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Full Length Research Paper

Alkali stress induced the accumulation and secretion of organic acids in wheat

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In this study, the seedlings of wheat were treated with salt stress (molar ratio of NaCl:Na_2SO_4 = 1:1) and alkali stress ($NaHCO_3$:Na_2CO_3 = 1:1). The contents of organic acids and inorganic ions in the seedlings then were measured, and the organic acids in root secretions also were analyzed to probe the roles of organic acids in wheat alkali tolerance. The results showed that the content of Na^+ increased greatly, whereas the contents of NO_3^-, SO_4^{2-} and $H_2PO_4^-$ decreased significantly. This caused a severe deficit of negative charge. The deficit of negative charge was remedied by greatly accumulated organic acids (OAs), with malic and citric acids as the dominant components in shoots. Therefore, the accumulation of OAs might result from the deficit of negative charge, and OA metabolic regulation might be a key pathway for keeping ionic balance and stable the pH. Secretion of OAs such as lactic, acetic and formic acids was significantly stimulated by alkali stress, indicating that OA secretion may be important in pH adjustment outside roots.

Key words: Wheat, salt stress, alkali stress, organic acid, secretion, pH adjustment.

INTRODUCTION

The existence of alkali stress has been demonstrated clearly by a number of reports, which have shown it to be more severe than salt stress. In fact, salt and alkaline stress have been identified as two distinct types of stress that is well proven by numerous studies (Yang et al., 2007, 2008a, b, c, 2009, 2010). Salt stress in a soil generally involves osmotic stress and ion-induced injury (Munns, 2002). Comparison of alkali stress with salt stress reveals an added effect of alkali stress due to high pH. However, relatively, little attention has been given to alkali-stress. Even so, there have been some reports about high pH calcareous soils (Brand et al., 2002; Nuttall et al., 2003), alkaline soils (Hartung et al., 2002), alkaline salt stress (Shi and Yin, 1993; El-Samad and Shaddad, 1996; Campbell and Nishio, 2000; Yang et al., 2007) and mixed-salt stress (Shi and Sheng, 2005; Shi and Wang, 2005). Plants surviving alkaline soil need to maintain the stability of intracellular pH and regulate the pH outside roots, apart from osmotic adjustment and ion toxicity avoidance (Yang et al., 2007, 2008a, b, c). Thus, the pH adjustments *in vivo* and outside roots play the key role in plant alkali tolerance. A striking feature of plant tissues is that the total content of organic acids (OAs) is higher than in other organisms. It is an intermediate product of material and energy metabolism involving in abiotic stress responses (Ma et al., 2001).

In recent years, studies suggested that OAs were not accumulated in alkali-sensitive plants (Qu et al., 2004) and low alkali tolerant halophytes (Qu et al., 2003), while a great regulation of organic acids (Chen et al., 2009; Yang et al., 2008b) were found in alkali-tolerant halophytes. Previous studies had indicated that OAs is believed to be the key factor for alkali tolerance, especially for the intracelluler ionic homeostasis. Soil salinization and alkalization frequently co-occur, with alkalization causing severe problems in some areas. For example, in northeast China, more than 70% of the land area is alkaline grassland (Kawanabe and Zhu, 1991). Only a few alkali resistant plants can survive in these soils (Zheng and Li, 1999). Wheat (*Triticum aestivum*) is a salt-resistant crop (Zhao et al., 2002). The salt tolerance and its physiological mechanisms of wheat plants have been reported in many studies. However, a little is known about the mechanisms of alkali tolerance in wheat. It should be noted that the investigation on the mechanisms of salt- and alkali- tolerance of wheat is because it is important for utilization of saline fields. In this study, the wheat seedlings were treated with salt stress (molar ratio of $NaCl:Na_2SO_4$ = 1:1) and alkali stress (molar ratio of $NaHCO_3:Na_2CO_3$ = 1:1).

The contents of inorganic ion and organic acids in stressed plants then were measured to elucidate the mechanism by which wheat plants adapt to alkali stress.

MATERIALS AND METHODS

Plants materials

Seeds of wheat cultivar “Xiaobingmai 33” were provided by the Institute of Genetics and Cytology, Northeast Normal University. Wheat cultivar “Xiaobingmai 33”, a salt-resistant wheat cultivar was sown in plastic pots of 17-cm diameter. Each pot contained 15 seedlings. Seedlings were sufficiently watered with Hoagland nutrient solution once daily. All pots were placed outdoors and protected from rain. Temperatures during the experiment were 24 to 27°C during the day and 18 to 21°C at night (Yang et al., 2008c).

Simulated salt and alkaline conditions

Two neutral salts (NaCl and Na_2SO_4) were mixed in a 1:1 molar ratio and applied to the salt stress group. Tow alkaline salts (Na_2CO_3 and $NaHCO_3$) also were mixed in a 1:1 molar ratio, and applied to the alkali stress group. Within each group, two total salt concentrations were labeled: 30 and 60 mM. Salt stress groups were labeled S1 to S2, and alkali stress groups were labeled as A1 to A2. The pH of treatment solution was measured with a digital pH meter. In the salt stress and alkali stress groups, pH was 6.20 to 6.45 and 9.88 to 10.06, respectively.

Stress treatments

When the seedlings were 2 weeks old, 18 pots with seedlings growing uniformly were selected and randomly divided into 6 sets, 3 pots per set. One set was used as a control, a second set was used for growth index determination at the beginning of treatment, and the remaining 4 sets were used as various stress treatments. Each pot was considered a single replicate; therefore, there were three replicates per set. Stress treatments were performed once daily with the application of nutrient solutions containing the appropriate salts. All pots were watered thoroughly with 500 ml of treatment solution applied in three portions. Control plants were watered with nutrient solution. The entire treatment duration was 15 days.

Measurement of physiological indices

Plants were harvested in the morning after the final treatment, and were first washed with tap water followed by distilled water. Roots and shoots were separated, and fresh weights (FW) were determined per pot; the samples were set in baking oven at 105°C for 15 min and then set in vacuum drying oven at 40°C dehydration to constant weight called dry weight (DW). Relative growth rate (RGR) was calculated using the following formula:

$$RGR = \frac{\text{DW at the end of stress treatment} - \text{DW at the start of stress treatment}}{\text{Total treatment duration}}$$

Where, the DW values at the end of stress treatment were the sum of all materials in a pot, and expressed as mg (DW) $plant^{-1}d^{-1}$.

Dry samples of plant material (100 mg) were treated with 20 ml of deionized water at 100°C for 1 h, and the resulting extract was used to determine the contents of free inorganic ions and organic acids. The contents of NO_3^-, Cl^-, SO_4^{2-}, $H_2PO_4^-$ and oxalic acid was determined by ion chromatography using a DX-300 ion chromatographic system with an AS4A-SC ion-exchange column and a CDM-II electrical conductivity detector (mobile phase: $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM; DIONEX, Sunnyvale, USA). Other OAs was also determined by ion chromatography using the DX-300 ion chromatographic system with an ICE-AS6 ion-exclusion column, CDM-II electrical conductivity detector and an AMMS-ICE II anion suppressor (mobile phase: 0.4 mM heptafluorobutyric acid; DIONEX, Sunnyvale, USA). An atomic absorption spectrophotometer (TAS-990, Purkinje General, Beijing, China) was used to determine the levels of Na^+ and K^+.

Organic acid exudation experiment

Fifteen-day-old wheat seedlings growing in sand culture were removed and rinsed with sterile deionized water, then transferred to a beaker containing 250 ml of sterile nutrient solution for solution culture, with nutrient solution replaced daily. The roots were protected from light by wrapping the beakers with black paper. All beakers were placed in a growth chamber (27.0 ± 2°C day and 21.0 ± 2°C night, 16/8 h photoperiod at 240 μmol m^{-2} s^{-1}). After 15 days, the seedlings were rinsed with sterile deionized water and treated by transferring them to another beaker containing 250 ml of 10 mM salt (NaCl and Na_2SO_4 in 1:1 molar ratio) and alkali (Na_2CO_3 and $NaHCO_3$ in 1:1 molar ratio) treatment solutions. A 10 mM alkali treatment solution without plants was used as the control for pH. A beaker containing five seedlings was one replicate, with three replicates per treatment. During stress treatment, the photoperiod was 24 h of light. The pHs of each beaker were determined continuously with a digital pH meter. Two days after exposure to treatments, plants were harvested, and the treatment solution samples were freeze-dried. Freeze-dried solution samples were dissolved in 25 ml of deionized water. Then, the OAs in the solutions was determined with the methods described earlier.

Statistical analyses

Statistical analyses of data were performed using the statistical program SPSS 13.0. All data obtained were represented with the average of three replicates and the standard error (S.E.). The significance was tested at 5% level.

RESULTS

Growth

With increasing salinity, the RGR of wheat decreased, and the reducing extents under alkali stress ($p<0.05$) was greater than that under salt stress (Figure 1, $p<0.05$).

Ion accumulation

With increasing salinity, the Na^+ contents of shoots increased significantly, while K^+ content decreased ($P<0.05$), but the changes under alkali stress were

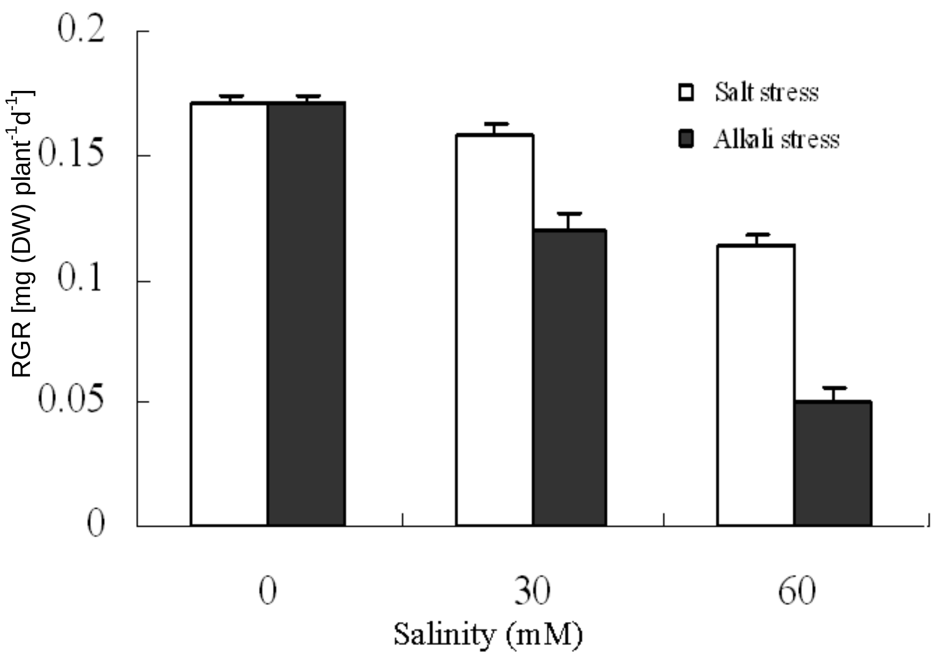

Figure 1. Effects of salt and alkali stresses on the relative growth rate (RGR) of wheat. The values are means (±SE) of three replicates.

greater than those under salt stress (Figure 2, $P<0.05$). The Cl^- content changed slightly under alkali stress ($P<0.05$), but increased greatly under salt stress ($P<0.05$). The SO_4^{2-} and $H_2PO_4^-$ contents in shoots increased under salt stress ($P<0.05$), but decreased under alkali stress. However, the NO_3^- content decreased under both stresses with increasing salinity levels, but the reducing extent was greater under alkali stress than under salt stress ($P<0.05$).

Organic acids

Malate, citrate, succinate, acetate, oxalate, formate and lactate were detected in the shoots of wheat. The malate and citrate contents were much higher than other OA. Salt stress slightly affected the accumulation of OAs (Figure 3), and even reduced OA accumulation. However, alkali stress strongly stimulated OA accumulation in shoots ($P<0.05$). Six OA (malate, citrate, acetate, oxalate, formate and lactate) were detected in wheat root, and the contents of malate, citrate, oxalate were much higher than other OA. The contents of all OA changed slightly under salt stress, but increased greatly under alikali stress (Figure 4, $P<0.05$).

OA secretion and pH outside root

The environment pH values outside wheat roots under salt stress were similar to control treatment with increasing treatment time (Figure 5), but the pH values outside wheat roots decreased very fast at fist 0 to 20 h under alkali stress, after this time point it could be stable. The results showed that the wheat roots may have a strong ability to regulate pH values outside wheat roots. Three OAs (acetate, formate and lactate) were detected in wheat root exudate, and the OA exudations were stimulated by alkali stress compared to salt stress, while the amount of secretion was very little, the concentration of total acid was less than 30 µmol/L (Figure 6).

DISCUSSION

In the present study, there was a significant decrease in RGR under both stresses, and the deleterious effect of alkali stress on wheat is more severe than that of salt stress (Yang et al., 2007, 2008a, b, c, 2009). With increasing salinity levels, the decrease in RGR were greater than under salt stress, indicating that salt and alkali stress are actually two distinct types of stress with two different mechanisms. Salt stress in a soil generally involves osmotic stress and ion-induced injury (Munns, 2002). Comparison of alkali stress with salt stress reveals an added high-pH effect of alkali stress. The high-pH environment surrounding the roots not only can directly endanger the root growth, membrane stability, cross-membrane potential and interfere in the function of root cells, but also cause some essential mineral ions like Ca^{2+},Mg^{2+}, HPO_4^{2-} and H_2PO_4 to precipitate (Shi and Zhao, 1997), leading to an indirectly effect on the plants

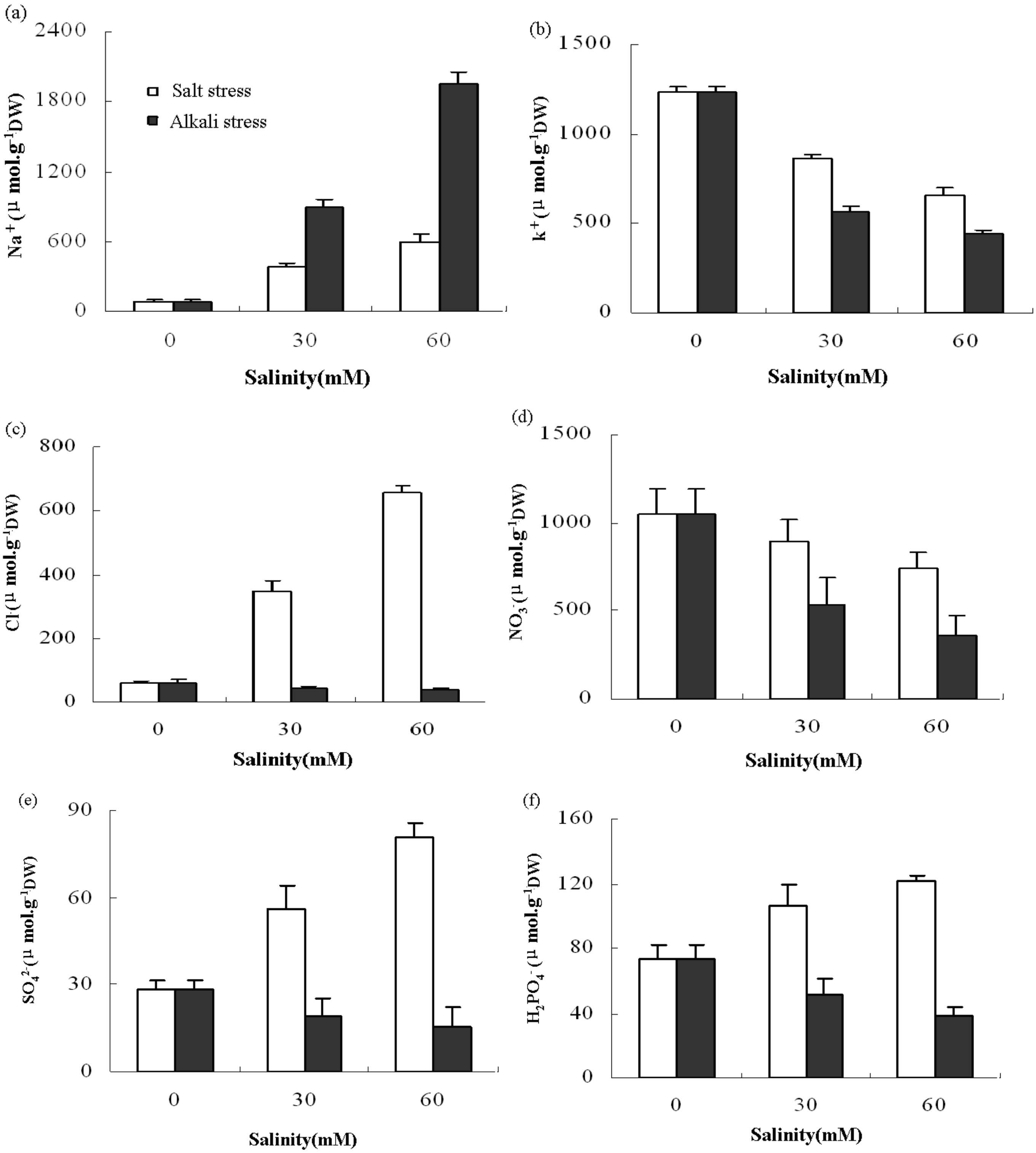

Figure 2. Effects of salt and alkali stresses on the contents of Na^+ (a), K^+ (b), Cl^- (c), NO_3^- (d), SO_4^{2-} (e) and $H_2PO_4^-$ (f) in shoots of wheat seedlings. The values are means (±SE) of three replicates.

by nutrition stress. Furthermore, the high-pH can cause the proton scarcity and damage the construction of cross-membrane potential, inhibiting the absorption of majority of ions. The decrease of inorganic anions contents may be due to the proton scarcity surrounding the roots. It is obvious that plants need to cope with high-pH for

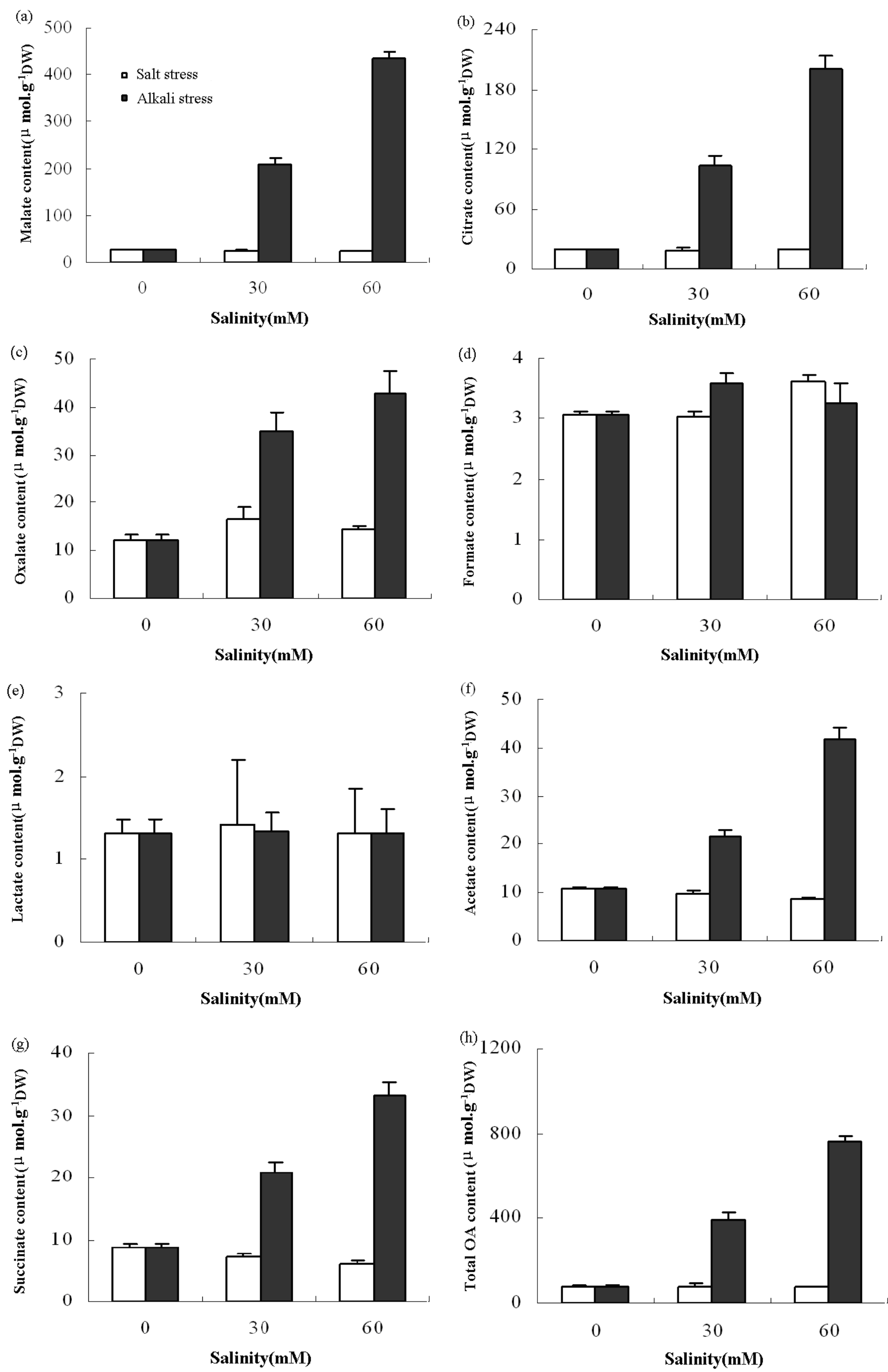

Figure 3. Effects of salt and alkali stresses on the contents of organic acids (OAs) in shoots of wheat. The values are means (±SE) of three replicates.

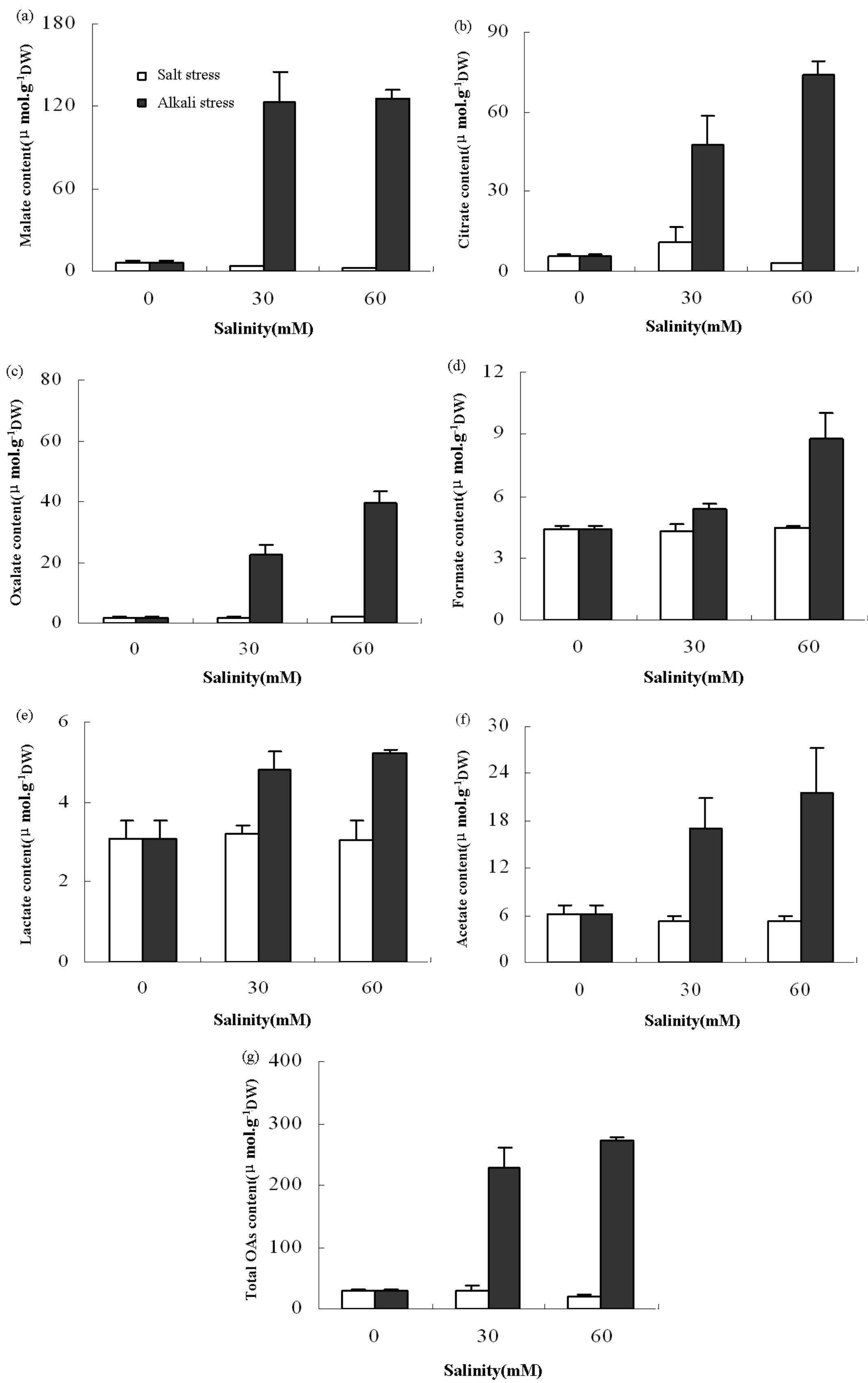

Figure 4. Effects of salt and alkali stresses on the contents of organic acids (OAs) in wheat roots. The values are means (±SE) of three replicates.

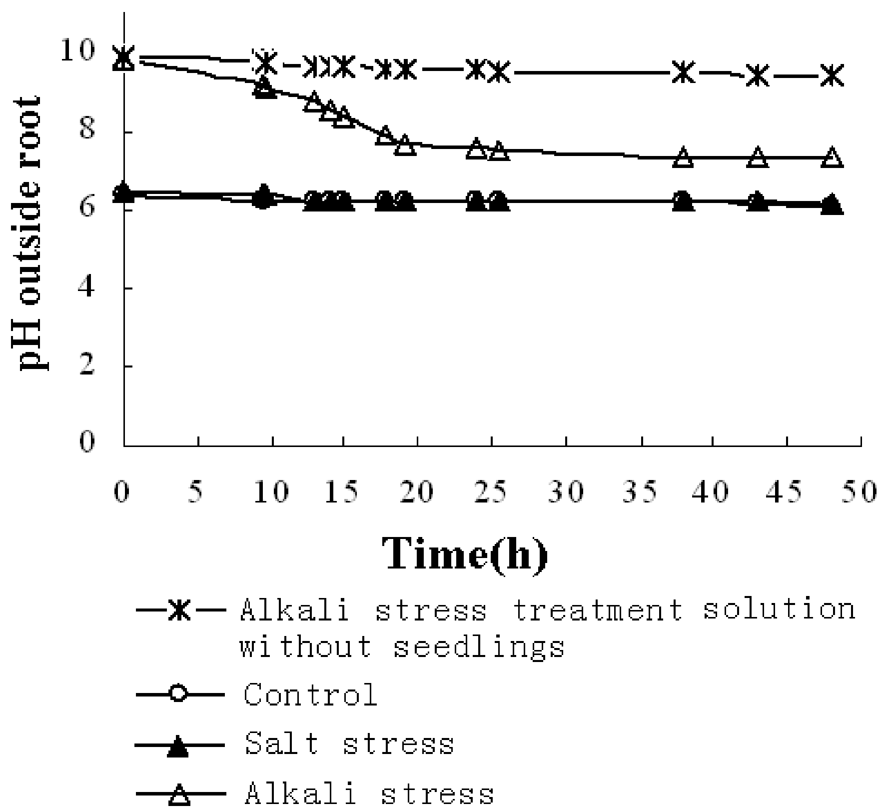

Figure 5. Change in pH outside wheat roots under salt stress and alkali stress. The values are means (±SE) of three replicates.

adapting to alkali stress. Therefore, to reveal the mechanism of alkali tolerance, we have to figure out the pH regulatory mechanisms.

Some reports revealed that halophytes in saline conditions could accumulate a large amount of inorganic ions (Na^+) in vacuoles to prevent the cells from being poisoned (Munns, 2002). Metabolism of Na^+ and K^+ plays a significant role in salt tolerance. Halophytes under salt–alkaline stress usually absorb Na^+ and simultaneously inhibit K^+ absorption (Short and Colmer, 1999; Khan et al., 2000a, b; Munns, 2002; Shi and Sheng, 2005; Shi and Wang, 2005). The high ratio of K^+/Na^+ can be considered as an important indicator for evaluating salt tolerance of plants. For many halophytes, the ratio of Na^+/K^+ increased with increasing salt concentrations under salt stress. In our study, the Na^+ content in wheat shoots increased and the K^+ content decreased under both stresses. However, the changes under alkali stress were greater than under salt stress. The changes in Na^+ and K^+ contents under alkali stress might result from the destructive effect of high pH on the ability of selective absorption of Na^+ and K^+, leading to an unbalance of ions. It appears that the accumulation of Na^+ and K^+ might be a special response to high pH but not to osmotic stress and ion toxicity, and should be further investigated.

Ionic imbalance is thought of the influx of superfluous Na^+ under salt stress (Niu et al., 1995; Blumwald, 2000; Parida and Das, 2005). However, under alkali stress, plants mainly absorb inorganic anions such as Cl^- (Ghoulam et al., 2002; Santa-Cruz et al., 2002), NO_3^- and SO_4^{2-}, or synthesized organic anions (Sagi et al., 1997) to keep ionic balance (Yang et al., 2007) and stable pH in shoots. Our results suggest that wheat can accumulate organic acids and inorganic ions as an essential approach to adjust pH. The accumulation of organic acids in wheat might be a response to the deficiency of inorganic ions. The results showed that the content of Na^+ greatly increased, whereas the content of NO_3-, SO_4^{2-} and $H_2PO_4^-$ decreased significantly. This caused a severe deficit of negative charge. The deficit of negative charge was remedied by greatly accumulated organic acids (OAs), with malic and citric acids dominant components in shoots. Therefore, the accumulation of OAs might result from the deficit of negative charge, and OA metabolic regulation might be a key pathway for keeping ionic balance and stable the pH. Our results further revealed that citric and malic acids were the main metabolite of wheat, with relatively high contents in the shoots. High content of oxalic acid was also observed in the roots while the content of other organic acids was lower in wheat.

This is contrast to that observed in *Kochia sieversiana*

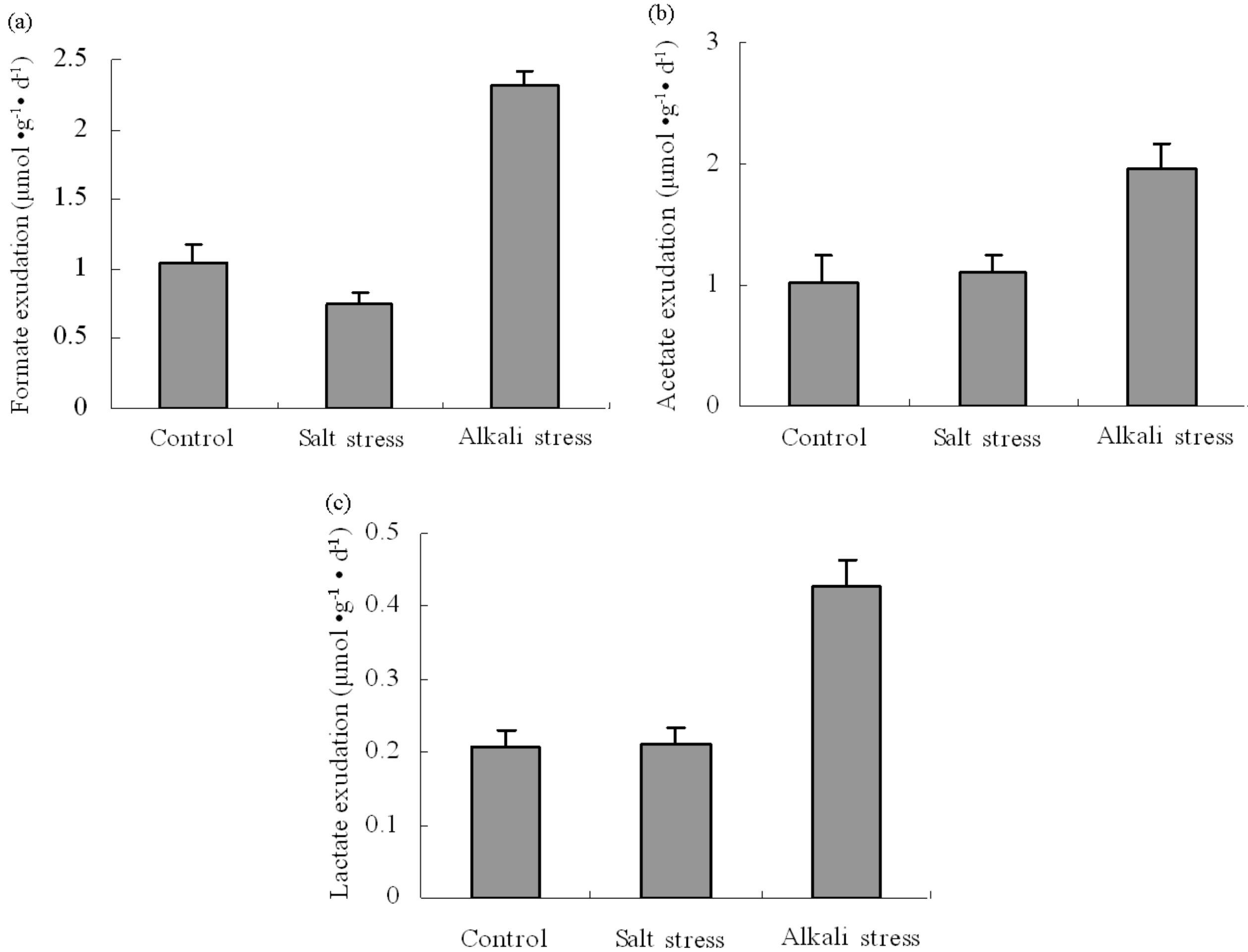

Figure 6. Organic acid exudation by wheat roots under salt and alkali stresses. The values are means (±SE) of three replicates.

(Yang et al., 2007) and *Suaeda glauca* (Yang et al., 2008a) where a higher accumulation of oxalic acid was reported under alkali stress together with minimal contents of other organic acids which were hardly detected. These phenomena indicated that OA metabolism adjustment may play different roles in different plant species.

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