CHAPTER 3

Salt-induced respiration and its role in salt adaptation

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3.1 Introduction

Respiration forms the core of plant intermediary metabolism and plays a pivotal role in the growth and metabolism of all photosynthetic organisms. It produces energy and various metabolites that are used in biosynthetic processes and are essential for growth, maintenance, and carbon balance of all plant cells (McCabe *et al*., 2000; Raghavendra and Padmasree, 2003).

In general, any stress will incur increased maintenance costs due to metabolic perturbations in organisms. Salt stress leads to water deficit, ion imbalances and oxidative stress. Electrolyte accumulation and osmolyte synthesis, for turgor regulation, are key determinants of salt tolerance, which incur metabolic costs in terms of respiratory ATP. In leaf cells, approximately seven moles of ATP are needed to accumulate one mole of NaCl as an osmoticum, whereas the amount of ATP required to synthesize one mole of an organic compatible solute is an order of magnitude higher (Munns and Tester, 2008). Increasing salt concentration in the external medium has been reported to bring about an increase in the respiratory oxygen uptake in glycophytes as well as halophytes (Lundegardh, 1955; Rains, 1972; Takemura *et al*., 2000). High salinity resulted in significant increase in respiratory oxygen uptake in excised leaf and hypocotyl of the mangrove species *Avicennia marina* and *Aegiceras corniculatum* (Burchett *et al*., 1989). The increased respiratory rate could possibly provide the energy required for osmolyte synthesis, ion accumulation and vacuolar compartmentation (Jolivet *et al*., 1990). However, enhanced respiration is also considered a pathological response to stress, since

less salt tolerant species exhibited increased respiration (Burchett *et al*., 1989; Lopez-Hoffmann *et al*., 2006).

The synthesis of ATP in respiring organisms is coupled to the flow of electrons through the mitochondrial electron transport chain to a terminal electron acceptor. Plants are equipped with two different respiratory electron transport pathways from ubiquinone (Q) to O_2 (Maxwell *et al*., 1999; Yip and Vanlerberghe, 2001). The cynanide-sensitive cytochrome c oxidase mediated (COX) pathway is primarily used for ATP generation from ADP and inorganic P (Pi) by the process of oxidative phosphorylation. Alternatively, a non-phosphorylating pathway involving alternative oxidase (AOX), branches at the level of Q and donates electrons directly to O_2 to form water in a single four-electron step (Siedow and Moore, 1993). This cyanide-resistant pathway bypasses two of three proton coupling sites and thus most of the energy is lost as heat.

Stress-induced physical changes in membrane components may restrict electron transport and bring about the accumulation of reactive oxygen species (ROS), which can result in mitochondrial oxidative damage (Geraldes-Laakso and Arrabaça, 1997; Maxwell *et al*., 1999). Electron complex I and III of mitochondrial electron transport (mtETC) regulate the reduction level of mitochondrial ubiquinone pool, which is the primary determinant of mtROS output (Rhoads *et al*., 2006). An increase in activity/expression of antioxidants in mitochondria is observed when plants are subjected to salinity stress (Slesak *et al*., 2002; Wang *et al*., 2004). However, a metabolically cheaper option is to prevent ROS generation by transporting electrons via the AOX pathway (thereby preventing over-reduction of the respiratory electron transport components). The AOX gene is encoded by a small family of nuclear genes that exhibit highly conserved regions, suggesting a

pivotal role of AOX in plant functioning (Vanlerberghe and McIntosh, 1997). The most characteristic function of AOX pathway is seen in thermogenic respiration of aroid spadices (Meeuse, 1975). However, AOX is also known to be activated as an early response to metabolic imbalances (Clifton *et al*., 2005) induced by conditions of either chilling (Purvis and Shewfelt, 1993), drought (Bartoli *et al.* 2005), salt stress (Geraldes-Laakso and Arrabaça, 1997) or pathogen attack (Simons *et al*., 1999). It is thought to provide a mechanism for optimizing metabolic efficiency for regulating growth under stress conditions (Arnholdt-Schmitt *et al*., 2006). Superoxide radicals and H_2O_2 can induce the expression of AOX genes (Wagner, 1995), suggesting that AOX pathway may be induced when mitochondria experience oxidative stress. In addition, under limiting conditions of ADP and/or Pi to COX pathway, AOX functions to maintain respiratory electron flux to O_2, thereby permitting TCA cycle to operate and provide carbon skeletons for other cellular processes (Lambers and Steingrover, 1978).

In this chapter we studied the leaf respiration rates in fresh water irrigated plants of *B. cylindrica* grown in the greenhouse (FW plants) and salt adapted plants collected from the sea coast (SW plants). Further, respiration rates were also studied when the leaves of these two types of plants were immersed in 4 M NaCl to try and uncover the possible salt adaptive strategies, which may not be prominent when plants are exposed to gradual increases in salt concentration (Ottow *et al*., 2005).

Effect of various cations and anions, dehydration stress and ion transport inhibitors on respiration, as well as the contribution of COX and AOX pathway in FW and SW plants was also studied. The objective of these studies was to gain some insights on respiratory metabolism in this facultative mangrove.

3.2 Materials and Methods

3.2.1 Plant material

Mature viviparous 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. Propagules were then sown in polythene bags, containing garden soil and organic manure in the same ratio, and irrigated with fresh water 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.

Saplings of uniform size (eight-leaf stage) collected from the seacoast (Mumbai) were called seawater (SW) plants. These plants were brought to the laboratory in buckets along with the seabed soil and seawater in which they were growing and used for measurements directly. The second pair of leaves from the shoot apex was used for respiratory O_2 uptake measurements.

3.2.2 Leaf respiration measurements

The respiratory O_2 uptake of leaf pieces was monitored polarographically using a Clark-type oxygen electrode (Hansatech, Norfolk, UK). Temperature of the measurement chamber was controlled by circulating water from a water bath maintained at 25ºC. The second pair of leaves from the shoot apex was used for all respiratory O_2 uptake measurements. Leaves of FW and SW were cut into small pieces (approximately 0.5 x 0.5 cm) by a sharp razor blade. Approximately 100 mg of leaf tissue was placed in

oxygen electrode cuvette for measurement of O_2 uptake. O_2 uptake was monitored in rapidly stirred and ambient air saturated solution (2.5 ml), during which the O_2 levels were never below 50-60% of the original levels. Solutions were continuously stirred using a magnetic flea. Cuvette was sealed properly with plunger and the O_2 electrode was covered with a black cloth cover to block out light during the respiratory O_2 measurements. The slope of the O_2 depletion curve provided a measurement of O_2 uptake by leaf tissue, from which the respiration rate was calculated on fresh weight basis of the sample. Respiratory O_2 uptake was expressed in terms of nmoles O_2 consumed s^{-1} g^{-1} fresh weight. The rates of O_2 uptake were calculated assuming that the concentration of oxygen in the air-saturated buffer at 25º C was 0.23 μmoles ml^{-1}.

3.2.3 Measurement of O_2 uptake using different concentrations of NaCl

Leaf pieces (100 mg) of FW and SW plants were immersed in either 10 mM Tris-HCl buffer, pH 7.5 control or NaCl solutions of different concentrations (0.5 M, 1 M, 2 M, 3 M, and 4 M NaCl) prepared in 10 mM Tris-HCl buffer, pH 7.5. O_2 uptake was monitored in the respective solutions (2.5 ml) for 3-5 min (after aerating the solutions in the measurement chamber) during which the O_2 levels were never below 50-60% of the original levels. The respiration rate was determined every 30 min for a total period of 2 h.

3.2.4 Study of PEG- induced dehydration stress on respiration

Dehydration stress was imposed by polyethylene glycol (PEG), a neutral active polymer used widely in plant water deficit studies. Leaf pieces (100 mg) of FW and SW plants were immersed in 30% PEG 8000 (Sigma Aldrich, USA) or 4 M NaCl solution prepared in 10 mM Tris-HCl (pH 7.5) for 30 min and then used for O_2 uptake measurements in

these respective solutions. 10 mM Tris-HCl buffer (pH 7.5) was used as control for FW plants and seawater for SW plants.

3.2.5 Effect of other cations and anions on respiration

The ion-specific effects on respiration rates of leaves of FW plants were studied by substituting 4 M NaCl with solutions of other cations and anions. These cations were used at similar concentrations (4 M in case of monovalent and divalent cations) or at equivalent charge concentrations (e.g. 2 M for divalent cations; 1.33 M for trivalent cations). Anion solutions were similarly prepared, but were restricted by their maximum solubility, (e.g. 1.9 M Na_2SO_4 and 1 M Na phosphate (NaP- added as a mixture of mono- and di-basic sodium phosphate)). All solutions were prepared in 10 mM Tris-HCl buffer (pH 7.5). Ion specific effects on respiration were studied by measuring the respiration rate in 100 mg fresh leaf pieces of FW plants after 5 min of incubation in solutions containing the respective cations or anions.

3.2.6 Respiration in presence of ion transport inhibitors

Amiloride, an inhibitor of Na^+/H^+ antiporter, and DIDS, anion channel blocker were taken to study the effect of ion transport inhibitors on respiratory O_2 uptake in FW plants. Stock solution of 15 mM amiloride and 1 mM DIDS was prepared freshly in double-distilled water. Leaf pieces (100 mg) of FW plants were immersed in aqueous solutions of 1 mM amiloride (3,5-diamino-6-chloro-N-(diaminomethylene) pyrazinecarboxamide monohydrochloride, dihydrate; Hoechst, India) or 0.1 mM DIDS (4,4'-diisothiocyanato stilbene 2,2' disulfonic acid; Sigma Aldrich, USA) for 30 min. The leaf pieces were transferred to either 10 mM Tris-HCl buffer, pH 7.5 or 4 M NaCl solutions for 5 min, 30 min or 1 h after which O_2 uptake was measured in the respective solutions.

3.2.7 Studies on contribution of cytochrome c oxidase (COX) and alternative oxidase (AOX) pathways to respiration

KCN and salicylhydroxamic acid (SHAM) were used to inhibit the COX and AOX pathways respectively. Both KCN and SHAM were obtained from Sigma Aldrich, USA. 1 M stock solution (pH 7.0) of KCN was prepared by dissolving in double-distilled water and neutralized with 2 M HCl. SHAM was dissolved in 2 M KOH and then neutralized with 2 M HCl to get a stock solution of 50 mM according to Nagel *et al.* (2001). The optimum concentration of these inhibitors was determined from titration experiments in which 100 mg leaf pieces of FW plants were immersed in varying concentrations ranging from 0 to 20 mM of KCN and SHAM for 1 h and then O_2 uptake was measured in 10 mM Tris-HCl (pH 7.5). Based on titration results, 15 mM KCN and 10 mM SHAM, which elicited a maximum response, were used for determination of cytochrome and alternative pathways in subsequent experiments. The inhibitors were added individually or together to 10 mM Tris-HCl buffer, pH 7.5 (for FW plants), seawater (for SW plants) and 4 M NaCl solutions (for both FW and SW plants). Leaf pieces (100 mg) were immersed in the above solutions for 1 h. The pieces were then transferred to the respective solutions but without inhibitors for O_2 uptake measurements. Contribution of the COX and AOX pathway to total respiration was determined as per Vani and Raghavendra (1994).

2,4-Dinitrophenol ($C_6H_4N_2O_5$, DNP; Sigma Aldrich USA) was used for uncoupling respiration. 10 mM DNP stock solution (dissolved in 2 M KOH and neutralized with 2 M HCl) was added in 10 mM Tris-HCl buffer, pH 7.5 (for FW plants), seawater (for SW plants) and 4 M NaCl solutions (for both FW and SW plants). 100 mg leaf pieces were

immersed in these solutions for 1 h and then used for measuring O_2 uptake in the respective solutions without DNP.

3.2.8 ROS measurements

In vivo generation of ROS was examined with the cell permeable probe 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma, USA). The dye permeates membranes and is retained by cells after cleavage of the acetate moiety by cellular esterases. Fluorescence develops upon oxidation of the dye by H_2O_2, peroxyl radical, or peroxynitrite anion (Tarpey and Fridovich, 2001). Stock solution of the dye was prepared in DMSO (dimethyl sulfoxide) and kept in the dark at -20ºC. About 25 mg leaf tissue was immersed for 30 min in solutions of 15 mM KCN or 10 mM SHAM added in 10mM Tris-HCl buffer, pH 7.5 (for FW plants), seawater (for SW plants) and 4 M NaCl solutions (for both FW and SW plants). After 30 min, DCFH-DA was added to each solution to a final concentration of 5 μM and samples were incubated in dark for a further 30 min. The solutions were then decanted and the leaf samples frozen in liquid N_2, homogenized and thawed. Freeze thawing was repeated at least thrice and then the tissues were suspended in 200 μl of 10 mM Tris-HCl buffer, pH 7.5. The samples were centrifuged at 9838 x g (Kubota 1710, Japan) for 5 min at 4°C and 100 μl of the supernatant from each sample was taken in a 96-well ELISA plate. Fluorescence measurements were made using a spectrofluorometer (Thermo, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The relative fluorescence values were expressed in terms of per mg protein, after measuring the protein levels in the same samples using Bradford's method (1976).

3.2.9 Statistical analysis

All observations were made from the 2nd pair of leaves of at least five independent FW and SW plants. Experiments were repeated at least thrice. Values were expressed as means along with their standard deviations. The data was subjected to statistical analysis using one-way ANOVA and Tukey's pairwise comparison test was used to determine differences between pairs of treatments. Difference between respiration rates obtained in presence or absence of different cations and anions as compared to controls was analyzed by Student's t test.

3.3 Results

3.3.1 Effect of salt (NaCl) concentration on respiration rate

Time course studies on the effect of increasing concentrations of NaCl on respiratory oxygen consumption over time 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 (***Fig 3.1***). A significant increase in respiratory oxygen uptake was observed almost immediately after subjecting the leaves of FW or SW plants to 4 M NaCl. The increase in respiratory activity was about five and three-fold after 5 min of in 4 M NaCl, in FW (***Fig 3.1a***) and SW (***Fig 3.1b***) plants respectively as compared to the leaves not subjected to salt treatment. After one hour incubation in 4 M NaCl, about two-fold increase in O_2 consumption was observed in both FW and SW leaves as compared to their non salt controls. No significant alterations in respiration were observed at salt concentrations ranging from 0-2 M salt.

3.3.2 Effect of PEG-induced dehydration stress on respiration rate

Leaves of FW and SW plants were immersed in 30% PEG (which exerts an osmotic pressure of -2 MPa) solution (instead of 4 M NaCl) and respiration was measured after 30 min. PEG-induced dehydration stress led to about 50% reduction in respiratory rates in both FW and SW plants (***Fig 3.2***). While about three and two-fold increase in respiration was measured in the leaf tissue of FW and SW plants respectively, with 4 M NaCl treatment.

3.3.3 Effect of various cations and anions on respiration rate

Respiration rates were also enhanced in the presence of 4 M solution of KCl and to some extent with LiCl and CsCl, but not in the presence of RbCl, divalent cations (Ca^{2+} and Mg^{2+}), or trivalent cations (Al^{3+}) at equivalent concentrations in terms of charge (***Fig 3.3***). On the other hand increased respiration was only observed in the presence of Cl^- but not in the presence of $CH3COO^-$, SO_4^{2-} or PO_4^{3-} at equivalent concentrations.

3.3.4 Effect of ion transport inhibitors on respiration rate

On pre-treating leaves of FW plants with amiloride, an inhibitor of Na^+/H^+ antiport, before subjecting them to 4 M salt treatment, a significant decrease in the respiratory rate was observed in comparison to the respiratory rate of 4 M NaCl-treated leaves (***Table 3.1***). However pretreatment with DIDS, an anion channel blocker led to an inhibition of respiratory rates in leaves irrespective of whether they were subjected to salt treatment or not.

3.3.5 Effect of respiratory inhibitors and an uncoupler on respiration rate

Inhibitors like KCN and SHAM that block either the COX or AOX pathway, cannot be used to assess the activities of the two respiratory pathways in plants (Day *et al*., 1996).

The only reliable way to assess the engagement of the AOX pathway is based on the differential oxygen-isotope-fractionation technique based on different ability of the two terminal oxidases to fractionate $^{18}O_2$ (Guy *et al*., 1989; Krause and Lambers, 2001; McDonald *et al*., 2003), which was beyond the scope of this study. Therefore, these inhibitors were only used to gain information about the KCN-resistant and the SHAM-resistant components as affected by various treatments.

The concentration of each inhibitor at which maximum inhibition of respiration occurred was 15 mM for KCN and 10 mM for SHAM (***Fig 3.4***). These concentrations were used in subsequent experiments with FW and SW plants subjected to 4 M salt treatment. KCN inhibited respiration in the leaves of both FW and SW plants that were immersed either in 10 mM Tris-HCl buffer pH 7.5 or in 4 M NaCl (***Fig 3.5a***).

Treatment of leaves with 10 mM SHAM led to a significant decrease in respiration only in the FW plants treated with 4 M salt. When used together, KCN and SHAM inhibited respiration further as compared to the inhibition observed with either of these inhibitors. When respiration was uncoupled from oxidative phosphorylation using 0.5 mM DNP, there was a 300% increase in respiration in leaves of the FW plants immersed in 10 mM Tris-HCl buffer pH 7.5, but only a 125% increase when the leaves were immersed in 4 M NaCl instead of buffer (***Fig 3.5b***). The respiration increases in leaves of SW plants immersed in seawater or 4 M NaCl were 177% and 172% respectively.

3.3.6 ROS generation during respiration

ROS generation observed in terms of fluorescence arising from oxidation of DCFH-DA occurred in both FW and SW plants (***Fig 3.6***). In FW leaves it was almost twice that observed in SW leaves. Leaves of FW, but not SW plants showed a two-fold increase in

the production of ROS when immersed in a solution of 4 M NaCl. When the leaves of FW plants were incubated in 4 M NaCl containing respiration inhibitors, KCN led to a slight reduction in the ROS generated in response to 4 M NaCl, but SHAM led to a two-fold increase in ROS generation over that observed with 4 M salt. ROS production in SW plants did not alter significantly upon similar treatments.

3.4 Discussion

The dark respiration rates in leaves of FW and SW plants were similar, indicating that these plants did not show any apparent metabolic perturbations in spite of the variation in their external and internal salt content. Subjecting the leaves of FW and SW plants to increasing salt concentrations led to increased respiration rates, with 3-fold higher rate of respiration observed as early as 5 min of 3 M and 4 M salt exposure. Salt-stimulated respiration (Lundegardh, 1955) has been observed in various glycophytes and halophytes (Livne and Levin, 1967; Luttge *et al*. 1971, Luttge and Higinbotham, 1979; Burchett *et al*., 1989).

The increase in respiration was specific to monovalent cations especially Na^+ and K^+ and to Cl^-. It has been reported that Cl^- influx into plants under salt stress occurs passively through anion channels and usually accompanies transport of the corresponding cation (Lorenzen *et al*., 2004). More recently the presence of cation-Cl^- co-transporters, which play an important role in maintaining ion homeostasis in animals, has been reported in plants (Colmenero-Flores *et al*., 2007). Hence the transport of Cl^- and cations like Na^+ and K^+ appear to be interdependent. Na^+ or Cl^- are known to exert toxic effects if allowed to accumulate in the cytoplasm and are therefore sequestered to the vacuoles, which is an

important adaptive strategy in halophytes (Glenn *et al*., 1999). Sequestration of Na^+ to vacuoles is carried out by a salt-inducible Na^+/H^+ antiporter, which is driven by the activities of H^+-ATPases and H^+-PPases (Blumwald *et al*., 2000; Parida and Das, 2005). It has been proposed that the positive membrane potential generated across the tonoplast by activities of ATPases and H^+-PPases is dissipated by Cl^- transport through a uniport (Plant *et al*., 1994). Hence respiratory energy requirements would be necessary for the uptake and sequestration of Na^+ or Cl^-. In our experiments, blocking Na^+ transport with amiloride led to a reduction in the salt-induced respiration surge, which was suggestive of energy requirements for Na^+ transport. However the relationship between modified cellular ionic status and proton pumping activity or ATP levels still remains speculative. Inhibition of anion transport with DIDS led to a reduction of respiration in the presence or absence of salt. DIDS is reported to block the voltage dependent anion channels in outer mitochondrial membrane, which play a role in regulating apoptosis (Godbole *et al*., 2003), and this could account for the DIDS-induced inhibition of respiration irrespective of the salt concentration.

Salt-induced increase in respiration was not due to the dehydration effect of salt, since a significant reduction in respiratory O_2 uptake was observed in PEG treated leaves of FW and SW plants. Drought stress led to reduced dark respiration rates in bean and pepper leaves (González-Meler *et al*., 1997) but not in soybean leaves (Ribas-Carbo *et al*., 2005). When whole plants of *B. cylindrica* were exposed to 4 M salt solution (by dipping the roots in salt solution) for 48 h, no change in leaf respiration rate was observed in SW plants (see Chapter 2). Hence whole plant responses to high salt differed from the responses shown by leaves exposed to high salt in *B. cylindrica*. This may be because salt

exclusion by roots, which has been reported for *Bruguiera* species (Takemura *et al*., 2000) could alter the actual salt concentration experienced by leaves. Sensitivity of respiration to stress conditions was also shown to vary at the tissue and whole plant level in several other plants (Sesay *et al*., 1986; Burchett *et al*., 1989; Hu *et al*., 2006).

The COX pathway appeared to contribute to most of the respiratory oxygen uptake in the FW and SW plants. However when treated with 4 M NaCl, the FW plants showed a significant contribution of the AOX pathway, while the SW plants did not. The electron partitioning through the alternative pathway increased from about 10% under well-watered or mild water-stress conditions to near 40% under severe water stress in soybean leaves (Ribas-Carbo *et al*., 2005). The AOX pathway activity in dehydration-acclimated leaves of wheat was seen to be lower than that in the non-acclimated controls, and the COX pathway activity accounted for most of the respiratory O_2 uptake in the acclimated plants (Zagdańska, 1995). The flux of electrons via different respiratory pathways highlights the striking metabolic flexibility of respiration in response to stress.

Uncouplers abolish the electrochemical proton gradient across the inner mitochondrial membrane and thereby allow maximum electron flux through the COX pathway (Elthon and Stewart, 1983). Stimulation in respiratory O_2 uptake by uncouplers has been observed in plants (Beevers, 1953; Honda, 1955; Krause and Lambers, 2001). A 300% increase in respiration rate observed in FW leaves treated with DNP suggested that COX was the main respiratory pathway. Although uncoupled respiration with 4 M NaCl treatment was also enhanced in the FW leaves, the increase was not as much as that seen in control FW leaves. It could therefore be concluded that a non-phosphorylating pathway might be engaged under harsh salt stress in FW plants. SW leaves showed

almost similar enhancement of respiratory O_2 uptake irrespective of whether leaves were exposed to 4 M NaCl or not.

Involvement of the AOX pathway in FW and SW plants (not subjected to 4 M salt) was inferred when KCN and SHAM together led to a further decrease in respiration than the decrease seen with KCN alone. AOX pathway is known to be activated as an early response to metabolic imbalances and is thought to have adaptive significance in stress situations (Purvis and Shewfelt, 1993; Day e*t al*., 1995; Clifton *et al*., 2005; Arnholdt-Schmitt *et al*., 2006). A major function of the AOX is to balance the requirements of carbon metabolism and mitochondrial electron transport (Vanlerberghe and McIntosh, 1997). Under stress conditions, the activity of the AOX pathway allows the TCA cycle to continue providing carbon skeletons for metabolism and this is particularly important in the synthesis of compatible solutes (Mckenzie and McIntosh, 1999). Along with other mitochondrial uncoupling proteins, the AOX pathway is thought to function as a sink for reducing power and dissipates proton gradients developed across mitochondrial membrane, thus preventing the generation of ROS (Millar and Day, 1997; Maxwell *et al*., 1999; Møller, 2001; Bartoli *et al*., 2005; Borecky and Vercesi, 2005). Since AOX respiration is nonphosphorylating, it is unlikely to play a role in salt sequestration, which requires ATP or PPi. On the other hand, since excess respiratory activity is known to generate ROS, the AOX pathway may play a role in preventing ROS accumulation thereby protecting cells from oxidative damage (Noctor *et al*., 2006; Navrot *et al*., 2007). This role was observed in FW plants subjected to 4 M salt treatment where ROS generation was significantly higher than that in plants not treated with salt or in the SW plants subjected to 4 M salt. That the ROS were associated with increased respiration was

confirmed by pretreating leaves with KCN, which led to a decrease in respiration as well as ROS generation. On the other hand, when 4 M salt treated FW leaves were pretreated with the AOX inhibitor SHAM, generation of ROS was significantly increased over that observed with 4 M salt treatment alone, indicating that AOX played a role in reducing ROS accumulation in FW plants in the presence of high salt. Participation of the AOX pathway has been reported under increasing salinity in wheat (Kong *et al*., 2001), but not in halophytes. In *Populus* plants subjected to salt shock, AOX activity was much higher than that observed when the plants were subjected to a gradual increase in salt concentration (Ottow *et al*., 2005). This may explain the lack of AOX engagement in SW plants subjected to 4 M NaCl treatment, where the salt shock was milder than that experienced by the FW plants. The use of leaves from FW plants and the use of very high salt concentrations played an important role in getting some indications of the salt adaptation mechanisms in *B. cylindrica*, since the responses were exaggerated as compared to those in salt-adapted plants.

The possible salt adaptation strategies in *B. cylindrica* plants were salt sequestration and prevention of oxidative damage. These two mechanisms also appear to play an important role in adaptive responses of glycophytes to salt stress. Rice plants overexpressing the Na^+/H^+ antiporter (Hui *et al*., 2007) or expressing a salt-induced voltage dependent anion channel from *Pennisetum glaucum* (Desai *et al*., 2006) showed better salt tolerance. Similarly an increase in antioxidant metabolism was observed in halophytes subjected to salt stress (Wang *et al.*, 2004; Jithesh *et al.*, 2006). Rice transgenics over-expressing SOD exhibited better salinity tolerance (Tanaka *et al.*, 1999).

However the cause underlying the rapid increase in respiration (within 5 min) on exposure to high salt remains unexplained. Fundamental information is still lacking on how respiration and the processes supporting it are physiologically controlled. In particular, more information is needed on the *in vivo* cell-specific mechanisms by which partitioning of electrons between the phosphorylating versus non-energy conserving pathways of mitochondrial electron transport react to changes in glycolytic and tricarboxylic acid (TCA) cycle fluxes in response to changing environments and different catabolic and anabolic cellular demands.