Salt-induced respiration in *Bruguiera cylindrica* – role in salt transport and protection against oxidative damage

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ABSTRACT

Leaves of *Bruguiera cylindrica* plants grown in the greenhouse and irrigated with fresh water (FW plants) and those from salt-adapted plants from the seacoast (SW plants) showed about 5-fold and 3-fold increase in respiration, respectively, when immersed in 4M NaCl solution. The increase in respiration was not due to dehydration effect of high salt concentration, since PEG-imposed dehydration stress to the leaves led to an inhibition of respiration rates in both FW and 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 1mM amiloride, an inhibitor of the Na^+ / H^+ antiporter, reduced the NaCl-induced respiration surge. At least some part of the observed respiratory increase could therefore be for providing energy for ion transport, since the Na^+ / H^+ antiport activity is driven by activities of the tonoplast and plasma membrane H^+ -ATPases and H^+ -PPases. Respiration of the leaves from both FW and SW plants was accounted for by the COX pathway and was inhibited by KCN. But 4M NaCl-induced increase in FW, but not SW plants, was inhibited by the AOX inhibitor, SHAM. Also, generation of ROS was reduced by treatment with KCN, but increased with SHAM. This pointed to a protective role of AOX in reducing ROS generation during salt-induced respiration. Our results indicated that NaCl-induced increase in leaf respiration of *B. cylindrica* plants irrigated with fresh water was required for (a) salt transport and (b) reducing the harmful effects of ROS that are known to accompany increased respiratory activity. [Physiol. Mol. Biol. Plants 2008; 14(3) : 217-226] E-mail : sujata@unipune.ernet.in

Key words : Alternative oxidase, *Bruguiera cylindrica*, cytochrome oxidase, Na^+/H^+ antiport activity, reactive oxygen species, oxidative damage

Abbreviations : AOX (alternative oxidase); COX (cytochrome oxidase); DIDS (4,4'-diisothiocyanato stilbene 2,2' disulfonic acid); DNP (2,4-dinitrophenol); FW plants (fresh water plants); PEG (polyethylene glycol); KCN (potassium cyanide); ROS (reactive oxygen species); SHAM (salicylhydroxamic acid); SW plants (sea water plants)

Bruguiera species are known to accumulate salt to levels higher than that of sea water (Takemura *et al.*, 2000; Kura-Hotta *et al.*, 2001). Salt is thought to act as a cheap and easily available osmoticum, which maintains an inward flow of water into the plant (Shabala, 2000; Pardo and Quintero, 2002). Na^+ uptake is largely mediated through the K^+ transporter (Blumwald, 2000) and non-specific cation channels (Demidchik and Maathuis, 2007). Cl^- ions on the other hand are known to move passively through anion channels and their transport is coupled with the transport of corresponding cations (Lorenzen *et al.*, 2004). Salt accumulation is known to affect ion homeostasis by decreasing the levels of internal K^+ and

Ca^{2+} (Serrano *et al.*, 1999) and also causes metabolic toxicity because most enzymes of halophytes are as sensitive to salt as those of glycophytes (Flowers *et al.*, 1977). Removal of salt from the cytoplasm is therefore essential for maintaining the cellular metabolic activity. Halophytes are known to sequester Na^+ to the vacuole using energy-dependent mechanisms involving tonoplast H^+ -ATPases, H^+ -pyrophosphatases and Na^+/H^+ antiporters (Parks *et al.*, 2002; Vera-Estrella *et al.*, 2005). Cl^- sequestration to vacuoles is also reported (Glenn *et al.*, 1999). Increased osmotic concentration of the vacuole is counterbalanced by accumulation of compatible osmolytes in the cytoplasm, which include mannitol, proline and quaternary ammonium compounds (Hu *et al.*, 2000). Synthesis of compatible solutes like cyclitols and organic acids has been reported in

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Bruguiera gymnorhiza subjected to high salt concentration (Sugihara *et al.*, 2000). Besides osmotic adjustment, synthesis of protective compounds that play a role in preventing salt-induced oxidative damage also plays an important role in the adaptation of halophytes to the saline environment (Cheesemann *et al.*, 1997; Parida *et al.*, 2004; M'rah *et al.*, 2007).

All these processes incur metabolic costs that would require additional ATP generation when plants are subjected to salt stress. 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). However respiration increase is also thought to arise as a pathological response to stress, since the increase is greater in the less salt tolerant species (Burchett, 1989; Lopez-Hoffmann *et al.*, 2006). Plants are known to be equipped with two different respiratory electron transport pathways (Yip and Vanlerberghe, 2001; Maxwell *et al.*, 1999). The cytochrome oxidase mediated (COX) pathway is primarily used for ATP generation. The alternative oxidase (AOX) pathway is known to be activated as an early response to metabolic imbalances (Clifton *et al.*, 2005) and is thought to provide a mechanism for optimizing metabolic efficiency for regulating growth under stress conditions (Arnholdt-Schmitt *et al.*, 2006). An important role ascribed to the AOX pathway is preventing over-reduction of the respiratory electron transport which may lead to generation of reactive oxygen species (ROS) (Maxwell *et al.*, 1999; Møller, 2001; Umbach *et al.*, 2005).

In the present paper we have 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). *B. cylindrica* is a facultative mangrove and can grow in soils irrigated with fresh water. Further, respiration rates were also studied when the leaves of these two types of plants were immersed in 4M 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 (see Ottow *et al.*, 2005).

MATERIALS AND METHODS

Plant material and growth conditions

Mature propagules of *B. cylindrica* L. 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, 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. Irradiance in the greenhouse varied between 75-150 µmol m⁻² s⁻¹ during different times of the day. Saplings of uniform size (eight-leaf stage) were selected from the greenhouse-grown plants for the experiments and were called fresh water grown (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 O₂ 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 used for measuring O₂ uptake.

Water Content

Two leaves each from five independent FW and SW plants were used for determining the water content. The leaves were cut into three pieces (about 2 cm² area), weighed immediately (fresh weight) and then immersed in petri dishes containing water for 16 h at 4°C. The leaf pieces were weighed again (turgor weight) after placing them briefly between blotting paper to remove the surface water. Finally the leaf pieces were dried for 48 h at 80 °C and weighed (dry weight).

Water content was calculated as:

$$\frac{\text{Fresh weight}-\text{Dry weight}}{\text{Dry weight}} \times 100$$

Relative water content (RWC) was calculated as:

$$\text{RWC (\%)} = \frac{\text{Fresh weight}-\text{Dry weight}}{\text{Turgor weight}-\text{Dry weight}} \times 100$$

Na and Cl content

Leaves of FW and SW plants were dried for 48 h at 80 °C, weighed (dry weight) and powdered. 1g of dry leaf powder was heated in a furnace at 400 °C for 3h. 100mg of ash was resuspended in water and the Na and Cl contents measured using an atomic absorption spectrophotometer (Chemito 201, India). The content of these ions was expressed in terms of mg⁻¹ dry weight.

Osmometry and electrolytic conductivity

Two leaves each from five independent FW and SW plants were ground in a mortar and pestle, centrifuged at $9676 \times g$ (Kubota 1710, Japan) for 10 min and the supernatant used as cell sap. Osmolarity of the cell sap was measured using a vapor pressure osmometer (Wescor 5500, USA). To measure electrolytic conductivity (EC), the cell sap was diluted 500-fold with 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} .

Leaf Respiration Measurements

The respiratory O_2 uptake of leaf pieces was monitored polarographically in the dark at 25°C using a Clark-type oxygen electrode (Hansatech, Norfolk, UK). O_2 uptake was monitored in freshly aerated solutions for 3–5 min, during which the O_2 levels were never below 50–60% of the original levels. Leaves of FW and SW plants were immersed in NaCl solutions ranging from 0.5M to 4M prepared in 10 mM Tris-HCl buffer, pH 7.5 and the respiration rate determined every 30 min for a total period of 2 h.

Effect of PEG-induced dehydration stress on respiration

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

Effect of other cations and anions on respiration

Ion specific effects on respiration were studied by measuring the respiration rate in 100mg fresh leaf pieces immediately after immersing them in aerated salt solutions of different cations and anions. 4M solutions each of NaCl, KCl, CsCl, LiCl and RbCl, 2M and 4M solutions each of CaCl_2 and MgCl_2 and 4M and 1.33M of AlCl_3 were prepared in 10 mM Tris-HCl buffer (pH 7.5). 4M solution of CH_3COONa , 1.9M of Na_2SO_4 and 1M of Na_2PO_4 were also prepared in 10 mM Tris-HCl buffer (pH 7.5).

Effect of ion transport inhibitors on respiration

100mg leaf pieces of FW and SW plants were immersed in aqueous solutions of 1mM amiloride (3,5-diamino-6-chloro-N-(diaminomethylene) pyrazinecarboxamide monohydrochloride, dihydrate; Hoechst, India) or 0.1mM

DIDS (4,4'-diisothiocyanato stilbene 2,2'-disulfonic acid; Sigma, USA) for 30min, after which they were transferred to 10mM Tris-HCl buffer, pH 7.5 (for FW plants), sea water (for SW plants) and 4M NaCl solutions (for both FW and SW plants) for further 5min, 30min or 1h and then used for O_2 uptake measurements.

Contribution of COX and AOX pathways to respiration

KCN and salicylhydroxamic acid (SHAM) were used to inhibit the COX and AOX pathways respectively. The optimum concentration of these inhibitors was determined from titration experiments in which 100mg leaf pieces were immersed in varying concentrations ranging from 0 to 20mM of KCN (dissolved in water and neutralized with 2M HCl) and SHAM (dissolved in 2M KOH and neutralized with 2M HCl (Nagel *et al.*, 2001) respectively for 1h and O_2 uptake measured. Based on titration results, 15mM KCN and 10 mM SHAM were used for subsequent experiments. The inhibitors were added individually or together to 10mM Tris-HCl buffer, pH 7.5 (for FW plants), sea water (for SW plants) and 4M NaCl solutions (for both FW and SW plants). Leaf pieces were immersed in the above solutions for 1h prior to O_2 uptake measurements. Contribution of the COX and AOX pathway to total respiration was determined as per Vani and Raghavendra (1994).

Dinitrophenol (DNP) was used for uncoupling respiration. 0.5M DNP solution (dissolved in 2M KOH and neutralized with 2M HCl) was prepared in 10mM Tris-HCl buffer, pH 7.5 (for FW plants), sea water (for SW plants) and 4M NaCl solutions (for both FW and SW plants). Leaf pieces were immersed in these solutions for 1h and then used for measuring O_2 uptake.

ROS measurements

In vivo generation of ROS was examined with the cell-permeable probe 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA; Sigma). 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 , peroxy radical, or peroxyxynitrite anion (Tarpey and Fridovich, 2001). Stock solution of the dye was prepared in DMSO (dimethyl sulfoxide) and kept in the dark at -20°C . About 25 mg leaf tissue was immersed for 30min in solutions of 15 mM KCN or 10 mM SHAM prepared in 10mM Tris-HCl buffer, pH 7.5 (for FW plants), sea water (for SW plants) and 4M NaCl solutions (for both FW and SW plants). After 30min, DCFH-DA was added to each solution to a final concentration of $5\mu\text{M}$ and samples were incubated in dark for a further 30min. The solutions

were then decanted and the leaf samples frozen in liquid N₂, homogenized and thawed. Freeze thawing was repeated at least thrice and then the tissues were suspended in 200 μ L of 10mM 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 μ L 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).

Statistical analysis

All observations were made from the 2nd pair of leaves of at least 3 independent FW and SW plants. 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. Student's t test was used to compare the respiration rates obtained with each cation and anion with the control.

RESULTS

Water and salt status of FW and SW plants

Leaves of *B. cylindrica* plants growing in the greenhouse and irrigated with fresh water (FW plants) showed 16% less water content than the leaves of plants growing on the sea coast (SW plants) (Table I). Osmolarity, electrolytic conductivity, as well as the internal Na and Cl content of SW plants was about twice the values observed with FW plants.

Effect of salt on respiration

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 3M and 4M NaCl (Fig 1).

A significant increase in respiratory oxygen uptake was observed almost immediately after subjecting the leaves of FW or SW plants to 4M NaCl. The increase in respiratory activity was about 2-fold after 1h incubation in 4M NaCl, in both FW (Fig 1a) and SW

Table 1. Water and salt content in leaves of *B. cylindrica* plants. Osmolarity and EC measurements were made using the leaf sap. Values represent means of at least 3 independent experiments along with their SD.

	FW plants	SW plants
Water content (%)	71.4 $\pm$ 3.6	83.1 $\pm$ 0.6
RWC (%)	79.4 $\pm$ 2.9	95.5 $\pm$ 4.1
Na (mg g ⁻¹ dw)	10.6 $\pm$ 4.8	23.9 $\pm$ 2.4
Cl (mg g ⁻¹ dw)	23.2 $\pm$ 9.4	47.9 $\pm$ 0.4
Measured osmolarity (mmols Kg ⁻¹)	1030 $\pm$ 78	2292 $\pm$ 281
EC (mS cm ⁻¹)	64.3 $\pm$ 7.83	128.1 $\pm$ 25

Fig. 1. Effect of NaCl concentration on respiration rate of *B. cylindrica* leaves over a 2h incubation period. 100mg leaf pieces were immersed in NaCl solutions of varying concentrations prepared in 10mM Tris-HCl buffer (pH 7.5) and used for O₂ uptake measurements using a Clark type oxygen electrode. (a) Fresh water irrigated (FW) plants, (b) Plants growing on the sea coast (SW). Each point represents respiration measurements of leaf samples from 3 independent plants.

Table 2. Effect of amiloride, an inhibitor of Na^+/H^+ antiporter and DIDS, an anion channel blocker, on leaf respiration of FW plants of *B. cylindrica*. 100mg leaf pieces were incubated in 1mM amiloride or 0.1mM DIDS for 30min, after which the leaf pieces were immersed in 10mM Tris-HCl buffer (pH 7.5) or 4M NaCl prepared in 10mM Tris-HCl buffer (pH 7.5) for varying time periods. Values represent mean $\pm$ SD of three independent experiments. Values differing significantly ($P=0.05$) from their respective controls are indicated by asterisks.

	FW (Time min)			FW+4M NaCl (Time min)		
	5	30	60	5	30	60
Control	0.6 $\pm$ 0.05	1.4 $\pm$ 0.00	2.5 $\pm$ 0.12	3.3 $\pm$ 0.20	4.2 $\pm$ 0.17	5.5 $\pm$ 0.19
1mM Amiloride	0.9 $\pm$ 0.26	1.2 $\pm$ 0.48	2.0 $\pm$ 0.54	2.1 $\pm$ 0.91*	3.1 $\pm$ 0.54*	3.0 $\pm$ 0.42*
0.1mM DIDS	0.6 $\pm$ 0.12	1.0 $\pm$ 0.02*	1.5 $\pm$ 0.08	1.6 $\pm$ 0.13*	2.9 $\pm$ 0.35*	4.0 $\pm$ 0.44*

(Fig 1b) plants as compared to the leaves not subjected to salt treatment. No significant alterations in respiration were observed at salt concentrations ranging from 0-2M salt.

Effect of PEG-induced dehydration stress on respiration

Leaves of FW and SW plants were immersed in 30% PEG (which exerts an osmotic pressure of -2 MPa) solution (instead of 4M NaCl) and respiration measured after 30min. PEG-induced dehydration stress led to about 50% reduction in respiratory rates in both FW and SW plants (Fig 2).

Fig. 2. Effect of 4M NaCl and 30% PEG on respiration of *B. cylindrica* leaves. 100mg leaf pieces of FW and SW plants were immersed in 4M NaCl or PEG solution prepared in 10mM Tris-HCl buffer (pH 7.5) for 30 min before O_2 uptake measurements. Values are means $\pm$ SD of three independent experiments. Values differing significantly ($P=0.05$) from their respective controls are indicated by asterisks.

Ion-specific effects on respiration

The ion-specific effects on respiration rates of leaves of FW plants were studied by substituting 4M NaCl with solutions of other cations and anions. Respiration rates were also enhanced in the presence 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). On the other hand increased respiration was only observed in the presence of Cl^- but not in the presence of CH_3COO^- , SO_4^{2-} or PO_4^{3-} at equivalent concentrations. On pretreating leaves of FW plants with

Fig. 3. Effect of various cations and anions on leaf respiration of *B. cylindrica*. 100mg leaf pieces of FW plants were immersed in solutions of different cations and anions prepared in 10mM Tris-HCl buffer (pH 7.5) and O_2 uptake measurements were carried out after about 5min. Values represent mean $\pm$ SD of three independent experiments. Values differing significantly ($P=0.01$) from their respective controls are indicated by asterisks.

amiloride, an inhibitor of Na^+ / H^+ antiport, before subjecting them to 4M salt treatment, a significant decrease in the respiratory rate was observed in comparison to the respiratory rate of 4M-treated leaves (Table II). 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.

Effect of inhibitors and an uncoupler on respiration

Initially a titration experiment was carried out in which respiration was monitored after 1h incubation of leaves of FW plants in varying concentrations of KCN (Fig 4a) or SHAM (Fig 4b) added to either 10mM Tris-HCl buffer pH7.5 or 4M NaCl. The concentration of each inhibitor at which maximum inhibition of respiration occurred was

Fig. 4. Titration of *B. cylindrica* leaf pieces with respiratory inhibitors. 100 mg leaf pieces of FW plants were immersed in 10mM Tris-HCl buffer (pH 7.5) or 4M NaCl with (a) 0-20mM KCN and (b) 0-20mM SHAM for 1h followed by O_2 uptake measurements. Values represent mean $\pm$ SD of three independent experiments.

15mM for KCN and 10mM for SHAM. These concentrations were used in subsequent experiments with FW and SW plants subjected to 4M salt treatment. KCN inhibited respiration in the leaves of both FW and SW plants that were immersed either in 10mM Tris-HCl buffer pH7.5 or in 4M NaCl (Fig 5a). However treatment of leaves with 10mM SHAM led to a significant decrease in respiration only in the FW plants treated with 4M 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 0.5mM DNP, there was a 300%

Fig. 5. Effect of KCN, SHAM and DNP on respiration of *B. cylindrica* leaves. 100mg leaf pieces of FW and SW plants were incubated for 1h in (a) 15mM KCN, 10mM SHAM or a mixture of both and (b) 0.5mM DNP solutions prepared in 10mM Tris-HCl buffer (pH 7.5) (for FW plants), seawater (SW plants), or 4M NaCl (for both FW and SW plants) followed by O_2 uptake measurements. Values represent mean $\pm$ SD of 3-5 independent experiments. Values differing significantly ($P = 0.05$) from their respective controls are indicated by asterisks.

increase in respiration in leaves of the FW plants immersed in 10mM Tris-HCl buffer pH7.5, but only a 125% increase when the leaves were immersed in 4M NaCl instead of buffer (Fig 5b). The respiration increases in leaves of SW plants immersed in 10mM TRIS-HCl buffer pH7.5 or 4M NaCl were 177% and 172% respectively.

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

Fig. 6. Intracellular ROS distribution in leaves of FW and SW plants of *B.cylindrica* in the presence of KCN or SHAM. 25mg leaf pieces were incubated in 10mM Tris-HCl buffer (pH 7.5) or 4M NaCl in the presence or absence of 15mM KCN or 10mM SHAM for 30 min. 5mM DCFH-DA was then added to each sample and these were incubated for a further 30min. Fluorescence measurements of oxidized DCFH-DA in leaf extracts prepared in 10mM Tris-HCl buffer (pH 7.5) were carried out spectrofluorimetrically. Values represent means $\pm$ SD of three independent experiments. Values differing significantly ($P = 0.05$) from the respective controls (10mM Tris-HCl or 4M NaCl) are indicated by asterisks.

DISCUSSION

The leaf respiration rates of FW and SW plants were similar, indicating that leaves of these plants had achieved homeostasis 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 and 4 M salt exposure. Salt as well as osmotic signaling has been reported to occur within seconds of exposing plants to these stress factors and is shown to be mediated through cGMP and Ca^{2+} (Donaldson *et al.*, 2004). Signaling events at the plasma membrane, which by their very nature must be rapid, have been reported to bring about an alteration in the phosphorylation status of pyruvate dehydrogenase (Miernyk *et al.*, 2006). However, respiration is universally considered to be regulated by the utilization of ATP or by proton leakage, i.e. by respiratory control, and not by modification of component enzymes, which are always in far excess in the kinetic sense. Thus signaling events that imply modification of component enzymes would not account for this rapid stimulation of respiration.

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^- cotransporters, 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 known to occur through the Na^+ / H^+ antiport, which is driven by the activities of H^+ ATPases and H^+ PPases (Parks *et al.*, 2002; Tester and Davenport 2003). 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 could be envisaged in 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 increase, which was

suggestive of energy requirements during salt transport. However the relation between modified cellular ionic status to proton pumping activity or the 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 also reported to block the voltage dependent anion channel in outer mitochondrial membrane, which plays 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.

The COX pathway appeared to contribute to most of the respiratory oxygen uptake in the FW and SW plants. However when treated with 4M NaCl, the FW plants showed a significant contribution of the AOX pathway, while the SW plants did not. Involvement of the AOX pathway in FW and SW plants (not subjected to 4M salt) was however suggested from the observation that use of KCN and SHAM together led to further decreases in the KCN inhibited respiration. AOX pathway is known to be activated as an early response to metabolic imbalances and is thought to have adaptive significance in stress situations (Clifton *et al.*, 2005; Arnholdt-Schmitt *et al.*, 2006). 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 reactive oxygen species (ROS) (Møller, 2001; Bartoli *et al.*, 2005; Borecky and Vercesi, 2005). Since AOX respiration is non-phosphorylating, 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 may play a role in preventing ROS accumulation by tetravalent reduction of oxygen by reduced ubiquinone, to form water, thereby protecting cells from oxidative damage (Noctor *et al.*, 2006; Navrot *et al.*, 2007). This role was observed in FW plants subjected to 4M salt treatment where ROS generation was significantly higher than that in plants not treated with salt or in the SW plants subjected to 4M 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 leaves were pretreated with the AOX inhibitor SHAM, the generation of ROS was further increased over that observed with 4M salt, indicating that AOX played a role in reducing ROS accumulation in FW plants in the presence of high salt. Engagement of the AOX pathway has been reported under increasing salinity in wheat (Kong *et al.*, 2004), 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 4M NaCl treatment, where the salt shock was milder than that experienced by the FW plants.

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 FW and SW plants (results not shown). Hence whole plant responses to high salt differed from the responses shown by leaves exposed to high salt. This may be because salt exclusion by roots has been reported for *Bruguiera* species (Takemura *et al.*, 2000), which would alter the actual salt concentration experienced by leaves. The use of leaves from fresh water grown plants, and treatments of very high salt concentrations played an important role in getting some indications of the salt adaptation mechanisms in mangroves since the responses were exaggerated as compared to those in salt-adapted plants.

CONCLUSION

The possible salt adaptation strategies used by *B. cylindrica* plants were salt sequestration and prevention of oxidative damage. These two mechanisms also appear to play an important role in adaptive responses to salt stress in glycophytes. For example wheat or *Populus* plants exposed to salt stress showed an increase in AOX respiration (Kong *et al.*, 2001; Ottow *et al.*, 2005). Similarly 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. However the cause underlying the rapid increase in respiration (within 5 min) on exposure to high salt remains unexplained.

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REFERENCES

- Arnholdt-Schmitt, B., Costa, J.H. and de Melo, D.F. (2006). AOX- a functional marker for efficient cell reprogramming under stress? *Trends Plant Sci.*, 11: 281-287.
- Bartoli, C.G., Gomez, F., Gergoff, G., Guiamet, J.J. and Puntarulo, S. (2005). Up-regulation of the mitochondrial alternative oxidase pathway enhances photosynthetic electron transport under drought conditions. *J. Exp. Botany*, 56: 1269-1276.
- Blumwald, E. (2000). Sodium transport and salt tolerance in plants. *Curr. Opin. Cell Biol.*, 12: 431-434.
- Borecky, J. and Vercesi A.E. (2005). Plant uncoupling mitochondrial protein and alternative oxidase: energy metabolism and stress. *Bioscience Reports*, 24: 269-283.
- Bradford, M.M. (1976). A rapid and sensitive method for quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem.*, 72: 248-254.
- Burchett, M.D., Clarke, C.J., Field, C.D. and Pulkownik, A. (1989). Growth and respiration in two mangrove species at a range of salinities. *Physiologia Plantarum*, 75: 299-303.
- Cheeseman, J.M., Herendeen, L.B., Cheeseman, A.T. and Clough, B.F. (1997). Photosynthesis and photoprotection in mangroves under field conditions. *Plant Cell Environ.*, 20: 579-588.
- Clifton, R., Lister, R., Parker, K.L., Sappl, P.G., Elhafez, D., Millar, A.H., Day, D.A. and Whelan, J. (2005). Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Mol. Biol.*, 58: 193-212.
- Colmenero-Flores, J.M., Martinez, G., Gamba, G., Vazquez, N., Iglesias, D.J., Brumos, J. and Talon, M. (2007). Identification and functional characterization of cation-chloride cotransporter in plants. *The Plant Journal*, 50: 278-292.
- Demidchik, V. and Maathuis, F.J.M. (2007). Physiological roles of nonselective cation channels in plants: from salt stress to signaling and development. *New Phytol.*, 175: 387-404.
- Desai, M.K., Mishra, R.N., Verma, D., Nair, S., Sopory, S.K. and Reddy, M.K. (2006) Structural and functional analysis of a salt stress inducible gene encoding voltage dependent anion channel (VDAC) from pearl millet (*Pennisetum glaucum*). *Plant Physiol. Biochem.*, 44: 483-493.
- Donaldson, L., Ludidi, N., Knight, M.R., Gehring, C. and Denby, K. (2004) Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. *FEBS Letters*, 569: 317-320.
- Flowers, T.J., Troke, P.F. and Yeo, A.R. (1977.) The mechanism of salt tolerance in halophytes. *Annu. Rev. Plant Physiol.*, 28: 89-121.
- Glenn, E.P., Brown, J.J. and Blumwald, E. (1999). Salt tolerance and crop potential of halophytes. *Critical Rev. Plant Sci.*, 18(2): 227-255.
- Godbole, A., Varghese, J., Sarin, A. and Mathew, M.K. (2003). VDAC is a conserved element of death pathways in plant and animal systems. *Biochim. Biophys. Acta*, 1642: 87-96.
- Hui, C., Rui, A., Jiang-Hua, T., Xiang-Huan, C., Fu Shun, H., Jia, C. and Xue-Chen, W. (2007). Over-expression of a vacuolar Na⁺/H⁺ antiporter gene improves salt tolerance in upland rice. *Mol. Breeding.*, 19: 215-225.
- Hu, Y.C., Schnyder, H. and Schmidhalter, U. (2000). Carbohydrate deposition and partitioning in elongating leaves of wheat under saline soil conditions. *Aust. J. Plant Physiol.*, 27: 363-370.
- Kong, Y., Zhou, G. and Wang, Y. (2001). Physiological characteristics and alternative respiratory pathway under salt stress in two wheat cultivars differing in salt tolerance. *Russian J. Plant Physiol.*, 48(5): 595-600.
- Kura-Hotta, M., Mimura, M., Tsujimura, T., Washitani-Nemoto, S. and Mimura, T. (2001). High salt-treatment-induced Na⁺ extrusion and low salt-treatment-induced Na⁺ accumulation in suspension-cultured cells of the mangrove plant. *Bruguiera sexangula*. *Plant Cell Environ.*, 24: 1105-1112.
- Lopez-Hoffman, L., DeNoyer, J.L., Monroe, I.E., Shaftel, R., Anten, N.P.R., Martinez-Ramos, M. and Ackerley, D.D. (2006). Mangrove seedling net photosynthesis, growth and survivorship are interactively affected by salinity and light. *Biotropica*, 38: 606-616.
- Lorenzen, I., Aberle, T. and Plieth, C. (2004). Salt stress-induced chloride flux: a study using transgenic *Arabidopsis* expressing a fluorescent anion probe. *The Plant Journal*, 38: 539-544.
- Lundegardh, H. (1955). Mechanisms of absorption, transport, accumulation, and secretion of ions. *Annu. Rev. Plant Physiol.*, 16: 1-24.
- Maxwell, D.P., Wang, Y. and McIntosh, L. (1999). The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc. Natl. Acad. Sci.*, 96: 8271-8276.
- Miernyk, J.A., Szurmak, B., Tovar-Mendez, A., Randall, D.D., Muszynska, G. 2007. Is there a signal transduction pathway that links events at the plasma membrane to the phosphorylation state of the mitochondrial pyruvate dehydrogenase complex? *Physiol. Plantarum.*, 129: 104-113
- Møller, I.M.. (2001). Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 52: 561-591.
- M'rah, S., Ouerghi, Z., Eymery, F., Rey, P., Hajji, M., Grignon, C. and Lachaâl, M. (2007). Efficiency of biochemical protection against toxic effects of accumulated salt differentiates *Thellungiella halophila* from *Arabidopsis thaliana*. *J. Plant Physiol.*, 164: 375-384.
- Nagel, O.W., Waldron, S. and Jones, H.G. (2001). An off-line implementation of the stable isotope technique for measurements of alternative respiratory pathway activities. *Plant Physiol.*, 127: 1279-1286.

- Navrot, N., Rouhier, N., Gelhaye, E. and Jacquot, J.P. (2007). Reactive oxygen species generation and antioxidant systems in plant mitochondria. *Physiol. Plant*, 129: 185-195.
- Noctor, G., De Paepe, R. and Foyer, C.H. (2006). Mitochondrial redox biology and homeostasis in plants. *Trends Plant Sci.*, 12: 125-134.
- Ottow, E.A., Brinke, R.M., Teichmann, T., Fritz, E., Kaiser, W., Brosché, M., Kangasjärvi, J., Jiang, X. and Polle, A. (2005). *Populus euphratica* displays apoplastic sodium accumulation, osmotic adjustment by decreases in calcium and soluble carbohydrates, and develops leaf succulence under salt stress. *Plant Physiol.*, 139: 1762-1772.
- Pardo, J.M. and Quintero, F.J. (2002). Plants and sodium: keeping company with the enemy. *Genome Biology Reviews*, 3(6): 1017.1-1017.4.
- Parida, A.K., Das, A.B. and Mohanty, P. (2004). Investigations on the antioxidant defense responses to NaCl stress in a mangrove, *Bruguiera parviflora*: Differential regulation of isoforms of some antioxidative enzymes. *Plant Growth Regulation*, 42: 213-226.
- Parks, G.E., Dietrich, M.A. and Schumaker, K.S. (2002). Increased vacuolar Na⁺/H⁺ activity in *Salicornia bigelovii* Torr. In response to NaCl. *J. Exp. Bot.*, 53: 1055-1065.
- Plant, P.J., Gelli, A. and Blumwald, E. (1994). Vacuolar chloride regulation of an anion- selective tonoplast channel. *J. Membrane Biol.*, 140: 1-12.
- Rains, D.W. (1972). Salt transport by plants in relation to salinity. *Annu. Rev. Plant Physiol.*, 23: 367-388.
- Serrano, R., Mulet, J.M., Rios, G., Marquez, J.A., Larrinoa, I.F., Leube, M.P., Mendizabal, I., Pascual-Ahuir, A., Proft, M., Ros, R. and Montesinos, C. (1999). A glimpse of the mechanisms of ion homeostasis during salt stress. *J. Exp. Bot.*, 50: 1023-1036.
- Shabala, S. (2000) Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. *Plant Cell Environment*, 23: 825-837.
- Sugihara, K., Hanagata, N., Dubinsky, Z., Baba, S. and Karube, I. (2000). Molecular characterization of cDNA encoding oxygen evolving enhancer protein 1 increased by salt treatment in the mangrove *Bruguiera gymnorhiza*. *Plant Cell Physiol.*, 41: 1279-1285.
- Takemura, T., Hanagata, N., Sugihara, K., Baba, S., Karube, I. and Dubinsky, Z. (2000). Physiological and biochemical responses to salt stress in the mangrove, *Bruguiera gymnorhiza*. *Aquatic Bot.*, 68: 15-28.
- Tarpey, M.M. and Fridovich, I. (2001). Methods of detection of vascular reactive oxygen species: nitric oxide, superoxide, hydrogen peroxide, and perperoxynitrite. *Circ. Res.*, 89: 224-236.
- Tester, M. and Davenport, R. (2003). Na⁺ tolerance and Na⁺ transport in higher plants. *Annals Bot.*, 91: 503-527.
- Umbach, A.L., Fiorani, F. and Siedow, J.N. (2005). Characterization of transformed *Arabidopsis* with altered alternative oxidase levels and analysis of effects on reactive oxygen species in tissue. *Plant Physiol.*, 139: 1806-1820.
- Vani, T. and Raghvendra, A.S. (1994). High mitochondrial activity but incomplete engagement of the cyanide-resistant alternative pathway in guard cell protoplasts of pea. *Plant Physiol.*, 105: 1263-1268.
- Vera-Estrella, R., Barkla, B.J., Garcia-Ramirez, L. and Pantoja, O. (2005). Salt stress in *Thellungiella halophila* activates Na⁺ transport mechanisms required for salinity tolerance. *Plant Physiol.*, 139: 1507-1517.
- Yeo, A.R. (1998). Molecular biology of salt tolerance in the context of whole-plant physiology. *J. Exp. Bot.*, 49: 915-929.
- Yip, J.Y.H. and Vanlerberghe, G.C. (2001). Mitochondrial alternative oxidase acts to dampen the generation of active oxygen species during a period of rapid respiration induced to support a high rate of nutrient uptake. *Physiologia Plantarum*, 112: 327-333.