect on  $A_L$  (Fig. 2a; Table 1). Plants grown with K and Ca addition showed lower  $A_L$  than those without K and Ca addition (Fig. 2a; Table 1), despite that lower NPQ and higher PSII efficiency were noticed in plants applied with K and Ca under salt stress condition (Table 2). Since the decreased  $A_L$  of cotton plants supplemented with K and Ca was accompanied with significantly lowered  $g_s$  at higher PPFD under salinity condition (Fig. 2b), this was an indicate that stomatal limitation might be responsible for the reduced  $A_L$ .

150 mM NaCl salinity increased stomatal limitation to photosynthesis of cotton plants (Table 2). However, applying K and Ca tended to alleviate this salinity-caused stomatal limitation on photosynthesis despite the effect was not statistically significant (Table 2). In chamber grown cotton plants, it was found that K status affect the rate of CO<sub>2</sub> assimilation, and stomatal limitation had a major effect on photosynthesis of the plants subjected to mild K deficiencies (Bednarsz et al., 1998). Salinity-induced K and Ca deficiencies and consequently lower ratio of K/Na and Ca/Na have also been reported as major factors to regulate stomatal movements through osmoregulation. Consistent with this, here, 150 mM NaCl salinity significantly increased leaf Na concentration ( $[Na]_{leaf}$ ) but decreased leaf K ( $[K]_{leaf}$ ) and Ca concentrations ( $[Ca]_{leaf}$ ), and the ratio of leaf K/Na ( $[K/Na]_{leaf}$ ), Ca/Na ( $[Ca/Na]_{leaf}$ ), and (K+Ca)/Na ( $[(K+Ca)/Na]_{leaf}$ ) (Fig. 4). While applying K and Ca apparently alleviated these reductions in  $[K]_{leaf}$ ,  $[Ca]_{leaf}$ ,  $[K/Na]_{leaf}$ ,  $[Ca/Na]_{leaf}$  and  $[(K+Ca)/Na]_{leaf}$  (Fig. 4a, c, d, e, f), which would reduce the salt-induced stomatal limitations on  $A_n$ .

Additionally, the enhanced  $V_{cmax}$  and  $J_{max}$ , and the decreased  $J_{max}/V_{cmax}$  could be linked to the increased leaf N concentration as

demonstrated in many C<sub>3</sub> plants (Makino et al., 1994; Nakano et al., 1997). Nevertheless, opposite results have been reported in rice, wheat and tobacco plants, where  $J_{max}/V_{cmax}$  increased with increasing leaf N concentration (Yamori et al., 2011). Limitations of  $V_{cmax}$ ,  $J_{max}$  and TPU on photosynthesis in P-deficient plants has also been observed in greenhouse shrubs (Poudyal et al., 2021). Similarly, in the present study, 150 mM NaCl salinity increased leaf nitrogen ( $[N]_{leaf}$ ) and leaf phosphorus concentration ( $[P]_{leaf}$ ), while applying K and Ca slightly alleviated this salinity-induced increments in  $[N]_{leaf}$  and  $[P]_{leaf}$  (Fig. 3a, b). The increased  $V_{cmax}$ ,  $J_{max}$  and TPU under these conditions apparently correlated positively to the enhanced  $[N]_{leaf}$  and  $[P]_{leaf}$ , and the decreased  $J_{max}/V_{cmax}$  was correlated negatively to the increased  $[N]_{leaf}$  and  $[P]_{leaf}$  (Fig. 6). In addition, 150 mM NaCl salinity decreased chloroplast CO<sub>2</sub> concentration ( $C_c$ ) despite increased mesophyll conductance ( $g_m$ ) (Table 3), perhaps because the increase in  $g_m$  was insufficient to compensate for change in  $A_n$  (Yamori et al., 2011). However, the plants grown with K and Ca addition showed higher  $C_c$  than those without K and Ca addition, though K+Ca treatment decreased  $g_m$  relative to O treatment (Table 3). This suggests that K and Ca supplement might be beneficial to alleviate lower  $C_c$  caused photosynthetic limitation under salinity stress.

The stomatal limitation to photosynthesis is mainly due to a lowered  $g_s$ , while the non-stomatal limitation to photosynthesis primarily affected by the balance of  $J_{max}$ ,  $V_{cmax}$  and  $C_c$ . For plants grown under stress conditions, a higher  $J_{max}/V_{cmax}$  ratio and lower  $C_c$  indicate the photosynthesis is mainly limited by RuBP carboxylation. Oppositely, a lower  $J_{max}/V_{cmax}$  ratio indicates photosynthesis is largely limited by RuBP regeneration (Yamori et al., 2010, 2011). In the present study, 150 mM NaCl salinity decreased  $g_s$  thus increased stomatal limitation ( $I_s$ ) on  $A_n$  (Fig. 1b; Fig. 6; Table 2). However, the decreased non-stomatal limitation ( $1-I_s$ ) on  $A_n$  (Table 2) resulted in the sustained  $A_n$  and significantly lower  $g_s$  and  $T_r$  (Fig. 1), and thereby increased intrinsic water use efficiency ( $WUE_i$ ) and instantaneous water use efficiency ( $WUE_{leaf}$ ) (Fig. 7a, b). The decreased non-stomatal limitation was accompanied with decreased  $J_{max}/V_{cmax}$  and  $C_c$ . In contrast, the enhanced  $J_{max}$ ,  $V_{cmax}$  (Table 3), and  $A_c$  negatively correlated with

decreased  $J_{\max}/V_{\max}$  and  $C_c$  (Fig. 6). Indicating that  $\text{CO}_2$  assimilation tended to be limited by RuBP regeneration in plants grown under 150 mM NaCl salinity.

## 5. Conclusion

The photosynthesis of cotton as affected by 150 mM NaCl salinity was mainly controlled by two mechanisms: stomatal and non-stomatal limitations. Decreased  $A_n$  under salt stress was mainly ascribed to the salinity-induced reduction in  $g_s$ , whereas, the enhanced  $g_m$ ,  $J_{\max}$ ,  $V_{\max}$  and TPU under salinity resulting in sustained  $A_n$  despite the significantly lowered  $g_s$  and  $T_r$ . Alleviated  $A_n$  by K and Ca addition under salt stress could be attributed to the sustained  $g_s$ . Collectively, applying K and Ca alleviated salinity-induced cotton growth limitation and improved water use efficiency of plant biomass. Therefore, K and Ca fertigation could be a promising management strategy for enhancing cotton production on saline soils.

## CRedit authorship contribution statement

**Yingying Ma:** Experiment design, experiment execution, data analyses and manuscript preparation. **Zuoqiang Yuan:** Data analyses and manuscript review. **Zhenhua Wei:** Data analyses and manuscript review. **Fei Yan:** Data analyses and manuscript review. **Xuezhi Liu:** Data acquisition and manuscript review. **Xiangnan Li:** Data acquisition and manuscript review. **Jingxiang Hou:** Data acquisition and manuscript review. **Zhanqing Hao:** Data analyses and manuscript review. **Fulai Liu:** Original research idea, experiment design, data analysis and manuscript review, guidance and management.

## Declaration of Competing 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.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2025.106092.

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