CHAPTER 2

The role of salt acclimation in the ability of *Bruguiera cylindrica* plants to withstand dehydration and osmotic stresses

CHAPTER 2: THE ROLE OF SALT ACCLIMATION IN THE ABILITY OF *BRUGUIERA CYLINDRICA* PLANTS TO WITHSTAND DEHYDRATION AND OSMOTIC STRESSES

2.1 Introduction

Abiotic stresses such as salt or drought stress primarily create hyperosmotic and hyperionic conditions, which are detrimental to the plant's survival. Hence osmotic and ionic adjustments are important aspects of salt acclimation and tolerance. Mangroves are a unique plant community thriving in tropical and sub-tropical shorelines. Their ability to survive under stressed environmental conditions such as soil salinity, intense light, water logging and nutrient availability, makes them interesting plant systems / models to study various aspects of salt acclimation and tolerance.

Osmotic adjustment is essential for preventing water loss from cells to the external saline environment, which has a more negative osmotic potential. Halophytes like *Atriplex vesicaria* and *Salicornia rubra* are known to accumulate Na^+ and Cl^- ions in vacuoles to facilitate osmotic adjustment (Flowers *et al*., 1977; Yeo and Flowers, 1980). Vacuolar compartmentation of Na^+ and $C1^-$ ions results in low cytosolic levels of these ions and prevents cytotoxicity, while facilitating osmotic adjustment required for cell expansion and maintenance of turgor.

However, vacuolar sequestration of ions by halophytes creates an osmotic imbalance within the cytoplasm. The halophytes can counter this imbalance by altering the relative volumes occupied by cytosol and vacuole in the cell (Binzel *et al*., 1988), or by synthesizing and accumulating large amounts of compatible solutes/metabolites that help in maintaining the hydrated state of the cytoplasm. These osmolytes include proline,

quaternary ammonia compounds like glycine-betaine, soluble sugars, sugar alcohols, and polyols (Tester and Davenport, 2003).

Proline accumulation under salt stress and water deficit conditions has been reported in glycophytes (Kavi Kishor *et al*., 2005). Enhanced accumulation of proline in response to increasing salt concentrations has been reported in halophytes like *Thellungiella halophila* and *Mesembryanthemum crystallinum* and *Bruguiera parviflora*, making it an important adaptive mechanism to high salinity (Kant *et al*. 2006; Parida *et al*., 2002; Sanada *et al*., 1995). Increased levels of proline accumulation in salt-stressed calli of *Suaeda nudiflora* suggested that besides its role as an osmolyte, proline also protected the callus cells from oxidative damage caused by free radicals during salt stress (Cherian and Reddy, 2003). *Avicennia marina* is known to accumulate glycine betaine (Ashihara *et al*., 1997) while *Aegiceras corniculatum* accumulates mannitol. Pinitol is a common osmolyte present in many members of family Rhizophoraceae (Popp *et al*., 1985). Thus accumulation of ionic and non-ionic solutes in halophytes is an important osmotic adjustment process that enables adaptation to the saline environment.

Osmotic and ionic stresses result in metabolic disturbances that lead to the generation of reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) (Smirnoff, 1993; Hernandez *et al*., 2000; Foyer and Noctor, 2003). ROS are by-products of important energy generating processes, photosynthesis and respiration and are generated in chloroplasts, mitochondria, and peroxisomes (Apel and Hirt, 2004). These ROS lead to indiscriminate damage to membrane lipids, proteins and DNA and cause injury to plants (Smirnoff, 1993). Membranes are the primary site of damage because ROS can react with polyunsaturated

fatty acids (PUFAs) and cause peroxidation of essential membrane lipids in plasma membrane or intracellular organelles (Stewart and Bewley, 1980). Peroxidation of plasma membrane lipids leads to the leakage of cellular contents, rapid desiccation and finally cell death. Intracellular membrane damage can affect mitochondrial respiration and plastid's carbon fixing ability (Scandalios, 1993).

However the ROS also act as stress signaling molecules and induce antioxidant protective mechanisms to eliminate or reduce ROS. These include (a) non-enzymatic antioxidant substrates that quench ROS (e.g. ascorbate, tocopherols, carotenoids) and (b) antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and a variety of general peroxidases (POX) (Hernandez *et al*., 2000). SOD (EC 1.15.1.1) is the first line of enzymatic defense against oxidative stress in plants and it occurs in most cellular compartments (mitochondria, chloroplast, cytosol) where O_2^- is produced. SODs are mutlimeric metalloproteins and catalyse the dismutation of O_2^- to H_2O_2. The principal H_2O_2-scavenging enzymes are ascorbate peroxidase (APX; EC 1.11.1.11) which is primarily located in chloroplast and cytosol, and catalase (CAT; EC 1.11.1.6) which is mainly produced in peroxisomes, glyoxisomes and mitochondria (Jithesh *et al*., 2006). The sub-cellular distribution of these enzymes suggests that chloroplastic APX scavenges H_2O_2 produced primarily during Mehler reaction, whereas CAT removes H_2O_2 generated during respiratory processes. Widely distributed in plants, the non-specific peroxidases (POX; EC 1.11.1.7) are also involved in H_2O_2 scavenging.

High levels of antioxidants are known to contribute towards oxidative stress tolerance in halophytes (Takemura *et al*., 2000; Parida *et al*., 2004) and changes in their activities and gene expression have been studied in various species like *B. parviflora*, *A. marina*, *T.*

halophila and *Suaeda salsa* (Parida *et al*., 2004; Taji *et al*., 2004; Wang *et al*., 2004; Jithesh *et al*., 2006).

Bruguiera cylindrica is a facultative mangrove and grows well in soils irrigated with fresh water besides salt water, which is its natural habitat. Using salt- or non-salt acclimated green house grown *B. cylindrica* plants, attempts have been made in this chapter to understand whether salt-acclimation mechanisms equip this mangrove species to cope with a short-term exposure to dehydration stress or extreme salt stress since plant responses to dehydration and salinity are often inter-related and the mechanisms overlap (Zhu, 2002; Sairam and Tyagi, 2004). Dehydration stress was imposed by an inert, non-ionic and non-toxic agent, polyethylene glycol (PEG). PEG, of more than 4000 molecular weight, induces water deficit conditions by decreasing the water potential of medium without being taken up by plants (Lawlor, 1970). PEG 8000 (30%) imparted an osmotic pressure similar to that of sea water (-2.0 MPa). For extreme salt stress, 4 M NaCl solution was used, which imparted almost nine times more (-17.8 MPa) osmotic pressure than that of sea water. Though mangroves survive naturally at sea water salt concentrations (~ 500 mM NaCl), significant changes in salt concentration occur due to climatic factors or inundation, exposing these plants to transiently high salinity (Kalir and Poljakoff-Mayber, 1981). Besides this, some of the salt acclimation strategies may not be prominent when plants are exposed to gradual increases in salt concentration (Ottow *et al*., 2005) and this led us to expose the plants to very high salt concentrations in order to uncover exaggerated responses. Studies have been carried out with respect to osmotic adjustment and antioxidant metabolism since these two strategies figure prominently in both salt and dehydration stress tolerance.

2.2 Materials and methods

2.2.1 Plant material

Mature propagules of *B. cylindrica* (L.) Blume were collected from the seacoast at Mumbai, India (19°13'45"N; 72°54'49"E). These propagules were washed under running tap water for 15 min., sown in polythene bags, containing garden soil and organic manure (1:1), and irrigated with either fresh water or 400 mM NaCl to field capacity. The propagules were maintained in the green house at 70-80% relative humidity (RH) and 25±2°C temperature. Light intensity in the greenhouse varied from 75-150 μmol m^{-2} s^{-1} during different hours of the day. Saplings of uniform size (eight-leaf stage) were selected from the greenhouse-grown plants for different experiments and were called fresh water (FW) plants and salt water (SW) plants, irrigated either with fresh water or 400 mM NaCl respectively.

2.2.2 Stress application

Desiccation stress was applied using 30% polyethylene glycol (PEG; Sigma Aldrich, USA) 8000 (which imparts an osmotic pressure of -2 MPa, equivalent to sea water) solution prepared in modified Murashige and Skoog's medium (½ MSM) (Naik *et al*., 2003). Osmotic stress was applied by using 4 M NaCl solution (which imparts an osmotic pressure of -17.8 MPa) in ½ MSM. Roots of FW plants were immersed in glass jars containing 250 ml of ½ MSM (controls), PEG or 4 M NaCl solutions. Roots of SW plants were immersed in 400 mM NaCl (controls), PEG or 4 M NaCl solutions. These plants were kept for 48 h in the green house. After 48 h, the second pair of fully expanded leaves from the shoot apex were harvested from five independent FW and SW plants and used for measurement of relative water content (RWC), osmometry, electrolytic

conductivity (EC), respiration, proline content, lipid peroxidation and antioxidant enzyme activities. Cation content of FW and SW plants was also estimated.

2.2.3 Cation Content

Leaves of FW and SW plants were dried at 80ºC for 48 h and powdered. The dry leaf powder was heated in a furnace at 400ºC for 3 h. 100 mg of ash was resuspended in double-distilled water and the content of Na^+, K^+ and Ca^{2+} were determined using atomic absorption spectrophotometer (Chemito 201, India). The ion contents were expressed in mg g^{-1} dry weight.

2.2.4 Relative Water Content

RWC was measured by Barrs and Weatherley (1962) method. Two leaves each from five independent FW and SW plants, growing with or without 30% PEG or 4 M NaCl, were used for determining RWC. The leaves were cut into three pieces (about 2 cm^2 area) and fresh weight (fw) was measured immediately after harvesting. The turgid weight (tw) was recorded after 24 h saturation at 4^{o}C. The leaf pieces were blotted dry to remove the surface water. Finally the leaf pieces were dried for 48 h in hot air oven at 65^{o}C and dry weight (dw) was measured. RWC was calculated by the following formula:

RWC (%) = [(fw-dw) / (tw-dw)]*100

2.2.5 Osmometry and electrolytic conductivity measurements

Leaves were ground in a mortar and pestle and centrifuged at 9838 x g (Kubota 1710, Japan) for 10 min. Supernatant was used as a source of cell sap. 10 µl of cell sap per sample was used for osmometry measurements. Osmolarity of the cell sap was measured using a vapor pressure osmometer (Wescor 5500, USA). Values were expressed in terms of osmotic pressure (MPa). The corresponding osmolarity was calculated by using the

formula $\pi = icRT$, where π is the osmotic pressure, i x c is the measured osmolarity (i being the Van't Hoff factor (which is 1.96 for NaCl)), c, the molar concentration (calculated by comparing the observed EC with that of the EC of known concentration of NaCl), R, the gas constant and T the absolute temperature (Moore, 1962). To measure electrolytic conductivity (EC), the cell sap was diluted 500-fold with distilled-water and placed in the conductivity cell until the cell was completely submerged in the sap solution. EC was measured using an electrolytic conductivity meter (ELICO, India) having a cell constant of 0.1 cm^{-1}. The EC for 0.05 M NaCl solution was used to derive the salt concentration in terms of NaCl equivalents in the sap solution. The corresponding osmotic pressure was calculated using same equation as mentioned above.

2.2.6 Proline Content

Free proline was estimated according to the method of Bates *et al.* (1973). Leaf tissue (0.5 g) from FW and SW plants was homogenized in 10 ml of 3% aqueous sulphosalicylic acid. The supernatant was treated with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid for 1 h in a boiling water bath. After cooling the solution in an ice bath, free proline was extracted in 4 ml of toluene by shaking the tubes vigorously for a min. Chromophore phase containing toluene was separated from aqueous phase and optical density of the solution was measured at 520 nm. A standard curve was used to determine the amount of free proline as μmoles g^{-1} dry weight.

2.2.7 Respiration measurements

Rate of oxygen consumption was measured with a Clarke-type oxygen electrode (Hansatech, Norfolk, UK) at 25^{o}C. Freshly cut leaf pieces of FW and SW plants weighing 100 mg were immediately immersed in 2.5 ml of aerated 10 mM Tris-HCl

buffer pH 7.5 in the measurement chamber and the rate of O_2 consumption was measured in the dark. Respiration rates were measured before the original O_2 levels dropped to 50% so as to avoid oxygen depletion effects. Respiratory O_2 uptake was expressed in terms of nmoles O_2 consumed s^{-1} g^{-1} fresh weight.

2.2.8 Lipid peroxidation

Accumulation of lipid peroxides was measured in terms of the amount of malondialdehyde (MDA) produced by thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968).

Leaf tissue (0.1 g) was homogenized in 1 ml of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 1205 x g (Kubota 1710, Japan) for 20 min. 4 ml of 20% TCA containing 0.5% TBA was added to 1 ml aliquot of supernatant. The mixture was heated at 95˚C for 30 min. The reaction was terminated by quick cooling on ice. The contents were centrifuged at 9838 x g for 15 min and absorbance was taken at 532 nm (Hitachi U-2000, Japan). A correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The MDA content was expressed in terms of μmols g^{-1} dry weight using an extinction coefficient (ε) of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

2.2.9 Enzyme extraction and assays

Leaf tissue (0.5 g) of the second pair of leaves from the shoot apex of FW and SW plants was homogenized in 4 ml of extraction buffer containing 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 mM EDTA, 0.056 μl (v/v) β-mercaptoethanol and 4% (w/v) polyvinylpyrrolidine (PVP), in a pre cooled mortar and pestle (Malan *et al*., 1990). The homogenate was centrifuged at 9838 x g (Kubota 1710, Japan) for 10 min at 4°C and

the supernatant was used for estimation of antioxidant enzymes. All steps in enzyme extraction preparation were carried out at 0-4ºC. Activities of CAT, APX, GDP and SOD were measured spectrophotometrically (Hitachi U-2000, Japan).

CAT was assayed spectrophotometrically by measuring the rate of disappearance of H_2O_2 as described by Volk and Feierabend, 1989. The reaction mixture (3 ml) was composed of 0.1 M potassium phosphate buffer and 30 µl enzyme extract. The reaction was started by adding 30% H_2O_2 and the activity was determined by monitoring decrease in absorbance at 240 nm (ε =36 M^{-1} cm^{-1}) for 3 min at interval of 30 s. The slope of the rate assay (ΔA) was used to determine enzyme activity, which was expressed as mKat g^{-1} dry weight.

APX activity was estimated spectrophotometrically by measuring the decrease in the absorbance due to conversion of exogenously added ascorbate (ε = 2.8 mM^{-1} cm^{-1}) to dehydroascorbate at 290 nm (Volk and Feierabend, 1989) for 3 min at interval of 30 s. The assay mixture (3 ml) contained 100 mM potassium phosphate buffer pH 7.5, 1 mM EDTA, 5 mM ascorbic acid and 20 µl enzyme extract. The reaction was initiated by 30% H_2O_2. APX activity was expressed in terms of mKat g^{-1} dry weight.

GDP activity was measured in terms of increase in absorbance at 470 nm due to the formation of tetra-guaiacol (ε = 26.6 mM^{-1} cm^{-1}) from guaiacol in the presence of H_2O_2 (Volk and Feierabend, 1989). The reaction mixture (3 ml) contained 100 mM potassium phosphate buffer pH 7.0, 3 mM guaicol and 15 µl enzyme extract. GDP activity was measured for 3 min at interval of 30 s and expressed as µKat g^{-1} dry weight.

SOD activity was determined in terms of inhibition of the rate of nitroblue tetrazolium (NBT) photochemical reduction (due to $O_2^{\cdot -}$ radicals generated by riboflavin in light)

(Beauchamp and Fridovich, 1971). The reaction mixture (3 ml) consisted of 50 mM potassium phosphate buffer pH 7.8 and 0.1 mM EDTA, to which a superoxide generating system containing 14.3 mM methionone, 82.5 µM NBT, and 2.2 µM riboflavin was added. Riboflavin was added in the last and tubes were shaken well. The reaction was started by adding 70 µl crude enzyme extract. Reaction mixture tubes were kept 25 cm away from a light source (1000 lux) for 7 min. The reaction was stopped by placing the tubes in dark. All the reactants along with 70 µl enzyme exract were incubated in dark as served as the respective blanks. The increase in absorbance due to formation of formazan was read spectrophotometrically at 560 nm. One unit of SOD activity was the amount of enzyme that inhibited NBT reduction by 50%. Activity of SOD was determined in Units g^{-1} dry weight.

2.2.10 Statistical analysis

Experiments were carried out at least thrice, during summer, rainy and winter season, each with three to six replicates. Data from one single experiment was taken into consideration for statistical analysis. Data from the other two sets of experiment which was not used for analysis, showed similar trends with reference to the parameters studied. Data was analyzed by one-way ANOVA (CROPSTAT Windows 7.2.2007.3, IRRI, Phillipines). Least significance difference (LSD) values were used to identify the means that differed significantly at P=0.05.

2.3 Results

2.3.1 Cation content

Cation analysis by atomic absorption spectrophotometry showed that both FW and SW plants had similar levels of Ca^{2+} ions. K^+ content of FW plants was higher than SW plants but not significant. However the Na^+ content in SW plants was twice that of FW plants (***Table 2.1***).

2.3.2 RWC and Osmotic Adjustment

Relative water content (%) of FW plants was lower than that of SW plants (***Table 2.2***). Both types of plants showed similar growth rates (data not shown) though the RWC of FW plants was about 15% lower than that of SW plants. When treated with PEG, RWC decreased significantly in the FW but not in SW plants. 4 M NaCl treatment led to an increase in RWC in both FW and SW plants, but the SW plants appeared hyperhydric since the RWC was over 100% (***Table 2.2***). The osmotic pressure of sap of SW plants was approximately twice that of the solution (400 mM NaCl = -2 MPa) used to irrigate them. Over three-fold increase was observed in the osmotic pressure of the sap of FW plants subjected to PEG treatment, but that of SW plants showed only a marginal increase. Salt treatment with 4 M NaCl led to an increase in osmotic pressure in both FW and SW plant sap. On measuring the electrolytic conductivity (EC) of the cell sap and deriving the osmotic pressure (in terms of NaCl equivalents), it was seen that the observed and derived osmotic pressure of SW and FW plants was similar indicating that ionic species accounted for almost all of the observed osmotic pressure (***Table 2.2***). However the derived osmotic pressure of the PEG- and 4 M NaCl-treated FW and SW

plants was lower than the respective measured values, suggesting the contribution of non-ionic species to the observed osmolarity.

2.3.3 Proline content

Proline levels were similar in FW and SW plants, but increased significantly in FW (2-fold) and SW (3-fold) plants in comparison to respective controls, when subjected to 4 M NaCl treatments. PEG treatment did not lead to significant changes in proline levels in FW and SW plants (***Table 2.2***).

2.3.4 Respiration rates

Respiration rates were significantly higher in SW plants as compared to the FW plants (***Table 2.3***). On application of PEG or 4 M NaCl treatment, the respiration rate of FW plants increased three and one fold respectively. However respiratory O_2 uptake did not alter with PEG or 4 M NaCl treatment in SW plants (***Table 2.3***).

2.3.5 Lipid peroxidation and antioxidant enzyme activities

The level of lipid peroxidation in the leaf tissue was measured in terms of MDA content. Levels of MDA in FW plants did not differ significantly from those of SW plants, but increased by 30% and 100% on treatment with 4 M NaCl or PEG respectively ***(Table 2.4***). No significant alteration in MDA levels was observed when SW plants were treated with PEG solution, and the levels halved when subjected to 4 M NaCl treatment.

Levels of CAT were almost half in the SW plants than in FW plants, while the levels of APX and GDP were similar in both plants (***Table 2.4***). SOD levels were 36% more in SW plants as compared to FW plants. After PEG treatment, there was a significant increase of 60 % and 20% in activities of CAT and SOD activity respectively, in SW plants. FW plants showed significant increase in GDP only. PEG treatment led to a

decrease in CAT and SOD activities in FW plants (***Table 2.4***). CAT activity was inhibited in 4 M NaCl treatment in both FW and SW plants significantly. Activity of GDP was observed to be enhanced two-fold and four-fold in FW and SW plants with 4 M NaCl treatment respectively (***Table 2.4***). Almost 50% decrease in SOD activity was seen in FW plants upon 4 M NaCl treatment, but remained unchanged in SW plants as compared to their respective controls.

2.4 Discussion

In saline ecosystems, halophytes are often subjected to salinity and drought. The physiological adaptations of mangroves to saline conditions are similar to the adaptations shown by plants to osmotic stress rather than to salt-specific effects and it has been suggested that plants showing drought resistance would also exhibit salinity tolerance (Munns, 2002). The success of plant adaptation to stress depends on an early sensing of the stress followed by an adequate reaction. For instance, plants show a stronger and faster defense response upon exposure to a pathogen, a phenomenon known as priming (Conrath *et al*., 2002). Priming can also be observed in plants exposed to abiotic stress. If plants have previously undergone an acclimation process, their reaction to the following stress is more successful (Lang and Palva, 1992; Knight *et al*., 1998; Sivritepe *et al*., 2003). In our experiments SW plants could be termed as salt-primed plants since they were acclimatized to saline conditions before they were exposed to short-term osmotic stresses. On the contrary, FW plants did not experience salt conditions previously and could be called as non-primed plants. Mangroves and halophytes are able to tolerate salt concentration even above sea water salinity (Flowers and Colmer, 2008; Ksouri *et al*.,

2010). Since salt stress is primarily known to exert osmotic effects, we attempted to understand whether the salt tolerance mechanisms of *B. cylindrica* enable the plant to tolerate PEG-mediated osmotic stress as well. Fluctuating salinity of the sea water can expose halophytes or mangroves to transiently very high salt concentrations (Kalir and Poljakoff-Mayber, 1981). Hence we also studied the effect of extreme salt stress imposed by 4 M NaCl on osmotic adjustments and antioxidant metabolism in FW and SW plants of mangrove *B. cylindrica*.

B. cylindrica plants irrigated with 400 mM NaCl accumulated about 1 M of salt (osmotic pressure = -2.75 MPa) in their leaves, as is also known to occur in several dicotyledonous halophytes and mangroves, where salt itself acts as an osmoticum for regulating water levels in cells (Matoh *et al*., 1987; Binzel *et al*., 1988; Glenn *et al.*, 1999; Takemura *et al*., 2000). However on subsequent exposure of the SW plants to desiccation stress (-2 MPa) or extreme salt stress (-17.8 MPa) it was observed that salt could not suffice as the osmoticum, since the measured osmolarity could not be accounted for by ionic species alone. Stress-induced accumulation of non-ionic compatible solutes in response to stress is known to occur in plant species, including mangroves and we studied proline accumulation as a representative compatible solute (Hanagata *et al.*, 1999; Takemura *et al*., 2000; Parida *et al*., 2002; Kavi Kishor *et al*., 2005). Proline accumulation was observed when the FW and SW plants, when subjected to 4 M NaCl treatment, but not in PEG treatment. *Bruguiera* spp. are primarily known to accumulate cyclitols and organic acids (Sugihara *et al*., 2000), besides proline (Parida *et al*., 2002). Although both drought and salinity induce water deficit at cellular, tissue, and whole plant levels, they may also have distinct effects on plant metabolism (Yu and Rengel, 1999; Munns, 2002; Hu *et al*.,

2007; Teixeira and Pereira, 2007). Hence FW and SW plants may accumulate osmolytes like cyclitols and organic acids other than proline under PEG stress, which we have not measured in our experiments. Compatible solutes are known to play an important role in osmotic adjustment and thereby stress tolerance. While halophytes are known to use Na^+ as an osmoticum in vacuole to lower cellular water potential, this necessitates the synthesis of compatible solutes in the cytoplasm to prevent water movement from cytoplasm to the vacuoles. On the other hand plants exposed to dehydration stress solely rely on synthesis of compatible solutes to maintain cell turgor. Synthesis of compatible solute enables survival of plants under stress conditions. However some reports suggest that accumulation of compatible solutes does not correlate to stress tolerance but are a symptom of salt susceptibility, since their synthesis involves large energy inputs, which affects the fitness of the plant (Rampino *et al.*, 2006; Chen *et al.*, 2007). Both FW and SW plants could survive harsh treatment with 4 M NaCl as long as 15 days (observations were made for this period). However, PEG stress led to leaf wilting, senescence, leaf shed and death of FW plants within a week. In SW plants, these symptoms were delayed, but eventually PEG-treated FW and SW plants died.

Exposure of plants to high salt concentrations is known to bring about an increase in the rate of respiratory O_2 uptake. Salt-induced respiration has been reported in plants (Rains, 1972) and is thought to play an important role in transport of salt across membranes. Accordingly, the SW plants showed higher respiratory rates than the FW plants. However the respiratory rate of FW plants but not SW plants increased in response to PEG and high salt treatments. *Dunaliella*, a marine alga, is known to accumulate high levels of glycerol to counteract salt stress and rate of dark respiration were seen to increase when

the alga was grown at 3 M salt concentration instead of 0.5 M, which is the normal salt concentration of sea water (Liska *et al*., 2004). The increased metabolic rates are thought to play an important role in the ability of this alga to survive at high salinity. However, in *B. cylindrica*, the increased respiration rates observed in FW plants treated with PEG and 4 M NaCl probably reflected metabolic perturbations caused by dehydration stress, rather than having a role in stress tolerance. Oxidative stress leads to increase in respirations and production of ROS (Tiwari *et al*., 2002). Therefore increased respiration is also correlated to the lower stress tolerance (Burchett *et al*., 1989), indicating that the higher respiration rate probably incurs a higher metabolic cost and reduces the fitness of the species.

Salt-induced osmotic and ionic disturbances cause oxidative damage resulting in accumulation of ROS (Ksouri *et al*., 2010). Salt-induced increase in respiration also enhances production of ROS (Halliwell and Gutteridge, 1999; Rhoads *et al*., 2006). An important adaptation of mangroves to saline conditions is the development of an efficient antioxidant metabolism to counteract salt-induced generation of ROS (Cheeseman *et al*., 1997; Parida *et al*., 2004). Oxidative damage was observed in terms of higher accumulation of lipid peroxides in the FW plants subjected to PEG and 4 M NaCl treatments. Lipid peroxides did not accumulate in SW plants subjected to similar treatments, which again pointed to the protective effect of salt priming in stress situations. The SW plants showed about 2-fold increases in SOD and CAT levels on exposure to desiccation stress, which could be correlated to the lower accumulation of lipid peroxides observed in these plants. Correspondingly the observed decrease in CAT and SOD in PEG-treated FW plants probably led to oxidative damage. A lack of increase

in levels of CAT, APX and SOD when FW plants were transferred to PEG or 4 M NaCl solution correlated to the increase in lipid peroxidation observed. Only GDP activity increased 28% and 70% respectively, when FW plants were subjected to PEG-induced dehydration and 4 M NaCl stress. This indicates that the increase in antioxidant enzyme activities occurred as a part of the active dehydration tolerance response rather than a secondary response to damage caused by dehydration stress.

Extreme osmotic stress imparted by 4 M NaCl led to drastic inhibition of CAT in FW and SW plants. Reduced CAT levels upon salt exposure have been reported in plants including mangroves and halophytes under stress conditions due to increased H_2O_2 levels (Feierabend *et al*., 1992; Parida *et al*., 2004). CAT activity decreased in the halophyte *Halimione portuiacoide*, exposed to media containing different concentrations (0 to 2 M) of NaCl but POX and SOD activities were unaffected. This decrease in activity of an enzyme under such conditions was attributed either to decrease of enzyme synthesis, or increase of enzyme degradation, or alternatively to conformational changes and reduced structural stability of the enzyme molecule (Kalir and Poljakoff-Mayber, 1981).

GDP did not seem to play a role in protection against oxidative stress in PEG-imposed dehydration and 4 M NaCl stressing FW plants. Higher levels of GDP were shown to correlate to growth retardation probably due to cell wall modifications, in rice (Lin and Kao, 2002) and to phenolic turnover accompanying leaf senescence, in *Rhizophora mangle* (Pearse *et al*., 2005) rather than its role in antioxidant metabolism.

The salt-primed (SW) plants of *B. cylindrica* seemed to cope better than their non-primed (FW) counterparts when exposed to desiccation stress or extreme salt stress. Salt priming of seeds has emerged as an effective method of improving stress tolerance in various

plants like tomato and canola (Cuartero *et al.*, 2006; Farhoudi *et al.*, 2007). The molecular mechanisms responsible for priming effects are thought to involve accumulation of signaling proteins or transcription factors, or epigenetic mechanisms like chromatin remodeling (Bruce *et al.*, 2007) both of which could bring about quicker and more potent responses to subsequent exposure to stress. Salt grown *B. cylindrica* plants were subjected to salt (400 mM) from the time of propagule germination till application of PEG or 4 M NaCl stress. This continuous exposure to salt may have enabled the plants to tolerate metabolic imbalances generated by PEG-mediated desiccation stress and stress imposed by a ten-fold higher salt concentration by an effect similar to that of salt priming of seeds. Salt priming may therefore be an important adaptive strategy for halophytes in their natural habitat, where they experience large seasonal variations in salt concentration (of sea water) and water availability. SW plants were able to withstand stress imposed by PEG (iso-osmotic to salt concentration to which the plants were adapted) or 4 M NaCl better than FW plants. Mechanisms underlying the better tolerance of SW plants could be attributed to better osmotic adjustment using salt and proline as osmoticum and an efficient antioxidant metabolism which prevented oxidative damage. Salt priming did not cause significant metabolic alterations in the absence of stress, but enabled SW plants to launch protective mechanisms when subjected to osmotic or ionic stress. Hence salt priming could be considered as an important adaptive strategy used by mangroves to cope with unpredictable variations in salinity in their natural habitat.