

## Physiological adaptation and gene expression analysis of *Casuarina equisetifolia* under salt stress

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### Abstract

*Casuarina equisetifolia* is widely planted in coastal areas of tropical and subtropical regions as windbreaks or to stabilize dunes against wind erosion due to its high salt tolerance and nitrogen-fixing ability. To investigate the mechanisms responsible for its salt tolerance, we examined growth, mineral composition, expression of genes for sodium ( $\text{Na}^+$ ) and potassium ( $\text{K}^+$ ) transport proteins, and antioxidant responses under NaCl treatments. Increasing NaCl concentrations inhibited lateral root elongation and decreased plant height, length of internodes, and numbers of branches and twigs. The  $\text{Na}^+$  content significantly increased whereas the  $\text{K}^+$  content significantly decreased in both shoots and roots with increasing external NaCl concentration, resulting in a significant increase in  $\text{Na}^+/\text{K}^+$  ratio. Most of the  $\text{Na}^+/\text{H}^+$  antiporter genes (*NHXs*) were obviously upregulated in roots after 24 and 168 h of salt stress, and *NHX7* was especially induced after 168 h. Almost all salt overly sensitive (*SOS*) genes were induced after 168-h treatment. Additionally, activities of superoxide dismutase, glutathione peroxidase, and catalase were significantly changed in shoots and roots under salt stress. Hence, we conclude that salinity tolerance of *C. equisetifolia* mainly relied on sequestering excess  $\text{Na}^+$  into vacuoles and on induced expression of *NHX* and *SOS* genes in roots and thus the maintenance of sufficient  $\text{K}^+$  content in shoots.

*Additional key words:* antiporter genes, catalase, glutathione peroxidase, ion homeostasis, potassium, salt overly sensitive, sodium, superoxide dismutase.

### Introduction

Salt stress is one of the most serious abiotic stresses worldwide. It causes osmotic stress, ionic toxicity, and nutritional imbalances, and even leads to plant death (Türkan and Demiral 2009). However, some plants like halophytes and semi-halophytes can grow at high salinity due to sequestering salts in cell vacuoles, synthesizing compatible solutes, and possessing a complex antioxidant defence systems (Zhu 2003). Discovering the mechanisms of salt stress tolerance and conferring them to crops and other economically important sensitive plants is desirable for sustainable food and wood production. Many studies have focused on elucidation of physiological, biochemical, and molecular mechanisms of ion exclusion, osmotic adjustment, and defence against

oxidative stress in salinity-tolerant species (Munns and Tester 2008). Many genes including those related to osmolyte synthesis or accumulation, signalling pathways regulation, oxidative protection, and regulating ion homeostasis have been recognized (Munns and Tester 2008, Yang *et al.* 2009). Among them, salt overly sensitive (*SOS*) pathway proteins and  $\text{Na}^+/\text{H}^+$  antiporters (*NHX*) were identified as the key to responses to salt stress by restricting the accumulation of toxic  $\text{Na}^+$ .

*Casuarina equisetifolia* of the family *Casuarinaceae* is extensively cultivated in coastal areas and on limestone soils near the shore of tropical and subtropical regions for sand stabilization, soil rehabilitation, and as shelterbelts. Thus, *C. equisetifolia* performed important ecosystem

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*Abbreviations:* Car - carotenoids; CAT - catalase; Chl - chlorophyll; d.m. - dry mass; EC - electrical conductivity; f.m. - fresh mass; GR - glutathione reductase; GSH-Px - glutathione peroxidase; MDA - malondialdehyde; *NHX* -  $\text{Na}^+/\text{H}^+$  antiporters; POD - peroxidase; qPCR - quantitative PCR; SOD - superoxide dismutase; *SOS* - salt overly sensitive; WC - water content.

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and coastal forests have recently gained great recognition, particularly after the devastating Southeast Asian tsunami (De Zoysa 2008). It is also an excellent raw material for the paper and pulp industries and it is preferred for use as poles and in scaffoldings (Zhong *et al.* 2010). As highly tolerant to salinity stress and being a non-halophyte, there is particular interest in study how this species responds and adapts to salinity stress. Recently, some clones of *C. equisetifolia* with high salt-tolerance were selected on basis of proline content, electrical conductivity (EC), peroxidase (POD) activity, and seed germination under salt stress (Yong *et al.* 2008, Wu *et al.* 2010). It was reported that *C. equisetifolia* seedlings could survive in 500 mM NaCl solution and proline was thought to be a major compatible solute to adjust osmotic pressure (Tani and Sasakawa 2003). Recently, several genes were identified in responses to cold stress and nitrogen fixation in *C. equisetifolia* (Obertello *et al.* 2007, Péret

*et al.* 2007, Li *et al.* 2016). However, few studies in this species have focused on ion toxicity and scavenging ROS in response and during adaptation to salinity, and no study has investigated molecular mechanisms and expression patterns of related genes mediating its adaptation to salinity stress.

For these reasons, Na<sup>+</sup> and K<sup>+</sup> content and activities of antioxidant enzymes superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), glutathione peroxidase (GSH-Px, EC 1.11.1.9), and glutathione reductase (GR, EC 1.6.4.2) were determined under different NaCl concentrations and treatment durations. Additionally, transcript analyses of the *HKT1*, *NHXs*, and *SOS* genes in roots were performed. The aim was to explore the effects of salinity on *C. equisetifolia* and to identify likely critical mechanisms in adaptation to high salinity such as osmotic tolerance or specific ion toxicity.

## Materials and methods

**Plants and treatments:** Seedlings of *Casuarina equisetifolia* L. clone A8 were cultured in a growth chamber of the Research Institute of Tropical Forestry for 8 weeks. For treatment with different NaCl concentrations, the plants were transferred to turf soil. After 1 week, plants were irrigated every 2 d with 0, 200, 400, or 600 mM NaCl solution and grown under a 16-h photoperiod, an irradiance of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , day/night temperatures of 24/18 °C, and a relative humidity of 70 % for 4 weeks. To test various durations of salt treatment, the roots of control plants were washed with clean water, and then the plants were treated with ½ Hoagland solution containing 200 mM NaCl for 0, 1, 6, 24, and 168 h. The roots and shoots were harvested and stored at -80 °C until analyses.

**Growth, water content, Na<sup>+</sup> and K<sup>+</sup> content, and photosynthetic pigments:** After different treatments, plant fresh mass (f.m.), shoot height, and basal stem diameter were recorded. Dry mass (d.m.) was determined after oven drying at 60 °C for 72 h, when constant mass was reached. The water content (WC) [%] was calculated as  $[(\text{f.m.} - \text{d.m.}) / \text{f.m.}] \times 100$ .

To determine Na<sup>+</sup> and K<sup>+</sup> content, samples were finely ground, and then digested with concentrated HNO<sub>3</sub> in a microwave system *CEM Mars 5* (Matthews, NC, USA). The extracts were analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES) (*Varian Vista-PRO RL*, Palo Alto, CA, USA).

Chlorophyll (Chl) *a*, Chl *b*, and carotenoids (Car) content was determined after extraction of fresh leaf tissue in 95 % (v/v) ethanol in darkness. The absorbance was measured with a UV-visible spectrophotometer (*UV2450*, Shimadzu, Kyoto, Japan) at 665, 649, and 470 nm. Quantities of Chl *a*, Chl *b*, and Car in the extracts

were calculated using the following formulae: Chl *a* =  $(13.95 \times A_{665}) - (6.88 \times A_{649})$ , Chl *b* =  $(24.96 \times A_{649}) - (7.32 \times A_{665})$ , and Car =  $[A_{470} \times 1000 - (2.05 \times \text{Chl } a) - (11.48 \times \text{Chl } b)] / 245$ .

## Antioxidant enzymes assay and malondialdehyde (MDA) content

To extract antioxidant enzymes, fresh shoot and root samples (0.1 g) were ground into a fine powder with liquid nitrogen, and then were homogenized in 0.9 cm<sup>3</sup> of ice-cold 10 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 12 000 g and 4 °C for 15 min, and the supernatants were used for determination of the activities of Cu-Zn SOD (which represents about 90 % of total SOD), CAT, POD, GSH-Px, and GR using assay kits (*Nanjing Jiancheng Bioengineering Institute*, Nanjing, China). All enzymes were detected with UV-Vis spectrophotometer and their activities were expressed as U g<sup>-1</sup>(f.m.). One unit of SOD was defined as the amount of enzyme that inhibits 50 % nitroblue tetrazolium photoreduction. One unit of CAT or POD was calculated as the enzyme activity that decomposed 1  $\mu\text{mol}$  of H<sub>2</sub>O<sub>2</sub> per minute. One unit of GSH-Px was defined as the enzyme activity that oxidizes 1  $\mu\text{mol}$  of NADPH per minute. One unit of GR was defined as the enzyme activity that reduced 1  $\mu\text{mol}$  of GSSG per minute.

Malondialdehyde (MDA) content was measured using MDA determination kit (*Nanjing Jiancheng Bioengineering Institute*) according to the manufacturer's instructions. Briefly, the shoot and root homogenates were centrifuged at 12 000 g and 4 °C for 15 min, and the supernatants were collected. The MDA and thiobarbituric acid (TBA) mixture was produced during the reaction of MDA in samples with TBA, and then this mixture was measured at 535 nm.

**RNA extraction, cDNA synthesis, and real-time PCR:**

Total RNA from each sample was isolated separately using the *RN38 EASYspin plus Plant RNA* kit (*Aidlab Biotech*, Beijing, China) following the manufacturer's instructions. RNA samples were treated with the *RNase-Free DNaseI* (*Qiagen*, Valencia, CA, USA) to eliminate residual genomic DNA. The purified RNA was quantified with a *NanoDrop 2000* spectrophotometer (*Thermo Fisher Scientific*, Wilmington, DE, USA) and RNA integrity was evaluated with an *Agilent 2100 Bioanalyzer* (*Agilent Technologies*, Santa Clara, CA, USA). Next, for each sample, 2.0 µg of total RNA was used as a template in cDNA synthesis with the *SuperScriptIII* reverse transcriptase (*Invitrogen*, Carlsbad, CA, USA) and cDNA products were diluted 25-fold for real time quantitative (q) PCR.

The sequences of *HKTI*, *NHX*, and *SOS* genes were obtained from the assembly of *C. equisetifolia* root transcriptome sequenced using *Illumina HiSeq* technology (SRP064226 in *NCBI SRA*). The gene primers were designed using the *Primer 3* software (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). The gene names, sequences, and primers used for real-time qPCR analysis are listed in Table 1. The real-time qPCR

was performed on an *Applied Biosystems 7500* using *SYBR Premix Ex Taq™* kit (*TaKaRa*, Shiga, Japan) following the manufacturer's instructions. Thermal cycling conditions were: 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and at 60 °C for 34 s. The dissociation curve was obtained by heating the amplicon from 60 to 95 °C. The *CaeUBC* and *CaeEF1a* combination was used as an internal control (Fan *et al.* 2017). Each sample was analyzed three times. The relative expression was calculated by  $\Delta\Delta CT$  method (Livak and Schmittgen 2001). Meanwhile, non-template controls and RT negative control were used for each sample.

**Statistical analysis:** All experiments were repeated at least three times, and data were analyzed by one-way *ANOVA* using *IBM SPSS Statistics 19 for Windows* (*IBM*, Armonk, NY, USA). Means were compared using the least significant difference (LSD) test at  $P < 0.05$ . To explore patterns of changes of different traits and gene expression, all data sets were normalized using Z-score transformation, and then clustered based on Euclidean distance. The heat maps were generated by using *MultiExperiment Viewer* software (<http://www.tm4.org/mev.html>).

**Results**

Increasing concentrations of NaCl produced obvious growth inhibition of *C. equisetifolia* plants. In comparison with control plants, shoot length was significantly decreased by 16.3, 24.4, and 23.1 % at 200, 400, and 600 mM NaCl, respectively (Table 1). However, shoot diameter showed no significant changes. After treatment with 200 and 400 mM NaCl for 4 weeks, the decrease in length of internodes, branches, and twigs was parallel to a reduction in the numbers of branches and twigs; however, no other visible symptoms of toxicity were observed (Fig. 1 Suppl.). Additionally, the decrease in root growth was also characterized by a reduction in numbers of lateral roots. The effect of high NaCl concentration of 600 mM resulted in considerable damage to plants, which were etiolated and had wilted leaves and black and decayed roots. For this reason, antioxidant enzyme activities in roots were not measured

at this concentration and 600 mM NaCl might be the highest concentration partially tolerated by this species.

The water content in shoots and roots was affected by NaCl treatment. The WC in roots significantly and progressively decreased with increasing NaCl concentrations. The WC in roots decreased to approximately 20 % when treated with 600 mM NaCl, and control roots had WC of 65.6 % whereas WC of control shoots was 86.8 % (Table 1). Besides, Chl *a*, Chl *b*, and Car content was not significantly reduced at 200 and 400 mM NaCl but decreased dramatically at 600 mM NaCl (Table 1).

The Na<sup>+</sup> content increased in both shoots and roots with increasing salinity and reached 17.6 and 16.0 mg g<sup>-1</sup>(d.m.), respectively, in plants exposed to 600 mM NaCl (Fig. 1A,B). The K<sup>+</sup> content slightly decreased in both shoots and roots at 200 and 400 mM NaCl, but severely decreased at 600 mM NaCl (Fig. 1A,B). Thus,

Table 1. Characteristics of *C. equisetifolia* plants grown at various salinity. Means  $\pm$  SEs,  $n = 3$ , different letters indicate significant differences between treatments using the LSD test at  $P < 0.05$ .

NaCl conc. [mM]	Shoot height [cm]	Shoot diameter [mm]	Water content [%]		Phytosynthetic pigments [mg g <sup>-1</sup> (f.m.)]		
			shoot	root	Chl <i>a</i>	Chl <i>b</i>	Car
0	47.89 $\pm$ 3.81a	2.87 $\pm$ 0.26	86.78 $\pm$ 0.90a	65.56 $\pm$ 3.61a	1.20 $\pm$ 0.09a	0.50 $\pm$ 0.02a	0.21 $\pm$ 0.02b
200	40.01 $\pm$ 3.38b	3.07 $\pm$ 0.38	88.10 $\pm$ 4.88a	58.57 $\pm$ 2.20b	1.30 $\pm$ 0.04a	0.54 $\pm$ 0.02a	0.23 $\pm$ 0.006ab
400	36.20 $\pm$ 2.83b	2.77 $\pm$ 0.41	83.42 $\pm$ 2.53ab	52.84 $\pm$ 1.59c	1.22 $\pm$ 0.03a	0.50 $\pm$ 0.02a	0.26 $\pm$ 0.007a
600	36.86 $\pm$ 5.25b	2.69 $\pm$ 0.48	78.65 $\pm$ 0.93b	19.70 $\pm$ 4.43d	0.53 $\pm$ 0.04b	0.42 $\pm$ 0.04b	0.08 $\pm$ 0.01c

$\text{Na}^+/\text{K}^+$  ratio in roots and shoots showed very significant increase with all NaCl concentrations compared with control plants (Fig. 1C). The highest  $\text{Na}^+/\text{K}^+$  was 3.269 and 7.503 in shoots and roots, respectively, which far exceeded 0.193 and 0.615 in controls. The  $\text{K}^+$  shoot/root ratio showed no significant change under salt stress; and  $\text{Na}^+$  shoot/root also showed no change when exposed to 200 and 400 mM NaCl, but, at 600 mM NaCl, there was a slight increase (Fig. 1D).

The  $\text{Na}^+$  content in roots increased after 1 h of treatment and significantly increased with treatment time. The  $\text{K}^+$  content decreased in roots after 24 and 168 h of NaCl treatment compared with controls (Fig. 2A). Also in shoots,  $\text{Na}^+$  content increased during 168 h of salt treatment, however,  $\text{K}^+$  content did not change (Fig. 2B).

The  $\text{Na}^+/\text{K}^+$  ratios clearly increased in roots and shoots with salt treatment time (Fig. 2C) and  $\text{Na}^+/\text{K}^+$  ratio was markedly higher in roots than in shoots (Fig. 2C). The  $\text{Na}^+$  shoot/root ratio significantly decreased under salt treatment and  $\text{K}^+$  shoot/root showed no change under less than 24 h of salt treatment (Fig. 2D). It was interesting that  $\text{K}^+$  shoot/root ratio significantly increased when plants were exposed to NaCl solution for 168 h (Fig. 2D). Hence, we concluded that a high shoot cytosolic  $\text{K}^+$  under salinity stress enabled maintaining enzymatic reactions, which is a characteristic feature of salt tolerance in *C. equisetifolia*. The  $\text{Na}^+$  content was significantly higher in roots compared with shoots suggesting that *C. equisetifolia* could sequester  $\text{Na}^+$  in root tissue to prevent sodium transfer to the shoot.

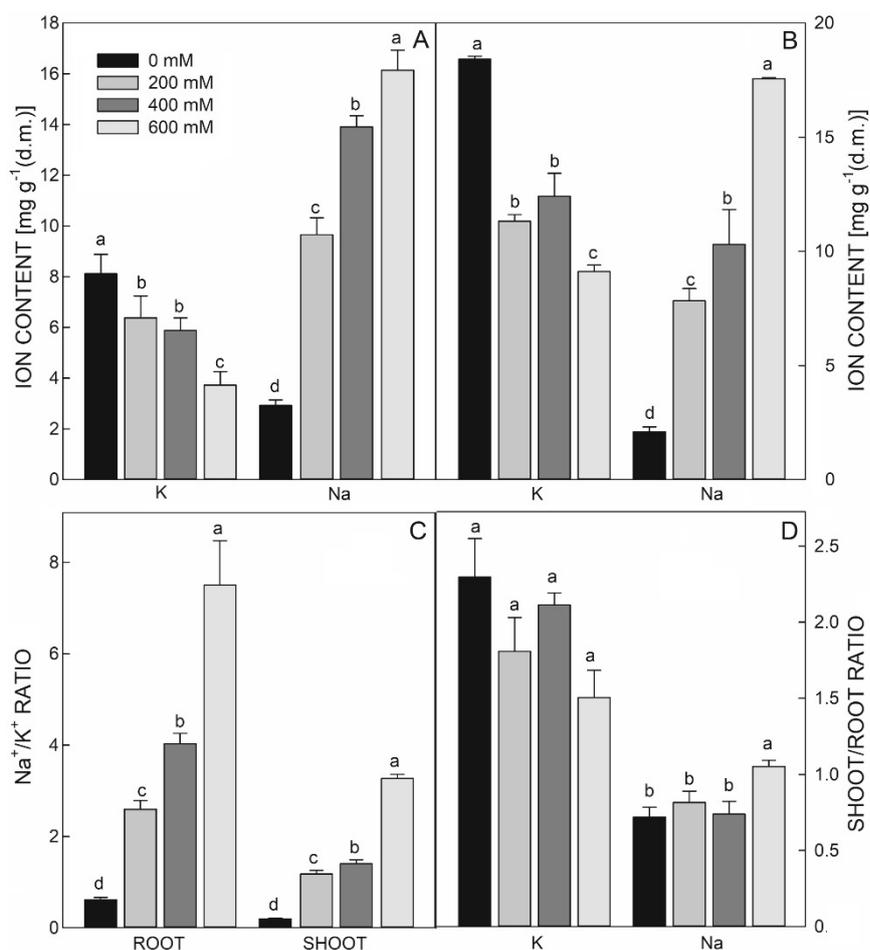


Fig. 1. Effects of various NaCl concentrations for 30 d on ion content in *Casuarina equisetifolia*. A -  $\text{Na}^+$  and  $\text{K}^+$  content in roots; B -  $\text{Na}^+$  and  $\text{K}^+$  content in shoots; C -  $\text{Na}^+/\text{K}^+$  ratio in shoots and roots; D - shoot/root ratio of  $\text{Na}^+$  and  $\text{K}^+$ . Means  $\pm$  SEs,  $n = 3$ , different letters indicate significant differences among treatments using the LSD test at  $P < 0.05$ .

Activities of SOD, CAT, GR, GSH-PX, and POD in shoots and roots changed as a consequence of NaCl treatment. In comparison with control, SOD activity in shoots significantly increased only at 200 mM NaCl (Fig. 3A). GSH-PX and CAT activities significantly

increased with increasing NaCl concentration with the exception of GSH-PX activity of shoots exposed to 600 mM NaCl, for which there was a 65.7 % reduction (Figs. 3B and 4B). Interestingly, GR activity in shoots gradually declined with increasing NaCl concentrations

(Fig. 3D), whereas POD activity did not change. In roots, GSH-PX and CAT activities significantly increased at 400 mM NaCl, whereas POD activity significantly increased with all salt concentrations compared with the controls. However, NaCl treatment had no effect on SOD and GR activities in roots.

In shoots, NaCl induced higher SOD activity after 6 h of treatment compared with the control (Fig. 4A). The same trends was observed for GSH-PX, CAT, and POD activities (Fig. 4B,C,E). Under longer treatment duration, the SOD, GSH-PX, CAT, and POD activities decreased. However, GR activity in shoots showed no significant change after salt exposure (Fig. 4D). In roots, SOD and CAT activities remained unchanged after 1, 6, and 24 h of salt stress, but decreased significantly after 168 h. The

GSH-PX and POD activities in roots decreased gradually with increasing duration of salt stress. Interestingly, GR activity in roots increased significantly after 1 h of salt stress compared with controls, however, after longer exposure, GR activity decreased to control level or less.

As an indicator of lipid peroxidation in plants under adverse environmental conditions, MDA content was altered by NaCl treatment. The MDA content significantly increased with NaCl treatments in shoots, but in roots, increases were only significant at 400 and 600 mM NaCl (Figs. 3F and 4F). MDA content in shoots at 600 mM NaCl was more than three times higher than the control value. In response to short-term salt treatment, the MDA content did not change significantly in roots but significantly decreased in shoots.

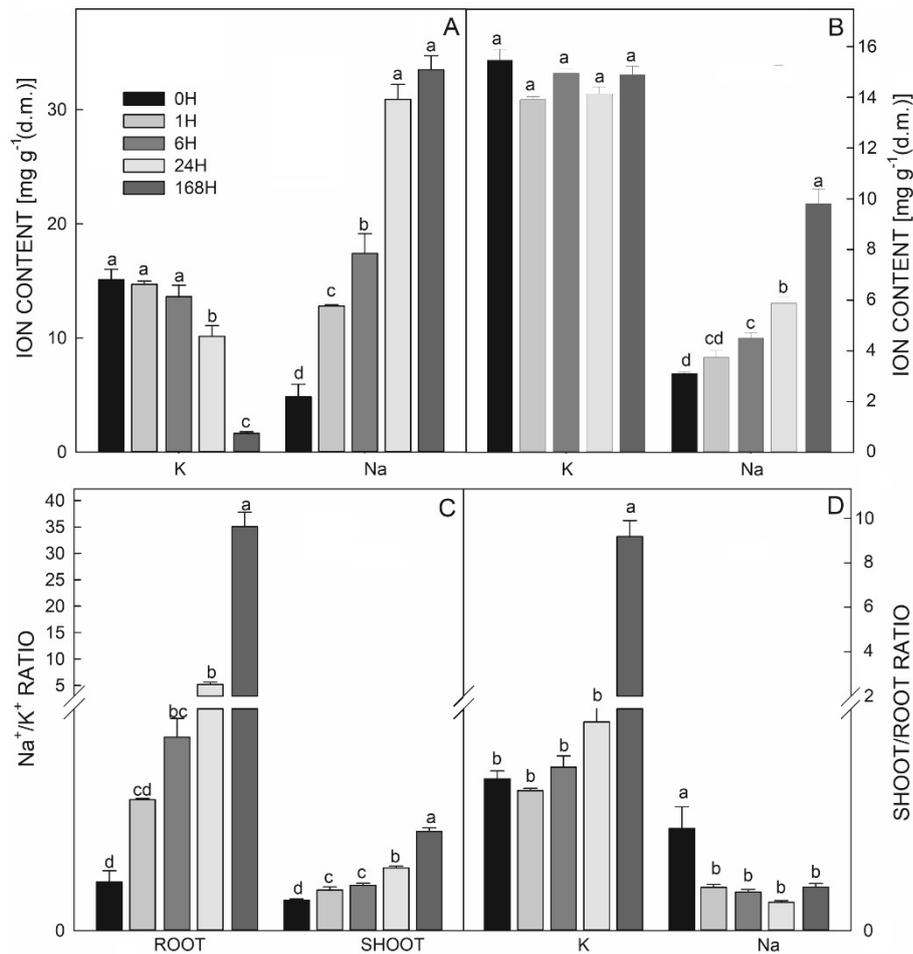


Fig. 2. Effect of different duration of 200 mM NaCl treatment on ion content in *C. equisetifolia*. A - Na<sup>+</sup> and K<sup>+</sup> content in roots; B - Na<sup>+</sup> and K<sup>+</sup> content in shoots; C - Na<sup>+</sup>/K<sup>+</sup> ratio in shoots and roots; D - shoot/root ratio of Na<sup>+</sup> and K<sup>+</sup>. Means ± SEs, n = 3, different letters indicate significant differences among treatments using the LSD test at P < 0.05.

To determine the mechanisms underlying differential Na<sup>+</sup> and K<sup>+</sup> accumulation in *C. equisetifolia*, expression profiles of genes encoding Na<sup>+</sup> and K<sup>+</sup> transport proteins were analyzed in roots. *CeqHKT1* and *NHX* gene family members including *CeqNHX1*, *CeqNHX2*, *CeqNHX3*,

*CeqNHX4*, *CeqNHX5*, *CeqNHX6*, *CeqNHX7/CeqSOS1*, and *CeqNHX8* were annotated as Na<sup>+</sup> and K<sup>+</sup> transport related genes based on our previous transcriptomic sequencing for *C. equisetifolia* (SRP064226 in NCBI SRA, <https://www.ncbi.nlm.nih.gov/sra/?term=SRP064226>).

These genes were strongly regulated under salt stress (Figs. 5,6). *CeqNHX1*, *CeqNHX5*, *CeqNHX6*, *CeqNHX7*, and *CeqNHX8* were obviously upregulated with increasing time of salt treatment, but mRNA content of *CeqNHX3* was reduced. It was interesting that *CeqNHX1*, *CeqNHX7*, and *CeqNHX8* notably responded to salt stress and *CeqNHX7* was only expressed at 168 h of salt treatment. However, *CeqHKT1* and *CeqNHX4* were

slightly downregulated at 24 h of salt stress treatment and *CeqNHX2* was downregulated at 1 h. The SOS salt stress signalling pathway is central to ion homeostasis under salt stress and plays a pivotal regulatory function in salt tolerance (Zhu 2000). One *SOS1* (*NHX7*), three *SOS2*, three *SOS3*, one *SOS4*, one *SOS5* and four *SOS6* genes were recognized in our previous transcriptomic sequencing for *C. equisetifolia* (SRP064226 in NCBI

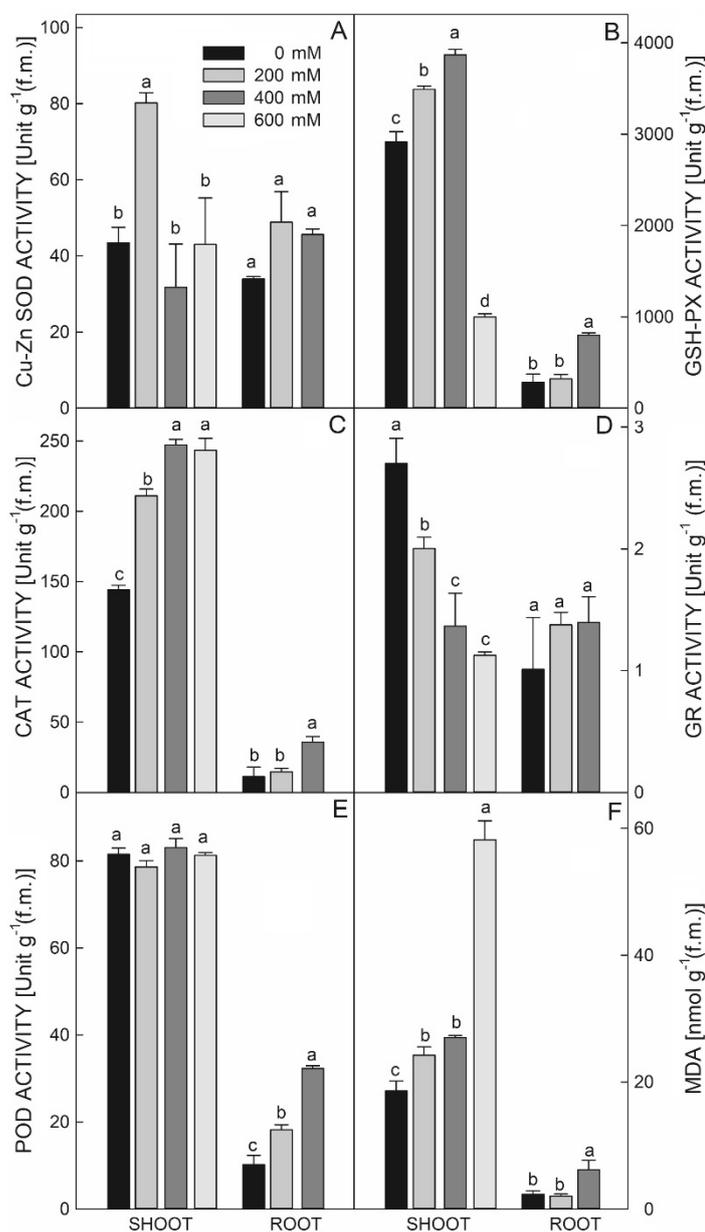


Fig. 3. Antioxidant enzyme activities and malondialdehyde (MDA) content in shoots and roots of *C. equisetifolia* subjected to NaCl treatments. Activities of A - superoxide dismutase (SOD), B - glutathione peroxidase (GSH-PX), C - catalase (CAT), D - glutathione reductase (GR), E - peroxidase (POD), and F - MDA content. Means  $\pm$  SEs,  $n = 3$ , different letters indicate significant differences among treatments using the LSD test at  $P < 0.05$ .

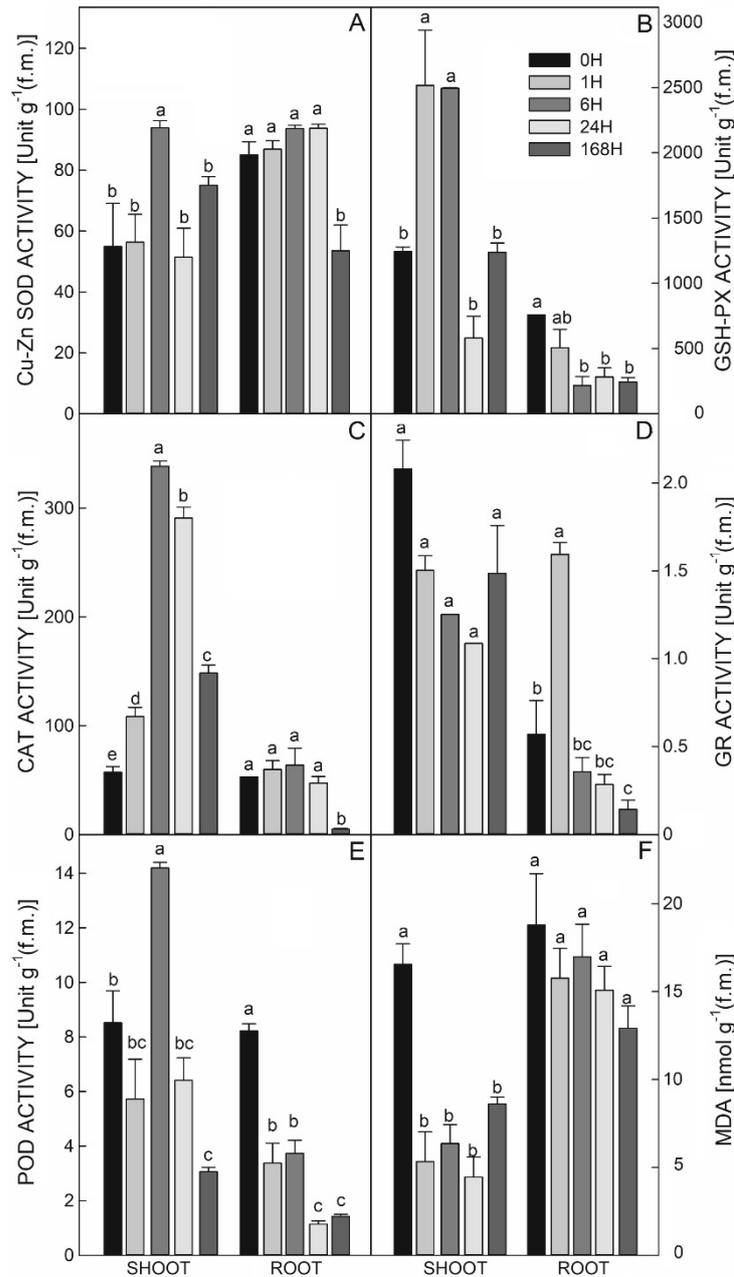


Fig. 4. Time course of antioxidant enzyme activities and MDA content in shoots and roots of *C. equisetifolia* grown under 200 mM NaCl. *A* - SOD, *B* - GSH-PX, *C* - CAT, *D* - GR, *E* - POD, *F* - MDA. Means  $\pm$  SEs,  $n = 3$ , different letters indicate significant differences among treatments using the LSD test at  $P < 0.05$ .

*SRA*). Thus, these genes were investigated here using real-time qPCR. In *C. equisetifolia* roots, expressions of almost all *SOS* genes were upregulated under salt treatment (Figs. 5 and 6). It was noteworthy that transcriptions of *CeqNHX7*, *CeqSOS2.3*, *CeqSOS3.2*, *CeqSOS6.1* and *CeqSOS6.3* were detected only when NaCl was applied. Notably, *CeqNHX7*, *CeqSOS3.2*, and *CeqSOS6.3* were induced only at 24- or 168-h salt treatments.

The traits were clustered into two main groups (group A and group B), each with three sub-groups along

different NaCl concentrations (Fig. 7). Overall, the values of traits in group A were greatly depressed by NaCl treatment. For example,  $K^+$  content in shoots, seedling height, and GR activity in shoots clustered in sub-group A-1; stem diameter and SOD activity in shoot clustered in sub-group A-2; and water content in both shoots and roots,  $K^+$  content in shoots, and Chl *a* and Chl *b* clustered in sub-group A-3. In contrast, the values of traits in group B were increased by NaCl treatment, especially MDA content and  $Na^+$  content in shoots belonged to sub-group B-3 and reached the highest values at 600 mM NaCl.

Similarly, changes in traits and gene expressions during NaCl treatments were also clustered into two main groups (group C and group D, Fig. 7B), with four and three sub-groups, respectively. Interestingly, in most sub-groups, the traits were always clustered with expression of genes encoding Na<sup>+</sup>/K<sup>+</sup> transport proteins in roots. For example, the activities of GSH-PX, MDA, and POD in roots were clustered with expression of *CeqNHX3* and *CeqSOS5* in sub-group C-1, but MDA activity and K<sup>+</sup> content in shoots were clustered with expression of *CeqHKT1* and *CeqSOS6.3* in sub-group C-2. Similar observations were also found in sub-groups D-1, D-2, and D-3. For example, Na<sup>+</sup> content in roots was clustered

with expression of *CeqSOS3.3* and *CeqSOS2.1* in sub-group D-2, and activities of SOD, CAT, and POD in shoots were clustered with expressions of *CeqNHX4*, *CeqSOS2.2*, and *CeqNHX2* in sub-group D-3. These results indicated the contributions of these genes to the corresponding traits in shoots and roots under salt treatments. Together, the dynamic changes of traits and gene expression reflected a well-organized, coordinated, and complex regulation network in *C. equisetifolia* in response to salt stress.

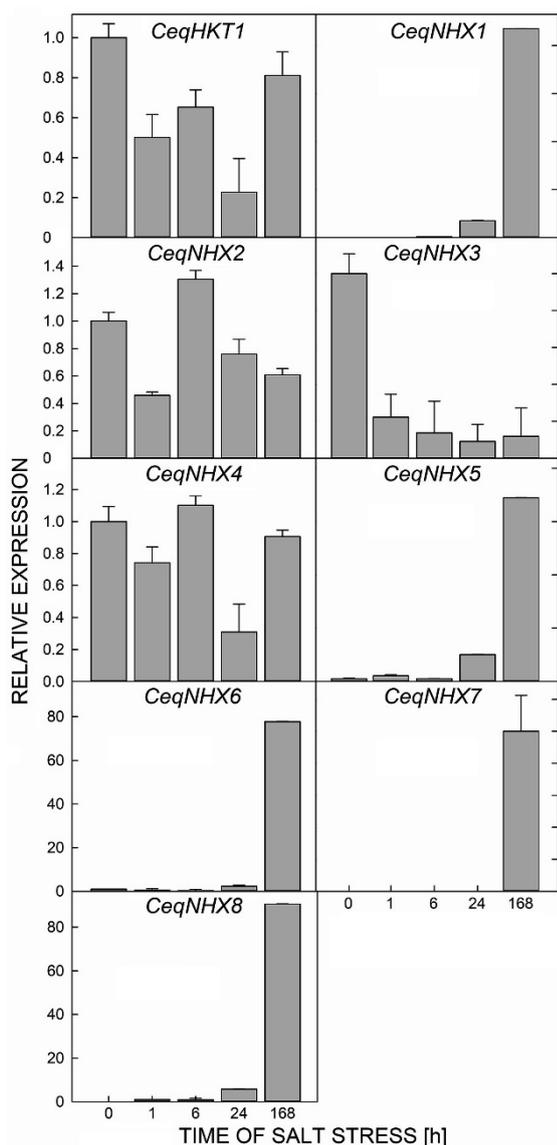


Fig. 5. Effect of different duration of 200 mM NaCl treatment on expressions of *CeqHKT1* and *CeqNHX* genes in roots of *C. equisetifolia*. Means ± SEs, n = 3.

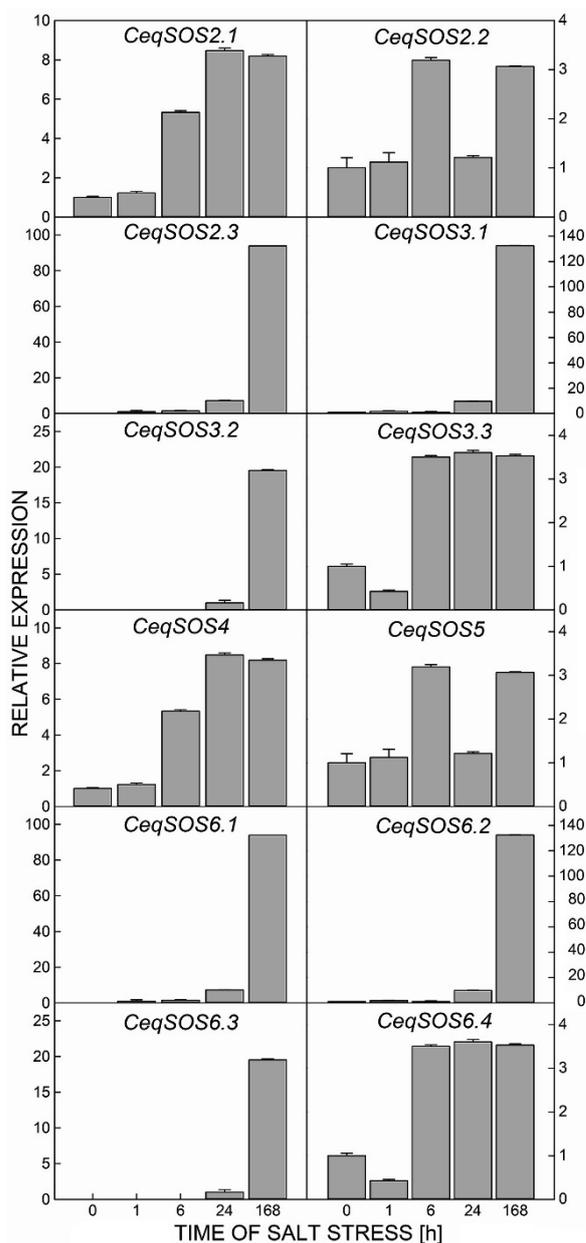


Fig. 6. Effect of different duration of 200 mM NaCl treatment on expressions of SOS genes in roots of *C. equisetifolia*. Means ± SEs, n = 3.

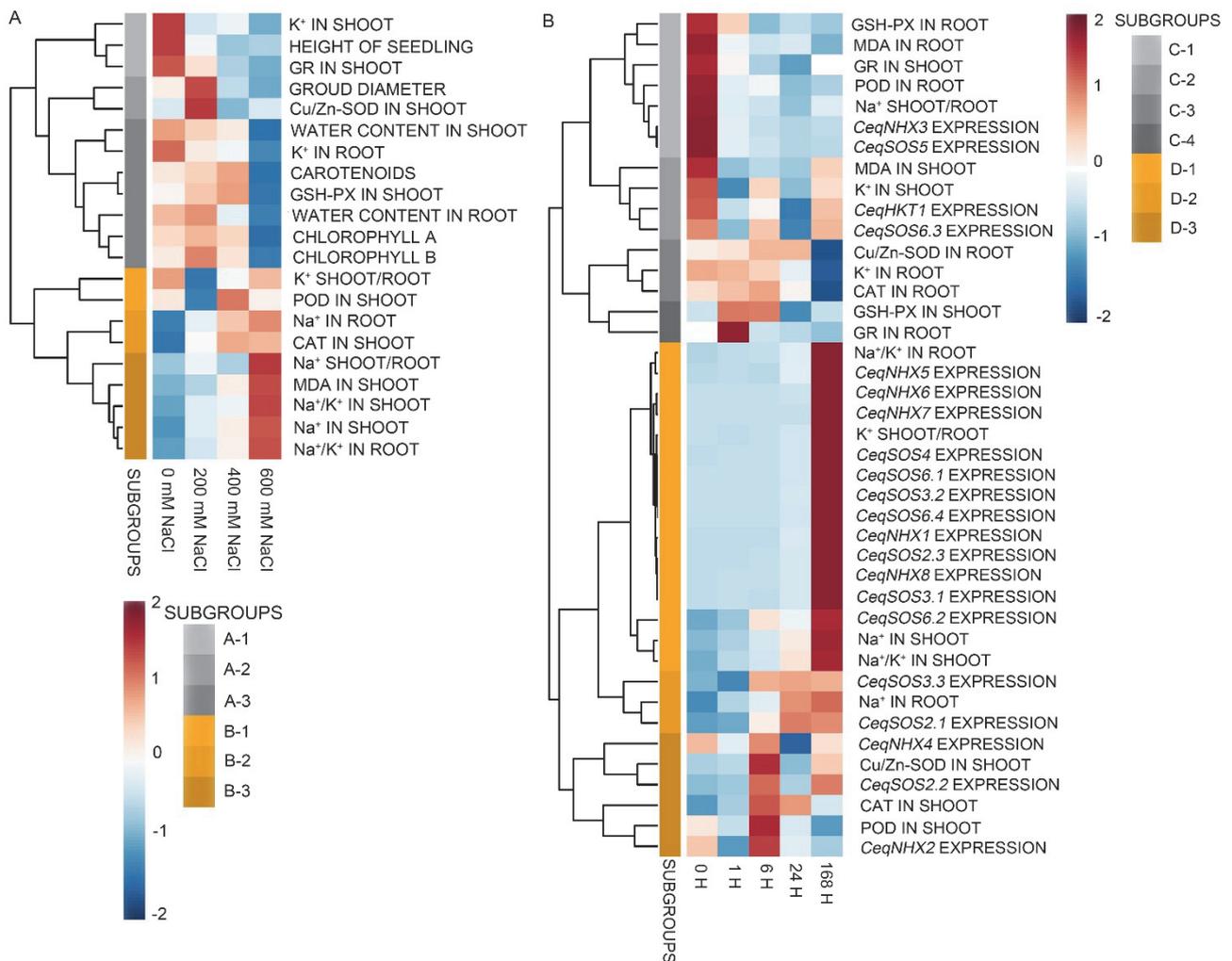


Fig. 7. Patterns of changes in different traits and gene expressions. *A* - Patterns for different NaCl concentrations. *B* - Patterns for different treatment duration. Values were normalized by Z-score transformation, and then clustered based on Euclidean distance. Different sub-groups are represented by different colours along the left side of the heatmap.

## Discussion

As highly salt stress tolerant, *C. equisetifolia* was used in the present study to elucidate its adaptation to salinity stress using physiological and transcriptional analyses. Under NaCl stress, higher Cu/Zn-SOD, GSH-PX, and CAT activities in roots and shoots, lower Na<sup>+</sup> content in shoots and lower Na<sup>+</sup>/K<sup>+</sup> ratios than in control were found. Salt stress increased the transcriptions of most *NHXs* and *SOS* genes.

Although no visual symptoms of NaCl toxicity were observed in *C. equisetifolia* shoots and roots when exposed to 200 and 400 mM NaCl solution, NaCl resulted in a significant reduction in their growth (fresh mass and number of branches and twigs). In *Brassica juncea* and *Panicum miliaceum*, the growth reduction under salt stress is connected with the inhibition of Chl biosynthesis and decreased Chl *a* and Chl *b* content

(Sabir *et al.* 2009, Mittal *et al.* 2012). It was surprising that Chl *a* and Chl *b* content was almost unchanged in our treatments with 200 and 400 mM NaCl. Hence, we speculated that *C. equisetifolia* plants tend to close stomata, subsequently leading to reduced photosynthesis and so plant growth. The roots were apparently damaged by 600 mM NaCl and the most affected were lateral roots. Our study suggests that salt stress inhibited plant growth by decreasing root growth and preventing formation and elongation of new twigs in *C. equisetifolia*.

At the biochemical level, antioxidant enzymes like SOD, CAT, POD, and GR play key roles in scavenging ROS produced in plant cells as byproducts of aerobic metabolism or as a result of disturbances in cell metabolic processes during salinity or other abiotic stresses (Apel and Hirt 2004). Recent studies show that CAT, SOD, GR,

and APX activities are mostly up-regulated under salt stress (Yıldızıtugay *et al.* 2011, Mittal *et al.* 2012, Sekmen *et al.* 2012). In the present study, CAT and GSH-PX activities in shoots increased significantly with increasing NaCl concentration and duration of treatment. Parallel to our findings, significantly increased activities of CAT and GSH-PX were found in *Suaeda salsa*, *Bruguiera gymnorhiza*, and 5-week-old tomato plants (Takemura *et al.* 2000, Caihong *et al.* 2005, Gapińska *et al.* 2008). Such results suggest that CAT and GSH-PX are key antioxidant enzymes acting synergistically in protecting cells against oxidative stress under salinity. The increase in SOD activity in shoots was observed after 6 h of 200 mM NaCl while SOD activity was not changed or decreased in other treatments. Hence, we conclude that SOD was essential at lower NaCl concentrations and during short periods for rapidly detoxifying superoxide anion in *C. equisetifolia* shoots. Similar results were also found in an endemic halophyte *Centaurea tuzgoluensis* under salt stress (Yıldızıtugay *et al.* 2011). Additionally, POD activity is essential in response to stress and significantly increases in tomato plants (Mittova *et al.* 2002, Chen and Heuer 2013), mulberry (Sudhakar *et al.* 2001) and alfalfa (Wang *et al.* 2009). In the present study, POD activity in roots of *C. equisetifolia* significantly increased with higher NaCl concentrations. However, POD activity in shoots did not significantly change with increasing NaCl concentration in accordance with results found in wheat (Perveen *et al.* 2011). Therefore, we suggest that POD probably contributed to a physical barrier against NaCl stress by participating in lignin biosynthesis, which also explained why POD activity did not significantly increase with higher Na<sup>+</sup> content in shoots.

The lower MDA content in shoots was observed in *C. equisetifolia* with increasing duration of salt stress, which also happens in cotton under low NaCl concentration (Meloni *et al.* 2003). We proposed that higher SOD, CAT, and GSH-PX activities rapidly detoxified ROS in *C. equisetifolia* shoots and then induced the lesser degree of membrane damage during short periods at lower NaCl concentrations.

Among salt-tolerant traits, the ability to restrict the transport and accumulation of Na<sup>+</sup> in shoots appears to be the most significant for adapting to salinity (Munns and Tester 2008). The *C. equisetifolia* plants accumulated

more Na<sup>+</sup> in roots with increasing NaCl concentrations and time and plants showed rather low Na<sup>+</sup> content in shoots. Although Na<sup>+</sup>/K<sup>+</sup> in shoots and roots significantly increased with increasing NaCl concentrations and treatment duration, *C. equisetifolia* had still sufficiently high K<sup>+</sup> content. The observed reduction in growth was caused by increased Na<sup>+</sup>/K<sup>+</sup> ratio, but it was not lethal for *C. equisetifolia*. This is consistent with previous studies in barley and *Gypsophila oblongeolata* (Widodo *et al.* 2009, Sekmen *et al.* 2012). Many halophytic plants can accumulate inorganic ions to concentrations equal to or greater than those of the surrounding root solution to facilitate water uptake from the medium (Bradley and Morris 1991, Aghaleh *et al.* 2009). The accumulation of Na<sup>+</sup> in the root endodermis and exodermis is enabled by the deposition of apoplastic barriers, and thus Na<sup>+</sup> is prevented from direct entry with external fluid into the stele and so to the shoot (Krishnamurthy *et al.* 2011). However, excessive Na<sup>+</sup> often interferes with K<sup>+</sup> accumulation, which results in impaired metabolic activities.

Various Na<sup>+</sup> transport proteins have been identified to restrict transport of Na<sup>+</sup> to shoots (Munns and Tester 2008). Among them, SOS pathway proteins and vacuolar NHX are considered as two efficient ways to reduce ion toxicity in plant cells by extrusion of Na<sup>+</sup> to the apoplast or external environment or sequestration of Na<sup>+</sup> in vacuoles (Munns and Tester 2008, Yang *et al.* 2009, Ji *et al.* 2013). To understand the mechanisms underlying limited Na<sup>+</sup> transport to the shoots in *C. equisetifolia*, we analyzed expressions of *CeqHKT1*, *CeqNHX*, and *CeqSOS* genes in this study. Expressions of all *CeqSOS* genes and *CeqNHX1*, *CeqNHX5*, *CeqNHX6*, *CeqNHX7*, and *CeqNHX8* genes in roots were upregulated under salt stress and expressions of most these genes was higher at 168-h salt treatment. This may be an important reason for the reduced shoot Na<sup>+</sup> content and salt damage. In previous studies, SOS signalling pathway activation was recognized as a key mechanism for Na<sup>+</sup> exclusion and ion homeostasis control (Zhu 2000, Ramezani *et al.* 2012, Feki *et al.* 2013, Zhao *et al.* 2016). The high expression of these genes at salt stress may be ascribed to signals originating from roots under salt stress, suggesting that *C. equisetifolia* roots could induce salt-tolerant genes to respond to salt stress.

## Conclusions

Salt stress in *C. equisetifolia* resulted in reduced shoot mass, shorter branches, and fewer twigs. Salinity induced several physiological and biochemical changes in *C. equisetifolia*, including small reduction in pigment contents, as well as decrease in K<sup>+</sup> content and increase in Na<sup>+</sup> accumulation and the Na<sup>+</sup>/K<sup>+</sup> ratio. The expression of all *CeqSOS* genes and *CeqNHX1*, *CeqNHX5*, *CeqNHX6*,

*CeqNHX7*, and *CeqNHX8* involved in Na<sup>+</sup> exclusion and ion homeostasis in roots were upregulated under salt stress. This may be important for high tolerance of *C. equisetifolia* to salinity stress. The salt tolerance of this species was also associated with increased activities of antioxidant enzymes CAT and GSH-PX.

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