

## Effects of salt and alkali stress on growth, accumulation of oxalic acid, and activity of oxalic acid-metabolizing enzymes in *Kochia sieversiana*

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### Abstract

*Kochia sieversiana* (Pall.) C.A. Mey. is a forage plant that can grow in extremely alkalized grasslands at pH 10 or higher. Accumulation of a large amount of oxalic acid (OxA) is a primary characteristic of *K. sieversiana*. In our study, seedlings of *K. sieversiana* were exposed to the following conditions: non-stress, salinity (200 mM, a molar ratio of NaCl and Na<sub>2</sub>SO<sub>4</sub> 1:1), and alkali stress (200 mM, a molar ratio of NaHCO<sub>3</sub> and Na<sub>2</sub>CO<sub>3</sub> 1:1). Growth, water content, content of organic acids (including OxA), Na<sup>+</sup>, and K<sup>+</sup>, and activities of some OxA metabolism-related enzymes were determined. Results show that glycolate oxidase was the key enzyme for OxA synthesis; however, the carboxylation of phosphoenolpyruvate (PEP) by PEP carboxylase (PEPC) probably played a minor role in the OxA-synthetic pathway. The pathway of L-ascorbic acid catabolism was not the main source of OxA accumulation, and the activity of oxalate oxidase (OxO) involved in OxA decomposition was not a limiting factor for inner OxA accumulation. Taken together, accumulation of a large amount of OxA are not related to the degradation and secretion function of OxO but largely depend upon its synthetic function.

*Additional key words:* ascorbic acid, glycolate oxidase, oxalate oxidase, phosphoenolpyruvate carboxylase.

### Introduction

Oxalic acid (OxA) is widely distributed in the plant kingdom, existing in soluble/insoluble states (Franceschi and Nakata 2005). It is a final metabolite, and OxA in plants not only adjust pH (Davies and Asker 1983) and Ca<sup>2+</sup> content within cells (Franceschi and Nakata 2005), but it is also related to nitrogen metabolism (Tian *et al.* 2008), ionic balance (Franceschi and Horner 1980), and other functions. In addition, it plays a crucial role in plant resistance to aluminum and heavy metals such as lead (Yang *et al.* 2000), copper (Mazen 2004), and cadmium (Hassan *et al.* 2008). Some plants (such as buckwheat, taro, stylo, and rice) exude and/or accumulate OxA or other organic acids *in vivo* to detoxify aluminum and lead (Cassol *et al.* 2016, Ma *et al.* 1997, Ma and Miyasaka 1998, Yang *et al.* 2000). Although there have been some reports concerning the relationship between OxA and

environmental stresses, the relation of OxA to salt-alkali stress has rarely been addressed.

*Kochia sieversiana* is a strongly salt/alkali-resistant forage plant preferred for treating the alkalized grasslands in northeastern China (Ma *et al.* 2011). Its young seedlings and seeds can be also used for traditional Chinese medicine (Zhai *et al.* 1996). Oxalic acid may be an important inner factor in the adaptability of *K. sieversiana* to salt/alkali. However, so far, the dynamics of OxA accumulation in *K. sieversiana* plants under salt and alkali stresses are not well understood. Clarification of the characteristics of OxA accumulation and its metabolic pathways would have an important scientific and economic value in saline/alkaline grassland management and recovery, and provide possibilities for acquisition and utilization of critical salt resistant genes.

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*Abbreviations:* GO - glycolate oxidase; ICL - isocitrate lyase; L-AA - L-ascorbic acid; OA - organic acid; OxA - oxalic acid; OxO - oxalate oxidase; PEPC - phosphoenolpyruvate carboxylase.

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To mimic the salt components and characteristics of alkalized soil in northeast China where *K. sieversiana* is widely distributed, two neutral ( $\text{NaCl} + \text{Na}_2\text{SO}_4$ ) and alkaline ( $\text{NaHCO}_3 + \text{Na}_2\text{CO}_3$ ) salt combinations were applied. The accumulation of OxA and the variation of

some OxA-related enzymes were dynamically monitored to provide a theoretical basis for ascertaining the special physiological mechanisms of the adaptability of *K. sieversiana* to salt/alkali stresses.

## Materials and methods

**Plants and treatments:** *Kochia sieversiana* (Pall.) C.A. Mey. seeds were collected from alkalized grasslands located in western Jilin Province, China ( $44^\circ40' - 44^\circ45' \text{N}$ ,  $123^\circ44' - 123^\circ47' \text{E}$ ). They were sown in 17-cm diameter plastic pots containing 2.5 kg of washed sand. Each pot contained 16 seedlings, and the seedlings were sufficiently watered with half-strength Hoagland nutrient solution every two days after emerging. Evaporated water was replenished with distilled water as needed. All experiments were performed outdoors from the middle of April to the end of May, and the seedlings were protected from rain.

To mimic the salt components and characteristics of alkaline soil in northeast China, two neutral salts were mixed at a 1:1 molar ratio ( $\text{NaCl} : \text{Na}_2\text{SO}_4$ ) and applied to the salt-stress group. Two alkaline salts were mixed in the same 1:1 molar ratio ( $\text{NaHCO}_3 : \text{Na}_2\text{CO}_3$ ) and applied to the alkali-stress group. The total salt concentration was 200 mM for both stress groups. This ensured that the concentration of  $\text{Na}^+$  and total ions for the salt- and alkali-stress groups were identical but possessed different pH and also ensured that the pH of the alkali stress solution was similar to that of the typical *K. sieversiana* habitat (9.80 - 10.10, Ma *et al.* 2011). The Hoagland nutrient solution was used for the control group. The pH of the salt stress, alkali stress, and control group solutions were 6.58, 9.96, and 6.56, respectively. The seedlings were subjected to stress treatments when they were six weeks old. Each pot was considered a single replicate, and each set contained three replicates. The plants were watered with 500 cm<sup>3</sup> of the nutrient solutions (with or without the salts) per pot twice a day, and samples were collected at 8, 16, 24, 36, 48, 72, 96, and 144 h.

The plants were washed with tap water followed by distilled water, and the water on the surface of the plants was blotted with filter paper. Fresh mature leaves (0.2 g) were collected and mixed separately to measure the activities of OxA-related enzymes. The other 5 g of shoots was freeze-dried, then analyzed to determine the content of organic acids (OAs), and  $\text{Na}^+$  and  $\text{K}^+$  ions. The other fresh samples were oven-dried at 85 °C for 15 min, then vacuum-dried at 40 °C to constant mass, and total dry mass (DM) was determined. Water content was calculated using the formula  $(\text{FM} - \text{DM})/\text{FM} \times 100$  and expressed as a percentage (Yang *et al.* 2008).

**Determination of organic acids and inorganic ions:** Samples of the freeze-dried plant material (100 mg) were mixed with 10 cm<sup>3</sup> of deionized water, ground, and then centrifuged at 3000 g for 10 min. The supernatant was collected and blended three times before it was added to the deionized water to bring the volume to 50 cm<sup>3</sup>. Oxalic acid was determined using ion chromatography (DX-300 ion chromatographic system; AS4A-SC ion-exchange column, CDM-II electrical conductivity detector, mobile phase:  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3 = 1.7/1.8 \text{ mM}$ ; Dionex, Sunnyvale, CA, USA). Other OAs (citric acid, malic acid, acetic acid, formic acid, lactic acid, and succinic acid) were also determined with DX-300 (ICE-AS6 ion exclusion column, AMMS-ICE II suppressor, mobile phase: 0.4 mM heptafluorobutyric acid).

An atomic absorption spectrophotometer (TAS-990, Purkinje General, Beijing, China) was used to determine  $\text{K}^+$  and  $\text{Na}^+$  content. The content of total L-ascorbic acid (L-AA) was detected with 2,4-nitrophenylhydrazine using a colorimetric method (Wang *et al.* 2004).

**Assay of enzyme activities:** Fresh leaf tissue samples (0.2 g) were ground fully in a chilled mortar with 2 cm<sup>3</sup> of a 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM  $\text{MgCl}_2$ , 0.1 % (v/v) Triton X-100, 10 % (v/v) glycerol, and 5 mM ethylenediaminetetraacetic acid disodium salt on ice. The homogenate was centrifuged at 15 000 g and 4 °C for 5 min. The supernatant was decanted and used for enzyme assays.

Glycolate oxidase (GO, EC 1.1.3.1) was assayed as described by Booker *et al.* (1997) with some modifications. A 1 cm<sup>3</sup> of assay mixture contained a 50 mM Tris-HCl buffer (pH 7.8), 10 mM phenylhydrazine HCl (pH 6.8), and 0.05 cm<sup>3</sup> of the enzyme extract. After incubating at 30 °C for 5 min, 0.1 cm<sup>3</sup> of glycolic acid (15 mM, neutralized to pH 7.0 with KOH) was added to start the reaction. Glycolate oxidase activity was determined by following the formation of glyoxylate phenylhydrazone (the coefficient of absorbance of 16.8 mM<sup>-1</sup> cm<sup>-1</sup>) at room temperature at 324 nm for 2 min after an initial lag phase of 1 min.

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was assayed as described by Yang *et al.* (2004) with slight modifications. A 1 cm<sup>3</sup> of reaction mixture contained 100 mM Tris-HCl (pH 8.4), 5 mM  $\text{MgCl}_2$ , 100 mM  $\text{NaHCO}_3$ , 25 mM phosphoenolpyruvate (PEP), 0.2 mM NADH, and 2 U of malate dehydrogenase (EC

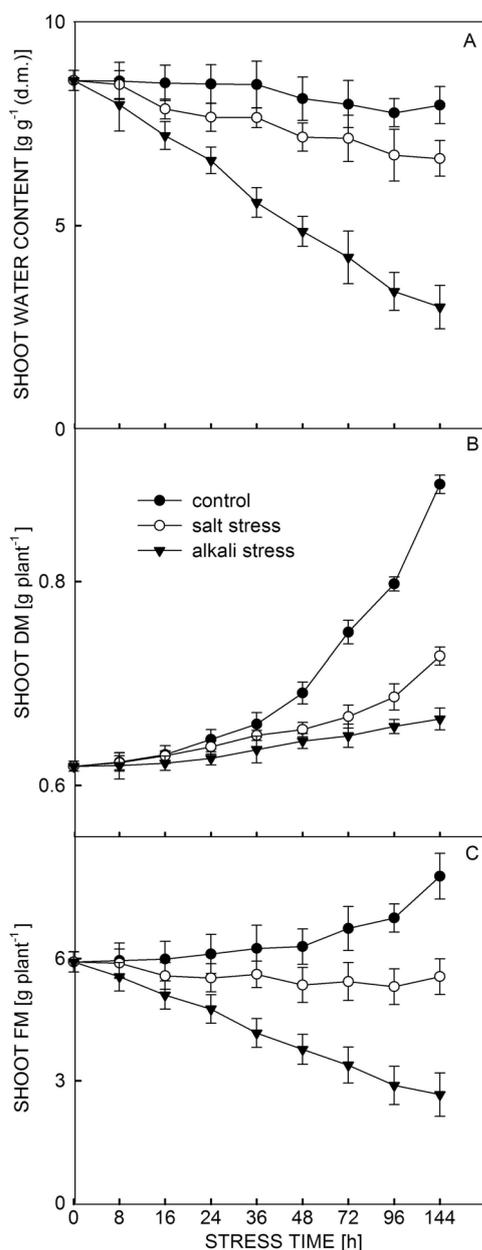


Fig. 1. Effects of salt and alkali stresses for 0 - 144 h on water content (A), dry mass (DM, B), and fresh mass (FM, C) in the shoots of six-week-old seedlings of *Kochia sieversiana*. Means  $\pm$  SEs,  $n = 3$ .

## Results

Water content of *K. sieversiana* shoots decreased under the salt or alkali stress ( $P < 0.01$ ) and the decrease was significantly greater under the alkali stress than under the salt stress (Fig. 1A). Nevertheless, the shoots maintained a relatively high water content (75 %) for a long period of the alkali stress (144 h). As *K. sieversiana* is not a succulent plant, it is worthwhile to study the plant specific mechanisms of salt and alkali tolerance.

1.1.1.37) (*Sigma-Aldrich*, Shanghai, China). After incubating at 30 °C for 10 min, 0.05 cm<sup>3</sup> of the enzyme extract was added to start the reaction. The activity of PEPC was determined by monitoring the disappearance of NADH at 340 nm at room temperature for 3 min.

Isocitrate lyase (ICL, EC 4.1.3.1) was assayed as described by Brock *et al.* (2001) with a slight modification. A 1 cm<sup>3</sup> of reaction mixture contained 2 mM 2-methyl-isocitrate, 2 mM MgCl<sub>2</sub>, 10 mM phenylhydrazine hydrochloride, 2 mM dithiothreitol, and 50 mM potassium phosphate (pH 7.0). After incubating at 25 °C for 10 min, 0.05 cm<sup>3</sup> of the enzyme extract was added. Isocitrate lyase activity was determined by following the formation of glyoxylate phenylhydrazone (the coefficient of absorbance of 16.8 mM<sup>-1</sup> cm<sup>-1</sup>) at room temperature at 324 nm for 4 min after an initial lag phase of 1 min.

Oxalate oxidase (OxO, EC 1.2.3.4) was assayed as described by Thakur and Goyal (2000) with some modifications. A 2 cm<sup>3</sup> of reaction mixture contained 80  $\mu$ M sodium succinate (pH 5.0), 1  $\mu$ M CuSO<sub>4</sub>, 1 mM OxA, and 0.15 cm<sup>3</sup> of the enzyme extract. After incubation at 40 °C for 5 min, 1.0 cm<sup>3</sup> of a colour reagent [50 mg of 4-aminophenazone, 100 mg of phenol, and 1 mg of horseradish peroxidase (EC 1.11.1.7; *Sigma-Aldrich*) per 100 cm<sup>3</sup> of a 0.4 M sodium phosphate buffer, pH 7.0] was added and maintained at room temperature in the dark for 15 min. Absorbance at 520 nm was recorded, and the amount of H<sub>2</sub>O<sub>2</sub> was calculated from a standard curve.

When the above mentioned enzyme activities were measured, the reaction mixture without enzyme extract was considered as a blank, and protein content of the enzyme extracts was determined by the method of Bradford (1976).

**Statistical analyses:** Statistical analysis of the data, which involved data processing and one-way analysis of variance, was performed using the SPSS v. 13.0 software (Chicago, IL, USA). The mean values were compared by a post-hoc least significant difference test. All of the acquired data were represented by the average of the three replicate measurements and a standard error (SE). The significance level was  $\alpha = 0.05$ . *F* values were calculated by analysis of variance.

Compared with the control ( $F = 25.43$ ,  $P < 0.01$ ), dry mass of *K. sieversiana* shoots rose slowly with increasing time under the salt stress ( $F = 5.62$ ,  $P < 0.01$ ), whereas the dry mass increased insignificantly under the alkali stress ( $P > 0.05$ ) (Fig. 1B). The fresh mass of shoots increased significantly with increasing time in the control ( $P < 0.01$ ), but increased insignificantly under the salt stress ( $P > 0.05$ ) and even decreased under the alkali

stress ( $P < 0.01$ ) (Fig. 1C). This may be related to the decreased water content under the alkali stress. Taken together, the salt/alkali stresses inhibited the growth of *K. sieversiana*, and the effects of the alkali stress were more severe than the salt stress.

Variations in content of OxA, malic acid, L-AA, and total organic acids were detected in shoots under the salt and alkali stresses (Fig. 2). In the control, OxA content did not change, however, under the stresses it increased significantly with increasing stress duration ( $P < 0.05$ ). The maximum content of OxA under the alkali and salt stresses was 3.02-fold and 1.28-fold higher, respectively, than in the control. In other words, both the salt stress and the alkali stress promoted accumulation of OxA to a various extent. In addition, our experiments indicate that OxA was not detected in *K. sieversiana* root exudates

under the salt/alkali stresses (data not shown). In the control, malic acid content was similar to that of OxA (Fig. 2B). Under the alkali stress, malic acid content increased sharply to the highest level at 96 h; however, its content decreased at 144 h. The maximum content of malic acid under the salt and alkali stresses was 2.07-fold and 14.5-fold higher, respectively, than in the control. So, the effect of the alkali stress on malic acid accumulation was much greater than that of the salt stress. Content of total L-AA was greater under the alkali stress than in the control (except at 144 h) (Fig. 2C). Although content of L-AA was low in *K. sieversiana* ( $< 2\%$  of OxA), L-AA could be considered the most important antioxidant as it plays an important role in protection against oxidative stress.

Content of  $K^+$  and  $Na^+$  showed no significant changes

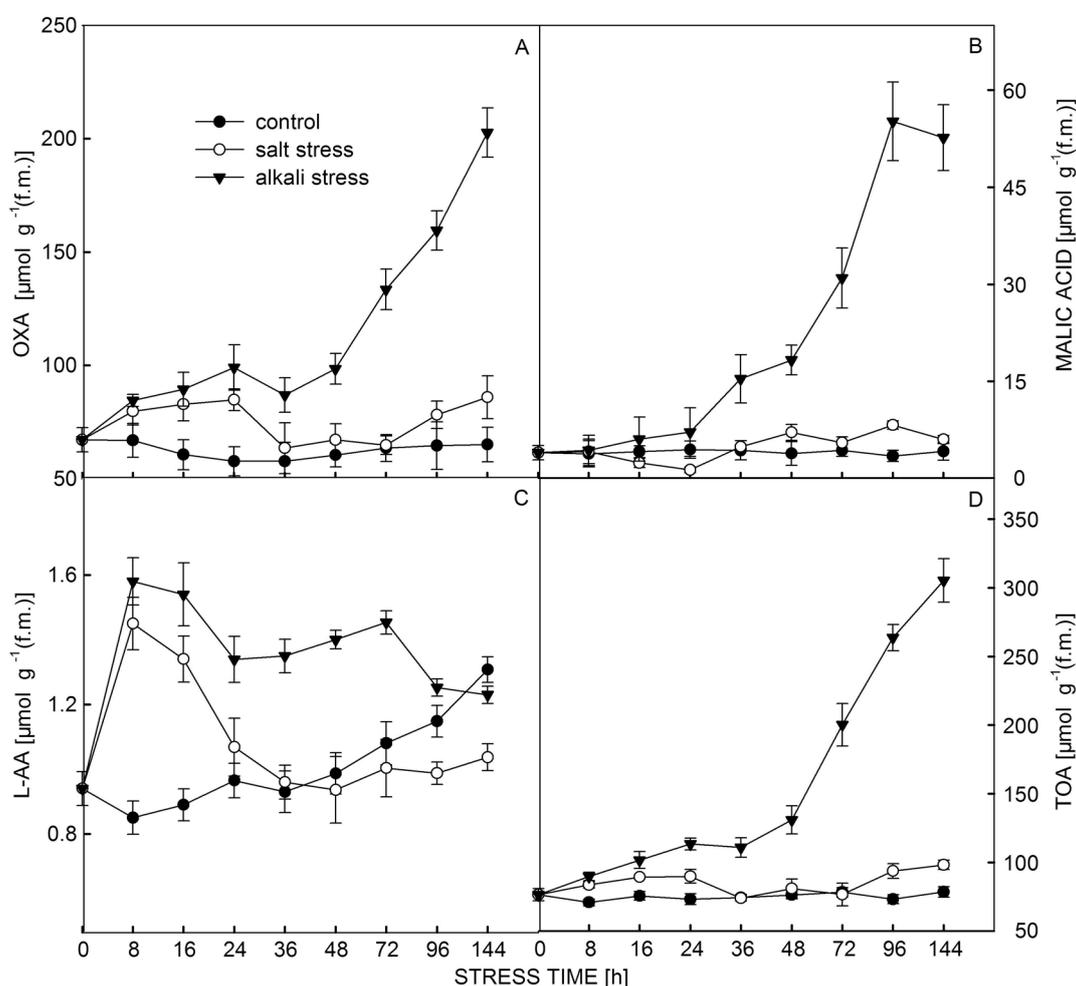


Fig. 2. Effects of salt and alkali stresses (0 - 144 h) on content of oxalic acid (OxA, A), malic acid (B), L-ascorbic acid (L-AA, C), and total organic acids (TOA, D) in shoots of *K. sieversiana*. Total organic acids include: OxA, citric acid, malic acid, acetic acid, formic acid, lactic acid, L-AA, and succinic acid. Means  $\pm$  SEs,  $n = 3$ .

in the control, however,  $Na^+$  content increased significantly ( $P < 0.01$ ) with increasing stress duration (Fig. 3A). The effects of both the stresses on  $Na^+$  content were similar up to 48 h, whereas the alkali stress

promoted more  $Na^+$  absorption than the salt stress after 48 h ( $Na^+$  content was 6.0-fold and 10.7-fold higher under the salt and alkali stresses than in the control at 144 h, respectively.) The variation of  $K^+$  content was more

complex in response to the salt and alkali stresses (Fig. 3B). Content of  $K^+$  increased initially, then decreased, and remained unchanged after 36 h under the salt stress; however, under the alkali stress, its content increased initially within 24 h, decreased shortly at 36 h, and then increased after 48 h.

Activities of OxA metabolism-related enzymes (GO, ICL, PEPC, and OxO) remained unchanged in the control. However, under both the stresses, activity of GO showed a rising trend with increasing stress time (Fig. 4A). The maximum activities of GO under the salt and alkali stresses were 1.42-fold and 1.82-fold higher than those of the control, respectively. Activity of ICL under both the stresses increased sharply during 24 - 36 h

( $P < 0.01$ ) and slowly after 36 h (salt stress:  $P > 0.05$ ; alkali stress:  $0.01 < P < 0.05$ ). Activities of PEPC in leaves showed no changes within 24 h under the salt and alkali stresses ( $P > 0.05$ ), but increased sharply after 24 h ( $P < 0.0001$ ), and the maximum activities were 5.85-fold and 2.67-fold higher than those of the control (Fig. 4C). Therefore, the salt stress promoted a higher activity of PEPC than the alkali stress. When the seedlings were subjected to the salt or alkali stresses for 8 h, OxO activities in leaves increased but they decreased with subsequent time. The minimum activities at 144 h decreased to 38.72 and 51.50 % of the control, respectively. Therefore, the inhibition of OxO activity by the salt stress was greater than by the alkali stress.

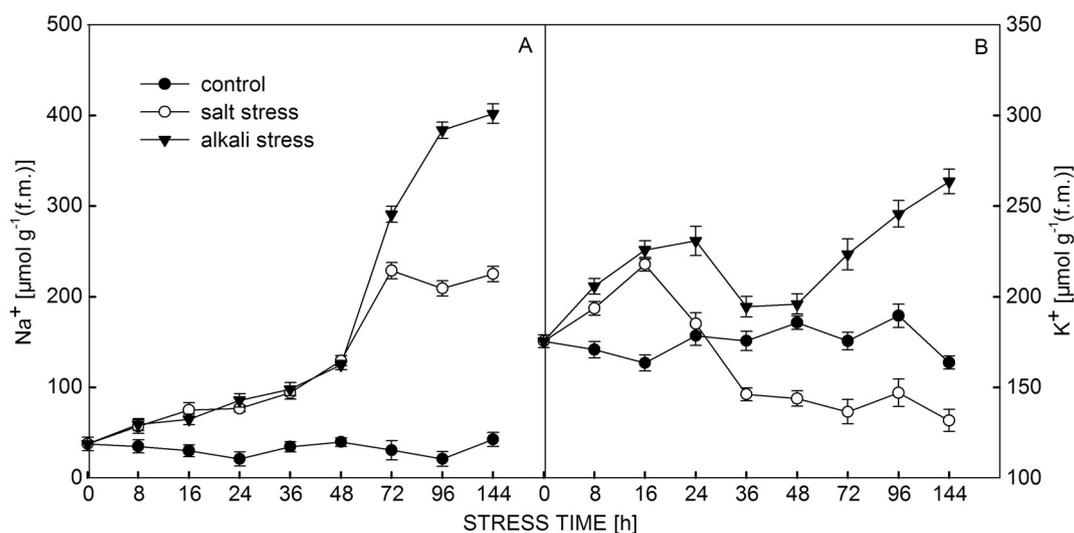


Fig. 3. Effects of salt and alkali stresses (0 - 144 h) on content of  $Na^+$  (A) and  $K^+$  (B) in shoots of *K. sieversiana*. Means  $\pm$  SEs,  $n = 3$ .

## Discussion

The most common and remarkable effect of salt stress is inhibition of growth (Naliwajski *et al.* 2014, Xu *et al.* 2014). Both the salt stress and the alkali stress adversely affected accumulation of fresh and dry masses in *K. sieversiana*, and the alkali stress inhibition was more apparent. Alkali stress has more complex and critically destructive effects on the environment than salt stress (Ma *et al.* 2011). *K. sieversiana* definitely consume more nutrients and energy to adapt to alkali stress; thus, the growth inhibition is more severe. This can be one of the most important survival strategies of *K. sieversiana* in response to stress.

Reducing water content is a rapid and efficient pathway for plants to respond to osmotic stress through a decrease in water potential (Lissner *et al.* 1999) and *K. sieversiana* was no exception (Fig. 1A). However, compared with *Phragmites australis* (Lissner *et al.* 1999), *K. sieversiana* maintained a relatively high water content over the stress time (up to 144 h).

Content of OxA was high (5.78 % of DM) under the control conditions, and OxA accumulation was stimulated by the salt and alkali stresses, especially the alkali stress (Fig. 2A). Accumulation of OxA may be due to a greater accumulation of  $Na^+$  and  $K^+$  (Reid and Smith 2001). Correlation analysis shows that the accumulation of OxA under the alkali stress was positively related to massive influxes of  $Na^+$  and  $K^+$  ( $R = 0.957$  and  $R = 0.870$ , respectively;  $P < 0.01$ ). A previous research has shown that the percent contribution of OxA to osmotic adjustment is the second after that of  $Na^+$  and  $K^+$  under salt and alkali stresses (Ma *et al.* 2011). Therefore, OxA was the most dominating counter ion to  $Na^+$  and  $K^+$ . Consequently, it is one of the important substances contributing to osmotic adjustment in *K. sieversiana*.

Oxalic acid is an ideal proton ( $H^+$ ) donor for an effective decrease of intracellular pH (Raven 1977). Our experimental results indicate that *K. sieversiana* tissue could maintain a constant pH around 6.6 - 6.7, when the

plants were under the salt or alkali stress (Ma *et al.* 2011). Being the main organic acid, OxA plays an important role in pH regulation (Davies and Asker 1983). Overall, OxA possibly plays an important role in contributing to osmotic adjustment and maintaining stable pH in *K. sieversiana*, and this is particularly important when plants are under alkali stress.

In *K. sieversiana* shoots, OxA is a key substance on which plant adaptability to salt/alkali stresses is based, and it accumulates mainly in mature leaves (Ma *et al.* 2011). Leaves are thought to be the main sites where OxA is synthesized (Libert and Franceschi 1987). Although the biosynthesis pathways are not entirely clear in plants, there are various metabolic pathways in nature (Fig. 5, Chakraborty *et al.* 2013). Therefore, activities of OxA-metabolic enzymes were determined only in mature leaves in this study.

Glycolate oxidase exists extensively within photosynthetic organisms, and it oxidizes glycolate to glyoxylate, and also glyoxylate to OxA (Millerd *et al.* 1962). Additionally, ICL can catalyze isocitrate cleavage. Activities of GO and ICL increased significantly ( $P < 0.01$ ) at 24 h of the salt stress and especially of the alkali stress. Correlation analysis indicates that activities of GO and ICL in leaves had significant and positive relations with content of OxA in shoots, however, the former ( $R = 0.726$ ) was more relevant than the latter ( $R = 0.511$ ). Therefore, GO could be considered as an important factor in OxA accumulation in plants. This is in agreement with some previous reports (Libert and Franceschi 1987) but not in agreement with Xu *et al.* (2006).

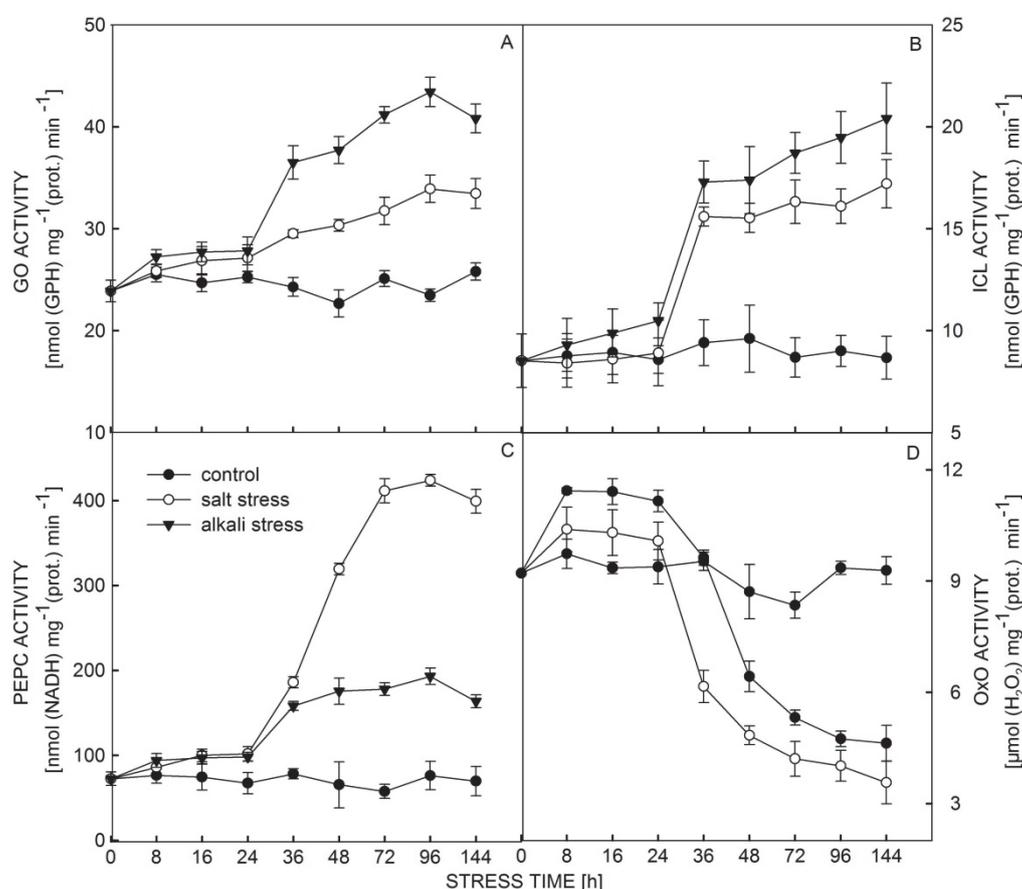


Fig. 4. Effects of salt and alkali stresses (1 - 144 h) on activities of oxalic acid-metabolizing enzymes in leaves of *K. sieversiana*: A - glycolate oxidase (GO); B - isocitrate lyase (ICL); C - phosphoenolpyruvate carboxylase (PEPC); D - oxalate oxidase (Oxo). (GPH - glyoxylate phenylhydrazine). Means  $\pm$  SEs,  $n = 3$ .

L-ascorbic acid is cleaved to yield OxA and L-(+)-tartaric acid in many plants (Debolt *et al.* 2007, Zhang *et al.* 2007). Nevertheless, biochemical data for this proposed pathway are lacking so far (Kazumi *et al.* 1992). No L-(+)-tartaric acid can be detected in any part

of *K. sieversiana* (Ma *et al.* 2011), and there is no negative correlation between the changes in content of OxA and L-AA ( $R = 0.227$ ). Moreover, content of L-AA in *K. sieversiana* shoots is very low (less than 2%). Taken together, the pathway of L-AA catabolism is

not the main source of OxA accumulation (Davey *et al.* 2000) also in *K. sieversiana*, however, this is not consistent with other previous studies (Kostman *et al.* 2001, Franceschi and Nakata 2005).

Activity of PEPC in leaves of the C<sub>4</sub> plant *K. sieversiana* was high in the control conditions and still increased significantly at 24 h of the salt and alkali stresses (Fig. 4C;  $P < 0.0001$ ). Activity of PEPC was significantly higher under the salt stress than under the alkali stress (Fig. 4C). An increased PEPC activity under salt or alkali stress may reflect the need of plants for an increased production of carbon skeletons in order to regulate osmotic potential and to maintain cell ionic neutrality by synthesis of OxA (Bouthour *et al.* 2012). In C<sub>4</sub> plants, PEP can be carboxylated by PEPC into oxaloacetate, which can be catalyzed by oxaloacetate hydrolase into OxA (Chang and Beevers 1968). However, under the stress conditions of this experiment, there was a negative correlation between the content of OxA and the activity of PEPC in leaves ( $R = -0.216$ ). Malate is also the precursor of OxA (Libert and Franceschi 1987), and the

content of OxA was positively associated with that of malate ( $R = 0.927$ ), which is the exact opposite of the report by Libert and Franceschi (1987), in which malate and OxA content in *Rheum officinale* leaves are negatively correlated with each other. Taken together, the possibility that either oxaloacetate or malate is the precursor of OxA can be ruled out. Therefore, PEPC is not a key enzyme in OxA accumulation.

Overall, the existence of an OxA biosynthetic pathway in plants remains controversial, but more researchers tend to believe that the predominant biosynthetic pathway of OxA may vary from species to species (Xu *et al.* 2006). Synthesis and accumulation of OxA may be mainly promoted *via* increasing GO activity in *K. sieversiana*. However, it has been reported that OxA is an inhibitor of GO (Burrows and Tyril 2012), and OxA synthesized in large amounts may be adverse to continuous OxA synthesis *via* GO. In fact, newly-formed free OxA or a soluble form in leaves can always form insoluble calcium OxA, which helps to synthesize and accumulate OxA continuously.

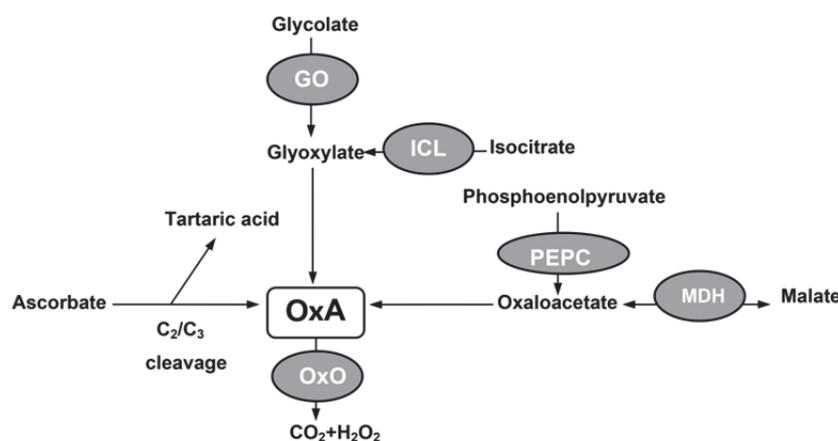


Fig. 5. Oxalic acid (OxA) biosynthesis. Glycolate, glyoxylate, isocitrate, oxaloacetate, and ascorbate are the precursors of OxA synthesis in plants. GO - glycolate oxidase, PEPC - phosphoenolpyruvate carboxylase, ICL - isocitrate lyase, OxO - oxalate oxidase, MDH - malate dehydrogenase.

Other studies have shown that OxA is not an end product of metabolism and continues to be oxidized and degraded into H<sub>2</sub>O<sub>2</sub> and CO<sub>2</sub> (Lane *et al.* 1993). However, OxO may be an important OxA-degrading enzyme in plants (Chen *et al.* 2007). Oxalate oxidase was the only enzyme whose activity decreased under the salt/alkali stresses, and the inhibition of OxO activity by the salt stress was greater than by the alkali stress (Fig. 4D). In other words, OxA had a slower decomposition rate under the salt stress than under the alkali stress, whereas content of OxA in shoots was significantly lower under the salt stress than under the alkali stress (Fig. 2A). Correlation analysis also shows that there was a minor negative correlation between the content of OxA and the activity of OxO ( $R = -0.242$ ), and OxA decomposition was not a limiting factor for inner OxA accumulation. Moreover,

H<sub>2</sub>O<sub>2</sub>, which is formed by OxO using OxA as substrate, induces expression of some related genes such as salt-resistant genes (Hurkman and Tanaka 1996) and heavy metal-resistant genes (Berna and Bernier 1999) to increase plant resistance. The effects of alkali stress on plants are more severe than salt stress, and a higher activity of OxO under alkali stress can induce more H<sub>2</sub>O<sub>2</sub>, which might be related to an increased alkali-resistance by inducing expression of some related genes. This hypothesis deserves further investigation.

In conclusion, content of OxA in the plant tissue was controlled by its synthesis, degradation, and secretion. Oxalic acid in *K. sieversiana* was synthesized in leaves, and its accumulation was mainly determined by activity of GO. Activity of OxO involved in OxA decomposition was not a limiting factor for inner OxA accumulation.

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