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ORIGINAL ARTICLES

Antioxidative Defense Potential to Salinity in the Euhalophyte *Salicornia brachiata*

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Abstract The antioxidative defense mechanism to salinity was assessed by monitoring the activities of some antioxidative enzymes and levels of antioxidants in an obligate halophyte, *Salicornia brachiata*, subjected to varying levels of NaCl (0, 200, 400, and 600 mM) under hydroponic culture. In the shoots of *S. brachiata*, salt treatment preferentially enhanced the activities of ascorbate peroxidase (APX), guaiacol peroxidase (POX), glutathione reductase (GR), and superoxide dismutase (SOD), whereas it induced the decrease of catalase (CAT) activity. Similarly, salinity caused an increase in total glutathione content (GSH + GSSG) and a decrease in total ascorbate content. Growth of *S. brachiata* was optimum at 200 mM NaCl and decreased with further increase in salinity. Salinity caused an increase in Na^+ content and a decrease in K^+ content of shoots. Proline levels did not change at low (0-200 mM NaCl) or moderate (400 mM NaCl) salinities, whereas a significant increase in proline level was observed at high salinity (600 mM NaCl). Accumulation of Na^+ may have a certain role in osmotic homeostasis under low and moderate salinities in *S. brachiata*. Parameters of oxidative stress such as malondialdehyde (MDA), a product of lipid peroxidation, and H_2O_2 concentrations decreased at low salinity (200 mM NaCl) and increased at moderate (400 mM NaCl) and high salinities (600 mM NaCl). As a whole, our results suggest that the capacity to limit ionic and oxidative damage by the elevated levels of certain antioxidative enzymes and antioxidant molecules is important for salt tolerance of *S. brachiata*.

Keywords Antioxidative enzymes · *Salicornia brachiata* · Lipid peroxidation · Halophyte · Sodium chloride

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Introduction

Environmental stresses such as high light intensity, temperature extremes, drought, high salinity, herbicide treatment or mineral deficiencies induce oxidative stress in plants through an enhanced generation of reactive oxygen species (ROS) (Khanna-Chopra and Selote 2007). The ROS include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl free radical ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) (Mittler 2002). These cytotoxic ROS cause peroxidation of membrane lipids, denaturation of proteins, mutation of DNA, and various types of cellular oxidative damage (Azevedo Neto and others 2006). Plant cells are protected against the detrimental effects of ROS by a complex antioxidant system comprising nonenzymic and enzymic antioxidants (Foyer and Noctor 2003). Ascorbate (AsA) and glutathione (GSH) serve as potent nonenzymic antioxidants within the cell. AsA scavenges the most dangerous forms of ROS, that is, $^{\bullet}OH$, $O_2^{\bullet-}$, and H_2O_2, and dismutates H_2O_2 through the action of ascorbate peroxidase (Asada 2006). Reduced glutathione (GSH) participates in the regeneration of ascorbate via dehydroascorbate reductase (DHAR). It also reacts with singlet oxygen and $^{\bullet}OH$ radicals to protect protein thiol groups, thus representing the major cytoplasmic thiol disulfide redox buffer in the plants (Asada 2006). The enzymic antioxidants

include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POX), enzymes of the ascorbate-glutathione cycle ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Foyer and Noctor 2003). The enzyme SOD dismutates $O_2^{\bullet -}$ to H_2O_2 and is present in the cytosol and different cell organelles. Catalases eliminate H_2O_2 by breaking it down to H_2O and O_2 and do not require any reducing equivalent (Sharma and Dubey 2005). Peroxidases that are located in the cytosol, vacuole, and extracellular space scavenge H_2O_2 by oxidation of substrates. POXs require the phenolic compound guaiacol as the electron donor to decompose H_2O_2 (Asada 2006).

In higher plants, there are several distinct isoforms of APX that convert H_2O_2 to H_2O using ascorbate as an electron donor and they are localized in the cytosol and various organelles (Madhusudhan and others 2003). APX plays an important role in protecting cells against the damaging effect of H_2O_2. Because ascorbate is oxidized to monodehydroascorbate in APX-catalyzed H_2O_2 decomposition, a system for regeneration of ascorbate is necessary. A regenerating cycle involving AsA, GSH, and the specific enzymes MDHAR, DHAR, and GR is present in plant cells, that is, the ascorbate-glutathione cycle or the Halliwell-Asada pathway (Asada 2006). In this cycle some of the monodehydroascorbate is regenerated by NAD(P)H in a reaction catalyzed by MDHAR, but the remainder undergoes spontaneous dismutation to ascorbate and dehydroascorbate. DHAR catalyzes the reduction of DHA to AsA by oxidizing GSH. For most of the cellular functions GSH must be available in its reduced form (GSH). The reduction of oxidized glutathione (GSSG) to GSH is carried out by GR in a NADPH-dependent reaction (Foyer and Noctor 2003).

When plants are subjected to environmental stresses, the balance between the production of ROS and the quenching activity of the antioxidants becomes upset, often resulting in oxidative damage. Plants with high levels of antioxidants, either constitutive or induced, have been reported to provide sufficient resistance against oxidative damage (Parida and others 2004b).

Salinity is one of the major constraints affecting crop productivity in various regions of the world. Halophytes are known for their unique ability to tolerate high salinity, and these plants provide viable organisms for studying the mechanisms they use to handle high salt concentrations. *Salicornia brachiata* Roxb. is a stem succulent and leafless euhalophyte that belongs to Chenopodiaceae. It is one of the most salt-tolerant plant species in the world and requires an adequate concentration of NaCl for optimal growth and development (Reddy and others 1993). It has been reported that salinity induces the accumulation of low-molecular-weight organic compounds such as glycinebetaine, proline, and sugars in the cytoplasm that act as osmolytes and osmoprotectors in *Salicornia* (Moghaieb and others 2004). As oxidative stress is regarded as a key component in the injury of plants from abiotic stresses, the enzymes of the ascorbate-glutathione cycle constitute important components of the antioxidative defense system of plants. Although several studies have reported antioxidative defense mechanisms to combat environmental stresses in glycophytes (Hernandez and others 2000; Lee and others 2001; Dash and Mohanty 2002; Sharma and Dubey 2005; Azevedo Neto and others 2006; Khanna-Chopra and Selote 2007), there are very few reports on antioxidative defense systems in halophytes (Parida and others 2004b; Ben Amor and others 2005; Surowka and others 2007). The present study aimed to examine the possible involvement of ionic and oxidative stress and alteration in the status of nonenzymic and enzymic components of the antioxidant defense mechanism through study of some key enzymes of the ascorbate-glutathione cycle, ions, and proline levels in an obligate halophyte *S. brachiata* subjected to various levels of salinity. A detailed study of the activity behavior of antioxidative enzymes that are induced under salinity stress and are related to salt tolerance is essential to identifying the genes that encode them; this in turn may yield new leads to produce transgenic salt-tolerant crops using genes from *Salicornia* employing biotechnological approaches.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of *Salicornia brachiata* Roxb. were collected from salt marshes of Bhavnagar, Gujarat, India (latitude 21°35′ N and longitude 72°16′ E). Seeds were sown in plastic pots (25 cm × 10 cm) filled with soil:sand:peat (2:1:1). Seedlings were raised in the experimental greenhouse under nonsaline conditions by irrigating them with tap water and were exposed to daylight with photosynthetic active radiance (PAR) of 800–1000 (μmol m^{-2} s^{-1}). Two-month-old healthy seedlings of uniform size were selected for hydroponic culture in Hoagland's nutrient medium supplemented with NaCl (0, 200, 400, and 600 mM). The hydroponic cultures were maintained in a culture room under a photoperiod of 14 h at light intensities of 350 μmol m^{-2} s^{-1}, a room temperature of 22 ± 2°C, and relative humidity of 60%. The cultures were aerated continuously with an air bubbler. The nutrient solution was replaced with freshly prepared solution at 7-day intervals. The shoots were harvested after 0, 7, 14, and 21 days of

treatment from different sets of plants for assay of various antioxidants and antioxidative enzymes.

Measurement of Growth Parameters

Plant height and fresh and dry weights of shoots and roots of control and NaCl-treated plants were measured after 21 days of NaCl treatment. For measurement of fresh and dry weights, shoots and roots from control and NaCl-treated plants were excised and the fresh weight was recorded. Subsequently, these plant parts were wrapped in preweighed aluminum sheets and kept in an incubator at 80°C for 48 h and cooled in a desiccator before the dry weight was recorded.

Estimation of Ion Contents in Shoots

The shoot samples were dried in an oven at 80°C for 48 h for analysis of ions. After drying, the samples were homogenized and then ground shoot material (1 g) was placed in a 100-ml volumetric flask, a 10-ml mixture of HNO_3 and $HClO_4$ (9:4) was added, and the content of the flask was mixed by swirling. The flask was placed on a hot plate on low in a digestion chamber and then heated at higher temperature until the production of red NO_2 fumes ceased. The contents were further evaporated until the volume was reduced to 3–5 ml. Completion of digestion was confirmed when the liquid became colorless. After cooling the volumetric flask, 20 ml deionized water was added, the volume was made up to 100 ml, and the solution was filtered through Whatman No. 1 filter paper. Aliquots of this solution were used for the determination of Na^+ and K^+ content of shoots by inductively coupled plasma atomic emission spectrometry (Optima 2000, PerkinElmer, Waltham, MA, USA).

Estimation of Proline

After 21 days of NaCl treatment, shoot samples were harvested for estimation of proline, which was done following the method of Ringel and others (2003) using ninhydrin reagent. The absorbance was measured at 520 nm.

Preparation of Enzyme Extract and Assay of Enzyme Activity

Shoot tissues (0.5 g) were ground to a fine powder in liquid N_2 and then homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.05% (w/v) Triton X-100, and 5% (w/v) polyvinylpolypyrrolidone (PVPP) using a chilled pestle and mortar. The homogenate was centrifuged at 15,000*g* for 15 min at 4°C and the supernatants were collected and used for the assays of catalase, guaiacol peroxidase, glutathione reductase, and superoxide dismutase (SOD). For ascorbate peroxidase (APX) activity, separate extraction was done using the buffer mentioned above except it contained an additional 5 mM ascorbate to protect APX activity. Protein concentrations in the enzyme extract were determined by the method of Bradford (1976).

Superoxide Dismutase (EC 1.15.1.1)

Superoxide dismutase (SOD) activity was measured by its ability to inhibit photoreduction of nitroblue tetrazolium (NBT) (Beyer and Fridovich 1987). The reaction mixture (3 ml) contained 50 mM KPO_4 (pH 7.8), 9.9 mM L-methionine, 58 μM NBT, 0.025% Triton X-100, 2.4 μM riboflavin, and 50 μl of enzyme extract. Riboflavin was added last and the test tubes containing reaction mixture were incubated for 10 min under 300 μmol m^{-2} s^{-1} irradiance at 25°C. The increase in absorbance due to formazan formation was read at 560 nm. The reaction mixture with no enzyme developed maximum color because of the maximum rate of reduction of NBT. Nonirradiated reaction mixture was used as the control because it did not develop color. One unit of SOD was defined as the amount of enzyme that inhibits 50% NBT photoreduction.

Catalase (EC 1.11.1.6)

Catalase (CAT) activity was determined spectrophotometrically by measuring the disappearance rate of H_2O_2 at 240 nm, taking $\Delta\varepsilon$ at 240 nm as 43.6 M^{-1} cm^{-1} (Patterson and others 1984). The reaction mixture contained 50 mM potassium phosphate (pH 7.0) and 10.5 mM H_2O_2 (Miyagawa and others 2000). The reaction was run at 25°C for 2 min after adding the enzyme extract containing 20 μg of protein, and the initial linear rate of decrease in absorbance at 240 nm was used to calculate the activity.

Ascorbate Peroxidase (EC 1.11.1.11)

Ascorbate peroxidase (APX) was assayed as described by Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbic acid, and 0.1 mM H_2O_2. The reaction was started at 25°C by the addition of H_2O_2 after adding the enzyme extract containing 50 μg of protein. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient 2.8 mM^{-1} cm^{-1}.

Guaiacol Peroxidase (EC 1.11.17)

Guaiacol peroxidase (POX) activity was measured spectrophotometrically at 25°C by following the method of

Jebara and others (2005). The reaction mixture (2 ml) consisted of 50 mM potassium phosphate (pH 7.0), 9 mM guaiacol, and 19 mM H_2O_2. The reaction was started by the addition of an enzyme extract equivalent to 5 µg of protein. The formation of tetraguaiacol was measured at 470 nm ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$).

Glutathione Reductase (EC 1.6.4.2)

Glutathione reductase (GR) activity was determined at 25°C by measuring the rate of reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as the increase in absorbance at 412 nm ($\varepsilon = 14.15\ mM^{-1}\ cm^{-1}$) according to the method of Smith and others (1988). The reaction mixture (1 ml) consisted of 100 mM KPO_4 (pH 7.5), 1 mM EDTA, 1 mM oxidized glutathione (GSSG), 0.75 mM DTNB, 0.1 mM NADPH, and the enzyme. NADPH was added to start the reaction.

For CAT, APX, POX, and GR, one unit of enzyme was defined as the amount of enzyme necessary to decompose 1 µmol of substrate per minute at 25°C.

Measurement of Ascorbic Acid and Glutathione Levels

Ascorbic Acid

Total ascorbate (AsA + DHA) and ascorbate (AsA) were measured based on the reduction of ferric to ferrous ion with ascorbic acid in acid solution followed by the formation of the red chelate between the ferrous ion and bathophenanthroline (Wang and others 1991). Shoot tissue (0.2 g) was homogenized in 2 ml of cold 5% trichloroacetic acid (TCA) containing 4% PVPP. The homogenate was filtered through four layers of cheesecloth and centrifuged at 16,000*g* for 10 min at 4°C. The supernatant was used for total ascorbate (AsA + DHA) and reduced ascorbate (AsA) assay. The reaction mixture for AsA contained 20% ethanol, 4% TCA, 0.04% H_3PO_4-ethanol, 0.1% bathophenanthroline-ethanol, and 0.003% $FeCl_3$-ethanol. The reaction mixture was incubated at 30°C for 90 min for the Fe^{2+}-bathophenanthroline complex to develop and the A_{534} was recorded. Total AsA was determined through a reduction of DHA to AsA by dithiothreitol, after which 0.24% N-ethylmaleimide-ethanol was added in addition to the reaction mixture used for estimating AsA and the color developed was recorded at A_{534}. DHA was measured from the difference of total ascorbate and AsA values. A standard curve in the range of 0–10 µmol AsA was used.

Glutathione

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined according to the method of Griffith (1980). Shoot tissues (0.5 g) from control and NaCl-treated plants were ground using a pestle and mortar in liquid nitrogen with 1 ml of 5% sulfosalicylic acid and centrifuged at 10,000*g* for 5 min. A 300-µl aliquot of the supernatant was removed and neutralized to pH 7.0 by adding 18 µl of 7.5 M triethanolamine. A 150-µl sample was used for the determination of total glutathione (GSH + GSSG). Another 150-µl sample was pretreated with 3 µl of 2-vinylpyridine for 60 min at 20°C to mask GSH and to allow the determination of GSSG alone. Fifty-microliter aliquots of both types of samples were mixed with 700 µl of 0.3 M NADPH, 100 µl of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and 150 µl of 125 mM $NaPO_4$-6.3 mM EDTA buffer, pH 6.5. Ten microliters of glutathione reductase (50 U ml^{-1}) was added and the change in absorbance at 412 nm was monitored. A standard curve prepared by using GSH and GSSG was used in the calculation of the amounts of total glutathione, reduced GSH (total GSH - oxidized GSSG), and GSSG.

Hydrogen Peroxide (H_2O_2) Levels

Hydrogen peroxide contents were determined by the peroxidase-coupled assay according to Veljovic-Jovanovic and others (2002). Shoot samples (100 mg) were ground to a fine powder in liquid nitrogen and the powder was extracted in 2 ml of 1 M $HClO_4$ in the presence of 5% insoluble PVPP at 4°C. The homogenates were centrifuged at 12,000*g* for 10 min at 4°C and the supernatant was neutralized with 5 M K_2CO_3 to pH 5.6 in the presence of 50 µl of 0.3 M phosphate buffer (pH 5.6). The homogenate was centrifuged at 12,000*g* for 1 min to remove $KClO_4$. The sample was incubated prior to assay for 10 min with 1 U ascorbate oxidase (Sigma, St. Louis, MO, USA) to oxidize ascorbate prior to assay. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 3.3 mM 3-(dimethylamino) benzoic acid, 0.07 mM 3-methyl-2-benzothiazolinonehydazone, and 0.3 U horseradish peroxidise (Sigma). The reaction was initiated by addition of 100 µl of the sample. The absorbance change at 590 nm was monitored at 25°C. H_2O_2 content in the extract was calculated from a standard curve prepared by using 2–12 nmol H_2O_2.

Lipid Peroxidation

The extent of lipid peroxidation was estimated by determining the concentration of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction following the method of Draper and Hardley (1990). Shoot material (0.5 g) was homogenized in 2 ml of 0.1% (w/v) TCA solution. The homogenate was centrifuged at 15,000*g* for 10 min and 1 ml of the supernatant obtained was added to 4 ml of 0.5% (w/v) TBA in 20% (w/v) TCA. The

mixture was incubated at 90°C for 30 min and the reaction was stopped by placing the reaction tubes in an ice water bath. Samples were centrifuged at 10,000*g* for 5 min and the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The concentration of MDA was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}.

All the spectrophotometric analyses were performed using a UV-visible spectrophotometer (SpectramaxPlus 384, Molecular Devices, Sunnyvale, CA, USA) and Soft-Max Pro v5.2 software (Molecular Devices) at Kinetic and Endpoint modes.

Statistical Analysis

Statistical analysis of the results was performed according to Duncan's multiple-range tests. Data were subjected to a two-way ANOVA and the least significant difference at $P \leq 0.01$ was determined following the method of Sokal and Rohlf (1995).

Results

Plant Growth

The effects of NaCl on plant growth, assessed by plant height and fresh and dry matter of the shoots and roots, were studied after 21 days of exposure to varying concentrations of NaCl (Table 1). Plant height and fresh and dry weights were maximal in the plants treated with 200 mM NaCl and decreased progressively with increasing salt concentration The results showed that *S. brachiata* required 200 mM NaCl to express its maximal growth potential (Table 1).

Ion Homeostasis in *S. brachiata* Under Salinity

To confirm whether the ion and organic solutes determine osmotic homeostasis, we measured the Na^+, K^+, and proline contents in shoots. After 21 days of NaCl treatment, the Na^+ content of shoots increased significantly with an increase in salt concentration and decreased levels of K^+ were noted (Table 2). Proline content increased significantly at 600 mM NaCl compared to that at 0 mM NaCl. However, there was no significant difference in proline contents between 0, 200, and 400 mM NaCl-treated plants of *S. brachiata* (Table 2).

Effects of Salinity on Antioxidative Enzymes

Superoxide Dismutase (SOD)

Figure 1 shows the changes in SOD activity during exposure to varying concentrations of NaCl. SOD activity increased significantly with increasing concentrations of NaCl. After 21 days of exposure, SOD activity in *S. brachiata* increased by 135, 144, and 149% in 200, 400, and 600 mM NaCl-treated plants, respectively, compared to controls (Fig. 1).

Catalase (CAT)

The catalase activity increased marginally (4%) at a low concentration (200 mM) of NaCl and decreased with further increase in salinity (400 and 600 mM, Fig. 2). The maximum decline in catalase activity after 21 days of exposure was 19 and 25% in 400 and 600 mM NaCl-treated plants, respectively, whereas no loss in CAT activity was observed in control (0 mM NaCl-treated) plants during the 21-day period of experimentation (Fig. 2).

Ascorbate Peroxidase (APX)

In contrast to CAT, APX activity was enhanced significantly with an increase in salt concentration (Fig. 3). A rapid enhancement in APX activity was observed in the first week of NaCl treatment (39, 62, and 78% in 200, 400, and 600 mM NaCl-treated plants, respectively), after which it increased steadily (Fig. 3). At the end of the experimental period (21 days), APX activity increased by 87, 91.6, and 96.9% in 200, 400, and 600 mM NaCl-treated plants, respectively, compared to controls (Fig. 3). The

Table 1 Effects of various concentrations of NaCl (0–600 mM) on growth parameters of *S. brachiata* measured after 21 days of NaCl treatment

NaCl (mM)	Plant height (cm $plant^{-1}$)	Shoot FW (g $plant^{-1}$)	Root FW (mg $plant^{-1}$)	Shoot DW (mg $plant^{-1}$)	Root DW (mg $plant^{-1}$)
0	9.3 ± 0.4a	0.48 ± 0.04a	57.2 ± 5.8a	41.4 ± 3.5a	13.6 ± 1.2a
200	12.65 ± 0.5b	1.56 ± 0.07b	90.5 ± 6.7b	116.7 ± 7.4b	20.5 ± 1.5b
400	10.2 ± 0.3c	1.01 ± 0.05c	75.5 ± 5.2c	73.5 ± 5.5c	17.2 ± 1.7c
600	7.85 ± 0.3d	0.55 ± 0.05d	67.0 ± 4.9d	62.9 ± 6.9d	15.7 ± 0.8c
LSD ($P = 0.01$)	0.71	0.10	10.51	11.13	2.48

Values are mean ± SE, $n = 3$. Different letters indicate statistically different means at $P \leq 0.01$

Table 2 Effects of varying concentrations of NaCl (0–600 mM) on ions and proline levels in shoots of *S. brachiata* measured after 21 days of NaCl treatment

NaCl (mM)	Na^+ (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	Na^+/K^+	Proline (μg g^{-1} FW)
0	59.2 ± 1.5a	23.12 ± 1.02a	2.56 ± 0.16[a]	3.2 ± 0.02[a]
200	124.65 ± 1.4b	16.25 ± 1.23b	7.67 ± 0.35b	3.5 ± 0.03[a]
400	162.18 ± 2.1c	10.17 ± 0.72c	15.94 ± 0.45c	3.7 ± 0.02[a]
600	189.27 ± 2.5d	9.76 ± 0.55d	19.39 ± 0.73d	8.1 ± 0.32b
LSD ($P = 0.01$)	5.29	2.51	1.30	0.55

The values are mean ± SE. Different letters indicate statistically different means at $P \leq 0.01$

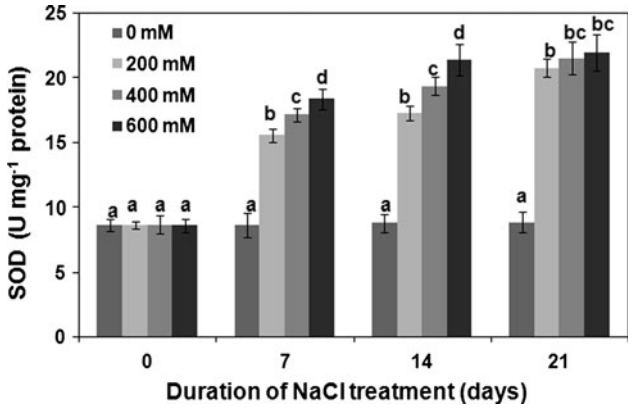

Fig. 1 Effects of different concentrations of NaCl on the activity of superoxide dismutase (SOD) in *S. brachiata* measured as a function of days of NaCl treatment. Enzyme activity was expressed as units per mg protein (U mg^{-1} protein). One unit of SOD was defined as the amount of enzyme that inhibits 50% NBT photoreduction. Values are mean ± SE. Letters beside the symbols indicate statistically different means at $P \leq 0.01$

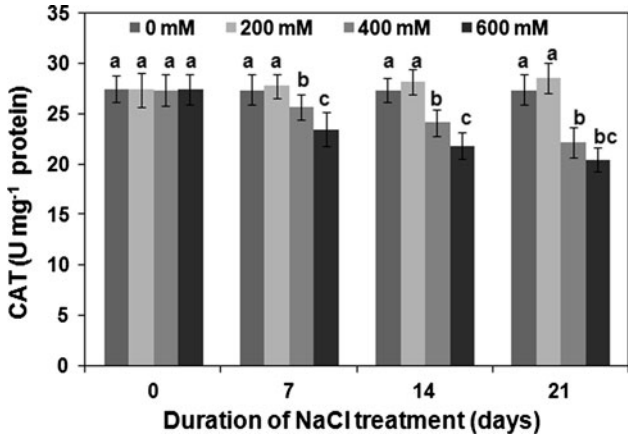

Fig. 2 Effects of different concentrations of NaCl on the activity of catalase (CAT) in *S. brachiata* measured as a function of days of NaCl treatment. Enzyme activity was expressed as units per mg protein (U mg^{-1} protein). One unit of CAT was defined as the amount of enzyme necessary to decompose 1 μmol of substrate per minute at 25°C. Values are mean ± SE. Letters beside the symbols indicate statistically different means at $P \leq 0.01$

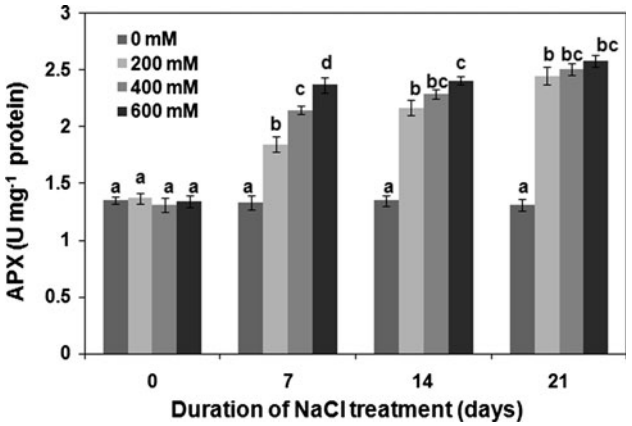

Fig. 3 Effects of different concentrations of NaCl on the activity of ascorbate peroxidase (APX) in *S. brachiata* measured as a function of days of NaCl treatment. Enzyme activity was expressed as units per mg protein (U mg^{-1} protein). One unit of APX was defined as the amount of enzyme necessary to decompose 1 μmol of substrate per minute at 25°C. Values are mean ± SE. Letters beside the symbols indicate statistically different means at $P \leq 0.01$

control plants showed a more or less uniform APX activity throughout the experiment.

Guaiacol Peroxidase (POX)

POX activity increased with increasing salt concentration (Fig. 4). A rapid increase in POX activity was observed in the first week of NaCl treatment (59, 116, and 158% in 200, 400, and 600 mM NaCl-treated plants, respectively, compared to 0 mM), after which POX activity increased steadily (Fig. 4). After 21 days, POX-specific activity increased by 156, 192, and 208% in 200, 400, and 600 mM NaCl-treated plants, respectively, compared with controls.

Glutathione Reductase (GR)

The effect of varying concentrations of NaCl on the GR activity of *S. brachiata* is shown in Fig. 5. As is evident, GR activity increased significantly with increasing

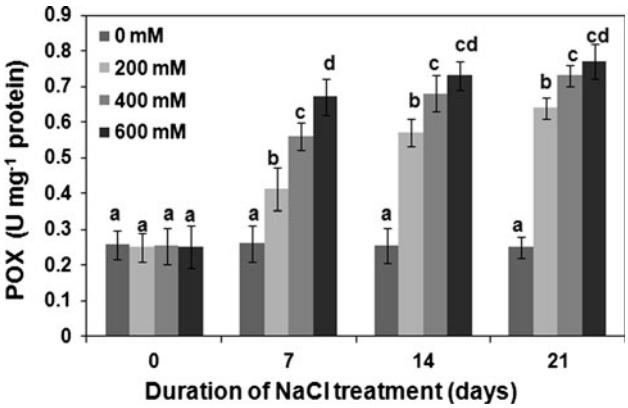

Fig. 4 Effects of different concentrations of NaCl on the activity of guaiacol peroxidase (POX) in *S. brachiata* measured as a function of days of NaCl treatment. Enzyme activity was expressed as units per mg protein (U mg^{-1} protein). One unit of POX was defined as the amount of enzyme necessary to decompose 1 μmol of substrate per minute at 25°C. Values are mean ± SE. Letters beside the symbols indicate statistically different means at $P \leq 0.01$

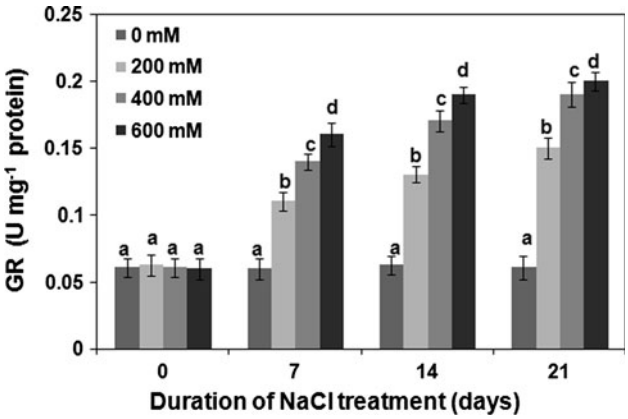

Fig. 5 Effects of different concentrations of NaCl on the activity of glutathione reductase (GR) in *S. brachiata* measured as a function of days of NaCl treatment. Enzyme activity was expressed as units per mg protein (U mg^{-1} protein). One unit of GR was defined as the amount of enzyme necessary to decompose 1 μmol of substrate per minute at 25°C. Values are mean ± SE. Letters beside the symbols indicate statistically different means at $P \leq 0.01$

concentration of NaCl. After 21 days of exposure, GR activity increased by 150, 216, and 233% in 200, 400, and 600 mM NaCl-treated plants, respectively, compared to control-grown plants.

Effects of Salinity on Ascorbate and Glutathione Pools

The total ascorbate concentration decreased significantly with increase in salinity (Table 3). The same pattern was observed for reduced ascorbate. DHA content decreased under low salinity (200 mM NaCl), whereas it increased under moderate (400 mM) and high salinities (600 mM, Table 3). The ASA/DHA ratio increased under low salinity (200 mM NaCl) and decreased under moderate (400 mM) and high salinities (600 mM, Table 3).

The total glutathione content increased significantly with increasing concentration of NaCl (Table 4). A similar pattern was observed for reduced glutathione (GSH). However, oxidized glutathione (GSSG) levels remained unaffected by salinity (Table 4). As a result, the GSH/GSSG ratio followed the same pattern as that of total glutathione content (Table 4).

H_2O_2 Level and Lipid Peroxidation

H_2O_2 content in the control leaf did not show much change during the 21-day period of the experiment (Table 5). However, it decreased at low salinity (200 mM NaCl) and increased at moderate (400 mM) and high salinities (600 mM NaCl). After 21 days of NaCl treatment, H_2O_2 content decreased by 19% in 200 mM NaCl-treated plants and increased by 19.5 and 24.6% in 400 and 600 mM NaCl-treated plants, respectively, compared to controls (Table 5).

Lipid peroxidation (MDA content) in the shoots of *S. brachiata* was correlated with salt-induced growth inhibition (Table 5). MDA content in *S. brachiata* decreased by 19% at low salinity (200 mM NaCl) and increased significantly to 67 and 123% at 400 and 600 mM NaCl, respectively, compared to the controls after 21 days of NaCl treatment (Table 5).

Discussion

The exposure to NaCl imposes oxidative stress in glycophytes as well as halophytes due to changes in the osmotic and ionic environment of the cell (Allakhverdiev and others 2000; Hasegawa and others 2000). *S. brachiata* thrived with 200 mM NaCl rather than without NaCl, which indicates its capability to counter both osmotic and ionic stresses. The enhancement in growth at 200 mM NaCl was not a simple result of altering osmotic pressure: some ionic effects specifically contributed to the growth of *S. brachiata*. Intracellular ion homeostasis is fundamental to the physiology of living cells. High salinity and osmotic stress disturb ion homeostasis. Accumulation of ions and synthesis of organic solutes in the cytoplast can re-establish cellular osmotic homeostasis. To confirm whether the ion and organic solutes determine osmotic homeostasis, we measured the contents of Na^+, K^+, and proline. Treatment with NaCl induced an increase in Na^+ content and a decrease in K^+ content in shoots of *S. brachiata*. Similar results have been reported in a number of plants (Khan 2001; Koyro 2006). Although Na^+ competition led to the

Table 3 Effects of varying concentrations of NaCl (0–600 mM) on reduced ascorbate (AsA), dehydroascorbate (DHA), and total ascorbate (AsA + DHA) contents in shoots of *S. brachiata* seedlings after different periods of NaCl treatment

Duration of treatment (days)	NaCl (mM)	AsA (μmol g^{-1} FW)	DHA (μmol g^{-1} FW)	Total ascorbate (μmol g^{-1} FW)	AsA/DHA
0	0	2.93 ± 0.02a	0.31 ± 0.03a	3.24 ± 0.06[a]	9.46 ± 0.66a
7	0	2.91 ± 0.03a	0.30 ± 0.02a	3.22 ± 0.06[a]	9.71 ± 1.50a
	200	2.83 ± 0.04a	0.27 ± 0.03a	3.11 ± 0.07[a]	10.5 ± 1.33a
	400	2.36 ± 0.02b	0.37 ± 0.02b	2.73 ± 0.04b	6.37 ± 1.03b
	600	2.04 ± 0.03c	0.41 ± 0.03c	2.46 ± 0.07c	4.97 ± 1.05c
	LSD ($P = 0.01$)	0.09	0.06	0.17	3.31
14	0	2.92 ± 0.02a	0.32 ± 0.03a	3.25 ± 0.05[a]	9.12 ± 0.66a
	200	2.76 ± 0.02b	0.25 ± 0.02a	3.02 ± 0.05b	11.04 ± 1.00a
	400	2.17 ± 0.04c	0.46 ± 0.04b	2.63 ± 0.08c	4.72 ± 1.02b
	600	1.91 ± 0.02d	0.57 ± 0.02c	2.48 ± 0.05d	3.35 ± 1.05c
	LSD ($P = 0.01$)	0.09	0.09	0.15	2.60
21	0	2.95 ± 0.03a	0.32 ± 0.02a	3.28 ± 0.05[a]	9.21 ± 1.50a
	200	2.68 ± 0.04b	0.23 ± 0.03b	2.91 ± 0.08b	11.65 ± 1.33a
	400	2.08 ± 0.03c	0.49 ± 0.02c	2.57 ± 0.05c	4.24 ± 1.50b
	600	1.79 ± 0.02d	0.71 ± 0.03d	2.50 ± 0.05d	2.52 ± 0.61c
	LSD ($P = 0.01$)	0.09	0.09	0.15	3.53

The values are mean ± SE. Different letters indicate statistically different means at $P \leq 0.01$

Table 4 Effects of varying concentrations of NaCl (0–600 mM) on reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (GSH + GSSG) contents in shoots of *S. brachiata* seedlings after different periods of NaCl treatment

Duration of treatment (days)	NaCl (mM)	GSH (nmol g^{-1} FW)	GSSG (nmol g^{-1} FW)	Total glutathione (nmol g^{-1} FW)	GSH/GSSG
0	0	187 ± 8.5a	27.1 ± 2.5a	214 ± 11a	6.90 ± 0.3a
7	0	186 ± 7.5a	26.85 ± 3.5a	213 ± 11	6.94 ± 0.4a
	200	267 ± 8.5b	27.05 ± 2.5a	294 ± 11b	9.88 ± 0.6b
	400	285 ± 9.0c	27.45 ± 3.0a	312 ± 12c	10.4 ± 0.5c
	600	298 ± 7.5d	27.55 ± 2.5a	326 ± 10d	10.83 ± 0.4d
	LSD ($P = 0.01$)	22.36	7.968	30.24	1.32
14	0	188 ± 8.5a	27.0 ± 2.0a	215 ± 10.5a	6.96 ± 0.2a
	200	281 ± 8.0b	27.15 ± 2.5a	308 ± 10.5b	10.36 ± 0.5b
	400	297 ± 7.5c	27.25 ± 2.0a	324 ± 9.5c	10.91 ± 0.7c
	600	307 ± 9.0d	26.95 ± 3.0a	334 ± 12d	11.41 ± 0.3d
	LSD ($P = 0.01$)	22.68	6.61	29.25	1.28
21	0	187 ± 7.0a	27.05 ± 2.0a	214 ± 9.0a	6.93 ± 0.4a
	200	291 ± 8.0b	27.25 ± 2.5a	318 ± 10.5b	10.67 ± 0.6b
	400	316 ± 9.0c	27.10 ± 2.5a	343 ± 11.5c	11.66 ± 0.7c
	600	328 ± 7.5d	27.05 ± 2.5a	355 ± 10.0d	12.14 ± 0.5d
	LSD ($P = 0.01$)	21.70	6.54	28.22	1.54

The values are mean ± SE. Different letters indicate statistically different means at $P \leq 0.01$

decrease of K^+ to some extent, the content of K^+ was still maintained at a relatively high level; this may have a role in protecting the plant photosynthetic apparatus from salt stress (Delfine and others 1998). There are several reports of unchanged levels of K^+ under salt stress in spinach (Delfine and others 1998), *Suaeda salsa* (Zhao and others 2003), and *Bruguiera parviflora* (Parida and others 2004a).Under typical physiological conditions, plants maintain a low Na^+/K^+ ratio in their cytosol, with relatively high K^+ (100–200 mM) and low Na^+ concentration (1–10 mM). This Na^+/K^+ ratio is optimal for many metabolic functions in cells (Brumwald and others 2000).

Table 5 Effects of varying concentrations of NaCl (0–600 mM) on H_2O_2 and MDA contents in shoots of *S. brachiata* seedlings after different periods of NaCl treatment

Duration of treatment (days)	NaCl (mM)	H_2O_2 (nmol g^{-1} FW)	MDA (nmol g^{-1} FW)
0	0	72.8 ± 2.8a	43.5 ± 2.1a
7	0	73.1 ± 2.3a	43.1 ± 2.4a
	200	69.7 ± 2.8a	38.3 ± 2.7a
	400	76.1 ± 2.4a	46.58 ± 2.5a
	600	79.5 ± 2.5a	48.06 ± 2.3a
	LSD ($P = 0.01$)	6.87	6.80
14	0	73.5 ± 1.9a	43.5 ± 2.1a
	200	65.2 ± 3.1b	36.8 ± 3.0a
	400	81.3 ± 2.8c	68.7 ± 2.5b
	600	85.7 ± 3.2d	89.5 ± 3.1c
	LSD ($P = 0.01$)	7.67	7.42
21	0	73.2 ± 2.5a	43.7 ± 2.5a
	200	61.5 ± 2.5b	35.4 ± 2.6b
	400	87.5 ± 3.1c	72.9 ± 3.2c
	600	91.27 ± 3.5d	97.4 ± 3.6d
	LSD ($P = 0.01$)	8.04	8.25

The values are mean ± SE. Different letters indicate statistically different means at $P \leq 0.01$

Although the ratio of Na^+/K^+ increased under salt stress in *S. brachiata* (Table 2), most of the Na^+ may have been compartmentalized into vacuoles to contribute to osmotic adjustment. Previous studies have shown that Na^+ uptake plays a central role in determining the salt tolerance in both dicotyledenous and monocytyledonous halophytes (Glenn and others 1999). Halophytes use the controlled uptake of Na^+ (balanced by Cl^- and others anions) into cell vacuoles to drive water into the plant against a low external water potential (Glenn and others 1999). In *S. brachiata* the content of Na^+ rather than proline significantly increased under salt treatments. It suggests that Na^+ was the main contributor to osmotic adjustment under salt stress in *S. brachiata*. The use of organic solutes for osmotic adjustment is an energy-consuming process at the cellular level (Flowers and Colmer 2008) and synthesis of proline costs more energy compared to the uptake of Na^+. It has been reported that proline levels increase significantly in leaves of the nonsecretor mangrove *B. parviflora* (Parida and others 2002) and in the salt secretor mangrove *Aegiceras corniculatum* (Parida and others 2004c). Moghaieb and others (2004) showed that *Suaeda maritima* plants accumulate higher levels of proline in their leaves under salt conditions than *Salicornia europaea* plants. However, *S. europaea* plants exhibit higher betaine content in their leaves than *S. maritima* (Moghaieb and others 2004). It is possible that different halophytes deal with osmotic stress by accumulating different organic solutes. In the meantime, the ability of Na^+ to adjust ion homeostasis is limited. In our study, when salt concentrations reached 600 mM, seedling growth was inhibited compared with that under 200 mM NaCl, whereas the content of proline (Table 2) increased significantly. We speculated that significant amounts of Na^+ from the roots into the cytoplasm could not be transported to the vacuoles anymore under 600 mM NaCl. The accumulated sodium in the cytoplasm might induce the synthesis of some compatible solutes, as seen by higher proline accumulation under severe salt stress (Table 2). This could further protect the enzymatic system in the cytoplasm from ionic damage to some extent. In *S. brachiata*, 200 mM NaCl did not induce an increase in proline. Proline produced under high salt treatments possibly plays a more important role in protecting the enzymatic system in the cytoplast but not in adjusting the osmotic homeostasis in *S. brachiata*.

An important consequence of salinity stress in plants is the excessive generation of highly toxic superoxide radicals ($O_2^{\bullet -}$), particularly in chloroplasts and mitochondria (Mittler 2002). Superoxide radicals generated by oxidative metabolism are dismutated into H_2O_2 and O_2 by superoxide dismutase (SOD). The long-term exposure of *S. brachiata* to salinity induced a 135–149% increase in the SOD activity (Fig. 1). This increased SOD activity with increasing salinity is in agreement with the results reported about other plants (Lee and others 2001; Sekmen and others 2007). However, our results contrast with those of Santos and others (2001) who reported decreased levels of SOD in sunflower under salt stress. It has also been reported that the cytosolic CuZnSOD (*sod1*) transcript remains unaltered in response to NaCl stress in *Avicennia marina* (Jithesh and others 2006). It is therefore evident that SOD represents an important protective mechanism against possible NaCl-induced reactive oxygen species (ROS) production in *S. brachiata*.

Our results indicated a decline in catalase (CAT) activity under high salinity, which suggests that CAT appears not to be an effective scavenger of H_2O_2 in *S. brachiata*. This enzyme has a relatively poor affinity for H_2O_2 and in the presence of light undergoes photoinactivation with subsequent degradation (Shang and Feierabend 1999). A decline in CAT activity under salt stress in sunflower (Santos and others 2001), rice (Lee and others 2001), and maize (Azevedo Neto and others 2006), under drought stress in rice (Sharma and Dubey 2005), and under photooxidative stress in tobacco chloroplasts (Miyagawa and others 2000) agrees with our results in *S. brachiata*. However, our results contrast with those of Takemura and others (2000) who reported an inductive response in CAT

activity in the mangrove *Bruguiera gymnorrhiza* under salt stress. It has also been reported that expression of the antioxidant gene catalase (*cat1*) was induced in response to NaCl stress in *Avicennia marina* (Jithesh and others 2006). In our experiments, an increase in H_2O_2 levels was observed at high salinity (400 and 600 mM) in shoots of *S. brachiata*. The increase in H_2O_2 is possibly due to the decreased activity of CAT (Lee and others 2001; Parida and others 2004b). In transgenic tobacco with low CAT activity, H_2O_2 arising from photorespiration was an important mediator of cellular toxicity during NaCl stress (Van Breusegem and others 2001). However, H_2O_2 seems to be an important signal molecule by acting as a direct link between environmental stress and key adaptive responses (Foyer and Noctor 2003).

Ascorbate peroxidase (APX) prevents the accumulation of excess H_2O_2 in cells via the ascorbate-glutathione pathway (Asada 2006). An increase in APX activity was observed in salt-treated seedlings of *S. brachiata*, with a concomitant decrease in H_2O_2 concentration at low salinity (200 mM NaCl) and a marginal increase (19–24%) at high salinity (400 and 600 mM). This suggests a key role for APX in the detoxification of H_2O_2 under salinity. Similar to our results, a salt-induced increase in APX activity has also been reported in many plants (Hernandez and others 2000; Sreenivasulu and others 2000; Lee and others 2001; Azevedo Neto and others 2006).

Guaiacol-specific peroxidase (POX) has been used as an indicator for stresses such as high temperature, salinity, and drought (Hernandez and others 2000; Sreenivasulu and others 2000; Lee and others 2001; Dash and Mohanty 2002; Parida and others 2004b; Sharma and Dubey 2005). POX protects cells against harmful concentrations of hydroperoxides. Unlike the report by Hernandez and others (2001) that found only nonsignificant changes in POX activity in two pea cultivars that differed in NaCl tolerance, our results showed that salinity significantly increased POX activity in *S. brachiata*. Our results agree with the earlier reports of salt-induced increase in peroxidase activity in foxtail millet (Sreenivasulu and others 2000) and rice (Lee and others 2001). In *S. brachiata*, this enzyme plays a crucial role in the defense mechanisms against oxidative stress, possibly in cooperation with additional antioxidant enzymes, by controlling appropriate H_2O_2 concentrations.

Like APX, glutathione reductase (GR) is essential for maintaining the redox state of ascorbate and glutathione (Asada 2006). GR plays an important role in controlling endogenous H_2O_2 content through an oxidoreduction cycle involving glutathione and ascorbate (Foyer and Noctor 2009). The GR activity in *S. brachiata* increased by 233% at high salinity compared to the control. This result is in contrast to that of rice leaf in which no significant alteration in GR activity was seen (Lee and others 2001). Our results indicate that GR might have an important role in the antioxidant defense system against salt exposure in *S. brachiata*.

Ascorbate and glutathione are key nonenzymatic antioxidants in plants mainly through their involvement in the ascorbate-glutathione cycle, an important part of the H_2O_2 detoxification pathway (Foyer and Noctor 2003). The results of this study show that the content of reduced ascorbate (AsA) and total ascorbate decreased in *S. brachiata* with increase in salinity. Such a loss in ascorbate suggests that the amount regenerated under high salinity is insufficient or that ascorbate synthesis is lower than ascorbate catabolism. Our results agree with those of Hernandez and others (2000), who reported a larger decrease of AsA in a NaCl-sensitive versus a NaCl-tolerant pea cultivar. The gradual decrease of AsA content in *S. brachiata* could in part be due to its participation in the reduction of H_2O_2 to H_2O through the increase in APX activity. The change in the AsA/DHA ratio, an important indicator of the redox status of the cells, is one of the first signs of oxidative stress (Foyer and others 2006). The AsA/DHA ratios in *S. brachiata* increased at low salinity (200 mM NaCl) and decreased at the high NaCl levels (400 and 600 mM). The growth optimum (200 mM NaCl) of *S. brachiata* was correlated with a higher redox status. Confirming our results, Ashraf and Harris (2004) showed that the salt-tolerant cultivars of cotton also had a higher AsA/DHA ratio than the salt-sensitive lines under salt stress conditions. In *S. brachiata* DHA levels increased at high salinity (400 and 600 mM NaCl). This suggests a higher rate of AsA oxidation under high salinity, which seems to be accompanied by a decrease in the rate of its synthesis.

Like ascorbate, glutathione is a multifunctional compound with important functions outside the antioxidative system (Foyer and others 2006). Reduced glutathione (GSH) plays an important role in the antioxidant defense system of plants because it not only participates in the regeneration of ascorbate via dehydroascorbate reductase, but it can also react with singlet oxygen and the $^{\bullet}OH$ radical and protect protein thiol groups (Asada 2006). We observed that unlike ascorbate, the constitutive glutathione content increased significantly with increasing salinity in *S. brachiata*. Furthermore, the enhanced GSH contents were matched by an increased GSH/GSSG ratio, which also fits well with the pattern (induction and/or maintenance and reduction) of GR activity observed.

MDA, a product of lipid peroxidation in plants exposed to adverse environmental conditions, is a reliable indicator of free radical formation in tissue (Hernandez and Almansa 2002). We observed that MDA concentration decreased under low salinity (200 mM NaCl) and increased at high salinity (400 and 600 mM NaCl). Our results showed that the growth optimum at 200 mM NaCl was concomitant

with lower MDA content. The lower MDA content and higher growth rate at 200 mM NaCl in *S. brachiata* may be due to a higher efficiency of the antioxidative defense system at this concentration of salt. This again suggests that oxidative damage due to salinization of *S. brachiata* is largely controlled by the antioxidative enzymes. Similar results that correlate lipid peroxidation to antioxidative system activity were also reported by other researchers (Hernandez and Almansa 2002; Bor and others 2003; Azevedo Neto and others 2006; Sekmen and others 2007).

In summary, our results clearly indicate that high salinity induces an overproduction of $O_2^{\bullet-}$ in *S. brachiata*, which is counterbalanced by increased activity of SOD. Increased SOD activity induces an overproduction of H_2O_2. The increased activity of ascorbate peroxidase, guaiacol peroxidase, and glutathione reductase maintains appropriate levels of H_2O_2. Our data strongly suggest that induction of antioxidant defenses, which include maintenance of the ascorbate-glutathione redox pool and upregulation of antioxidant enzymes, is at least one component of the tolerance mechanism of *S. brachiata* to long-term salinity as evidenced by the growth behavior of the plants. Maintenance of osmotic homeostasis by accumulation of Na^+ also has a role in salt tolerance of *S. brachiata*. Our data strongly support a salt-induced increase in antioxidant enzyme activities in *S. brachiata.* Currently, it is not known whether the increase was due to an upregulation of the genes controlling the synthesis of these enzymes or an increased activation of constitutive enzyme pools.

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