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Full Length Research Paper

Physiological and biochemical responses of halophyte *Kalidium foliatum* to salt stress

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In this study, the physiological and biochemical responses of a halophyte *Kalidium foliatum* to salinity were studied. In order to reflect salt-tolerance in *K. foliatum* and to analyze the physiological and biochemical mechanism for its salt tolerance, salinity threshold and biochemical parameters were studied. A halophyte, *Suaeda glauca*, which has strong salt resistance, was selected as a control and the changes in soluble sugar, malondialdehyde (MDA), proline, Na^+ and K^+, Na^+/K^+ ratio, and activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) were investigated. Four months old *K. foliatum* seedlings were subjected to 0, 0.15, 0.30, 0.45, 0.60, 0.75 and 0.90 M NaCl for 7 days. Salinity increased Na^+ content in *K. foliatum*, while Na^+/K^+ ratio did not quite change. Salinity also increased Na^+ content in *S. glauca*; however, Na^+/K^+ ratio changed with the increase of NaCl concentration. With the increase of NaCl concentration, MDA content in *K. foliatum* decreased, however, MDA content in *S. glauca* increased and this was related to enhanced activities of SOD, CAT and APX enzymes in *K. foliatum.* Content of soluble sugar and proline were lower in *K. foliatum* than in *S. glauca*. All these results suggest that there are different salt resistant metabolisms between *K. foliatum* and *S. glauca*, and salt treatments and salinity tolerance of *K. foliatum* might be closed related to increased capacity of antioxidative system to scavenge reactive oxygen species, stable absorption of K^+ and maintain Na^+/K^+ ratio.

Key words: *Kalidium foliatum, Suaeda glauca*, salinity stress, halophyte, salinity threshold, physiological adaptations.

INTRODUCTION

A study on global land use pattern revealed that 7% of the world's land areas, amounting to 1 000 million hectares, had become saline (Tester and Davenport, 2003; Munns, 2005), and the affected areas are still increasing. Irrigation systems are particularly prone to saline soil; about half of the existing irrigation lands in the world are under salt stress (Szabolcs, 1994). Salt stress severely impairs productivity and quality of products. High salt stress disrupts homeostasis in plant water potential and ion distribution at both cellular and whole plant level when plants grow under saline environment, which this in turn causes damage to biological molecules, growth arrest and even death (Munns and Tester, 2008).

Kalidium foliatum is a halophyte with succulent leaves. It widely distribute over South-East European, Central and Southwest Asia, Russia (South Siberia), East China, Mongolia and Kazakhstan (Ashraf, 1994). It can survive and grow well in high-saline conditions. However, there is little report on physiological characteristics of salt resistance in *K. foliatum*. Therefore, as part of our effort to explore some halophytes that have higher tolerance to salt as potential tool in combating saline soil, we attempted to evaluate the effects of salt stress on the halophyte *K. foliatum* using a halophyte *Suaeda salsa*, which has higher tolerance to salt as control. The results

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might help us to better understand physiological and biochemical mechanisms of salt tolerance in *K. foliatum*.

MATERIALS AND METHODS

Seeds of *K. foliatum* and *S. glauca* were collected from heavily saline soil in Bayannaoer City, Inner Mongolia, China and were grown in washed sand (pH 7.0) in a growth chamber with 30 000 lx light intensity for a period of 4 months. The seeds of each species were grown in 306 pots, in which 210 pots were for measurement of physiological parameters and 96 pots were for study of salinity threshold. Ten seeds per pot were cultivated on the Hoagland's nutrient solution with NaCl, evaporated water was replenished with Hoagland's nutrient solution every 2 days and also with distilled water when needed. The NaCl treatments in Hoagland's nutrient solution were: Control (no salinity treatment), 0.15, 0.30, 0.45, 0.60, 0.75 and 0.90 M NaCl. The experiment was arranged with four replicates. Temperatures during the experiment were in the range of 20 to 25℃ during the day and 18 to 20℃ at night. After 7 days of growth in salt treatment, plants were carefully removed from the hydroponics and washed with distilled water. All the experiments were repeated three times.

Salinity threshold

After 4 months growth, healthy and vigorous seedlings were selected for experimental treatments with 0.15, 0.30, 0.45, 0.60, 0.75, 0.90, 1.05, 1.20, 1.35, 1.50, 1.65, 1.80, 1.95, 2.10, 2.25, 2.40, 2.55, 2.70, 2.85, 3.00, 3.15, 3.30, 3.45, 3.60, 3.75, 3.90, 4.05, 4.20, 4.35 and 4.50 M NaCl for 12 days, and survival ratio of two halophytes was counted.

Organic osmotica

Total soluble sugars

Total soluble sugars were determined with anthrone reaction. Each 1.0 g of fresh leaf tissue was extracted with 25 ml of distilled water. The extract was incubated for 30 min at 100℃. The extract was used for the detection of total soluble sugars. Plant extract was mixed with 5 ml of anthrone, heated in a boiling water bath for 10 min, then cooled on ice for 10 min and incubated for 20 min at room temperature (25℃). The absorbance (OD) was read under visible light with wavelength 625 nm. Spectrophotometer (U2910, Hitachi, Japan) was used for all the following spectrophotometric reading unless specified. The soluble sugars concentration was determined from a standard curve.

Proline content

Each 1.0 g of fresh leaf tissue was homogenized with 5 ml of 3% sulfosalicylic acid and was extracted at 100℃ for 10 min. 2.0 ml of supernatant with 2.0 ml of distilled water, 2.0 ml of glacial acetic acid, 4.0 ml of acid ninhydrin solution (0.75 g ninhydrin in 30 ml of glacia acetic acid) and 2.0 ml of sulfosalicylic acid was extracted for 1 h at 100℃. The reaction was terminated on an ice bath. The reaction mixture was extracted with 4 ml of toluene mixed vigorously by passing a continuous stream of air for 2 min. Chromophore containing toluene was aspirated from the aqueous phase; 1.0 ml of chromophore containing toluene was warmed to room temperature and absorbance was read under visible light with wavelength 520 nm. The proline concentration was determined from a standard curve and was calculated on a fresh weight basis as follows:

Proline (ug) / Fresh weight (g) = (ug proline ml^{-1} × ml of toluene / 115.5) / (g of sample)

Malondialdehyde (MDA) content

MDA content was determined with thiobarbituric acid (TBA) reaction. In brief, 0.5 g of fresh leaf tissue was ground into homogenate and the homogenate was centrifuged at 3000 rpm for 10 min to get supernatant. Then 3.0 ml of 0.5% TBA was brought to 2.0 ml of supernatant for 15 min at 100℃. The reaction was terminated in an ice bath and reactant mixture was centrifugated at 1800 rpm for 10 min. The absorbance (OD) of supernatant was read under visible light with wavelength 532 and 600 nm. The concentration of MDA was calculated on a fresh weight basis as follows:

Malondialdehyde (nmol) / Fresh weight (g) = (OD532 - OD600) × A × (V / a) / 1.55×10^{-1} × W.

Where, A is the total volume of supernatant and TBA (5.0 ml); V is the total volume of resultant in reaction (5.0 ml); a is the supernatant volume (2.0 ml) and W is the Leaves' fresh weight (g).

Antioxidant enzyme activity determination

Superoxide dismutase (SOD) activity was measured spectrophotometrically as the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) at wavelength 560 nm. Fresh leaf tissue (1.0 g) with 3.0 ml of sodium phosphate buffer (50 mM, pH 7.8) was ground into homogenate on ice bath, then centrifugated at 4000 rpm at 4℃ for 50 min to get crude extract of SOD. The crude extract was shined in 3000 Lux for 15 min and then protected from light to stop reaction. The absorbance (OD) was read under visible light with wavelength 560 nm.

Ascorbate Peroxidase (APX) activity was measured spectrophotometrically as ascorbic acid (ASA) was oxidized and the absorbance (OD) of supernatant at 290 nm was read. Fresh leaf tissue (1.0 g) was ground in 3.0 ml K_2HPO_4-KH_2PO_4 buffer (50 mM, pH 7.0) into homogenate on ice bath, then centrifugated at 4000 rpm at 4℃ for 10 min to get crude extract of APX. 0.06 mM H_2O_2 was brought to reaction with 1.9 ml of K_2HPO_4-KH_2PO_4 buffer (50 mM, pH 7.0), 0.1 ml crude extract, 0.5 ml of ascorbic acid (ASA) and 0.5 ml of H_2O_2 (0.06 mM) for 30 s. The absorbance (OD) was read under an UV with wavelength 290 nm.

Peroxidase (POD) activity was determined by methyl catechol reaction. Fresh leaf tissue (2.5 g) with 4 ml phosphate buffer (50 mM, pH 5.5) was ground into homogenate on ice bath, then centrifugated in 3000 rpm at 4℃ for 10 min to get crude extract of POD. 1 ml of methyl catechol (50 mM) was brought to reaction with 2.9 ml of phosphate buffer (50 mM), 0.1 ml of crude extract and 1.0 ml of H_2O_2 (2%) for 3 min at 34℃. The absorbance (OD) was read under visible light with wavelength 470 nm.

Catalase (CAT) activity was determined by H_2O_2 reaction. Fresh leaf tissue (1.0 g) with 0.2 g of $CaCO_3$ and 2.0 ml of distilled water was ground into homogenate on ice bath, and then the homogenate was left standing to get supernatant. One ml of H_2O_2 (0.1 M) was brought to reaction with 10.0 ml of supernatant, 2.0 ml of H_2SO_4 (3.6 M), 0.2 ml of KI (20%, W/V), 0.2 ml of ammonium molybdate (10%, W/V) and 0.2 ml of starch (1%, W/V), then titrated with sodium hyposulfite (0.1 M) until achromatic color. Application amount of H_2O_2 and enzyme activity were calculated.

The contents of Na^+ and K^+

Na^+ and K^+ were measured by atomic absorption spectro-

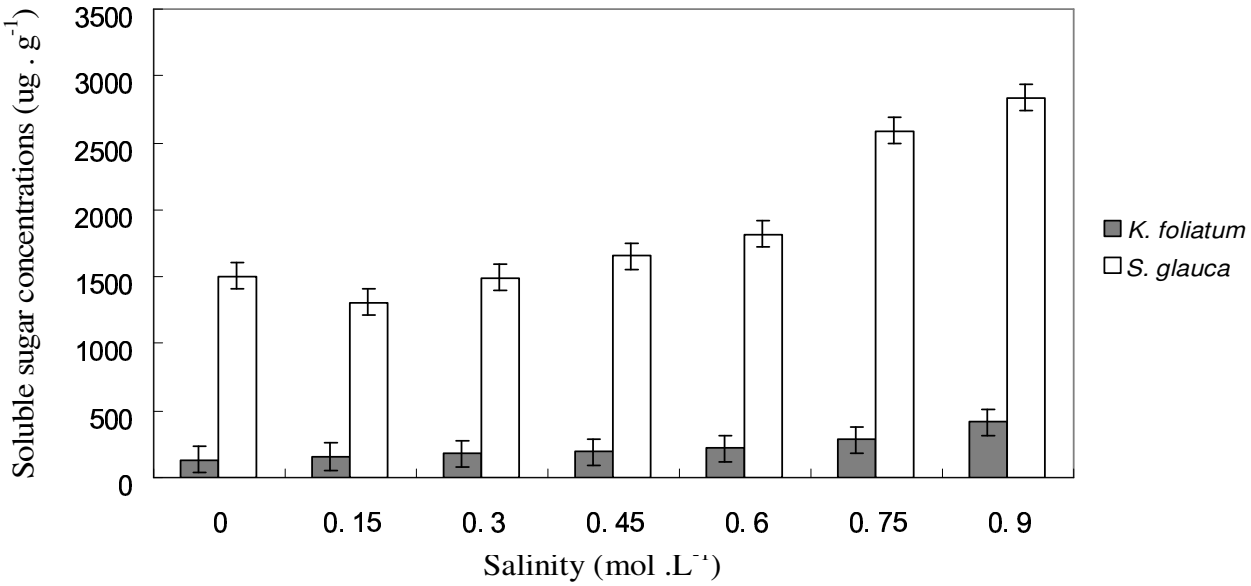

Figure 1. Effects of NaCl on soluble sugar contents in *K. foliatum* and *S. glauca*.

photometry (AAS). Fresh leaf tissue (0.2 g) was first washed with distilled water three times and then dried at 100°C to constant weight. The 10 ml of sulphuric acid was brought to reaction to nitrify into red brown at 500°C, and the H_2O_2 was added to fade (to make reaction solution clear), and then the extract was taken to determine free Na^+ and K^+ concentration. The atomic absorption spectrophotometer (Rayleigh, WFX-310, China) was used for the determination of sodium (Na) and potassium (K) concentrations with hydrochloric acid as mediator. Na^+/K^+ ratio was then calculated (Zhang and Liang, 2004).

Data analysis

All data were evaluated by one-way ANOVA using SPSS 16.0. Significance was tested at the 5% level, and Excel 2003 was used for drawing.

RESULTS

Salinity threshold

Salinity threshold is NaCl concentrations that can make 50% of plants lethally. The result shows that salinity threshold of *S.* glauca was 1.20 M NaCl, while that of *K.* foliatum was between 3.75 and 3.90 M NaCl. Hence, the salinity threshold of *K. foliatum* was significantly higher than that of *S. glauca*.

Organic osmotica

Total soluble sugar content

Total soluble sugar is an important osmotic adjustment solute. Total soluble sugar increased significantly in *S. glauca* leaves with increasing Na^+ of the grown medium, while no significant increase was found in *K. foliatum* leaves and soluble sugars contents in *S. glauca* leaves were remarkably higher than in *K. foliatum* ($P < 0.01$) (Figure 1).

Proline content

When salinity was below 0.45 M, no significant change in proline contents was observed. However, when salinity was raised above 0.45 M, the proline contents increased with rising salinity in *S. glauca*. The maximum proline contents were 2.6 times that of the control in *S. glauca* and 1.4 times that of the control in *K. foliatum*. The proline content in S. *glauca* was higher than in *K. foliatum* at all salt levels ($P < 0.05$) (Figure 2).

Malondialdehyde (MDA) content

MDA was used as an indicator of membrane lipid peroxidation, another indicator of stress in plant membranes. MDA contents in *K. foliatum* leaves decreased with increasing Na^+, while that in *S. glauca* leaves increased with increasing Na^+. MDA contents in *S. glauca* leaves were therefore remarkably higher than in *K. foliatum* leaves ($P < 0.01$) (Figure 3).

Antioxidant enzyme activity determination

The activity of superoxide dismutases (SOD)

In high salinity conditions, plants activate a number of

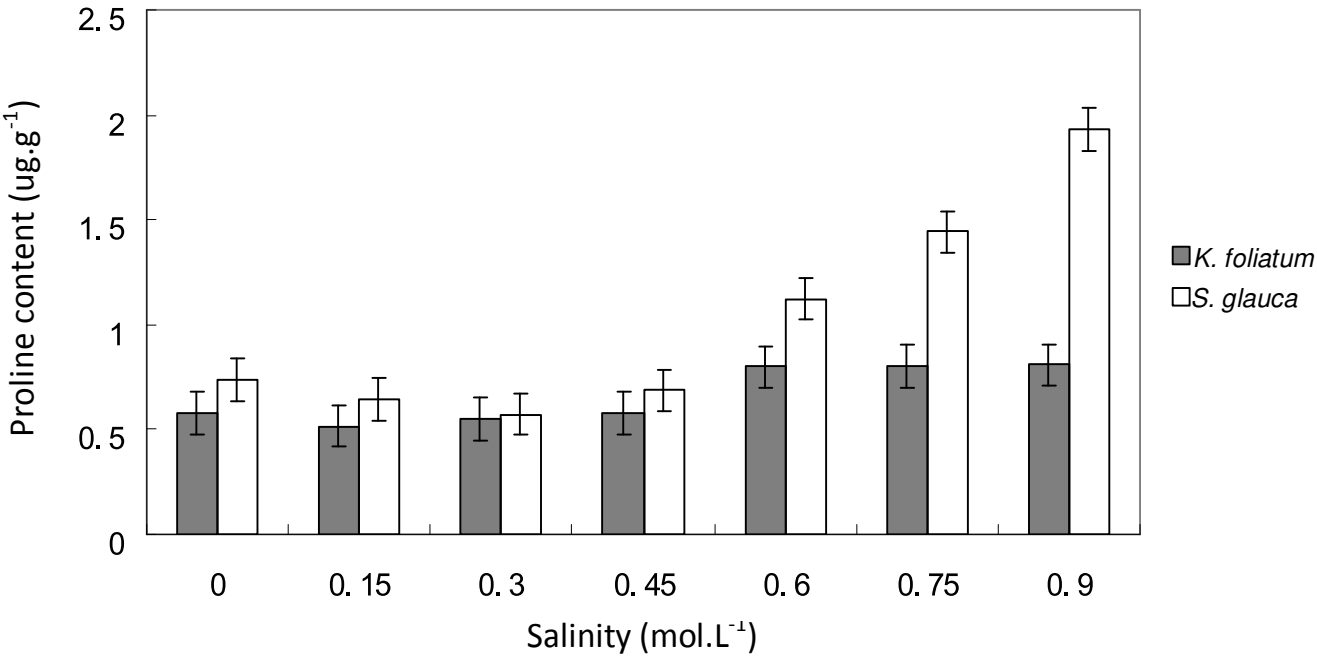

Figure 2. Proline contents in leaves of *K. foliatum* and *S. glauca* under salt stress.

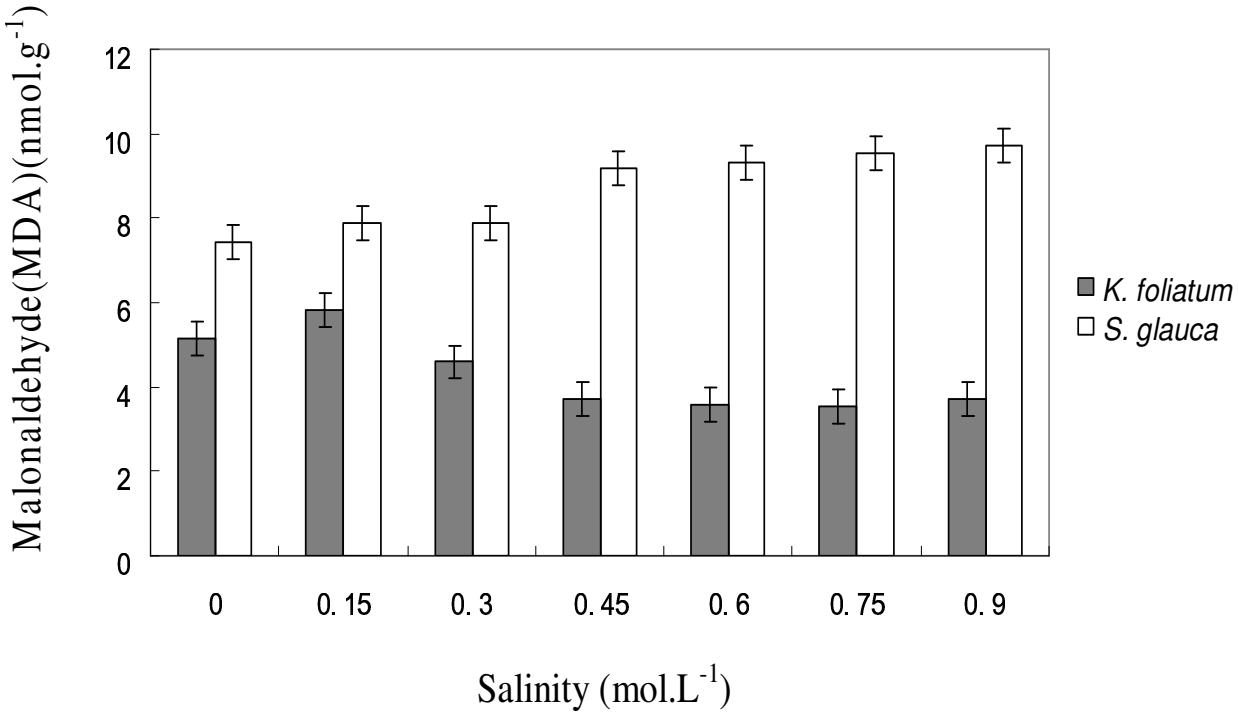

Figure 3. Malonaldehyde (MDA) contents in leaves of *K. foliatum* and *S. glauca* under salt stress.

antioxidant enzymes that protect against potentially cytotoxic reactive oxygen species (ROS). The activity of superoxide dismutases (SOD) in *K. foliatum* increased with increasing Na^+. The superoxide dismutases (SOD) activity of the control in *S. glauca* was relatively high when salinity was below 0.45 M, but decreased when salinity was raised above 0.45 M. The superoxide dismutases (SOD) activity increased with rising salinity and there was no much difference between *K. foliatum* and *S. glauca* (Figure 4).

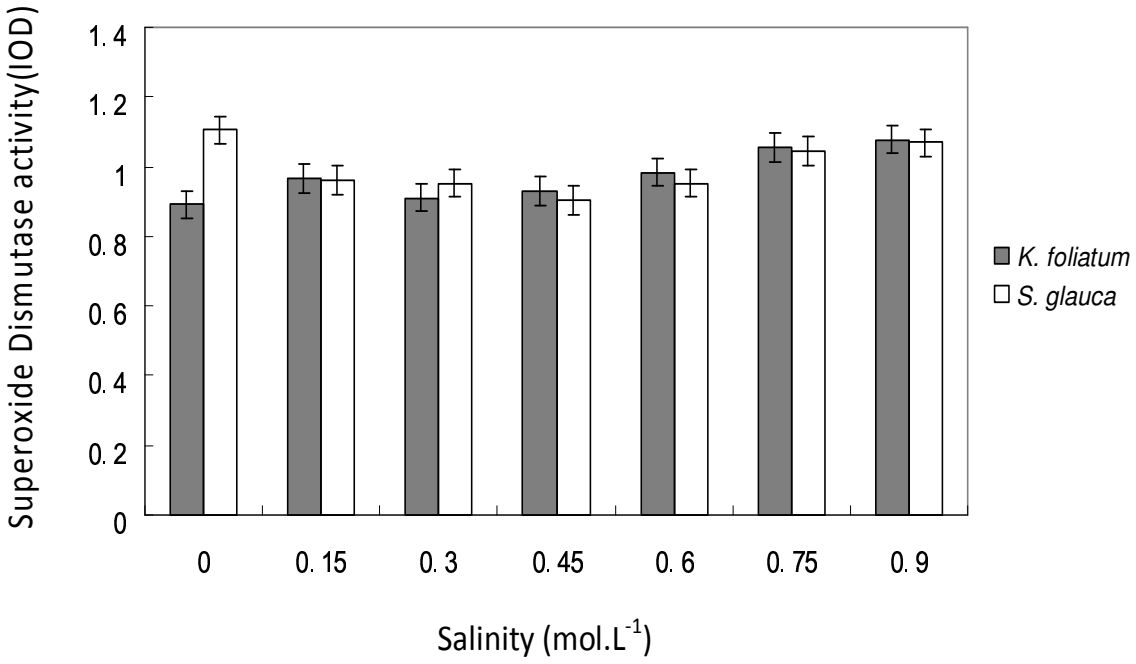

Figure 4. Superoxide dismutase activity in leaves of *K. foliatum* and *S. glauca* under salt stress. IOD: Integral absorbance.

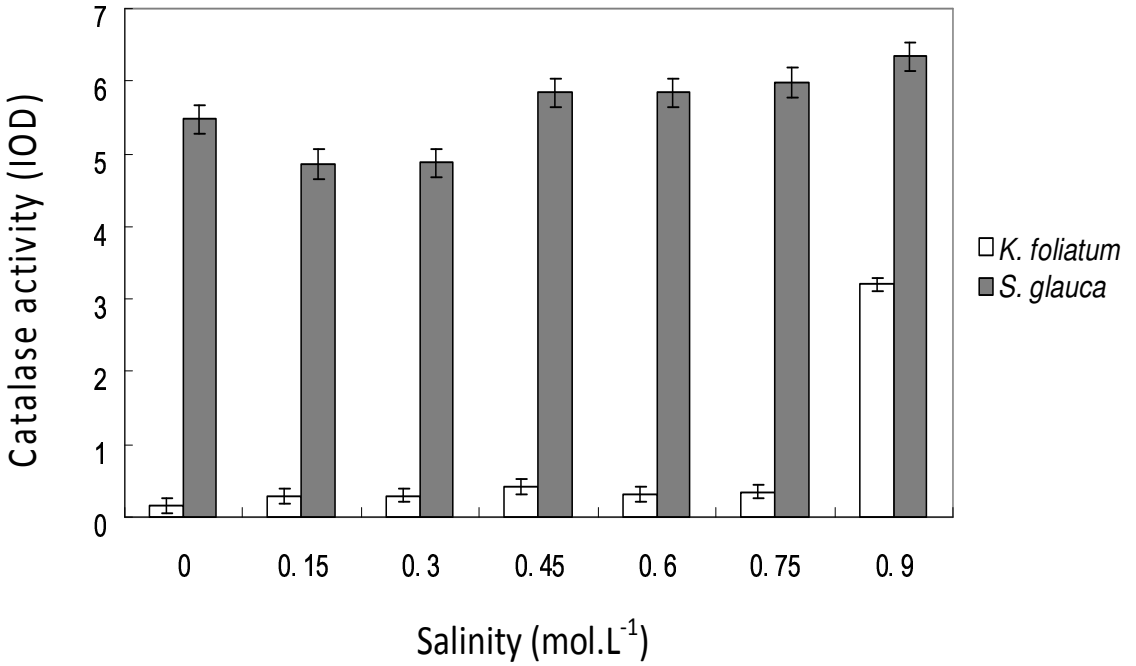

Figure 5. Catalase activity in leaves of *K. foliatum* and *S. glauca* under salt stress. IOD: Integral absorbance.

The activity of catalase (CAT)

The CAT activity of the control in *K. foliatum* was relatively high and the activity of catalase (CAT) increased with increasing Na^+. When salinity was below 0.90 M, no significant change was observed in the CAT activity of *S. glauca*, however, when salinity was at 0.90 M, the catalase (CAT) activity increased remarkably. The catalase activity in *K. foliatum* was remarkably higher than *S. glauca* ($P < 0.01$) (Figure 5).

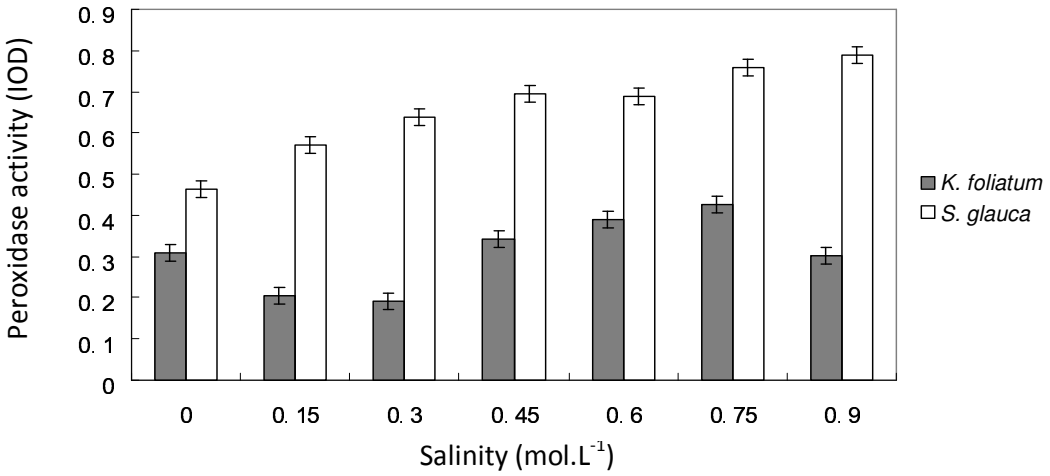

Figure 6. Peroxidase activity in leaves of *K. foliatum* and *S. glauca* under salt stress. IOD: Integral absorbance.

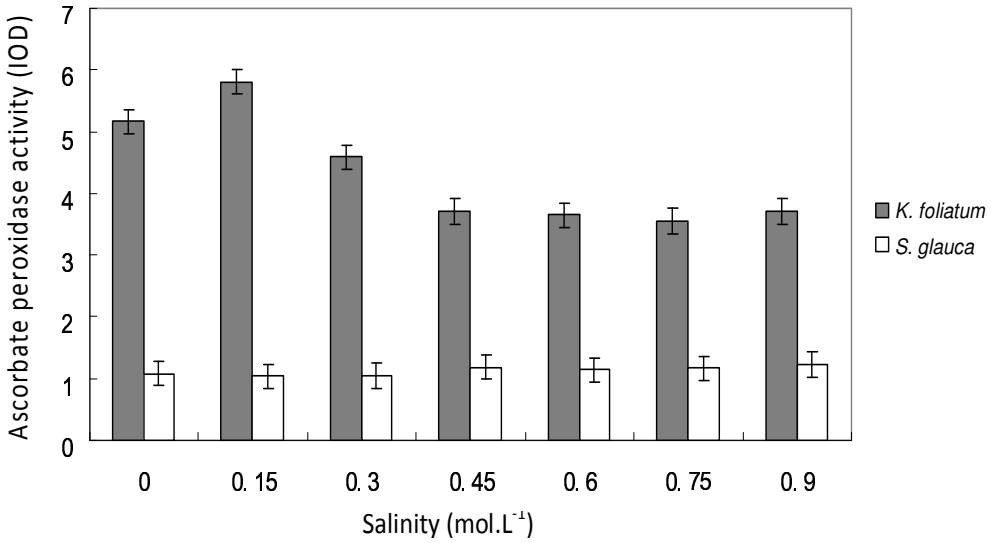

Figure 7. Ascorbate peroxidase activity in leaves of *K. foliatum* and *S. glauca* under salt stress. IOD: Integral absorbance.

The activity of peroxidase (POD)

The POD activity increased significantly in *S. glauca* with increasing Na^+. The POD activity of the control in *K. foliatum* was relatively high, when salinity was below 0.30 M, but decreased when salinity was raised above 0.30 M. Thus, its activity increased with rising salinity. The peroxidase activity in *S. glauca* was remarkably higher than *K. foliatum* ($P < 0.01$) (Figure 6).

The activity of ascorbate peroxidase (APX)

The APX activity of the control in *K. foliatum* was relatively high and decreased significantly, while the APX activity remained relatively unchanged in *S. glauca* with increasing salinity. The activity of ascorbate peroxidase in *K. foliatum* was remarkably higher than in *S. glauca* ($P < 0.01$) (Figure 7).

The contents of Na^+, K^+ and Na^+/K^+ ratio

Na^+ content of the two species increased with increasing Na^+. The overall increasing trends were similar for *K. foliatum* and *S. glauca*, and Na^+ contents in *K. foliatum* leaves were remarkably higher than in *S. glauca* leaves ($P < 0.01$) (Figure 8). The K^+ contents of the control in *S.*

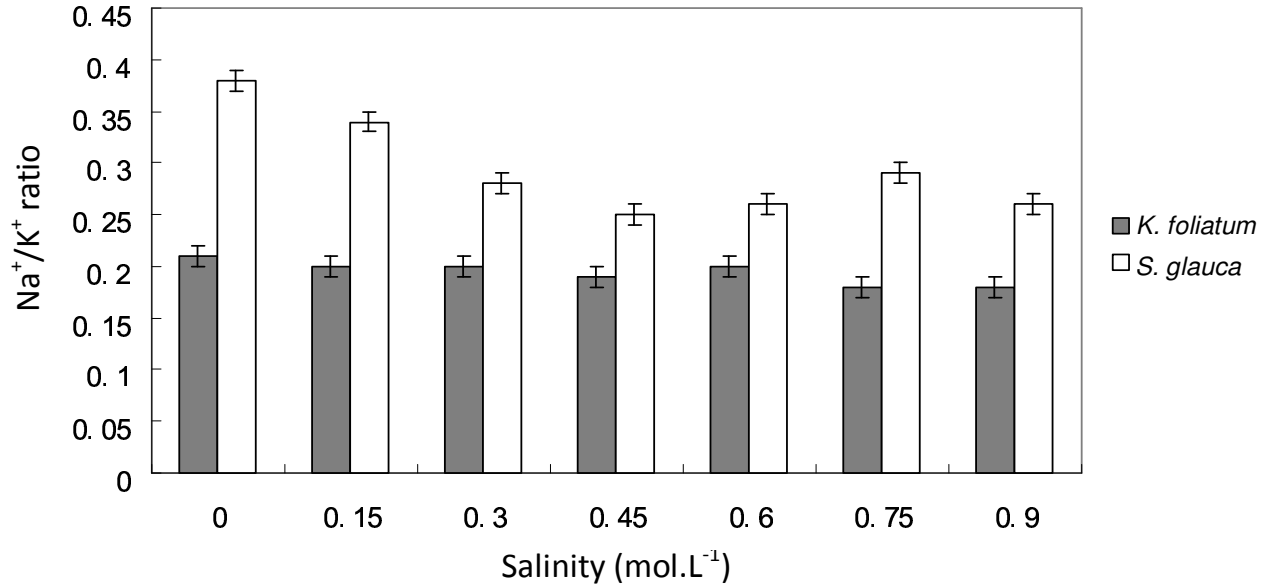

Figure 8. Na^+ contents in leaves of *K. foliatum* and *S. glauca* under salt stress.

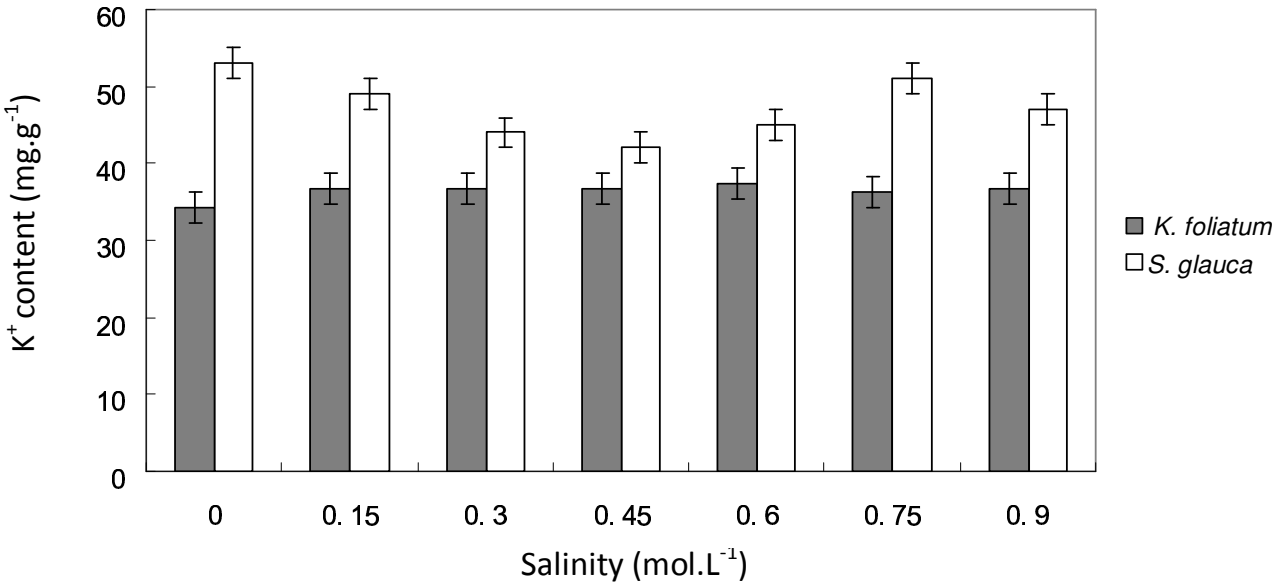

Figure 9. K^+ contents in leaves of *K. foliatum* and *S. glauca* under salt stress.

glauca were relatively high when salinity was below 0.45 M, but decreased with rising salinity when salinity was raised above 0.45 M. The K^+ contents increased with rising salinity. In contrast, no significant change was observed in K^+ contents of *K. foliatum* and the K^+ contents in *K. foliatum* leaves was remarkably lower than in *S. glauca* leaves ($P < 0.01$) (Figure 9).

The Na^+/K^+ ratio of the control in *S. glauca* was relatively high, and the Na^+/K^+ ratio also decreased with rising salinity. In contrast, no significant change was observed in Na^+/K^+ ratio of *K. foliatum*. The Na^+/K^+ ratio in *K. foliatum* was remarkably lower than in *S. glauca* ($P < 0.01$) (Figure 10).

DISCUSSION

Osmoregulation

A strong correlation between salt tolerance and total soluble sugar accumulation has been widely reported (Streeter et al., 2001; Taji et al., 2002). Total soluble

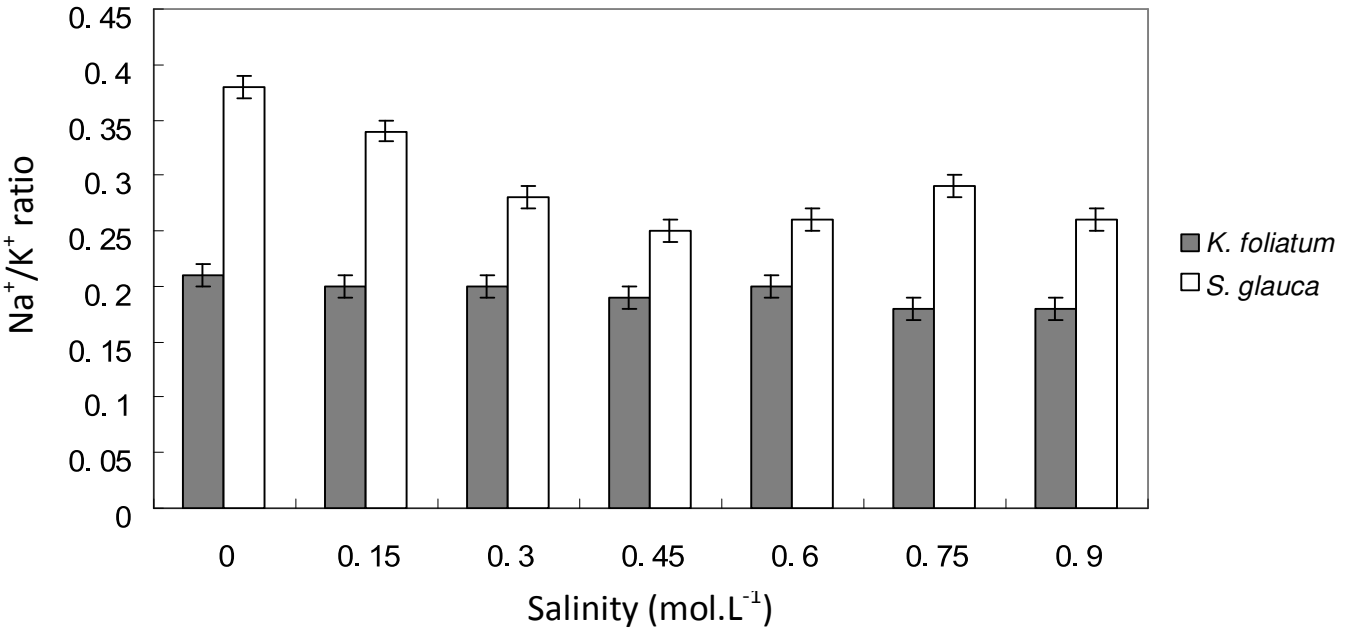

Figure 10. Na^+/K^+ ratio in leaves of *K. foliatum* and *S. glauca* under salt stress.

sugars were accumulated in relatively lower quantities in *K. foliatum* at higher salt levels than in *S. glauca*. Proline accumulation in response to environmental stresses has also been considered by a number of researchers as an adaptive trait of stress tolerance, and it is generally assumed that proline plays a considerable role in osmotic adjustment (Larher et al., 1993; Yoshiba et al., 1997). This higher proline accumulation in the salt tolerance can be appreciated as a further important factor of adaptation to salinity, as it has earlier been reported in a number of species (Ashraf, 1994; Ashraf and Harris, 2004; Mansour, 2000). *K. foliatum* showed a significantly lower accumulation of proline with increasing salt levels than did *S. glauca*.

Halophytes are also reported to accumulate large amounts of Na^+ under salt stress to perform osmotic adjustments (Moghaieb et al., 2004; Short and Colmer, 1999), simultaneously to inhibit K^+ absorption (Zhu, 2003) and to compartmentalize Na^+ into vacuoles to avoid Na^+ toxicity in cytosol (Khan et al., 2000). In the present work, we found that Na^+ content of *K. foliatum* was higher than that of *S. glauca*, while K^+ content of *K. foliatum* was lower than that of *S. glauca* and kept nearly unchanged with increasing salt concentration. This means there may be some different salt resistance mechanisms which could help *K. foliatum* to avoid the effect of increasing absorption of Na^+ on absorption of K^+. In the present work, we found that salinity threshold of *K. foliatum* was between 3.75 and 3.90 M NaCl, which was significantly higher than that of *S. glauca*, thus, depicting *K. foliatum* as a halophyte that can tolerate more salt than *S. glauca*. However, organic osmolytes such as total soluble sugars and proline in *K. foliatum* showed a significantly lower accumulation than in *S. glauca*. *K. foliatum* accumulated more Na^+ contents with salinity increase than S. glauca did, therefore, we suppose K. *foliatum* might perform mainly osmotic adjustments by Na^+ accumulation, and did not by total soluble sugars and proline.

Oxidative damage and antioxidative enzyme

Massive changes in ionic balance can cause damage to cellular membranes integrity and molecular structure due to the production of reactive oxygen species (ROS). The reactive oxygen species are highly reactive and in the absence of a protective mechanism in plants, can cause serious damage to different aspects of cell structure and function. MDA content, a product of lipid peroxidation, has been considered an indicator of oxidative damage. The halophytes depend against these reactive oxygen species by induction of activities of antioxidative enzymes such as superoxide dismutase, catalase, peroxidase and ascorbate peroxidase, which scavenge reactive oxygen species (Mittova et al., 2002).

Higher levels of SOD, CAT, POD and APX activities were detected in *K. foliatum*. This increase in the activity of antioxidative enzymes suggests its effective scavenging of the free radicals produced in *K. foliatum*. Lower MDA contents in *K. foliatum* might be associated with the minimum oxidative damage to membrane as a result of lower production of reactive oxygen species, which was found to be responsible for the reduction in the peroxidation of membrane lipids. Our results on the lower MDA content under salt stress in *K. foliatum* therefore suggest the presence of strong antioxidative defense

mechanism in *K.* foliatum.

In conclusion, the present study on the salt tolerance of K. foliatum confirms that *K. foliatum* showed high salt tolerance due to its higher activity of antioxidative enzymes, higher uptake of Na^+ accompanied by a stable uptake of K^+ and lower MDA contents. However, lower accumulation of soluble sugars and proline in *K. foliatum* tissues showed that some different salt resistance mechanism exist in *K. foliatum*. Since natural halophyte meet demands for stress tolerant plants breeding in modern agriculture, stress tolerant plants will prove very useful for revegetation of salt affected land by direct growth of such salt tolerant plants. In addition, genes from halophyte can be used for improving salt tolerance in salt sensitive plants through either conventional breeding or advanced molecular biology approaches.

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