ABSTRACT

Plant growth and productivity is severely affected by high salinity. Saline soils have high amount of soluble salts and exhibit electrical conductivity (EC_e) of more than 4 dS m^{-1}. High salinity leads to hyperionic and hyperosmotic stress at cellular level as well as whole plant level. Plants have developed an array of morphological, physiological, biochemical and molecular mechanisms to withstand salt stress. Presence of salt glands on leaves is a morphological adaptation shown by halophytes to salt stress. Other halophytes show physiological adaptations in which salt is removed from the cytoplasm to avoid metabolic toxicity. These include (a) Pumping of Na^+ ions into vacuoles by a vacuolar Na^+/H^+ antiporter, (b) Synthesis of compatible solutes such as amino acids, quarternary ammonium compounds, polyols etc. to counterbalance the osmotic potential in cytoplasm, (c) Increase in respiration rate, which provides ATP for vacuolar sequestration of salt and osmolyte synthesis, (d) Detoxification of reactive oxygen species (ROS) that may be generated as a consequence of metabolic impairment caused by salt stress.

Mangroves are woody trees and shrubs that grow in estuarine or brackish environment of tropical and sub-tropical coastal systems. Flourishing in hostile environmental conditions such as high salinity (with irregular periods of fresh water conditions), hypoxia, excess light and nutrient deficiencies, make mangroves ideal models for salt stress research.

In this thesis, we have compared some physiological responses of a mangrove *Bruguiera cylindrica* (L.) Blume to dehydration and high salt stress imposed on saplings irrigated with fresh water or salt water. The objective was to understand the salt adaptation mechanisms in this mangrove.

The aspects dealt with in this thesis are:

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

2. Salt-induced respiration and its role in salt adaptation

3. Identification and characterization of a plasma membrane Ca^{2+}/H^+ antiporter

4. Transcriptional regulation of ion-transporters

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

Mature propagules of *B. cylindrica* were collected from the seacoast at Mumbai, India. These were sown in garden soil and organic manure, and irrigated with either fresh water (FW plants) or 400 mM NaCl (SW) plants). Dehydration stress was applied to 6-8 leaved saplings using 30% PEG 8000 (-2 M Pa) and osmotic stress was applied using 4 M NaCl (- 17.8 M Pa).

After 48 h of stress application the relative water content (RWC) decreased significantly in FW but not in SW plants in response to PEG treatment and in both FW and SW plants treated with 4 M NaCl. Osmotic pressure derived from osmolarity measurements of sap of FW plants treated with PEG increased over three-fold while that of SW plants showed only a marginal increase. 4 M NaCl treatment led to increased osmotic pressure in both FW and SW plant sap. Osmotic pressure derived from EC measurements of PEG and 4 M NaCl treated FW and SW plants was lower than the corresponding measured values, suggesting the contribution of non-ionic species to the total osmotic pressure. Proline levels enhanced significantly in FW and SW plants subjected to 4 M NaCl treatment but not in response to PEG treatment. PEG or 4 M NaCl treatments led to increased respiration rates in FW plants but not in SW plants.

MDA levels increased in FW plants as compared to those in SW plants in response to PEG or 4 M NaCl treatment, indicating oxidative damage to the FW plants due to these stress treatments. Higher activities of CAT and SOD in SW plants in response to PEG treatment suggested their protective role against oxidative damage. Extreme salt stress led to a decrease in CAT and SOD levels in FW plants but not in SW plants. Our results indicated that SW plants performed better in terms of survival against desiccation and extreme salt stress than FW plants, probably because they were salt primed since early growth. Salt priming could be an adaptive strategy used by mangroves to withstand large seasonal fluctuations in the salt concentrations of their natural habitat.

2. Salt-induced respiration and its role in salt adaptation

We studied oxygen uptake rates in the leaves of FW and SW plants of *B. cylindrica* using various concentrations of salt from 0.5 M to 4 M NaCl to understand the relationship between respiration pathways and salt management strategies of *B.*

cylindrica. Saplings of from the greenhouse-grown plants were called fresh water grown (FW) plants while those collected from the seacoast (Mumbai) were called seawater grown (SW) plants. Leaves of FW and SW were used for measuring O_2 uptake.

The respiratory O_2 uptake of leaf pieces was monitored polarographically in the dark at 25ºC using a Clark-type oxygen electrode. Time course studies on the effect of increasing concentrations of NaCl on respiratory oxygen consumption showed that there was significant increase in respiration when leaf pieces of FW and SW plants were immersed in 3 M and 4 M NaCl. A maximum increase in respiratory oxygen uptake in response to 4 M NaCl treatment was 5-fold in FW plants and 3-fold in SW plants. The salt-induced increase in respiration rate was specific to monovalent cations, especially Na^+ and K^+, but not divalent or trivalent cations, and to Cl^-, but not other anions. Pretreatment of leaves of FW plants with 1 mM amiloride, an inhibitor of the Na^+/ H^+ antiporter reduced the NaCl-induced respiration surge. Respiratory O_2 uptake of the leaves from both FW and SW plants was accounted for by the cytochrome c oxidase (COX) pathway and was inhibited by KCN. However the increase in respiratory O_2 uptake in 4 M NaCl immersed FW leaves was also inhibited by the alternative oxidase (AOX) inhibitor, SHAM in addition to KCN.

The generation of ROS which generally accompanies high respiratory activity was monitored in terms of loss of fluorescence of cell-permeable probe 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) and was seen to decrease upon treatment with KCN. However treatment with SHAM led to an increase in ROS accumulation even though the respiratory O_2 uptake decreased in the 4 M NaCl treated FW leaves. This pointed to a protective role of AOX in reducing ROS generation during salt-induced respiration. The results indicated that NaCl-induced increase in leaf respiration of *B. cylindrica* plants was required for salt sequestration and for scavenging harmful ROS that are known to accompany increased respiratory activity.

3. Identification and characterization of a plasma membrane Ca^{2+}/H^+ antiporter

Attempts were made to study the activity of plasma membrane Na^+/H^+ and Ca^{+2}/H^+ antiporters by following the kinetics of ion-exchange across plasma membrane (PM) vesicles. PM-enriched vesicles were prepared from leaves of salt-adapted *B. cylindrica* plants (8-12 leaf stage) from seacoast. Aqueous two-phase partitioning in

the presence of a non-ionic surfactant, Brij 58 was used to prepare inside-out PM vesicles. Plasma membrane vesicles were shown to be transport-competent and formed proton gradients due to the activity of the plasma membrane-localized H^+-ATPase as detected by quenching of Acridine orange fluorescence.

The rate of ATP hydrolysis in PM vesicle fraction by PM H^+- translocating ATPases was studied in the presence of inhibitors that differentiate types of H^+-ATPases (from PM, tonoplasts and mitochondria) to determine the purity of PM fraction. Vanadate-sensitive (P-type) ATPase activity was inhibited by more than 70% in the isolated membrane fractions. In contrast, activities of nitrate-sensitive (V-type) ATPase (tonoplast) and azide-sensitive (F-type) ATPase for (mitochondria) were inhibited by approximately 23%. This showed the membrane fractions were predominantly of PM origin. Performing the ATPase assays in presence of increasing concentrations of ATP showed typical Michaelis-Menten type kinetics as seen from the Lineweaver-Burk plots. The mean K_m (ATP) value of the PM H^+-ATPase determined from the three PM preparations was 1.437 mM. The effect of different salt concentrations on the activity of P-type ATPase was seen. The activity remained unaltered at 100 mM NaCl treatment. NaCl concentrations above 100 mM, showed inhibition of ATPase activity.

We could detect Ca^{+2}/H^+ antiport activity but not Na^+/H^+ antiport activity in the plasma membrane vesicles. Antiporter activity was inferred from Ca^{+2}- induced collapse of the pH gradient. The Ca^{+2}/H^+ antiport activity in *B. cylindrica* plasma membranes exhibited saturation kinetics with a mean K_m of 12.8 µM, as estimated from Hanes-Woolf plot. The antiport in *B. cylindrica* leaves which is a moderately high capacity, high affinity antiporter, might have an important role in maintaining cytoplasmic Ca^{+2} concentrations under the saline environment in which this mangrove grows.

4. Transcriptional regulation of ion-transporters

By using semi-quantitative reverse transcription PCR (RT-PCR) technique, we also analyzed differential gene expression of primary plasma membrane transporters (PM H^+-ATPases) and ion transporters (Na^+/H^+ antiporter, Ca^{2+}/H^+ antiporter and K^+ transporter). cDNA sequences of known transporters were compared with those available in the EST library of *B. gymnorrhiza* using BLAST to obtain putative partial sequences corresponding to three transporters: (a) Na^+/H^+ antiporter (BP952176) (b)

Ca^{2+}/H^{+} antiporter (BP947531) and (c) K^{+} transporter (BP944372) and a H^{+}-pump (d) PM H^{+}-ATPase (BP949178).

RNA was extracted using TRI reagent from leaves of green house grown *B. cylindrica* plants, raised from vegetative propagules and irrigated either with fresh water (FW plants) or 400 mM NaCl (SW plants). The cDNA formed by reverse transcription of this RNA was subjected to PCR amplification for varying number of cycles using appropriate primers. The amplified products were sequenced to confirm that the amplification was specific to the respective cDNAs. The constitutively expressed 25S rRNA was used as a loading control. Moderate to high expression of Na^{+}/H^{+} antiport, Ca^{+2}/H^{+} antiport, K^{+} transporter and PM H^{+}-ATPase was observed in SW cDNA as compared to FW cDNA in different cycles of amplification. This suggested that transcriptional regulation of the antiporters may play a role in regulating ion homeostasis and salt tolerance in the mangrove *B. cylindrica*.