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MECHANISM OF SALT TOLERANCE IN *Vitex trifolia* LINN. var. *simplicifolia* CHAM: ION HOMEOSTASIS, OSMOTIC BALANCE, ANTIOXIDANT CAPACITY AND PHOTOSYNTHESIS

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ABSTRACT

Vitex trifolia Linn. var. *simplicifolia* Cham is a medicinal aromatic plant and perennial halophyte growing in the coastal areas around the Bohai Sea in China. The aim of this study was to investigate the salt tolerance mechanisms of *V. trifolia* when subjected to different concentrations of NaCl (0, 90, 180, 270, 360 and 450 mM) by measuring growth parameters, ion contents, proline, soluble sugar, soluble protein, malondialdehyde (MDA), photosynthetic pigment contents, chlorophyll fluorescence parameters and antioxidant enzyme activities. The plants died when the NaCl concentration reached 450 mM 20 days after salt stress. Biomass and shoot growth were inhibited by increasing salinity, while root growth was promoted at a NaCl concentration ranging from 90 to 270 mM. Na^+ and Cl^- accumulation was markedly promoted in both leaves and roots with increasing salinity, while no significant changes were observed in the K^+ concentration and K^+/Na^+ ratio in the leaves. Proline, soluble sugar and soluble protein contents increased significantly with increasing salinity. In order to eliminate the reactive oxygen species (ROS) produced by salt-induced oxidative stress, the activities of peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) were enhanced. Photosynthetic pigment contents and PSII activity did not significantly decrease under salt stress. The results indicate that the mechanism of salt tolerance in *V. trifolia* are by ion homeostasis, osmotic balance, antioxidant enzyme induction and photosynthesis adjustment.

Key words: antioxidant enzymes, ion homeostasis, osmotic balance, photosynthesis, salt tolerance

ABBREVIATIONS: RGR – relative growth rate; RWC – relative water content; FW – fresh weigh; TW – turgid weight; DW – dry weight; ROS – reactive oxygen species; MDA – malondialdehyde; SOD – superoxide dismutase; POD – peroxidase; CAT – catalase; APX – ascorbate peroxidase; Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; Chl – chlorophyll; Car – carotenoid; PSII – photosystem II; Fv/Fm – maximal photochemical efficiency of PSII in the dark; Yield – quantum efficiency of PSII; qP – photochemical quenching; qN – non-photochemical quenching

INTRODUCTION

Soil salinization is a serious environmental problem in many arid and semi-arid areas of the world and is responsible for decreasing crop productivity. It is reported that approximately 950 million ha of the world's land is affected by salinity [Zhao et al. 2013]. China accounts for 9.6% of the total (99 million ha)

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salinity-affected lands. The accumulation of salt in the soil severely affects the morphology, physiology and metabolism of plants, which reduces species and distribution of plants, ultimately leading to the loss of ecological function. As a substitute for saline soil remediation, more attention has been paid to the utilization and exploitation of halophytes due to their salt tolerance characteristics and ecological and potential economic values.

The plant characteristic for surviving and maintaining normal growth in a saline environment is called salt tolerance, and the salt tolerance is controlled by several factors including ion homeostasis, osmotic balance, antioxidant enzyme induction, photosynthesis adjustment, and sometimes a combination of these mechanisms. For halophytes, ion homeostasis and compartmentalization of toxic ions among the various tissues and the intracellular space are the most important salt tolerance mechanisms [Huchzermeyer et al. 2013]. The ability to compartmentalize Na^+ and Cl^- is considered the primary component of salt tolerance in halophytes [Shabala et al. 2013]. To cope with ionic toxicity, halophytes reduce Na^+ and Cl^- toxicity to the cytoplasm by sequestering them either into the outside apoplast or primarily into the vacuole [Xu et al. 2014]. In addition, the retention of K^+ in the cytoplasm is also an important factor for maintaining ion homeostasis [Percey et al. 2016].

ROS results as a secondary stress of high salinity, when the latter impairs the cellular metabolism. ROS over-production accelerates the decomposition of chlorophyll and reduces the activities of related enzymes during carbon assimilation, leading to a decline in plant photosynthesis, which affects normal plant growth and development [Singh et al. 2015]. To regulate the osmotic imbalance caused by excessive accumulation of salts in the soil solution, which highly reduces soil water potential, the accumulation of compatible solutes such as proline, soluble sugar and soluble protein in cytoplasm decrease cellular water potential to create a water potential gradient required to maintain water absorption going on in spite of high salinity [Shabala et al. 2011]. To prevent oxidative stress response caused by salt stress, plants have developed ROS scavenging mechanisms by enhancing enzymatic and non-enzymatic antioxidant enzyme activity [Flowers et al. 2008]. These ROS clearance mechanisms are used to maintain the structural integrity of cells and the normal functioning of various metabolic pathways.

Salinity also affects photosynthesis by reducing intercellular CO_2 concentrations caused by stomatal closure and ROS accumulation [Chaves et al. 2009], leading to a reduction in photosynthetic pigments and PSII activity. In many studies, a reduction in photosynthetic pigments has been reported due to pigment degradation and biosynthetic damage [Muchate et al. 2016]. It has also been reported that one reason for the reduction in PSII activity is plant exposure to excess energy, which has inevitable consequences on PSII if the dissipation mechanisms are not efficient enough [Qiu et al. 2003].

V. trifolia is a perennial halophyte distributed widely in coastal saline soil areas around the Bohai Sea in China. It belongs to Vitex in Verbenaceae and is a traditional aromatic and medicinal plant that has been widely studied for its aroma and medical use. Its natural habitat indicates that it has many outstanding properties in terms of salt tolerance. However, there is no report indicating how this species responds and adapts to salinity stress. For these reasons, *V. trifolia* was selected as a research material in the present study. With this aim, we determined *V. trifolia* growth, ion contents, osmolytes, lipid peroxidation, changes in the activities of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) and photosynthetic characteristics under salinity stress.

MATERIALS AND METHODS

Plant materials and treatment. Seeds from *V. trifolia* were collected from Binzhou city in Shandong Province (117°51'21.41"E and 38°15'52.73"N) in and around the Bohai Sea coastal region in China. The seeds were sterilized with 2% sodium hypochlorite for 5 min and rinsed thoroughly with deionized water. Then, the seeds were placed in Petri dishes with two filter papers (diameter 90 mm) that were kept moist. These Petri dishes were placed in climate chambers at 25°C for germination. The seedlings were transferred to plastic pots (130 × 97 × 116 mm) containing a potting mix (50% peat moss and 50% vermiculite; pH 6) when the two cotyledons had just fully expanded and

the pots were then placed in a greenhouse. The growth conditions were: a mean air temperature of 20–25°C, a 16-h photoperiod, and an average of 150 μmol·m^{-2}·s^{-1} of photosynthetically active radiation, a relative humidity of 45–65%.

After two months, healthy and uniform seedlings were selected and treated with various concentrations of NaCl. The salt was added to a quarter-strength Hoagland solution to provide final concentrations of 0 (control, ck), 90, 180, 270, 360, and 450 mM NaCl. To avoid osmotic shock, the higher NaCl concentrations (≥90 mM) were reached incrementally by 30 mM steps per day. The seedlings were subjected to 800 mL of 0, 90, 180, 270, 360 or 450 mM NaCl added to a quarter-strength Hoagland solution applied on days 1, 10, and 20. Salt treatment was maintained for 30 days. Thirty replicate seedlings were used in the experiments for each NaCl concentration, and each parameter was measured at least three times using five replicate seedlings. A completely randomized design was used in all the experiments.

Measurement of growth parameters. The root length of five seedlings were measured, and the shoot length of the other five seedlings were measured before the salinization experiment started. The experiment was repeated five times. The lengths of roots and shoots were measured once again after 30 days of salt treatment from five replicate seedlings. Next, the whole plants were oven dried at 105°C for 10 min and then dried at 70°C to a constant weight. The biomass was measured using an analytical balance. The relative growth rate (RGR) of shoot and root was determined according to the following formula of Hoffmann et al. [2002]:

$$RGR = (\ln G_2 - \ln G_1) / t_2 – t_1 \ (cm \cdot cm^{-1} \cdot day^{-1}),$$

where G_2 is the final crown width and root length, G_1 is the initial crown width and root length, $t_2–t_1$ is the duration of the experiment (days).

Measurement of relative water contents. Fresh leaf samples were collected, and their fresh weight (FW) was immediately taken. Then, the leaves were submerged in deionized water for 24 h to determine their turgid weight (TW). Finally, the leaf samples were dried completely to a constant weight in an oven at 70°C and weighed for their dry weight (DW). The relative water content (RWC) was calculated after salt stress for 0, 10, 20 and 30 days using the following formula:

$$RWC\ (\%) = ((FW – DW/TW – DW) \times 100).$$

Measurement of ion contents. After salt stress for 30 days, dried samples of leaf and root were ground into fine powder for measuring the Na^+, K^+ and $Cl^–$ contents. Next, 0.2 g powdered samples were digested in an H_2SO_4-H_2O_2 solution (All chemicals mentioned in the article were purchased from TianGen Biotechnology (Beijing, China) Co., Ltd.). The extract, which was concentrated in H_2SO_4–H_2O_2, was diluted to a specified volume with distilled deionized water to determine the elemental contents. The Na^+ and K^+ contents were measured using an atomic absorption spectrophotometer (SpectAA-220, Varian, Australia). For the determination of $Cl^–$ content, 0.1 g of powdered sample was added to 20 mL of deionized water at 100°C for 1.5 h, the $Cl^–$ content was determined by an automatic potentiometric titrator (ZD-2, Leici, Shanghai, China) as described by Qi et al. [2013].

Measurement of proline contents. The proline content was determined according to the method of Bates et al. [1973]. Fresh leaf material (0.2 g) was homogenized in 5 mL of 3% (w/v) sulphosalicylic acid and extracted at 100°C for 10 min. Next, the homogenates were centrifuged at 13 000 g for 10 min. The supernatant (2 mL) was reacted with 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent. The reaction mixture was incubated in a digital circulating water bath at 100°C for 30 min and then cooled in an ice bath to terminate the reaction. After cooling, the mixture was extracted with 4 mL of toluene, and the absorbance of the organic phase was measured at 520 nm on an UV-visible spectrophotometer (Thermo Scientific BioMate 3S, Thermo Scientific Ltd., USA). A calibration curve was adapted using a proline solution of known concentration.

Measurement of soluble sugar contents. The soluble sugar content was determined by the anthrone method as described by Li et al. [2000]. Fresh leaf sample (0.2 g) was added to 10 mL of distilled water and boiled for 30 min for the extraction. After cooling in an ice bath, 0.5 mL of boiled sample was mixed with 1.5 mL of distilled water, 0.5 mL of anthrone

solution and 5 mL of concentrated sulphuric acid, then the mixture was placed in a boiling bath for 1 min. After cooling to room temperature, absorbance was measured at 630 nm using an UV-visible spectrophotometer. The final soluble sugar concentration was expressed as % glucose equivalents compared with the standard curve.

Measurement of soluble protein contents. The soluble protein content was measured according to Bradford [1976] using G-250 (Thermo Scientific, USA). Fresh leaf sample (0.5 g) was added to 2 mL of distilled water and homogenized in a mortar. The homogenate was washed with 6 mL of distilled water and then collected in the centrifuge tube. Then, the homogenate was extracted for 30 min at room temperature followed by centrifugation at 12 000 g for 20 min. The supernatant was transferred to a volumetric flask and fixed with distilled water to scale for the sample extraction buffer. Next, 0.1 mL of supernatant was fully mixed with 5 mL of Bradford reagent. The absorbance was measured at 595 nm after 2 min, and the final protein contents were expressed as $mg \cdot g^{-1}$ FW.

Measurement of lipid peroxidation. Lipid peroxidation was determined in terms of the malondialdehyde (MDA) contents [Li et al. 2000]. Fresh leaf sample (0.5 g) was homogenized in 5 mL of 5% trichloroacetic acid followed by centrifugation at 10 000 g for 10 min at 4°C. Then, 2 mL of supernatant was added to 2 mL of 0.67% 2-thiobarbituric acid, the reaction mixture was incubated at 100°C for 30 min, then it was cooled in an ice bath to terminate the reaction. After centrifugation at 10 000 g for 5 min, the absorbance of the supernatant was measured at 532 nm, 600 nm and 450 nm. The concentration of MDA was calculated based on the following formula: $C\ (\mu mol \cdot l^{-1}) = 6.45\ (A532 - A600) - 0.56\ A450$. The final MDA contents were expressed as $\mu mol \cdot g^{-1}$ FW.

Enzyme extraction and activity assay. Fresh leaf sample (0.2 g) was homogenized in 6 ml of 50 mM sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 1% PVPP (w/v). The homogenate was centrifuged at 4°C at 13 000 g for 20 min, and the resulting supernatant was used as a crude enzyme to determine the antioxidant enzyme activities. All enzyme activities were expressed as units$\cdot min^{-1} \cdot g^{-1}$ FW.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by inhibiting the photochemical reduction of nitroblue tetrazolium (NBT) according to Zhu et al. [1990] with minor modifications. The 3-mL reaction mixture contained 1.7 mL of sodium phosphate buffer (50 mM, pH 7.8), 0.3 mL of Met buffer (130 mM), 0.3 mL of NBT buffer (750 $\mu mol \cdot l^{-1}$), 0.3 mL of EDTA-Na2 buffer (100 $\mu mol \cdot l^{-1}$), 0.3 mL of lactoflavin (20 $\mu mol \cdot l^{-1}$) and 0.1 mL of enzyme extract. The reaction mixture was illuminated at a light intensity of 4000 x for 20 min. The reaction mixture with 0.1 mL of enzyme extract was incubated in the dark served as the dark blank. SOD activity per unit was defined as the amount of enzyme required to inhibit the NBT photochemical reduction by 50% at 560 nm.

The peroxidase (POD, EC 1.11.1.7) activity was measured by using the guaiacol method according to Zhou and Leul [1999] with some modifications. The 5-mL reaction mixture contained 2.9 mL of sodium phosphate buffer (200 mM, pH 6.0), 1.0 mL of 2% H_2O_2, 1.0 mL of guaiacol (50 mM) and 0.1 mL of enzyme extract. The absorbance was measured at 470 nm. POD activity per unit was the amount of enzyme required for the absorbance value to increase by 1 per min.

The catalase activity (CAT, EC 1.11.1.6) was estimated with the Aebi [1984] method with some modifications. A reaction mixture (3 mL) contained 2.5 mL of sodium phosphate buffer (50 mM, pH 7.0), 0.3 mL of H_2O_2 (100 mM) and 0.2 mL of enzyme extract was prepared, the absorbance was measured at 240 nm. CAT activity per unit was the amount of enzyme required for the absorbance value to increase by 1 per min.

Ascorbate peroxidase (APX, EC 1.11.1.11) was determined according to Nakano and Asada [1981] with some modifications. The reaction mixture (3 ml) contained 1.8 ml of sodium phosphate buffer (50 mM, pH 7.0), 0.1 ml of ascorbate (15 mM), 1 ml of H_2O_2 (0.3 mM) and 0.1 ml of enzyme extract. The change in the absorbance at 290 nm was recorded 30 s after the addition of H_2O_2. APX activity per unit was the amount of enzyme required for the absorbance value to increase by 1 per min.

Measurement of photosynthetic pigments. Fresh leaf sample (0.2 g) was extracted in the dark with 10 mL of 95% ethanol (v/v) until the tissues from 10, 20 and 30 days after salt treatment became completely white. The absorbance was measured at 663 nm,

647 nm and 470 nm using an UV-visible spectrophotometer. Estimation of the photosynthetic pigments was calculated according to Lichtenthaler and Wellburn [1983] using the following equations.

$$\text{Chl } a \text{ } (\mu g \cdot ml^{-1}) = 13.95A665 - 6.88A649$$

$$\text{Chl } b \text{ } (\mu g \cdot ml^{-1}) = 24.9\ A649 - 7.32A665$$

$$\text{Car } (\mu g \cdot ml^{-1}) =$$

$$= (1000A470 - 2.05\text{Chl } a - 114.8\text{Chl } b)/245$$

Measurement of chlorophyll fluorescence. After 0, 10, 20, and 30 days of salt treatment, the chlorophyll fluorescence parameters of the 3rd or 4th fully expanded leaves from the apical meristem were examined using a pulse-amplitude modulation fluorometer (PAM-2500, Walz, Germany). Then the leaves were fixed on the leaf clip and the measurements always occurred in the same area of the leaf for five replicate seedlings. The leaves were dark-adapted for 30 min before the measurements. The minimal fluorescence of the dark-adapted state (Fo) was measured by a modulated pulse (0.15 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Maximal fluorescence (Fm) was measured by a saturating actinic light pulse (4000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 0.8 s) in the dark-adapted state. The steady state fluorescence yield (Fs) was recorded after the plants were illuminated with ambient light conditions for 30 min.

The maximal fluorescence of the light-adapted state (Fm') was measured by a saturating actinic light pulse (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 0.7 s). The minimal fluorescence in the light-adapted state (Fo') was recorded by illumination with a far-red light after switching the actinic light illumination off. The maximal photochemical efficiency of PSII in the dark (Fv/Fm = (Fm – Fo)/Fm) was determined by the method in Van Kooten and Snel [1990], and the PSII quantum efficiency (Yield = (Fm' – Fs)/Fm') was determined by the method in Genty et al. [1989]. Photochemical quenching (qP = Fm' – Fs/Fv') and non-photochemical quenching (qN = (Fv – Fv')/Fv) were determined by the method in Schreiber et al. [1986].

Data analysis. The results were statistically analysed by one-way ANOVA using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Duncan's test was applied for the mean separation for the significant differences among treatments at the $P < 0.05$ significance level. All the results are means of at least three independent experiments.

RESULTS

Growth and RWC. After salt stress treatment for 20 and 30 days, plants with NaCl concentrations that reached 450 mM withered and died. The biomass and RGR of the crown width decreased significantly with the increase in salinity (Tab. 1). The RGR was markedly increased initially (90–270 mM) and was then inhibited (360 mM), reaching maximum values at a salt concentration of 270 mM, which resulted in approximately 1.3-fold higher values compared with the control. The root RGR values were all higher than the control (Tab. 1). The leaf RWC under salt stress was not significantly different from the control (Tab. 1).

Ion contents. Generally, the Na^+ contents increased significantly in the leaves and roots in response to the concentrations compared with the control, though

Table 1. Effects of different salt treatments for 30 days on the growth and relative water contents in *V. trifolia*. Values are the means ±SE. Different letters in the same column indicate a significant difference based on Duncan's test ($P < 0.05$)

Treatment (mM)	Biomass (g)	RGR of stem ($cm \cdot cm^{-1} \cdot day^{-1}$)	RGR of root ($cm \cdot cm^{-1} \cdot day^{-1}$)	Leaf relative water content (%)
ck	4.90 ±0.071 a	0.015 ±0.00040 a	0.0074 ±0.00045 d	82.04 ±5.27 a
90	1.12 ±0.025 b	0.013 ±0.00024 b	0.0096 ±0.0011 c	81.57 ±0.68 a
180	0.57 ±0.011 c	0.012 ±0.00085 c	0.014 ±0.00038 b	78.46 ±1.14 ab
270	0.41 ±0.0084 d	0.0092 ±0.00062 d	0.017 ±0.00057 a	76.39 ±1.15 ab
360	0.36 ±0.0062 e	0.0042 ±0.00084 e	0.012 ±0.00065 c	72.11 ±2.76 b

Table 2. Effects of different salt treatments for 30 days on Na^+, K^+, Cl^- and K^+/Na^+. Values are the means ±SE. Different letters in the same column indicate a significant difference based on Duncan's test ($P < 0.05$)

Treatment (mM)	Na^+ (mg·g^{-1})		K^+ (mg·g^{-1})		Cl^- (mg·g^{-1})		K^+/Na^+	
	root	leaf	root	leaf	root	leaf	root	leaf
ck	2.19 ±0.077 c	1.95 ±0.067 c	14.43 ±0.31 ab	15.76 ±0.40 a	0.023 ±0.00096 d	0.027 ±0.0011 e	6.60 ±0.18 a	8.10 ±0.43 a
90	2.59 ±0.084 b	3.10 ±0.081 b	13.76 ±0.57 b	16.22 ±0.57 a	0.053 ±0.00075 c	0.082 ±0.0012 d	5.31 ±0.10 b	5.23 ±0.061 b
180	2.68 ±0.10 b	3.10 ±0.13 b	14.47 ±0.44 ab	16.13 ±0.56 a	0.069 ±0.00084 b	0.088 ±0.00064 c	5.40 ±0.041 b	5.21 ±0.22 b
270	3.27 ±0.051 a	3.73 ±0.10 a	15.38 ±0.49 ab	16.33 ±0.91 a	0.10 ±0.0010 a	0.14 ±0.0010 a	4.71 ±0.094 c	4.38 ±0.26 b
360	3.29 ±0.084 a	3.74 ±0.061 a	15.60 ±0.58 a	16.55 ±0.41 a	0.11 ±0.0016 a	0.12 ±0.00072 b	4.75 ±0.11 c	4.43 ±0.17 b

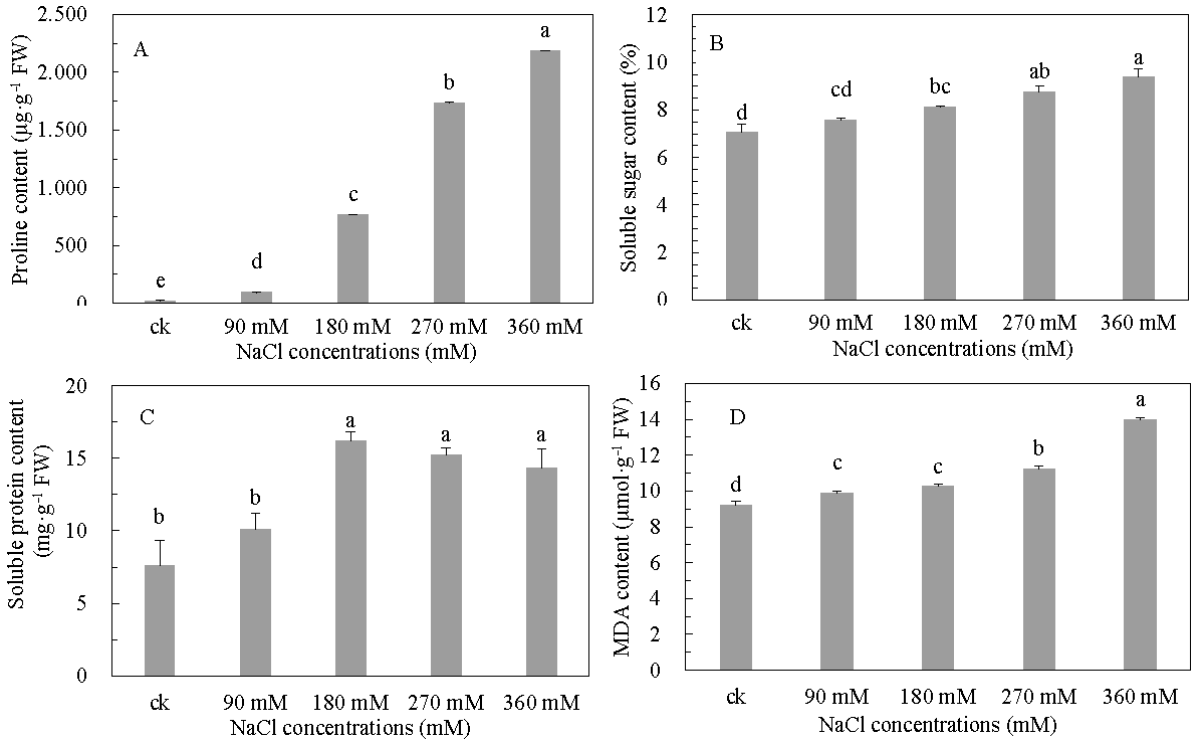

Fig. 1. Effects of different salt treatments on the proline, soluble sugar, soluble protein and MDA contents after 30 days of salt stress. Values are the means ±SE**.** Different letters on the error bars indicate significant differences among the various treatments based on Duncan's test ($P < 0.05$)

there were no significant differences between the 90-mM and 180-mM or 270-mM and 360-mM salt exposure. The Na^+ contents in the roots and leaves reached a maximum at the 360-mM salt concentration, which resulted in approximately 1.5- and 1.9-fold higher values compared with the control respectively. The Na^+ contents were higher in the leaves than in the roots at the same salt concentrations (Tab. 2).

There were no significant differences of the K^+ contents among the various salt concentrations. The K^+ contents were higher in the leaves than in the roots the same as Na^+ (Tab. 2). The Cl^- contents in the leaves and roots were markedly increased with the increase of salt stress, and were reached a maximum value at the salt concentrations of 270-mM and 360-mM, which were 5.2 and 4.8 times compared with the control

respectively. Similar to the Na^+ and K^+ distributions, the Cl^- contents were higher in the leaves than in the roots (Tab. 2).

The K^+/Na^+ ratio in leaves was inhibited by the increase in salinity, and was reached a minimum value at the 270-mM salt concentration. The K^+/Na^+ ratio in leaves was decreased by about 46% compared to the control, but there were no significant differences between the various salt concentrations. Similarly, the K^+/Na^+ ratio was inhibited by the increase in the salinity in the roots compared with the control. The results showed that there were no significant differences between the 90-mM and 180-mM or 270-mM and 360-mM salt concentrations. The minimum value was obtained at the 270-mM salt concentration, which resulted in approximately 29% lower values compared with the control (Tab. 2).

Proline, soluble sugar, soluble protein and MDA contents. The proline contents were markedly increased with the increase in salt concentrations, reaching a maximum value at the 360 mM salt concentration, with approximately 98-fold higher values than the control (Fig. 1*A*). The soluble sugar contents increased significantly with the increase in salinity compared with the control, except at a salinity of 90 mM. The soluble sugar contents reached a maximum value at a salinity of 360 mM and had 1.3-fold higher values than the control (Fig. 1*B*). Similar to the soluble sugar content trend, the soluble protein contents increased significantly with the increase in salinity compared with the control, except at a salinity of 90 mM. The soluble protein contents reached a maximum value at a salinity of 180 mM, and this value was 2.1-fold higher than that of the control (Fig. 1*C*). The MDA

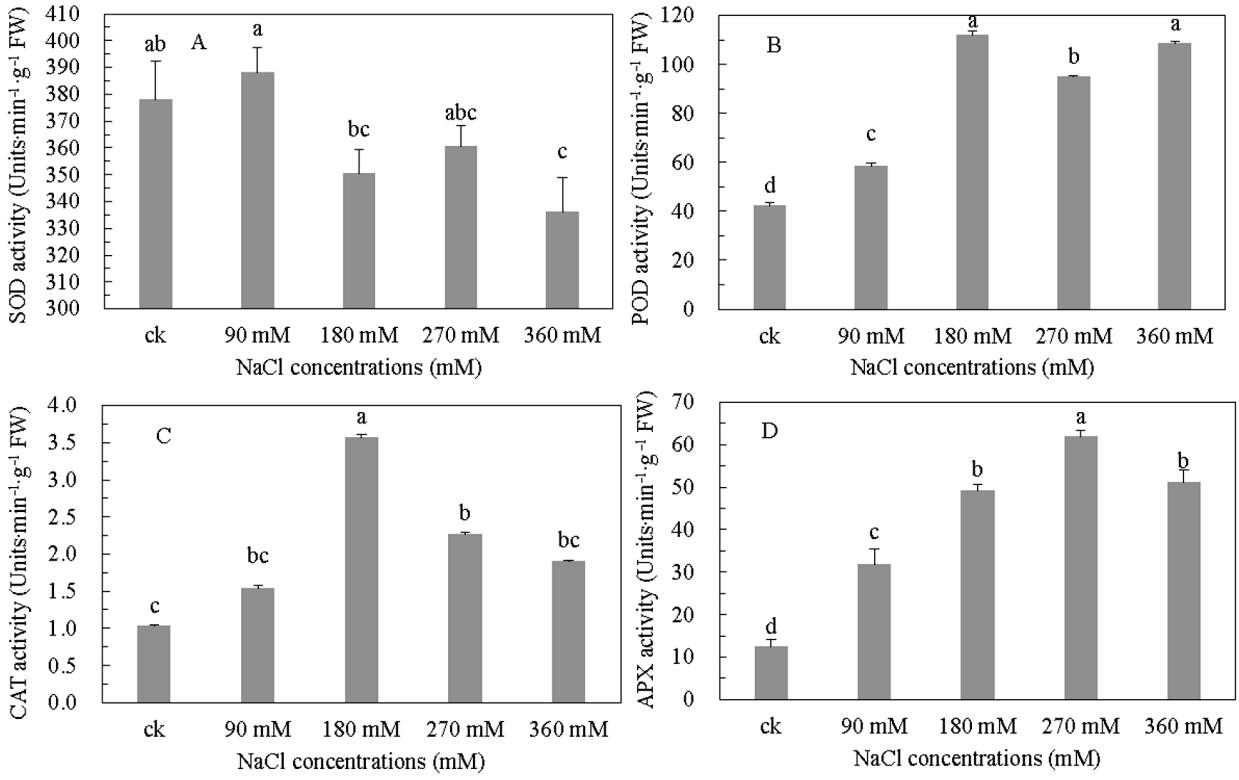

Fig. 2. Effects of different salt treatments on SOD, POD, CAT and APX activity after 30 days of salt stress. Values are the means ±SE. Different letters on the error bars indicate significant differences among the various treatments based on Duncan's test ($P < 0.05$)

contents increased significantly with the increase in the salt concentration, though there were no significant differences between the 90-mM and 180-mM salt concentrations compared with the control. The highest MDA contents were detected at the 360 mM salt concentration, with approximately 1.5-fold higher values compared with the control (Fig. 1*D*).

Antioxidant enzymes. SOD activity decreased with increasing salt concentrations, and there were no significant differences between the 90-mM to 270-mM salt concentrations compared with the control. SOD activity reached a minimum value at a salinity of 360 mM and was 11% lower than the control (Fig. 2*A*). In contrast, POD, CAT and APX activities were markedly increased with the increase in salinity, with their maximum values occurring at the 180, 270 and 360 mM salt concentrations, which resulted in approximately 2.7-fold, 3.5-fold and 5-fold higher values, respectively, compared with the control (Fig. 2*B–D*).

Photosynthetic pigments. After salt stress treatment for 10 days, the Chl *a*, Chl *b*, and total Chl contents were not significantly affected by 90-mM to 360-mM salt concentrations compared with the control, though these contents were significantly inhibited when the salt concentration reached 450 mM. The Car contents were not significantly affected by 90-mM to 270-mM salt concentrations compared with the control, though they were significantly inhibited when the salt concentration ranged from 360 mM to 450 mM (Tab. 3). After salt stress for 20 days, the Chl *a* and total Chl contents were not significantly affected by salt stress compared with the control. Chl *b* contents increased significantly when the salt concentration ranged from 90 mM to 180 mM but decreased when the salinity ranged from 270 mM to 360 mM, com-

Table 3. Effects of different salt treatments on photosynthetic pigments after 10, 20 and 30 days of salt stress. Values are the means ±SE. Different letters in the same column indicate a significant difference based on Duncan's test ($P < 0.05$)

Time of treatment (days)	NaCl concentration (mM)	Chlorophyll *a* ($mg \cdot g^{-1}$ FW)	Chlorophyll *b* ($mg \cdot g^{-1}$ FW)	Total chlorophyll ($mg \cdot g^{-1}$ FW)	Carotenoid ($mg \cdot g^{-1}$ FW)
10	ck	0.93 ±0.014 a	0.30 ±0.0062 a	1.24 ±0.020 a	0.19 ±0.0049 cd
	90	0.91 ±0.022 a	0.30 ±0.0083 a	1.21 ±0.031 a	0.18 ±0.0033 d
	180	0.91 ±0.021 a	0.30 ±0.0088 a	1.21 ±0.030 a	0.19 ±0.0028 bc
	270	0.88 ±0.0048 a	0.30 ±0.0020 a	1.18 ±0.0069 a	0.20 ±0.00010 abc
	360	0.88 ±0.028 a	0.31 ±0.012 a	1.19 ±0.040 a	0.20 ±0.0037 ab
	450	0.73 ±0.0077 b	0.26 ±0.0015 b	0.99 ±0.0092 b	0.20 ±0.00061 a
20	ck	0.78 ±0.012 a	0.24 ±0.0017 b	1.02 ±0.012 a	0.17 ±0.0027 b
	90	0.84 ±0.037 a	0.27 ±0.0012 a	1.11 ±0.052 a	0.18 ±0.0069 ab
	180	0.87 ±0.060 a	0.26 ±0.0067 a	1.13 ±0.081 a	0.19 ±0.0050 ab
	270	0.79 ±0.025 a	0.23 ±0.0053 bc	1.02 ±0.030 a	0.19 ±0.0079 ab
	360	0.78 ±0.018 a	0.23 ±0.00056 c	0.99 ±0.062 a	0.19 ±0.0039 a
30	ck	0.77 ±0.038 a	0.24 ±0.015 b	1.01 ±0.053 bc	0.18 ±0.0072 c
	90	0.81 ±0.020 a	0.28 ±0.016 a	1.09 ±0.033 ab	0.21 ±0.0084 b
	180	0.84 ±0.025 a	0.29 ±0.013 a	1.13 ±0.039 a	0.25 ±0.0054 a
	270	0.69 ±0.011 b	0.23 ±0.0046 b	1.00 ±0.018 bc	0.24 ±0.0028 a
	360	0.68 ±0.013 b	0.22 ±0.011 b	0.97 ±0.018 c	0.25 ±0.0068 a

pared with the control. The Car contents increased with the increase in salinity, and there were no significant differences, except for when the salt concentration was 360 mM (Tab. 3). After salt stress for 30 days, the Chl *a*, Chl *b*, and total Chl contents increased initially and then decreased compared with the control; these values were significantly affected at salt concentrations of 270–360 mM, 90–180 mM and 180 mM, respectively, compared with the control. The Car contents were markedly increased by increasing salinity, compared with the control, reaching a maximum value at a salt concentration of 360 mM (Tab. 3).

Chlorophyll fluorescence. After salt stress for 10 days, Fv/Fm decreased with increasing salinity, and the difference was significant at higher salinity concentrations (Fig. 3*A*). Similar trends were observed for the Yield and qP, while qN was not influenced by the increase in salinity (Fig. 3*B–D*). When the salt stress was prolonged and lasted for 20 days, the Fv/Fm value was similar to that after salt stress for 10 d, but there were no significant differences at the 90-mM to 270-mM salt concentrations (Fig. 3*A*). However, the Yield, qP and qN trends were not the same as when treated with salt stress for 10 days; these values markedly increased with the increased salinity, compared with the control, when the salinity ranged from 180 mM to 360 mM (Fig. 3*B–D*).

The changes in Fv/Fm were always the same, even with the extended salt stress time periods. Similar trends were observed after salt stress treatment for 30 days (Fig. 3*A*). In the last stage of the salt stress treatment, the Yield initially increased and then decreased with increasing salinity; a significant difference was only observed when the salinity was higher than 360 mM (Fig. 3*B*). The qP increased initially and then decreased; this value was higher than the control when

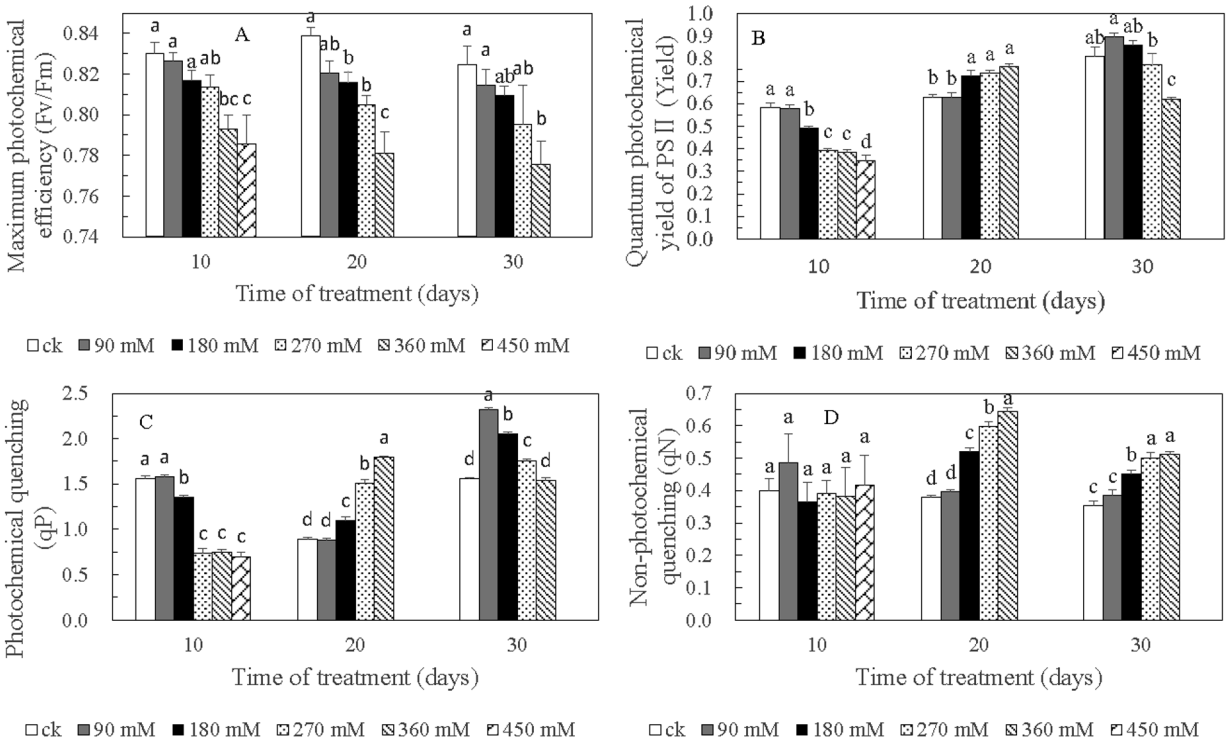

Fig. 3. Effects of different salt treatments on chlorophyll fluorescence parameters after 10, 20 and 30 days of salt stress. Values are the means ±SE. Different letters on the error bar indicate significant differences among the various treatments based on Duncan's test ($P < 0.05$)

the salinity ranged from 180 mM to 270 mM, and significant differences were observed (Fig. 3*C*).The results for qN were similar after salt stress for 20 days, and significant differences were observed at higher salinities (180–360 mM) (Fig. 3*D*).

DISCUSSION

The mechanism of salt tolerance in *V. trifolia* is discussed in the present study. The biomass and shoot RGR of *V. trifolia* were inhibited with the increase of salinity, while the contents of Na^+ and Cl^- were increased with the increase of salt stress. The above results indicated that the plant growth of *V. trifolia* was affected by the effects of osmotic and ionic stress. In other studies, some halophytes were insensitive to salt stress, such as *Chenopodium quinoa* and *Atriplex halimus* [Becker et al. 2017, Hamdani et al. 2017]. Some plants like *Prosopis strombulifera* and *Atriplex prostrate* [Llanes et al. 2014, Bueno et al. 2015] were stressed when subjected to moderate NaCl concentrations (10–500 mM). The root RGR of *V. trifolia* increased with the increase of salt stress, which may be related to the absorption and maintenance of K^+ in the roots. Steady-state regulation of K^+ is a key factor in ionic steady-state, osmotic regulation, and salt tolerance.

Preventing Na^+ and Cl^- accumulation and increasing K^+ absorption is an important mechanism for resisting salt stress [Munns and Tester 2008]. The contents of Na^+ and Cl^- in both the leaves and roots of *V. trifolia* were gradually increased with the increase of salinity, and the contents of Na^+ and Cl^- in leaves were higher than that of roots. This result indicated that the ions of *V. trifolia* were accumulated as a penetrant to reduce the water potential, thereby maintaining water absorption in plants under salt stress. This confirmed that the plants have the ability to maintain K^+ contents and hence avoid salt toxicity caused by the accumulation of Na^+ and Cl^- [Munns and Tester 2008]. Furthermore, a key feature salt tolerance in plants is the maintenance of a high cytosolic K^+/Na^+ ratio [Amor et al. 2005]. The K^+/Na^+ ratio in the leaves did not change significantly under different salt concentrations, indicating that the leaves have a stronger capacity for selective K^+ absorption relative to Na^+ absorption. Similar results were also found in the studies with halophytes such as *Sesuvium portulacastrum*, *Cakile maritime* and *Centaurea tuzgoluensis* [Megdiche et al. 2007, Yildiztugay et al. 2011, Muchate et al. 2016].

Organic osmolytes such as proline, soluble sugar and soluble protein are accumulated under salt stress and are used to regulate osmotic adjustment and maintain cell turgor, and scavenge ROS [Farooq et al. 2009, Slama et al. 2015, Anjum et al. 2017]. Reports indicated that proline maintains osmotic adjustment as an osmoprotectant, helped to stabilize cellular structures and functions as an antioxidant and a protector of photosynthetic machinery [Verbruggen and Hermans 2008, Ashraf et al. 2017, Mansour and Ali 2017]. In addition, soluble sugar and soluble protein contents increased significantly under salinity indicating that these organic osmolytes may play an important role in osmotic adjustment under salt stress. Similar results were also observed in the study with the halophyte *Sesuvium portulacastrum*, where the proline and soluble sugar contents increased significantly at NaCl stress [Lokhande et al. 2010, Rajaravindran and Natarajan 2012]. In contrast, in the halophyte *Suaeda salsa*, a decrease in soluble sugar was observed under salinity stress [Liu et al. 2006].

The concentration of MDA is used to indicate the degree of oxidative damage, so it is an indicator of membrane lipid peroxidation caused by salt stress [Bor et al. 2003, Pérez López et al. 2009]. In halophytes, proper membrane functions are associated with lower levels of MDA, and unchanged MDA content is an important characteristic of salt tolerance in halophytes [Seckin et al. 2010, Yıldıztugay et al. 2011]. The MDA contents of *V. trifolia* increased significantly compared to the control. It is suggested that *V. trifolia* had been damaged by oxidation under salt stress. In addition, the change of MDA in many halophytes, like *L. bicolor*, *Crithmum maritimum* and *Sesuvium portulacastrum*, under salt stress is different from that in *V. trifolia* [Amor et al. 2005, Liu et al. 2008, Rajaravindran et al. 2012].

In order to eliminate salt-induced ROS, the expression and activity of antioxidant enzymes are enhanced as a prime component of the defense mechanism of halophytes [Shabala 2013]. Nevertheless, Munns and Tester [2008] noted that salt tolerance and the induction of antioxidant enzymes did not always have a direct correlation. In this study, *V. trifolia* exhibited different responses to salinity treatment with respect to the activities of antioxidant enzymes. In general, the activity

of POD, CAT and APX increased, but the activity of SOD decreased compared to the control. The results show that not SOD, but POD, CAT and APX play a role in the defense mechanism for scavenging ROS. Similar results have been reported in other halophytes such as *Cakile maritime* [Amor et al. 2005] and *Glycine soja* [Chen et al. 2013], whereby the activities of POD, CAT and APX were increased with the increasing salinity level. At the same time, some different results have been found in some halophytes such as *Plantago maritime* and *Centaurea tuzgoluensis*, whereby the activity of SOD increased with increasing NaCl concentrations [Joseph et al. 2005, Yildiztugay et al. 2011].

High salinity severely impacts photosynthesis because ROS degrades chlorophyll and decreases photosynthesis levels, which in turn affects plant growth and development. In the present study, Chl *a*, Chl *b* and total Chl contents were inhibited only under 450 mM compared with the control after salt stress for 10 days. This result indicated that the chlorophyll was degraded and the photosynthesis was affected only under 450 mM. There were no significant decreases in the Chl *a*, Chl *b* and total Chl contents at 20 and 30 days after salinity stress, indicating that photosynthetic mechanism was regulated to adapt to salt stress. At the same time, carotenoid contents increased steadily after 10, 20 and 30 days of salinity stress, indicating that carotenoid was not affected by salt stress, and played a role in protecting photosynthetic apparatus. The changes of chlorophyll and carotenoids clearly demonstrated that the photosynthetic machinery in *V. trifolia* was not disturbed upon imposition of salt stress up to 360 mM NaCl. Ramani et al. [2006] reported an increase in the contents of Chl *a*, Chl *b* and carotenoids at 400 mM NaCl in *Sesuvium*. However, Megdiche et al. [2007] and Azti et al. [2016] showed a decrease in photosynthetic pigments in *Cakile maritime* and *Aeluropus littoralis.*

Chlorophyll fluorescence reflects the light energy used efficiently by PSII and has been widely used to analyse plant light absorption and utilization [Baker 2008, Guo and Tan 2015]. The photochemical efficiency of the PSII (Fv/Fm) is a classic parameter that reflects PSII stability and the degree of tolerance of plants to environmental factors. Yield is the actual photosynthetic efficiency of PSII in any light state, and qP is the quenching of fluorescence induced by photosynthesis; it reflects the level of photosynthetic activity and is an important indicator of the degree of PSII openness. As a main pathway for excess energy consumption, NPQ can accurately reflect the changes in heat dissipation and reflect the ability of light protection [Demiral and Türkan 2006, Hediye et al. 2007]. In the present study, the reduction in Fv/Fm reached a maximum at 20 days after salt stress, though the reduction was not significant after 30 days of salt stress. These results indicated that the photochemical efficiency of PSII was not significantly affected by salt stress throughout the experiment. In contrast, the qP trend was similar to that of Yield, which was most affected at 10 days and gradually adapted at 20 days. It is important to note that qN increased significantly with the increase in salt concentration at 20 and 30 days, reflecting the ability of dissipating excess light energy to increase the heat energy with increasing salinity. All of the above chlorophyll fluorescence parameters indicated that PSII of *V. trifolia* can maintain high activity under salinity stress. The similar results were found in halophytes like *Baccharis halimifolia* L. and *Myrica cerifera* L. [Zinnert et al. 2012] with the photosynthetic net rate limited mostly by stomatal closure rather than PSII damage. This is in contrast to the observations in *Chenopodium quinoa* and *Sarcocornia fruticosa* [Duarte et al. 2013, Percey et al. 2016] where the photosynthetic net rate was mostly limited by PSII damage. Furthermore, Qukarroum et al. [2015] reported that the decrease in PSII activity was associated with ROS. In the present study, there was no significant difference in the PSII activity with the increasing salinity level or the prolonged time of salt treatment, and was most likely related to the increase in antioxidant enzymes activity and removal of ROS.

CONCLUSION

Our results illustrate that salt tolerance mechanisms in *V. trifolia* are regulated by ion homeostasis, osmotic adjustment, and antioxidant defence system. Ion homeostasis maintenance is attributed to high K^+ contents in the leaves. Osmotic adjustment is retained by accumulating Na^+, Cl^-, proline, soluble sugar and soluble protein. POD, CAT and APX are effective in scavenging ROS generated by salt stress. In addition, the contents of photosynthetic pigments and the ac-

tivity of PSII do not decrease significantly under salt stress. It is suggested that *V. trifolia* responds to salinity by maintaining higher photosynthetic capacity and light utilization efficiency.

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