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SALT TOLERANCE AND MANAGEMENT OF MANGROVES: A COMPARATIVE ASSESSMENT OF SALT TOLERANCE LIMIT OF *BRUGUIERA PARVIFLORA* AND *BRUGUIERA GYMNORRHIZA* SEEDLINGS AFTER NaCl SHOCK

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Summary. Salinity tolerance and management strategies are many and they vary from species to species for mangroves. *Bruguiera zymnorrhiza* and *Bruguiera parviflora* of the family Rhizophoraceae are predominant non-secretor tree mangrove species providing characteristic forest-zonations in costal mangrove forests of India that are known to adapt to different salinity regimes. High doses of NaCl (500 mM) treatment applied to two-months old *B. gymnorrhiza* and *B. parviflora* grown as hydroponics for a short period (6 d) and subsequent recovery (7 d) revealed significant changes in physiological and anatomical parameters as well as antioxidative enzymes. Our results confirmed relatively high salt tolerant capacity of *B. parviflora* compared with *B. gymnorrhiza*. A significant increase of carotenoids, sugars, amino acids and polyphenol content was found after 6d of salt shock in both species. However, the osmo-regulatory adjustments were more pronounced in *B. gymnorrhiza* than in *B. parviflora*. The activities of catalase, ascorbate peroxidase and guiacol peroxidase were significantly increased upon salt treatment whereas chlorophyll content, RuBP carboxylase activity and total soluble protein content decreased significantly. The comparative analysis of biochemical and physiological parameters revealed that *B. gymnorrhiza* exhibited comparatively higher salt adaptive potential under high salt shock as compared to *B. parviflora* and therefore, this species can be recommended for afforestation of new mangrove vegetation in high salinity zones.

Key words: Antioxidative enzymes, mangrove, photosynthesis, RuBP carboxylase, salt stress, *Bruguiera*.

Abbreviations: AA – Ascorbic acid; APX – Ascorbate peroxidase; CAT – Catalase; EDTA – Ethylenediamine tetra-acetic acid (disodium salt); GPX – Guaiacol peroxidase; NBT – Nitro blue tetrazolium; PVP – Polyvinylpyrrolidone; RuBP carboxylase – Ribulose 1, 5-bisphosphate carboxylase; TCA – Trichloroacetic acid; TEMED – Tetra methyl ethylene diamine.

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INTRODUCTION

Mangrove forests are widely distributed in the inter-tidal zones of the tropical and subtropical areas of the globe (Tomlison et al., 1986). The East and West coasts of India have rich mangrove forests (Das et al., 1997). Bhitarkanika estuaries of Orissa in the East coast of India (latitude 20°4′N to 20°8′N; longitude 86°45′E to 87°50′E) contains luxuriant mangrove vegetations representing rich biodiversity of Indian mangrove flora (Das et al., 2005). Threats to mangrove vegetations are increasing worldwide due to the global climatic changes. *Bruguiera* species of the family Rhizophoraceae are abundantly found in Bhitarkanika mangrove forest and they are true, non-salt secreting tree mangroves. Six species of *Bruguieras* (*B. cylindrica, B. exaristata, B. gymnorrhiza, B. hainessi, B. parviflora* and *B. sexangula*) are found worldwide. Among them, *B. gymnorrhiza* and *B. parviflora* are commonly found in Bhitarkanika mangrove forest (Das et al., 1997). Effect of long-term exposure of *B. parviflora* to NaCl (100mM to 400mM) for 45 days on pigment content and other osmotic metabolites was earlier reported by our group (Parida et al. 2002). In addition, the changes in protein content as well as antioxidant enzymes were also reported (Sugihara et al 2000, Parida et al. 2004a, 2004b). Accumulation of salt in *B. sexangula* cells was recorded by Kura-Hotta et al. (2001). However, a comparative analysis of root, shoot and leaf anatomical changes, and the adaptive response of photosynthesis and antioxidant enzymes in both species under short-term high salt exposure has not been reported, yet. Here we report a simple short-term high salt (500mM NaCl) exposure experiments using *B. gymnorrhiza* and *B. parviflora* and their subsequent recovery after transfer to normal culture medium as a tool to estimate the upper salt tolerance limit of the species. This experimental method seemed useful to access upper salt tolerance limit. It also provided clues for ascertaining the ability of the plants to survive in different zones, particularly in wetland marine estuary system such as Bhitarkanika and the adjacent degraded mangrove forest of Paradeep delta of Orissa. We used two main Rhiziophorian mangroves, *B. gymnorrhiza* and *B. parviflora* to study the effect of short-term high salt shock in hydroponics and their subsequent recovery on leaf relative water content, net CO_2 assimilation, root and stem anatomy as well as pigment content, amino acids, polyphenols, sugars, soluble protein content and antioxidative enzyme system. The salt shock protocol may be a useful method for testing salt tolerance limits of tree mangrove species for mangrove conservation and afforestation programs.

MATERIALS AND METHODS

Propagules of *Bruguiera gymnorrhiza* (BG) and *Bruguiera parviflora* (BP) were collected from the mangrove forest of Bhitarkanika, Orissa, India. Seedlings were raised on polypacks containing sand:soil:leaf mold (1:1:1) in a greenhouse under PAR of 677 to 1040 µmol $m^{-2}s^{-1}$, and were watered at two days interval with non-brackish water (Das et al., 1997). Healthy two-months old seedlings of uniform size (eight-leaf stage) were selected for hydroponics in full strength Hoagland nutrient medium (Hoagland

and Arnon, 1940). These cultures were aerated continuously and were maintained in a growth chamber at 22±2°C, 80% RH, 14 h photoperiod, and light intensity of 300 µmol $m^{-2}s^{-1}$. Sodium chloride (500 mM) was added to the Hoagland solution (pH 5.8-6.0) for treatment of both species while only control sets were maintained without NaCl. To prevent fungal growth, the culture medium was changed everyday. The 4th pair of leaves from the tip of the shoots of 0, 2, 4, 6-d treated and 7-d recovery plants with their respective controls was harvested for measurements of leaf relative water content and various biochemical, protein and enzyme analyses. The experiment was arranged in a complete randomized design and replicated thrice. The relative water content was calculated according to Beadles et al (1993). Net photosynthetic rate was determined using a PP-system photosynthetic gas analyzer (model TPS1, PP system, USA) under growth chamber condition (Panda et al., 2006). Transverse sections (TS) for anatomical studies were prepared with a sharp razor blade from roots, stems and leaves (three plants in each treatment and the experiment is replicated thrice) and immediately observed under light microscope (Nilkon Optiphot Japan). Photographs of TS were taken to record the direct effect of salt without losing much turgor of the cells in tissue.

For estimation of total chlorophylls, 0.5 g leaf tissue was homogenized in chilled 100% *N, N*-dimethylformamide (DMF) and pigment contents were calculated according to Lichtenthaler (1987). To estimate carotenoid content, 0.5 g leaf samples were homogenized in chilled 80% acetone and carotenoid content was estimated according to Arnon et al. (1949). Total soluble sugars were estimated according to the anthrone-sulphuric acid method of McCready et al. (1950) with some modifications (Aarrouf et al., 1999). To extract and analyze free amino acids, 0.5 g of leaves was homogenized in 70% ethanol with a mortar and pestle following the method of Moore and Stein (1948). Total polyphenols were analyzed according to the procedures of Chandler and Dodds (1983). The detailed procedures for isolation and estimation of chlorophyll, carotenoid, pigments, sugars, free amino acids, polyphenols were described earlier (Parida et al., 2002). Leaf tissues (0.5 g) were homogenized in 10% ice-cold TCA (trichloro acetic acid) using a pre-chilled mortar and pestle at 4°C. Homogenate was centrifuged at 10,000×*g* for 10 min at 4°C. Leaf protein sample containing 40 µg of protein with bromophenol blue dye was loaded in each well of gel cast. SDS gel was made according to Laemmli (1970) and polypeptides were stained with 0.5% Coomassie Briliant Blue R250 and photographed in gel scanner analyzed with Bio Rad (USA) Quantity One Software. Leaf sample (1 g) was homogenized with pre-chilled motor and pestle with 2 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM D-isoascorbic acid, 2% (w/v) PVPP (polyvinyl polypyrrolidone) and 0.05% (w/v) Triton X-100 following the procedure of Gossett et al. (1994). The homogenate was centrifuged at 10000×*g* for 10 min at 4°C. The supernatant was collected and used for the assay of catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and guaiacol peroxidase (GPX, EC 1.11.1.7).

RuBP carboxylase (EC. 4.1.1.39) activity was measured according to Yu-Chun et al. (1996). Leaves (0.5 g) were homogenized in a pre-chilled motor with 30 mg of PVPP, 0.2 g of acid washed sea sand and 4 ml of extraction buffer containing 100 mM Tris-HCl (pH 7.8), 1 mM EDTA-NaOH (pH 7.0), 5 mM dithiothreitol (DTT), 0.2% bovine serum albumin (BSA). The homogenate was centrifuged at 15000×*g* for 10 min. The supernatant was used immediately for determination of specific activity of RuBP carboxylase. The procedure was conducted at 0 to 4°C. RuBP carboxylase was assayed at 30°C in a medium containing 50 mM HEPES-KOH buffer (pH 8.0), 10 mM $NaHCO_3$, 0.2 mM NADH, 2.5mM ATP, 10 mM KCl, and 1 mM EDTA-NaOH (pH 7.0), 20 mM $MgCl_2$, 5 mM DTT, 5 mM phosphocreatine, 6 unit m/L 3-phosphoglyceric phosphorkinase and glyceraldehydes-3-phosphate dehydrogenase each, 20 unit m/L creatine phosphokinase and 50 µg of protein. The reaction mixture was incubated for 10 min at 28°C without RuBP, which was added to start the reaction with concentration 0.6 mM in a final volume of 1 ml. The absorbance was read at 340 nm. The activity was expressed as µmol NADH oxidized/min/mg protein by using the extinction coefficient 6.22 µmol/cm/s (Panda et al., 2006). RuBP and NADH were used from Sigma chemicals St Louis, Missouri USA.

Statistical analysis of the results was carried out according to Duncan's multiple range test. Data were subjected to a two-way analysis of variance (ANOVA) and the least significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ was determined following the procedure of Sokal and Rohlf (1969).

RESULTS AND DISCUSSION

Salt stress reduces plant's ability to take up water. High salinity affects the leaf water content. The exposure to 500 mM NaCl under hydroponics condition resulted in 2% and 4% decrease of relative water content in 6-d salt treated leaf samples of *B. gymnorrhiza* and *B. parviflor*, respectively. In *B. parviflora* the dry weight of salt treated samples increased from 0.240 g to 0.248 g whereas in *B. gymnorrhiza* the increase in dry weight was 47% as compared to control (Table 1). Takemura et al. (2000) reported accumulation of sodium in xylem sap of *B. gymnorrhiza.* The large enhancement in dry weight in BG within 6d seemed to be linked to internal salt accumulation as proposed by Takemura et al. (2000). However, the high amount of sodium in the cell vacuole sap might be another possible cause in NaCl-treated *B. gymnorrhiza* rather than *B. parviflora* – a possible adaptive mechanism by a rapid release of Na^+ in the vacuole sack as reported in *B. sexangula* (Kura-Hotta et al., 2001). Net photosynthetic rate (P_N) and RuBP carboxylase activity decreased upon exposure to salt treatment (500 mM NaCl) in both species. However, *B. parviflora* showed a significant decrease in photosynthetic rate from 25.51±1.89 µmol CO_2 $m^{-2}s^{-1}$ in control to 5.02 ±0.75 µmol CO_2 $m^{-2}s^{-1}$ in 500 mM NaCl treatment as compared to *B. gymnorrhiza* that showed a relatively low rate of net photosynthesis (10.12±1.21 µmol CO_2 $m^{-2}s^{-1}$ in control to 3.88±0.25 µmol CO_2 m^{-2} s^{-1} in treated plants). A significant decrease in RuBP carboxylase activity was noted in *B. parviflora* from 12.05±1.22 U/mg protein in control to

Table 1. Changes in fresh weight (FW) and dry weight (DW) of leaves (4th pair from the top of 2-months old seedlings) upon treatment of *B. parviflora* and *B. gymnorrhiza* with 500mM NaCl for 6 d.

Species/ treatment	FW [g]	DW [g]	Turgid wteight [g/leaf]	Relative water content [%]
Bruguiera parviflora				
Control (0d)	0.844±0.128	0.246±0.023	0.966±0.052	79.42±0.02
Control (6d)	0.850±0.167^{ns}	0.240±0.051^{ns}	0.974±0.031^{ns}	79.10±0.03^{ns}
Treated (6d)	0.890±0.145*	0.248±0.033^{ns}	1.071±0.012**	75.19±0.05*
Bruguiera gymnorrhiza				
Control (0d)	1.362±0.089^{ns}	0.456±0.034^{ns}	1.539±0.027^{ns}	81.20±0.03^{ns}
Control (6d)	1.307±0.138^{ns}	0.448±0.017^{ns}	1.495±0.033^{ns}	80.66±0.01^{ns}
Treated (6d)	1.783±0.145*	0.671±0.121*	2.134±0.055**	78.91±0.04*

^{ns} – not significant; * – Differences from control values are significant at $P \leq 0.05$; ** – Differences from control values are significant at $P \leq 0.01$.

4.82±0.72 U/mg protein in salt exposed samples. In control *B. gymnorrhiza* it was decreased from 9.12±0.45 U/mg protein in control to 5.95±0.44 U/mg protein in treated samples. It was evident that the rate of decrease of RuBP carboxylase activity was higher in *B. parviflora* than in *B. gymnorrhiza* upon salt exposure for 6d (data not shown) which might be due to the highly stable RuBP carboxylase enzyme in the latter case. Photosynthesis is strongly affected by salinity, mainly because of reduced stomatal conductivity, causing a decrease in intracellular CO_2 level (Downton et al., 1985; Seeman and Critchley, 1985). However, the decrease in net photosynthesis has also been shown to depend on non stomatal factors, which include electron transport and photophosphorylation activity (Keck and Boyer, 1974; Ortiz-Lopez et al., 1991). There is only a limited study on the changes in anatomy due to high salt exposure. The large air spaces were much more prominent in control *B. parviflora* (Fig. 1a) than in *B. gymnorrhiza* (Fig. 1c). Upon salt treatment, these air spaces got closed more in *B. parviflora* than in *B. gymnorrhiza* (Figs. 1b, 1d). We also noted a decrease of root parenchyma layer below epidermis in both species, however, that was more prominent in *B. parviflora* than in *B. gymnorrhiza.* Root anatomy of *B. parviflora* was more affected compared with *B. gymnorrhiza* as the air spaces in root got more closed in *B. parviflora* upon salinization. A comparison between the control stem samples of *B. parviflora* (Fig. 2a) and *B. gymnorrhiza* (Fig. 2c) indicated that in *B. gymnorrhiza*, one layer of chlorenchymatous cells, uniform parenchyma and packed xylem bundles were visible. In contrast, *B. parviflora* did not show chlorenchymatous cells, but exhibited the presence of collenchyma-type of cells and the vascular bundles were regularly spaced. Upon salt treatment, both species showed some deformities.

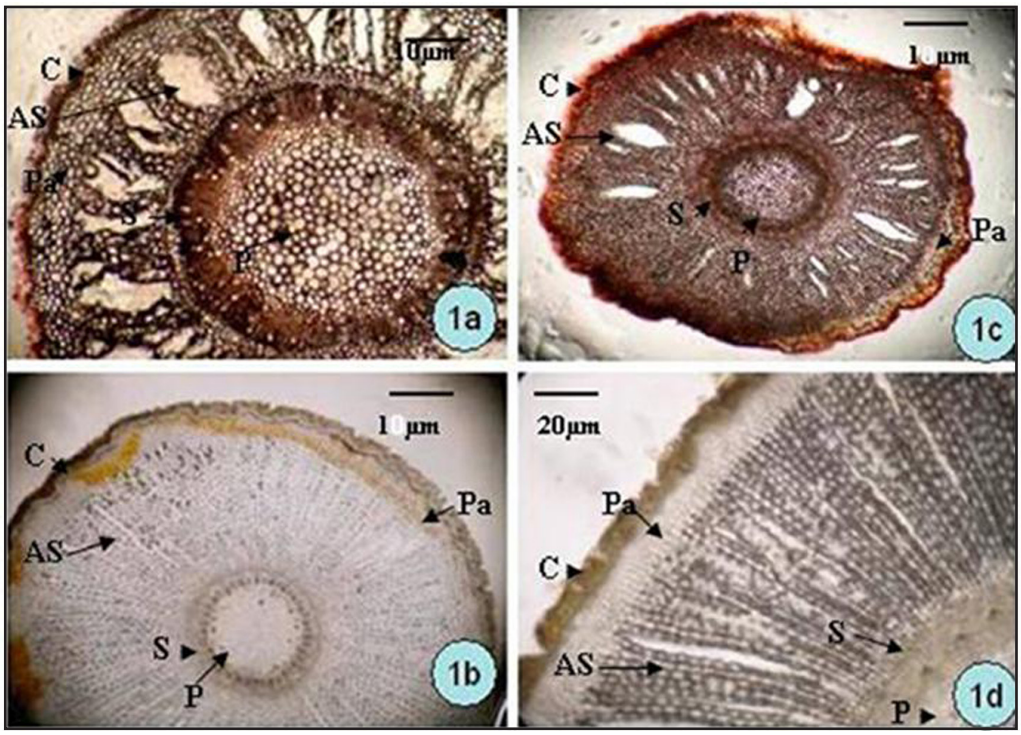

Fig. 1. Effect of application of 500 mM NaCl for a short period (6 d) on root anatomy (transverse section) of *B. parviflora* (BP). [control (a), treated (b)] and *B. gymnorrhiza* [control (c), treated]. C – cuticle; AS – air space; Pa – parenchyma; S – stellar region; P – pith.

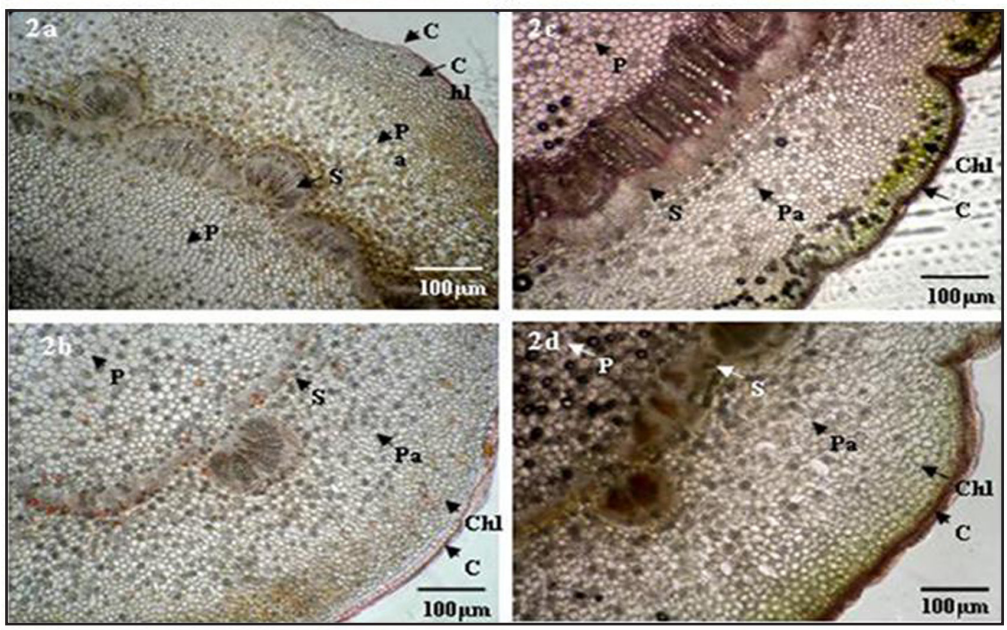

Fig. 2. Effect of application of 500 mM NaCl for a short period (6 d) on stem anatomy (transverse section) of *B. parviflora* [control (a), treated (b)] and *B. gymnorrhiza* [control (c), treated (d)]. C – cuticle; Chl – chlorechyma; Pa – parenchyma; S – stellar region; P – pith.

In *B. parviflora*, the steles got bulged and loosely spaced (Fig. 2c) while in *B. gymnorrhiza*, the stellar distortion was no more prominent (Fig. 2d). We also observed that salt treatment increased the aerenchymatous space and enlargement of parenchymatous cells in both species. The result on the stem anatomy agrees with the report of Xiao et al. (2009) where a decline of cortex thickness and pith radius were noted in *B. gymnorrhiza* seedlings in simulated semidiurnal tide condition. Leaf anatomy of both species exhibited some common characteristics. Leaf thickness, palisade parenchyma thickness, spongy parenchyma thickness and the palisade-spongy thickness ratio (35.9% 65.2%, 25.7%, 52.1% in *B. parviflora* and 30.1%, 56.2%, 20.2%, 46.9% in *B. gymnorrhiza,* respectively as compared to their respective controls) declined with increasing the duration of NaCl treatment (Fig. 3a - 3d), but the extent of reduction was higher in *B. parviflora* than in *B. gymnorrhiza*. A similar trend of reduction was recorded in *B. gymnorrhza* by Wang et al. (2007) upon 15% salinity under green house conditions or upon 15-27 PPT salinity (Nandy Datta et al., 2007).

In both *Bruguiera* species chlorophyll content decreased whereas carotenoid content increased upon exposure to salt shock (Fig. 4). Chlorophyll content decreased 4.9 times and 2.5 times in *B. parviflora* and *B. gymnorrhiza,* respectively indicating that the latter was relatively more tolerant to salt stress. Carotenoids are known to scavenge free radicals that get generated owing to excess excitation energy from chlorophyll

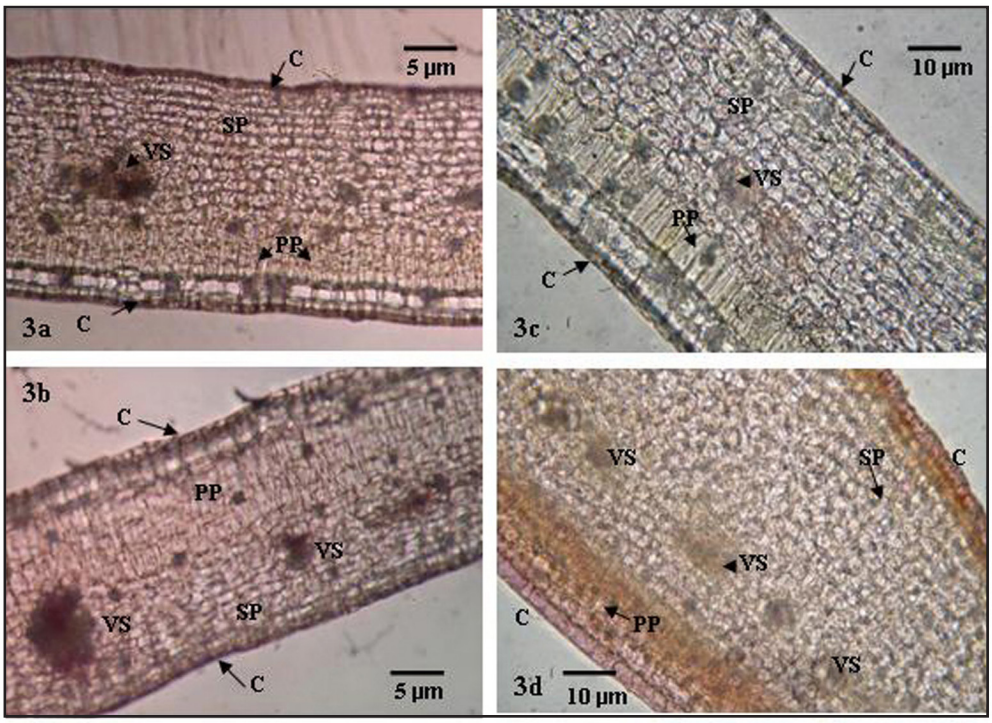

Fig. 3. Effect of application of 500 mM NaCl for a short period (6 d) on leaf anatomy (transverse section) of *B. parviflora* [control (a), treated (b)] and *B. gymnorrhiza* [control (c), treated (d)]. C – cuticle; PP – palisade parenchyma; SP – spongy parenchyma; VS – vascular system.

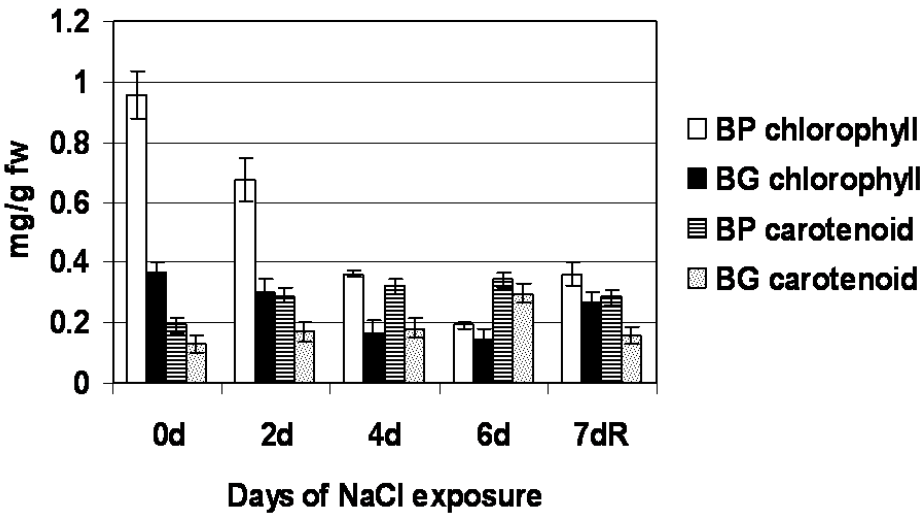

Fig. 4. Effect of application of 500 mM NaCl for a short period (6 d) on total chlorophyll and carotenoid content in leaves of *B. parviflora* and *B. gymnorrhiza*.

during photosynthesis (Arora et al., 2002). The increased carotenoid content in *B. gymnorrhiza* and *B. parviflora* upon salt shock could be an important feature of a salt tolerance mechanism. These findings also supported our earlier finding of long term (45 d) NaCl treatment (100 mM to 400mM) in *B. parviflora* (Parida et al., 2002). The *Chl* content increased after a 14-d long-term exposure and gradually decreased over a 45-d period while carotenoid content increased significantly during a short-term exposure (6d) to high NaCl (500 mM) treatment compared to the decrease found in long-term treatment (Parida et al., 2002). The short-term adjustment to high salt might be a possible adjustment phenomenon in terms of pigment synthesis rather than a stabilized mechanism found in long-term experiments. The results in Fig. 5 showed that total sugar content increased 1.6 times and 2.4 times in *B. parviflora* and *B. gymnorrhiza,* respectively after 6-d salt shock compared to controls. Salt exposure caused free amino acid and polyphenols content to increase by 4.8 times and 2.0 times in *B. gymnorrhiza,* respectively compared to their respective controls, whereas in *B. parviflora* the free amino acids and polyphenols increased 2.1 times and 1.06 times, respectively (Fig. 6). Compatible solutes like sugars, amino acids and polyphenols in both *Bruguiera* species increased upon salt shock, but the degree of changes was higher in *B. gymnorrhiza* than in *B. parviflora,* indicating that the former species possessed a better defense mechanism. Our earlier report on *B. parviflora* on long-term exposure also showed a dose-dependent increase of the osmolites (Parida et al., 2002), but in *B. gymnorrhiza* this was reported for the first time and it was higher as compared to *B. parviflora* even after a short-term exposure for 6 d. Almost all organisms, ranging from microbes to higher plants synthesize compatible solutes in response to osmotic stress (Burg et al., 1996). Out of these compatible solutes, sugar plays

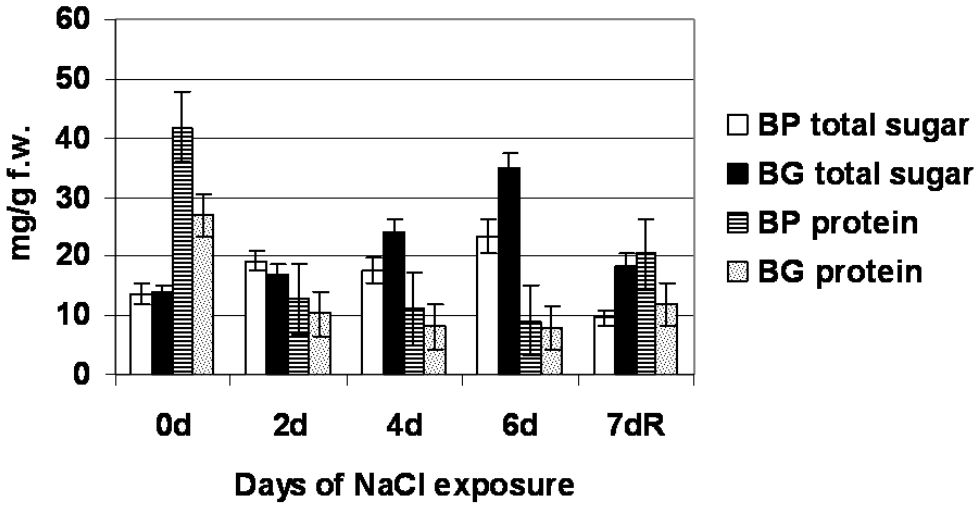

Fig. 5. Effect of application of 500 mM NaCl for a short period (6 d) on total sugar and total soluble protein content in leaves of *B. parviflora* and *B. gymnorrhiza*.

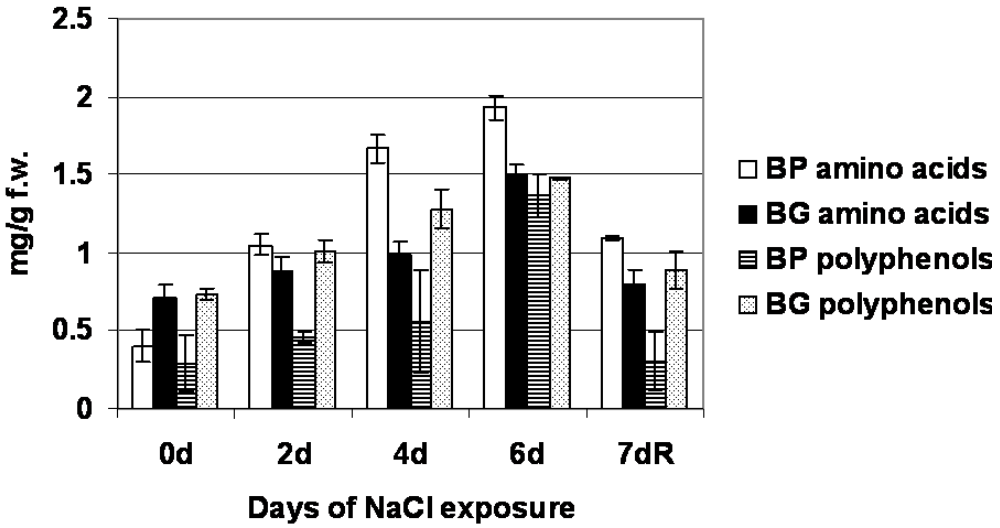

Fig. 6. Changes in free amino acids and polyphenol content in leaves of *B. parviflora* and *B. gymnorrhiza* upon exposure to 500 mM NaCl for 6 days.

an important role during osmotic stress tolerance by maintaining the membrane integrity (Manchanda and Garg, 2008). Total soluble protein content in *B. parviflora* decreased more rapidly in *B. gymnorrhiza*. The SDS-PAGE analysis showed that some proteins having apparent molecular mass of 16 kDa, 23 kDa, 33 kDa and 49 kDa were decreased more on the 4th d of salt treatment in *B. parviflora* as compared to *B. gymnorrhiza* (Fig. 7). Parida et al., (2002) reported earlier that exposure of *B. parviflora* to 400 mM NaCl for a long period of 45 d decreased the total soluble protein content as well as a 23 kDa protein shown in our laboratory

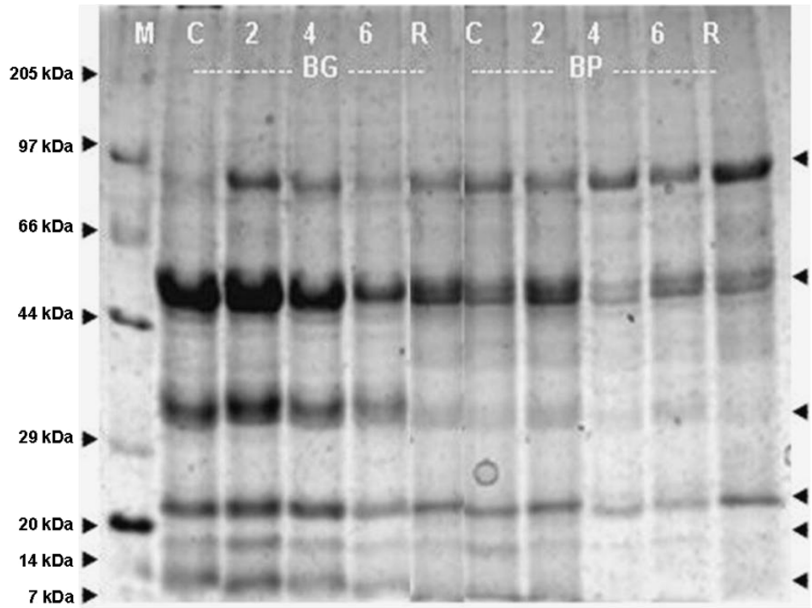

Fig. 7. SDS PAGE of total soluble leaf protein of *B. parviflora* and *B. gymnorrhiza* upon exposure to 500 mM NaCl for 0 d, 2 d, 4 d, 6 d and after 7-d recovery in Hoagland solution.

in long-term experiments (Parida et al., 2005). Sugihara et al. (2000) observed an enhancement in the level of 33 kDa manganese stabilizing protein only in salt-treated *B. gymnorrhiza* which was characterized as a light-harvesting complex protein. The molecular characterization of such novel salt-sensitive marker proteins with n-terminal amino acid sequencing might give insight in their involvement in various metabolic pathways.

Antioxidative enzymes are the true scavengers of reactive oxygen species (ROS), which are produced during osmotic or ionic stress conditions (Jithesh et al., 2006). In salt-treated *B. parviflora*, catalase activity increased 2.1 times compared to control whereas in *B. gymnorrhiza* the enhancement was 4.1-fold (Fig. 8). A significant variation was found in ascorbate peroxidase activity in both species. In *B. parviflora*, APX activity increased marginally whereas in *B. gymnorrhiza* the APX activity was enhanced 2.4 times upon exposure to salt (Fig. 9). Earlier, an increase of APX activity was noted in *B. parviflora* by our group which was dose-dependent and only one band was reported. It increased to 4-fold upon 45-d 400 mM NaCl treatment above control and it was 2 times higher after 7 days of treatment (Parida et al., 2004 b). This might be due to the up-regulation of this antioxidative enzyme for adaptive response of mangrove plants to high salt stress. This difference could be a species-specific marker, but it needs to be established. The ascorbate peroxidase plays an important role in regulation of intracellular level of H_2O_2 in higher plants (Van Breusegem et al., 2001). The enhanced APX activity in *B. gymnorrhiza* would possibly give additional protection against salt stress in this species of *Bruguiera*. Gel assay of antioxidative enzymes could clearly demonstrate the increase of APX activity only due to the increased quantity of that

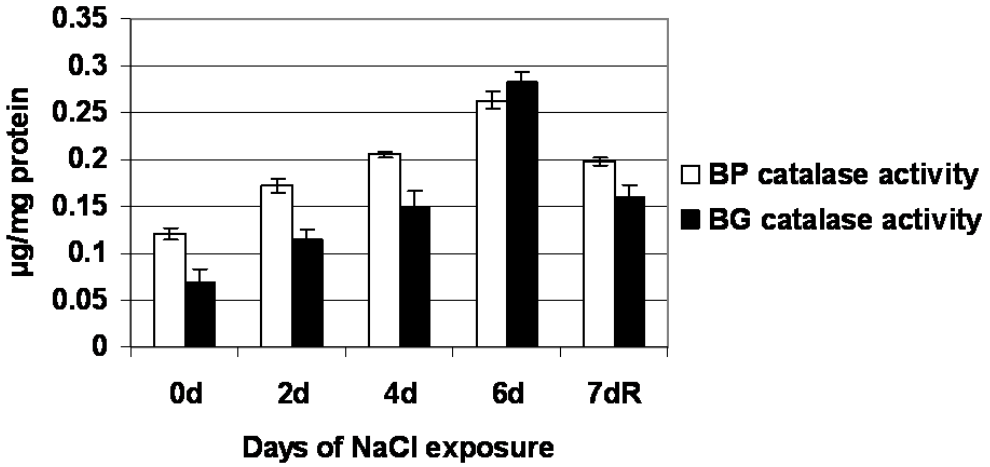

Fig. 8. Changes in catalase activity in leaves of *B. parviflora* and *B. gymnorrhiza* upon exposure to 500 mM NaCl for 6 d.

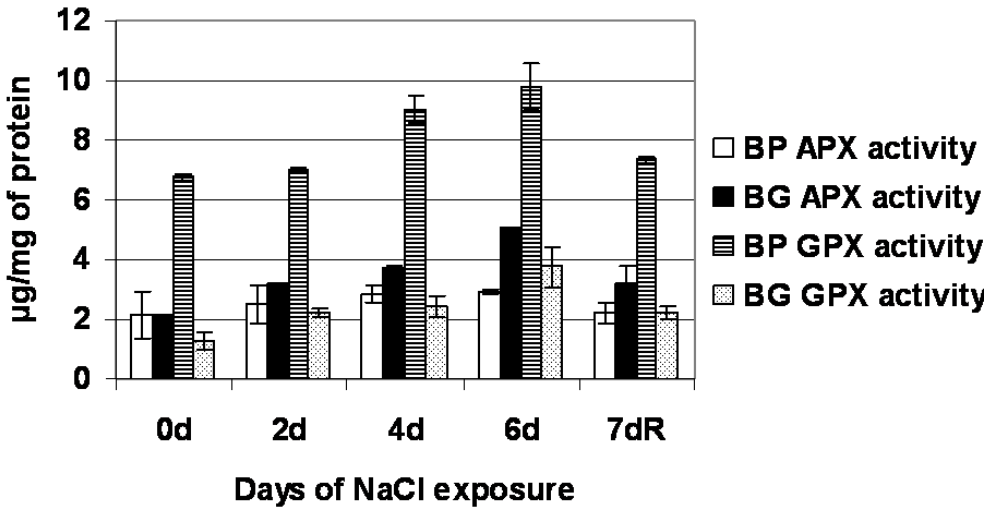

Fig. 9. Changes in ascorbate peroxidase activity and guiacol peroxidase activity in leaves of *B. parviflora* and *B. gymnorrhiza* upon exposure to 500 mM NaCl for 6d.

single form as evident in *B. parviflora* (Parida et al., 2004b) or to the increase of the number of isoforms of this enzyme in *B. gymnorrhiza.*

The GPX activity was higher in salt-treated *B. gymnorrhiza* than in *B. parviflora* (Fig. 9). Parida et al. (2004) reported a loss in catalase activity with salt in *B. parviflora* whereas an enhancement in both APX and GPX activity upon salt exposure was found. In *B. gymnorrhiza* the catalase activity was increased upon salinization up to sea water level (Takemura et al., 2000).

In summary, the data in the present study demonstrated that out of the two species of *Bruguiera* studied, *B. gymnorrhiza* exhibited a higher adaptive potential under salinity stress as judged by the photosynthetic carbon assimilation,

antioxidant defense metabolism and accumulation of osmoprotectants as compared to *B. parviflora*. The short-term salt shock and subsequent recovery provide a relatively fast and simple protocol for assessing tolerance limit of plants. A few selected physiological and biochemical parameters could be rapidly assayed. Our study showed that *B. gymnorrhiza* was relatively more salt resistant than *B. parviflora* and this seemed to reflect in their zonation patterns.

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