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PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF A MEDICINAL HALOPHYTE *LIMONIUM BICOLOR* (BAG.) KUNTZE TO SALT-STRESS

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Abstract

Limonium bicolor (Bag.) Kuntze is a perennial herb belonging to the Plumbaginaceae family. It is a typical recretohalophyte as well as a medicinal plant, distributing at saline soil areas in coastal areas and grasslands. In this paper,physiological mechanisms of *L. bicolor* to defend salt stress and effects of salinity on medicinal ingredients were investigated. The effects of different NaCl concentrations on the number of salt glands, Na^+ content, dry weight and water content in tissues, gas exchange parameters involving net CO_2 assimilation rate, stomatal conductance, intercellular CO_2 concentration and transpiration rate, malondialdehydecontent and electrolyte leakage, activities of superoxide dismutase, peroxidase and catalase and accumulations of secondary metabolites such as total phenolic, total flavonoid, gallic acid and myricetrin of leaves were determined. The results show that 100 and 200 mM NaCl induced facilitated effects in *L. bicolor* reflected in the increase in dry weight, tissue water content, net CO_2 assimilation rate, the number of salt glands, activity of superoxide dismutase, and content of gallic acid and myricetrin. The 300 mM NaCl treatment resulted in obviously decline in gas exchange parameters, and significant increases in Na^+ levels, malondialdehyde level and electrolyte leakage. It was suggested that increased salt tolerance of *L. bicolor* was due to the corresponding resistancemechanisms involving an increased number of salt glands, enhanced activities of antioxidant enzymes, and an accelerated accumulation of secondary metabolites. What's more, the results on effects of salinity on medicinal ingredients in *L. bicolor* under different salt concentrations could provide theoretical basis for the standardization cultivation technique of *L. bicolor*.

Key words:*Limonium bicolor*, Plumbaginaceae, Medicinal Halophyte, Malondialdehyde content

Introduction

Soil salinization is one of the major factors limiting plant growth and development. The increased salinity of arable lands will probably lead toadevastating global effect, resulting in up to 50% landloss by the middle of the 21st century (Llanes *et al*., 2016). As salt levels in soil rise, the uptake of water by plants decreases. Moreover if roots take up large quantities of sodium and chloride ions, the plants metabolism, including photosynthesis becomes impaired, and growth is stunted (Amor*et al*., 2005; Mäser *et al*., 2002; Flowers & Yeo, 1995).

Plants have evolved various protective mechanisms to defend salt injury. With special mechanisms of salt tolerance, halophytes can resist high salt environments by osmotic adjustment, compartmentation of toxic ions, ion homeostasis, upregulation of anti-oxidative enzymes and the synthesis of secondary metabolites (Parida & Das, 2005). Cultivation of these plants ondegraded saline lands with brackish water would sparearable land and fresh water for conventional agriculture(Abideen *et al*., 2015).

Phenolic compounds, which include phenolic acid, flavonoids, tannins and phenolic diterpenes, are classed as secondary metabolites and are intermediates in the non-enzymatic phenylpropanoid pathway, which is part of the defensive armory of plants. There is much current interest in their role in enabling plants to withstand environmental stresses (Zheng *et al*., 2001). They are active against free radicals, singlet oxygen and peroxide (Oueslati *et al*., 2010). Their ability to act as hydrogen or electron donors, to stabilize and delocalize unpaired electrons and to chelate transitional metals, is reported to underlie their role as anti-oxidants (Huang *et al*., 2005). Results in some recent studies show that the synthesis of phenolics depends on abiotic factors (Oueslati *et al*., 2010). Total phenolic content in the halophytic species *Cynara cardunculus* and the glycophyte *Raphanus sativus* dramatically increased under light salt treatment (Yuan *et al*., 2010; Falleh *et al*., 2008). Conversely, phenolic content in the glycophyte *Nigella sativa* decreased significantly under salt stress (Bourgou *et al*., 2008).

Limonium bicolor (Bag.) Kuntze, belonging to Plumbaginaceae, is a typical wild halophytegrowing in coastal saline-alkalisoil areas and grasslands in many parts of China with the most remarkable characteristic to be presence of salt-secreting structures (salt glands) on leaf surface. It was reported that salt glands of *L. bicolor* have four secretory pores which secrete NaCl (Feng *et al*., 2014). Itis also well known as a folk medicine with hemostatic effects. The active pharmaceutical ingredients in *Limonium* species are complex, containing amino acids, inorganic elements, sterol, flavonoids, alkaloids, polysaccharides, organic acids, volatile oils and other ingredients. Among them, gallic acid, a phenolic acid with antibacterial, antiviral and anticancer functions, and myricetrin, a natural bioflavonoid with antioxidant properties and potent anti-carcinogen and anti-mutagenic effects, are abundant in *L. bicolor* (Xie*et al*., 2011; Ong & Khoo, 1997).However, to date there are no reported studies assessing the part played by these chemicals in how *L. bicolor* responds to salt stress. Consequently, our knowledge of the action of phenolic compounds is rudimentary. In order to study the physiological mechanisms underlying the ability of *L. bicolor* to withstand salt stress and how salinity impacts on active pharmaceutical ingredients, we studied the effects of

varying salt concentrations on the following: the number of salt glands on leaves; Na^+ content; malondialdehyde (MDA)content; electrolyte leakage (EL); superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities of leaves; the dry weight (DW), water content and gas exchange parameters involving net CO_2 assimilation rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and the transpiration rate (*E*). What's more, the levels of the total phenolic, total flavonoid, gallic acid and myricetrin were also assessed. The results would help to evaluate the key mechanisms in salt stress and their effects on medicinal halophytes.

Materials and Methods

Plant material: *L. bicolor* seeds collected from Dongying in Shandong, China, were sown in pots containing a mixture of sand and turf peat (2:1 v/v). One-month-old seedlings were transferred to plastic pots (10 plants per pot) and irrigated with half-strength Hoagland nutrient solution (Hoagland & Arnon, 1950), maintained in a greenhouse with a mean temperature of 25±3°C, a relative humidity of 80±20%, and a natural light/dark cycle. After a month of growth, 12 pots of the two-month-old seedlings of similar size were equally divided into four groups. Hoagland nutrient solution supplemented with various concentrations of NaCl (0, 100, 200 and 300 mM) was used to irrigate the seedlings. We replicated each treatment three times using a randomized block arrangement. Salt concentrations were escalated by 50 mM NaCl every 12 h in order to prevent osmotic shock. A solution with a certain concentration of NaClwas added into each pot by adding twice the volume of the water-holding capacity every seven days. On other days 100 mL of Hoagland nutrient solution without added NaCl was added to each pot.The newly formed leaves in the navel of random seedlings in each repetition were cut and sampled after 21 days of salt treatment for experiments. Every experiment was performed in triplicate.

Assay ofdry weight andwater content. After 21 days of salt treatment, entire plants were harvested, weighed immediately to ascertain their fresh weight (FW) and oven dried at 60°C until of a constant dry weight (DW). The equation $[(FW - DW)/FW]\times 100\%$ was used to determine the water content of the entire plant.

Assay of gas exchange parameters. After 21 days of salt treatment, leaf gas exchange measurements were conducted for net CO_2 assimilation rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration rate (*E*). All measurements were carried out at 10–12 h using a CO_2/H_2O-gas exchange apparatus (Li-Cor 6400, Li-Cor Inc., Lincoln, USA).

Assay of electrolyte leakage. New leaves were cut from plants, washed three times with deionized water, and cut into discs of 0.5 cm. Five discs were put in one test tube, which was filled with 10 mL deionized water and placed in a vacuum at room temperature for 2 h. The initial conductivity (C_0) was then determined using a conductivity meter (DDS-307, Inc. Shanghai REX Instrument Factory, Shanghai, China). In order to completely disrupt the leaves tissues and free their electrolytes, the test tubes were sealed and boiled for 30 min. After they were cooled to room temperature, the conductivity (C_1) was measured. The formula $EL\ (\%) = (C_0/C_1) \times 100\%$ was used to calculate the relative EL.

Assay of MDA and Na^+ content and antioxidant enzyme activities. The newborn leaves in the navel of random seedlings in each repetition were cut and sampled after 21 days of salt treatment. The levels of malondialdehyde (MDA) and Na^+ and the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were determined with kits provided by the Nanjing Jiancheng Bioengineering Institute. A spectrophotometer (UV-2600, Unico Instruments Co., Ltd., Shanghai, China) was used for spectrophotometric analyses.

Assay of the number of salt glands. The number of salt glands were counted using an impression method as described by Hilu & Randall (1984). The surface of the newborn leaves was coated with a film of transparent nail polish. After 1 h, the film was removed using Scotch tape and fixed on a glass slide to be observed with an Eclipse 50i Microscope. Under 100-fold magnification, 10 different horizons were randomly selected and the number of salt glands was observed within each field with the same size.

Assay of content of total phenolic, total flavonoid, gallic acid and myricetrin. A portion of pulverized leaf powder (0.5 g) was extracted with 50 mL of 80% ethanol under reflux for 2 h, filtered and the process repeated. The combined filtrates were evaporated under reduced pressure at 50°C, then the residue was diluted with dimethyl sulfoxide to 10 mL. Total phenolic content was estimated using Folin-Ciocalteu colorimetric method with gallic acid as standard and total flavonoid content was measured using colorimetric method with rutin as standard (Atanassova*et al*., 2011).The absorbance was measured at 765 nm and 510 nmrespectively using a spectrophotometer (UV-2600, Unico Instruments Co., Ltd, Shanghai, China). The levels of gallic acid and myricetrin were determined by an HPLC system using sample solutions prepared before and filtered through a 0.22 μm filter (Millipore) on the basis of each chromatographically pure compound as a standard. Chromatographic analysis was performed using a Waters Breeze system with a spectrophotometer detector at 254 nm.Determination was performed at gradient elution on an Inertsil ODS-SP column (5μm, 4.6×250 mm) (Zhang *et al*., 2010).

Statistical analyses. All assays were carried out in triplicate. The results are expressed as means ± standard deviation (SD). The ANOVA test using the SPSS statistical program was employed. Means were separated by Duncan's Multiple Range test at $p< 0.05$ in order to determine the significance of values.

Results

Growth and water content: DW in *L. bicolor* was prominently induced by 100 and 200 mM NaCl but no significant differences at 300 mM NaCl compared to the control (Fig. 1a). The value reached a maximum at 200 mM NaCl and a minimum at 300 mM NaCl (ca. 127% and 86% of the control value, respectively). As the NaCl concentration increased, no significant change was observed in tissue water content except for the 100 mM NaCl treatment. When the plants were treated with 100 mM NaCl, the water content increased to 108% of the control (Fig. 1b).

Gas exchange parameters: *L. bicolor* showed different photosynthetic responses between different NaCl concentrations (Table. 1). P_N value increased at moderate NaCl concentrations (100 mM and 200 mM) but decreased at high NaCl concentration (300 mM) with the maximum at 100 mM NaCl (ca. 124% of the control value). The g_s showed no significant change as the salt concentration increased. However, the $g_s$decreased when the plants were treated with 300 mM NaCl. Similar to the g_s, the C_i was also lowered at the 300 mM NaCl concentration, with a minimum of 82% of the control value. Transpiration rate (*E*) gradually declined with increasing NaCl concentration. When the plants were treated with 300 mM NaCl, the reduction was 72% compared to the control.

MDA level and electrolyte leakage (EL): No significant change was observed in MDA level as the salt concentration increased. However, when the plants were treated with 300 mM NaCl, the MDA level increased to 179% of the control (Fig. 2). Membrane permeability was determined by measuring electrolyte leakage (EL). The EL increased as the NaCl concentration increased and reached a peak at 300 mM NaCl (ca. 152% of the control) (Fig. 3).

Na^+ level and the number of salt glands: Na^+ level was kept stable in leaves at 100 and 200 mM NaCl compared with the control. However, it was significantly increased under the 300 mM NaCl concentration (Fig. 4). Meanwhile, the number of salt glands either on the adaxial or abaxial surface of *L. bicolor* leaves increased considerably as the salt concentration rose, and then significantly decreased at 300 mM NaCl (Fig. 5). The number of salt glands on the adaxial and abaxial surface was 138% and 201%, respectively, of each control at 200 mM NaCl, and was 64% and 100%, respectively of each control at 300 mM NaCl. In addition, the total number of salt glands on the abaxial surface was more than on the adaxial surface.

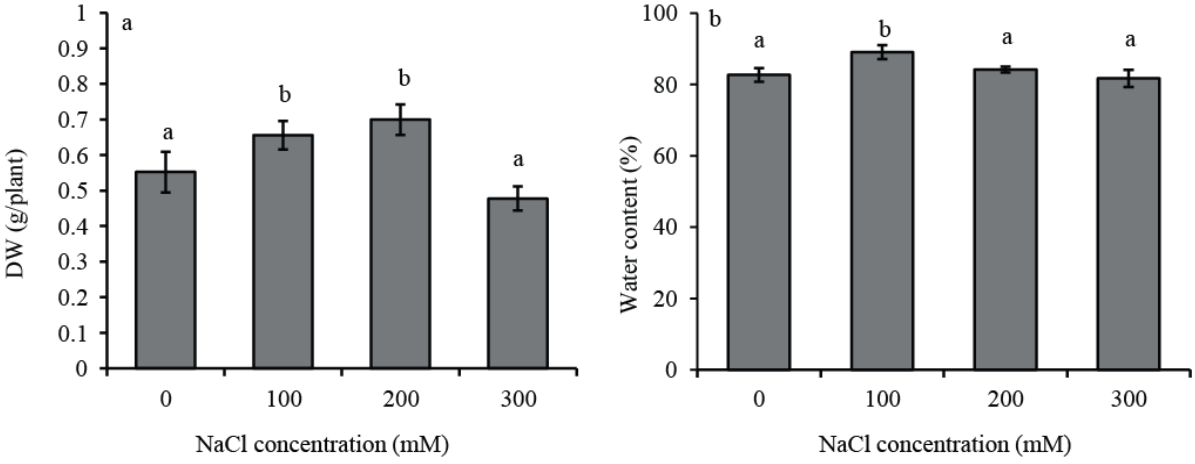

Fig. 1. Effects of NaCl on dry weight (DW) (a) and tissue water content (b) of the entire plant of *L. bicolor*. Each value is the mean ± SD of triplicates. Different letters indicate significant differences between treatments according to Duncan's multiple range test at $p < 0.05$.

Table 1. Effects of NaCl on net CO_2 assimilation rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration rate (*E*).

Treatment	P_N, mmol/(m^2 s)	g_s, mmol/(m^2 s)	C_i, µL/L	*E*, µmol/(m^2s)
Control	18.50±1.60b	0.41±0.03b	264.17±21.82b	9.25±1.73b
100 mM	23.02±1.05c	0.39±0.04b	241.67±9.42b	8.36±0.47ab
200 mM	21.52±1.88c	0.38±0.02b	255.33±24.61b	7.42±1.57ab
300 Mm	15.80±1.17a	0.30±0.03a	217.50±18.35a	6.63±0.74a

Each value is the mean ± SD of triplicates. Different letters indicate significant differences between treatments according to Duncan's multiple range at $p < 0.05$.

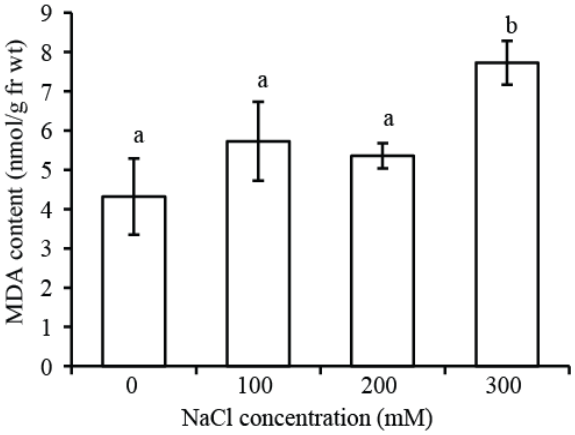

Fig. 2.Effects of NaCl on malondialdehyde (MDA) level in leaves of *L. bicolor*. Each value is the mean ± SD of triplicates. Different letters indicate significant differences between treatments according to Duncan's multiple range at $p < 0.05$.

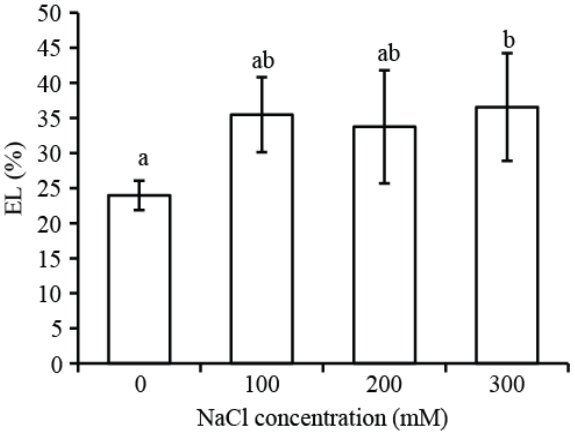

Fig. 3.Effects of NaCl on electrolyte leakage (EL) in leaves of *L. bicolor*. Each value is the mean ± SD of triplicates. Different letters indicate significant differences between treatments according to Duncan's multiple range at $p < 0.05$.

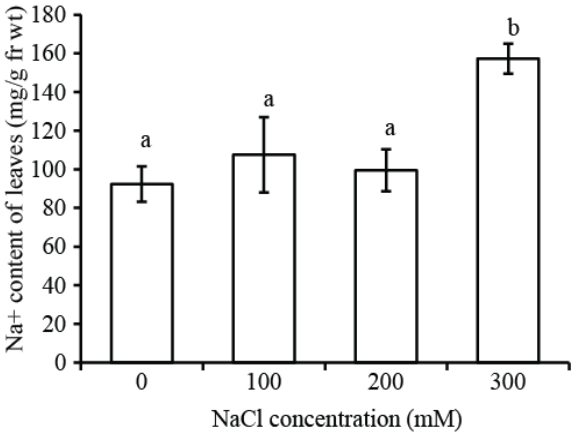

Fig. 4.Effects of NaCl on Na^+ accumulation in leaves of *L. bicolor*. Each value is the mean ± SD of triplicates. Different letters indicate significant differences between treatments according to Duncan's multiple range at $p < 0.05$.

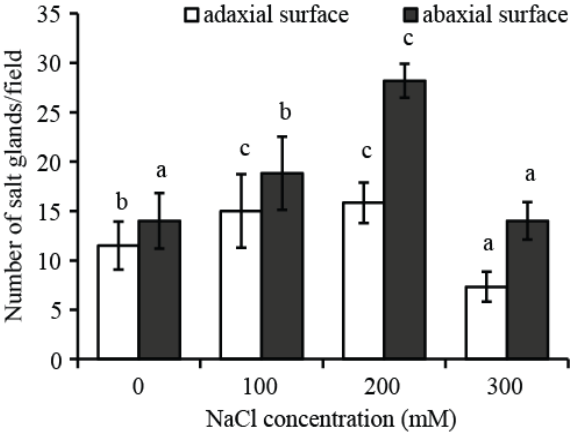

Fig. 5.Effects of NaCl on the number of salt glands on leaf surface of *L. bicolor*. Each value is the mean ± SD of ten repetitions. Different letters indicate significant differences between treatments according to Duncan's multiple range at $p < 0.05$.

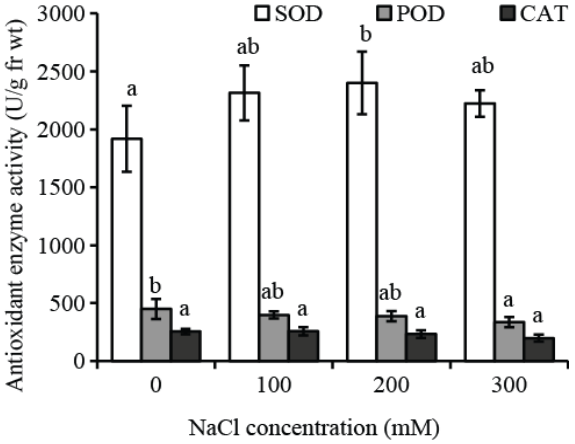

Fig. 6.Effects of NaCl on activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in leaves of *L. bicolor*. Each value is the mean ± SD of triplicates. Different letters indicate significant differences between treatments according to Duncan's multiple range at $p < 0.05$.

Antioxidantenzyme activity: As shown in Fig. 6, SOD activity increased initially, reaching peak values at 200 mM NaCl (ca. 125% of the control value). However, as the concentration of NaCl continued to rise, the activity of SOD decreased. POD activity decreased significantly at 300 mM NaCl. While CAT activity showed no obvious differences under different NaCl concentrations.

Levels of chemical components: NaCl stress induced various effects on the accumulation of different secondary metabolites in *L. bicolor*. Compared with the control, NaCl stress markedly decreased the level of total phenolics. The minimum level decreased to 79% of the control at 300 mM NaCl (Table 2). The effects of NaCl stress on total flavonoids were similar to those seen for total phenolics. When the plants were treated with 300 mM NaCl, the total flavonoid level decreased to 80% of the control. Accumulation of gallic acid and myricetrin changed significantly under different NaCl concentrations. The levels of gallic acid and myricetrin first increased and then decreased, reaching peak values (ca. 119% and 109% of the control, respectively) at 200 mM NaCl.

Table 2. Effects of NaCl on chemical component levels (total phenolic, total flavonoid, gallic acid and myricetrin) in leaves of *L. bicolor*.

Treatment	Total phenolic content, (mg/g dry wt)	Total flavonoid content, (mg/g dry wt)	Gallic acid concent, (mg/g dry wt)	Myricetrin content, (mg/g dry wt)
Control	6.54±0.20b	2.17±0.18b	0.26±0.01a	0.32±0.03bc
100 mM	6.39±0.15b	2.12±0.13b	0.29±0.02ab	0.31±0.01b
200 mM	6.31±0.28b	2.04±0.24ab	0.31±0.03b	0.35±0.02c
300 Mm	5.18±0.19a	1.74±0.17a	0.27±0.02a	0.25±0.02a

Each value is the mean ± SD of triplicates. Different letters indicate significant differences between treatments according to Duncan's multiple range at $p < 0.05$.

Discussion

Compared to glycophytes, halophytes possess specialized mechanisms to resist and utilize large amount of salt via osmotic adjustment, compartmentation of toxic ions, ion homeostasis, upregulating anti-oxidative enzyme activities and synthesizing secondary metabolites. Halophytes usually show growth stimulation at moderate NaCl concentrations that would inhibit the normal growth of glycophytes (Malcolm *et al*., 2003). Consistent with this finding, *L. bicolor* responded to 100 and 200 mM NaCl by increased biomass production (Fig. 1a).Similar results have also been reported in *Atriplexhalimus,* another halophyte (Nedjimi, 2014). Tissue water content was improved using100 mM NaCl, indicating active metabolism at this level of salinity (Fig. 1b).

As a recretohalophyte,*L. bicolor* has evolved multicellular glands underlying the protective effect. Excess Na^+ is secreted out of the plant through these salt glands. In nature there are many kinds of halophytes like *Limonium* species which secrete salt through glands, such as *Zoysia*, *Rhizophora* and *Tamarix* (Zhou *et al*., 2001). In this study, the number of salt glands on either the adaxial or abaxial surface of *L. bicolor* leaves under 100 and 200 mM NaCl treatments was significantly higher than that under 0 and 300 mM NaCl treatments at the same size of field of vision under the microscope (Fig. 5). A large number of salt glands on leaves represent a high ability to secrete redundant salt following low sodium ion accumulation in leaves, which could result in a lower stress level. However, when the NaCl concentration overruns the upper limit that salt glands can hold, the number of the salt glands cannot increase with the increasing NaCl concentration. In this research, a 300 mM NaCl concentration obviously exceeds the upper limit. This may be a reasonable explanation for results in this paper that Na^+ accumulation in leaves was much higher with the 300 mM NaCl treatment than that in other treatments (Fig. 4). An excessive accumulation of Na^+ may also be the direct reason for other physiological and biochemical indexes at 300 mM NaCl.

Plant photosynthesis provides rich material and energy for plant growth. P_N, directly reflecting the assimilation ability of CO_2 per unit area of leaf, was significantly higher in the 100 and 200 mM NaCl treatments than the control,whereas the g_s, C_i and *E*showed no significant change as the salt concentration increased (Table 1). This is in accordance with previous findings, which have shown that P_N also increases at 200 mM NaCl in another halophyte *Sesuvium portulacastrum* (Rabhi *et al*., 2012). Increased P_N provides the necessary energy for plant growth and salt gland differentiation.

MDA concentration has often been used as a tool to assess the severity of oxidative stress (Parida & Das, 2005). Our data shows that a significant increase of MDA levels was found in the highest salinity treatment (300 mM NaCl), suggesting a high level of membrane lipid peroxidation of *L. bicolor* leaves under this NaCl concentration (Fig. 2). Membrane permeability, which can express the cellular membrane dysfunction due to salt stress, was determined by measuring EL (Nedjimi, 2014). In the present study, the EL in leaves of *L. bicolor* also increased significantly with the 300 mM NaCl treatment (Fig. 3). However, there was no significant change in MDA levels and EL in the 100 and 200 mM NaCl treatments compared to the control. Unchanged lipid peroxidation and membrane permeability seem to be a characteristic of tolerant plants, adapted to salinity (Shalata *et al*., 2001). Moreover, protective mechanisms utilized by plants to deal with reactive oxygen species (ROS) involve enzymatic antioxidant systems such as SOD, POD and CAT, and antioxidant compounds such as phenolic acids and flavonoids. SOD is an important scavenger of $O_2^{\cdot-}$.As a result of its enzymatic action, H_2O_2 and O_2 are produced. POD changes H_2O_2 to water in chloroplasts, and CAT eliminates H_2O_2 in the cytosol (Parida & Das, 2005; Zheng *et al*., 2001; Oueslati *et al*., 2010). In this study, SOD activity first increased and then decreased (Fig. 6). In contrast, there was no increase in POD and CAT activity under any NaCl treatment (Fig. 6). This could be explained by them not being active participants in the detoxification of ROS in *L. bicolor* under salt stress. Similar results have been found in other salt-treated plants, *Limonium aureum* (Linn.) Hill and *Daucus carota* L. (Bano *et al*., 2014; You *et al*., 2013).

Apart from the antioxidant enzymes, the involvement of phenolic compounds in stress alleviation has also been suggested. Phenolic compounds, including phenolic acids and flavonoids, are widely distributed in plants. They comprise a substantial group of organic compounds, with many and wide-ranging biological activities (Oueslati *et al*., 2010).Phenolic compounds

possess anti-oxidative properties, and their synthesis depends on abiotic factors.In traditional herbal medicine, the total phenolic levels and total flavonoid levels in*Cynara cardunculus* L. were enhanced by NaCl treatment compared to the control (Borgognone *et al*., 2014).In the present study, both total phenolic and total flavonoid levels showed no obvious change at the increased salt concentrations, except for the 300 mM NaCl treatment (Table 2). Accumulation of the two types of compounds under different salt concentrations showed the same trend, which is contrary to the trend for MDA (Fig. 2). This indicates that as more phenolic and flavonoid compounds accumulate, the less oxidative damage cell membranes of leaves suffered. Gallic acid and myricetrin, with antioxidant capacity, were abundant in *L. bicolor as* active pharmaceutical ingredients.In the present study, accumulation of gallic acid and myricetrin changed significantly under different NaCl concentrations (Table 2). Results show that the optimum NaCl concentration for the accumulation of gallic acid and myricetrin is 200 mM. From the results it can be observed that a 200 mM NaCl treatment which induced high biomass of *L. bicolor*, also facilitated the accumulation of gallic acid in the plant. These results indicate that gallic acid in *L. bicolor* may contribute to the plant growth under salt stress. However, further investigations are required to determine when and where phenolic compounds in *L. bicolor* are important in terms of resistance mechanisms.

In conclusion, the present study demonstrates the physiological and biochemical impacts of salt stress in the leaves of *L. bicolor*.*L. bicolor*, as a recretohalophyte, showed growth stimulation at moderate NaCl concentrations, and demonstrated a self-adjusting ability of Na^+ accumulation by its salt glands. However, a sharp reduction in the number of salt glands with the 300 mM NaCl treatment resulted in a high NaCl concentration that exceeded the upper limit that salt glands could hold. This caused excess Na^+accumulation, which resulted in decreases in gas exchange parameters, total phenolic levels and total flavonoid levels and significantly increased levels of MDA and EL. Results suggest that100 and 200 mM NaCl constitute the appropriate salt levels for the growth of *L. bicolor* and accumulation of gallic acid and myricetrin, which supports the assumption that plants response to stress are partly dependent on the systems for the production of secondary metabolites. In addition, similar togallic acid and myricetrin, SOD activity also showed a maximum at 200 mM NaCl. Finally, it seems apparent that as the salt concentration increased the corresponding resistantmechanisms gradually formed in *L. bicolor* including the increased number of salt glands, enhanced enzyme activities, and possibly also the accumulation of some secondary metabolites. What's more, there is an interesting hint that content of phenolic compounds in *L. bicolor* leaves may be raised through NaCl treatment. Researching on the production mechanisms of these secondary metabolites in *L. bicolor* under different salt concentration could provide theoretical basis for the standardization cultivation technique of *L. bicolor*.

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