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BRIEF COMMUNICATION

Antioxidative enzymes in two *in vitro* cultured *Salicornia* species in response to increasing salinity

M. AGHALEH[1]*, V. NIKNAM[2]*, H. EBRAHIMZADEH[1], and K. RAZAVI[3]

Department of Biology, Faculty of Science, Razi University, Kermanshah 14155-6343, Iran[1]
School of Biology, and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran 14155-6455, Iran[2]
National Institute of Genetic Engineering and Biotechnology, Tehran 14155-6343, Iran[3]

Abstract

The effects of salt stress on dry mass, lipid peroxidation, polyphenol and hydrogen peroxide content and activities of antioxidative enzymes were investigated in seedlings of *Salicornia persica* and *S. europaea* grown *in vitro*. Seeds were germinated under a broad range of NaCl concentrations (0, 100, 200, and 300 mM) on Murashige and Skoog medium for 45 d. Dry mass of both species increased at low (100 mM) salinity but decreased at higher NaCl concentrations. Malondialdehyde (MDA) content decreased at low salinity, whereas increased at 200 and 300 mM NaCl. H_2O_2 content in *S. europaea* was considerably enhanced by salinity, but it was not significantly affected in *S. persica*. The salt stress progressively enhanced the polyphenol content in *S. persica*, whereas in *S. europaea*, it increased with respect to the control only at higher salinities. In both species, the salinity progressively enhanced the superoxide dismutase (SOD) and peroxidase (POD) activities, whereas the CAT activity was only registered at the low salinity and the APX activity decreaseed in both species. The results indicate that *S. persica* exhibited a better protection mechanism against oxidative damage and it is more salt-tolerant than *S. europaea*.

Additional key words: ascorbate peroxidase, catalase, H_2O_2, malondialdehyde, peroxidase, *Salicornia europea*, *Salicornia persica*, superoxide dismutase.

The harmful effects of salinity on crop performance may be attributed to the ionic effect, osmotic effect, and alteration in ionic composition leading to deficiency of nutrients. As a consequence of these primary effects, a secondary stress, such as oxidative damage often occurs (*e.g.,* Sairam *et al.* 2002). One of the biochemical changes occurring when plants are subjected to salt stress is the production of reactive oxygen species (ROS). Halophytes, besides being able to regulate the ion and water movements, should have better antioxidant systems for the effective removal of ROS (Rout and Shaw 2001). The antioxidative enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) play a key role in removing ROS produced in plant cells as byproducts of normal cell metabolism or as a result of disturbance in the cell metabolic processes under abiotic stresses (Shaw *et al*. 2004). Therefore, the aim of this study was to evaluate whether the activities of antioxidative enzymes, lipid peroxidation, and content of polyphenols and H_2O_2 play a role in salinity tolerance of *Salicornia* species.

Seeds of *Salicornia persica* Akhani and *Salicornia europaea* L. were surface sterilized in a 10 % (m/v) sodium hypochlorite solution, containing a few drops of *Tween 20* for 5 min, followed by three fold washing with sterile distilled water. The seeds were germinated on Murashige and Skoog (1962; MS) media containing 0, 100, 200, and 300 mM NaCl under a 16-h photoperiod (white fluorescent lamps: irradiance of 33 μmol m^{-2} s^{-1}) and temperature of 25 ± 2 °C. *In vitro* formed shoots were maintained in a growth chamber for 45 d under above conditions.

The polyphenol content was determined by the Folin-Denis method (Folin and Denis 1912, Waterman and Mole 1994). The hydrogen peroxide (H_2O_2) content was determined according to Sergiev *et al*. (1997). The lipid

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Abbreviations: DTT - dithiothreitol; EDTA - ethylenediamine-N,N,N′,N′-tetraacetic acid; PVP - polyvinylpyrrolidone.
* Corresponding authors; fax: (+ 98) 831 81211869, e-mails: aghaleh@khayam.ut.ac.ir; niknamv@ut.ac.ir

peroxidation was measured in terms of malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction (Heath and Packer 1968).

Shoots of plants were homogenized in a chilled (4 °C) mortar using a 50 mM Tris-HCl buffer (pH 7.0) containing 10 mM EDTA, 2 mM $MgSO_4$, 20 mM dithiotreitol (DTT), 10 % (v/v) glycerol, and 2 % (m/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged twice at 13 000 *g* and 4 °C for 25 min, then the supernatant transferred to Eppendorf tubes and kept on ice at 4 °C for 60 min. The total protein content was measured using the Bradford (1976) method. The specific activities of catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), and superoxide dismutase (SOD, EC 1.15.1.1) were measured by the methods of Aebi (1974), Abeles and Biles (1999), Chen and Asada (1989), and Yu and Rengel (1999), respectively. SOD, CAT, POD, and APX isoforms were examined on a 10 % non-denaturating acrylamide gel using the methods of Laemmli (1970), Anderson *et al*. (1995), Fielding and Hall (1978), and Mittler and Zilinskas (1993), respectively. For SOD isoforms identification, assays were performed in the presence of selective inhibitors. KCN (3 mM) inhibited only CuZn SOD. H_2O_2 (5 mM) inhibited both Cu-Zn SOD and Fe SOD. Mn SOD was not inhibited by KCN or H_2O_2 (Lee *et al*. 2001).

The data were analyzed by using both one- and two-way analyses of variance (*ANOVA*) and the mean differences were compared by the lowest standard deviations (LSD) test. Each data point was the mean of three replicates.

Both the species showed an increase in the dry mass at the low salinity (100 mM NaCl) and a decrease at the higher salinities (Table 1). The salt stress progressively enhanced the polyphenol content in *S. persica*, whereas in *S. europaea*, there was a drop in the polyphenol content under the NaCl application in comparison to the control plants (Table 1). As illustrated in earlier studies, polyphenols can act as ROS scavengers, iron chelators, and enzyme modulators in plant cells and their content usually increases upon environmental stresses (Kirakosyan

Table 1. Changes in the dry mass [mg seedling^{-1}], content of MDA [nmol g^{-1}(f.m.)], H_2O_2 [μmol g^{-1}(f.m.)], and polyphenols [mg g^{-1}(d.m.)], and in specific activities of SOD [U mg^{-1}(protein)], CAT [μmol(H_2O_2) mg^{-1}(protein) min^{-1}], POX [μmol(benzidine) mg^{-1}(protein) min^{-1}], and APX [μmol(ascorbate) mg^{-1}(protein) min^{-1}] in *S. persica* and *S. europaea* treated with various concentrations of NaCl [mM] for 45 d. Means ± SE of three replicates. One unit of SOD was defined as the amount of enzyme which caused 50 % inhibition of NBT reduction. Different letters indicate significant differences ($P < 0.05$).

Species	NaCl	Dry mass	MDA	H_2O_2	Polyphenols	SOD	CAT	POX	APX
S. persica	0	2.37±0.2b	4.01±0.5b	1.20±0.4a	0.25±0.0d	14.5±2.4a	2.6±0.8b	0.63±0.03c	2.71±0.30a
	100	3.20±0.3a	4.06±0.2b	1.60±0.1a	0.28±0.0c	15.5±0.9a	6.2±0.4a	1.08±0.07bc	2.13±0.09a
	200	2.45±0.2b	4.42±0.8a	1.56±0.2a	0.31±0.0b	18.0±0.5a	3.9±0.2b	1.38±0.20b	0.71±0.08b
	300	2.37±0.2b	5.33±0.5a	1.81±0.2a	0.33±0.0a	20.4±2.1a	3.5±0.2b	2.09±0.20a	0.89±0.14b
S. europaea	0	2.27±0.2a	4.26±0.2b	0.70±0.1d	0.34±0.0a	12.9±0.5c	3.1±1.1a	1.02±0.02a	1.71±0.54a
	100	2.57±0.1a	4.48±0.4b	1.17±0.1c	0.28±0.0c	14.7±0.2bc	5.9±1.0a	0.78±0.13a	0.82±0.12b
	200	2.30±0.1a	5.74±0.1a	2.25±0.2b	0.31±0.0b	16.3±1.2b	5.8±1.0a	0.90±0.13a	0.80±0.07b
	300	1.75±0.1b	6.40±0.3a	2.66±0.1a	0.34±0.0a	19.4±0.7a	3.3±0.7a	0.75±0.12a	0.87±0.04b

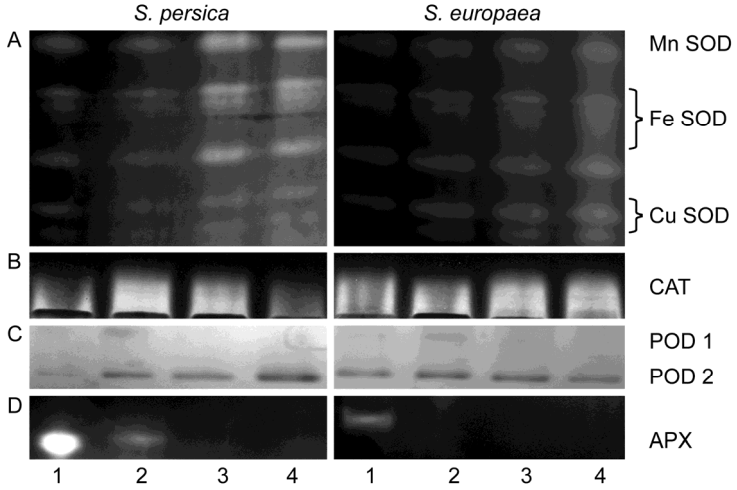

Fig. 1. Activity staining for SOD (*A*), CAT (*B*), POD (*C*), and APX (*D*) isoforms in *S. persica* and *S. europaea* subjected to 0, 100, 200, and 300 mM NaCl (*lanes* 1 to 4).

et al. 2004). The H_2O_2 content in *S. europea* was considerably increased by salinity (Table 1), but it was less affected in *S. persica*. In both the species, the low salinity (Table 1) had no significant effect on the MDA content in comparison to the control plants, whereas 200 and 300 mM NaCl caused an increase in the MDA content. In the present study, a lower MDA increase observed in salt-treated *S. persica* suggests better protection from oxidative damage. Shalata and Tal (1998) and Bor *et al*. (2003) found correlation between increased antioxidant enzyme activities and decreased lipid peroxidation under salt stress in salt tolerant *Lycopersicum pennellii* and *Beta maritime*.

In both *Salicornia* species, the total SOD activity was gradually enhanced by the salinity stress, and the highest SOD activity was recorded at 300 mM (Table 1). The analyses of SOD extracts on PAGE revealed six isoforms in both the species (Fig. 1*A*). Preincubation with the specific inhibitor revealed that two bands are CuZn SOD isoforms, three bands are Fe SOD, and one band is a Mn SOD isoform. The intensity of all the bands increased with increasing NaCl concentration.

The CAT activity increased in both the species at 100 mM NaCl. However, it decreased at 200 mM in *S. persica* and in both the species at 300 mM (Table 1). The analyses using PAGE revealed one CAT isoform in both species (Fig. 1*B*). In *S. persica,* the CAT band at low salinity was activated, but at the higher salinities, it was inhibited. In *S. europaea*, the band at 100 mM NaCl was inhibited, whereas at the higher salinities, this band was stimulated.

The total POD activity increased by the salinity in *S. persica* (Table 1). The POD activity revealed two isoforms of peroxidase on the gels in both the species (Fig. 1*C*). In both the species, the POD 2 band was strongly activated by the salinity, whereas POD 1 band was detected only at the low salinity. The total APX activity in both the species gradually declined with increasing salinity (Table 1). Gel analyses revealed one isoform in both the species (Fig. 1*D*), which was strongly inhibited by the salinity. The APX is not salt responsive in aquatic plants (Rout and Shaw 2001).

NaCl induced an increase in the cellular H_2O_2 content in both the species (Table 1). It is a circumstantial evidence of generation of $O_2^{\bullet-}$ under NaCl stress, since H_2O_2 is generated mainly by dismutation of $O_2^{\bullet-}$ catalyzed by SOD. H_2O_2 accumulation has been considered as a sign of oxidative stress, as its reaction with $O_2^{\bullet-}$ leads to the formation of highly reactive hydroxyl radical ($^{\bullet}OH$) causing peroxidative damage of biomolecules (Shaw *et al*. 2004, Kim *et al*. 2005). Hence, it is a requirement for plants to keep the content of both H_2O_2 and $O_2^{\bullet-}$ to a minimum in order to prevent the Fenton reaction from proceeding (Shaw *et al*. 2004, Mallik *et al*. 2011). A significant increase in the activities of SOD, CAT, and POD in the *Salicornia* species in response to the NaCl treatment protected the plants from NaCl induced oxidative stress.

In conclusion, based on the data obtained (dry mass, content of MDA, polyphenols, and H_2O_2, and activities of antioxidant enzymes), it is clear that *S. persica* is more salt-tolerant than *S. europaea*. It is possible that the better salt-tolerance of *S. persica* is associated with its ability to maintain higher activities of the antioxidant enzymes.

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