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Structural and functional role of salt glands of cogon grass (*Imperata cylindrica* (L.) Raeuschel) under salinity stress

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Abstract
Salt glands in Poaceae are often found in the Panicoid and Chloridoid grasses. *Imperata cylindrica* is a perennial panicoid grass with widespread distribution from non-saline to saline habitats. The efficient mechanism of salt gland is an essential property of the salt tolerance of any plant. Salt glands in *Imperata* are present and actively function to remove the toxic Na^+ ion from the cytoplasm of the mesophyll cells. SEM study reveal the presence of salt glands in both control and NaCl treated plants (200 mM NaCl for 3 days), but the density of salt glands is more in NaCl treated leaf samples. Also the vacuolarization of the cells is an important attribute for the sequestration of excess Na^+ ion. The concerted activity of these two mechanisms in *Imperata* is important for maintaining osmotic balance inside the cells. The time dependent lowering of H_2O_2 and O_2^- and increased accumulation of proline under NaCl stress was also observed in *Imperata*. This accounts for a favourable environment for other biological processes to occur. Also the lower electrolyte leakage and membrane lipid peroxidation accounts for hassle free functioning of salt glands under NaCl stress.

Key words : *Imperata cylindrica*, salinity, salt glands

Imperata cylindrica (L.) Raeuschel commonly known as cogon grass belongs to the sub-family Panicoideae of the family Poaceae. It is a major invasive perennial wild grass species growing in both dry and wetlands and has been reported to have flood tolerant potential (King and Grace, 2000). The species is also reported from saline or saline arid habitats (Hameed *et al.*, 2009). Salt tolerant grasses can cope up with the detrimental effects of salinity by a series of anatomical and physiological adaptations, such as by developing an extensive root system and salt secreting glands on the leaf surface (Marcum and Murdoch, 1990; Marcum *et al.*, 1998). Marcum (1999) in his studies on grasses and their salinity tolerance mechanisms pointed out that the tolerance is associated with Na^+ exclusion through the salt glands present on the surface of the leaves. The dissection of abiotic stress responsive genes in the closely related wild grass species for the development of salt tolerant cereal crops have been given due importance in the last decade (Tester and Bacic, 2005; Roy and Chakraborty, 2014). Tracing the physiological and biochemical adaptations in *Imperata* to salinity stress thus becomes the pre requisites in this context.

Salt glands are specialized epidermal structures found in leaves of various dicot and monocot plant families. In monocots the salt glands can be exclusively noticed in the subfamilies of Chloridoideae and Panicoideae of Poaceae (Marcum, 1999). The salt glands facilitate the secretion of excess salts from the cytoplasm, thus, plants with salt glands are capable of tolerating higher concentrations of salt as compared to the plants lacking salt glands (Flowers, 1985). In a plant with active salt glands, the secreted salt can be observed in the form of salt crystals on the leaf surface. Apart from Na^+ and Cl^-, the other ions secreted by salt glands include K^+, Mg^{2+}, Ca^{2+}, SO_4^{2-}, PO_4^{3-} and CO_3^{2-} (Haberlandt, 1914). Salt glands have been studied in several members of the Poaceae and have been reported to have a bicellular structure. These bicellular structures constitute an outer cap cell and a mesophyll-embedded basal cell (Naidoo and Naidoo, 1999, Oross and Thomson, 1982).

The aim of this study was to examine the morphological and anatomical adaptations of *Imperata cylindrica* under salinity stress. For this

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purpose, the Na^+ content, K^+ content and Na^+ exclusion was determined. The amount of salinity induced damage was seen in terms of H_2O_2 and O_2^- localization, electrolyte leakage and membrane lipid peroxidation. The accumulation of proline and relative water content was determined to understand the water status of the plants under stress. Above all the presence of salt glands was determined and the activities of the salt glands were used to understand the NaCl tolerance mechanism in *Imperata*.

Materials and Methods

Plant material and treatments

The rhizomes of *Imperata cylindrica* plants were collected from the NBU University campus and transferred to pots for vegetative propagation. The pots were watered regularly till the rhizomes developed fully grown plants. After that all the plants were carefully uprooted, the roots gently washed with distilled water and transferred to nutrient solution containing 0.1 X Hoagland solutions. The plants were then allowed to acclimatize in nutrient solution for 48 hours prior to salt shock experiments. Five treatments of different NaCl concentrations of 0, 10, 100, 200 and 500 mM were prepared by the addition of NaCl to the nutrient solutions in five different sets for all the plants.

Relative water content (RWC)

RWC was measured according to the standard protocol described by Barr and Weatherley (1962), and calculated by the following equation:

$$RWC\ (\%) = [(FW - DW) / (TW - DW)] \times 100$$

Where, FW - Sample fresh weight, TW - Sample turgid weight, DW - Sample dry weight

Morphological and ultrastructural changes

For visualization of salt glands and ultrastructural changes, light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) was performed. For light microscopy T.S. of leaf was cut and stained with toluidine blue. For SEM analysis, modified protocol of Pathan *et al.* (2008) was followed. Leaf samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 8-12 hr at 40 C. The samples were then washed thrice after every 1 hour with phosphate buffer. Then the leaf samples were dehydrated with a series of ethyl alcohol from 10 - 100%, followed by critical point drying. For SEM analysis the samples were coated with gold and examined under SEM (Hitachi S-530).

For TEM analysis, modified protocol of Campbell *et al.* (1990) was followed. Leaf samples were cut into 2 x 2 mm size and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 8-12 hr at 40 C and then washed thrice with phosphate buffer. After that post fixation was done with 2% OsO_4 and leaf samples were dehydrated in grades of ethyl alcohol and propylene oxide. Leaf samples were then embedded in LR White resin and ultrathin sections were cut with an ultra microtome (Leica UC6). The ultra thin sections were stained with uranyl acetate for 45 min and lead citrate for 15 min and observed under transmission electron microscope (Morgagni 268D).

Ion accumulation and exclusion

For Na^+ and K^+ ion estimation, leaf and root samples were homogenized in a mortar and pestle using liquid nitrogen in deionized water, following estimation of ions in a Flame Photometer. For standardization of the Flame photometer, standard solutions of sodium chloride (NaCl) and potassium chloride (KCl) was used respectively for Na^+ and K^+ ion estimation. For Na^+ ion exclusion from leaves, intact leaves of equivalent weight from different treatments were taken and dipped in equal volumes of deionized water for Na^+ estimation.

H_2O_2 and O_2^- localization

Hydrogen peroxide (H_2O_2) localization was done following the modified protocol of Thordal-Christensen *et al.* (1997). Leaf segments were incubated in 1mg/ml 3,3'diaminobenzidine (DAB)-HCl, pH 3.8 for 48 hours in dark chamber. The stained segments were then bleached in boiling ethanol/lactic acid/glycerol (4:1:1) for 5 min and examined under a compound microscope. H_2O_2 is

visualized as a reddish-brown colouration.

Superoxide anion (O_2^-) localization was examined using a modification of the Nitro blue tetrazolium (NBT) staining technique described by Romero-Puertas *et al.* (2004). Leaf segments were incubated in 0.05% (w/v) NBT in 10mM phosphate buffer (pH 7.5) at room temperature for 24 hours. The stained segments were then bleached in boiling ethanol/lactic acid/glycerol (4:1:1) for 5 min and examined under a compound microscope. The formation of a blue formazan precipitate indicates the reduction of NBT by superoxide.

Electrolyte leakage

Electrolyte leakage was measured using a conductivity meter as described by Lutts *et al.* (1996). Leaves segments were washed with deionized water and 1 g of leaves were cut into small pieces (about 1 cm^2) and then immersed in 20 mL deionized water and incubated at 25°C. After 24 h, electrical conductivity (EC1) of the solution was recorded. These samples were then autoclaved at 120°C for 20 min to release all electrolytes. Samples were then cooled to 25°C and the final electrical conductivity (EC2) was measured. The electrolyte leakage (EL) was calculated by the formula:

$$EL(\%) = EC1/EC2 \times 100$$

Membrane lipid peroxidation

Lipid peroxidation was measured in terms of accumulation of malondialdehyde (MDA) following the method of Health and Packer (1968). The sample was homogenized in 0.1% (w/v) TCA and centrifuged for 10 min at 10000 rpm. The supernatant was directly used for MDA estimation by adding 0.5% TBA followed by heating of the mixture for 30 min at 95°C and cooling it on ice. The absorbance of the sample was determined at 532 and 600 nm. Using an extinction coefficient of 155 $mM^{-1}cm^{-1}$ the concentration of MDA was calculated.

Proline accumulation

Proline accumulation in leaves and roots was estimated following the method of Bates *et al.* (1973). 1 gm tissue was homogenized in 3% sulfosalicyclic acid and estimation was done with ninhydrin solution by measuring the absorbance at 520 nm.

Results

Effect of NaCl on RWC

RWC of leaves and roots of *Imperata* decreased gradually with the increase in concentration of NaCl both after 24 and 72 hours of treatment (Fig. 1). The least RWC was recorded at 500 mM NaCl concentration. At 200mM the percentage of decrease in leaf RWC after 24 and 72 hours of treatment is 4.3 and 5.7 respectively and the percentage of decrease in root RWC after 24 and 72 hours of treatment is 8.4 and 10 respectively.

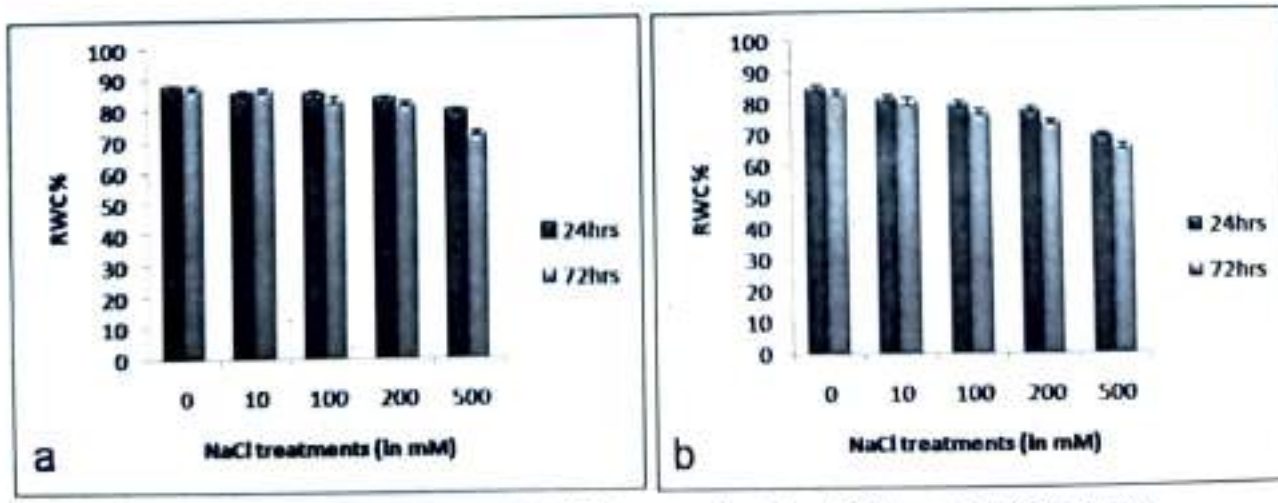

Figure 1 (a) RWC in leaves and (b) RWC in roots after 24 and 72 hours of NaCl treatments

Morphological and ultrastructural changes due to NaCl stress

The immediate response to salinity stress appears in the form of leaf rolling which was mediated by the presence of bulliform cells. However, no other visible symptoms of salt stress like leaf firing, browning and shrinkage appears in the NaCl treated plants up to 7 days of treatment. Apart from that the salt crystals appear on the leaf surfaces from 3 days of NaCl treatment (e" 200 mM) (Fig. 2a). Light microscopy of T.S. of leaf revealed the presence of typical Kranz anatomy as seen in all C4 plants. Specialized cells called salt glands were observed on the upper and lower epidermis of the leaves of the plants kept at 200 mM NaCl for 3 days (Fig. 2b). SEM study also revealed the presence of salt glands in both control and NaCl treated plants (200 mM NaCl for 3 days). The presence of salt glands can be distinctively pointed out from the trichomes and papillae by their broader and oval tips. The density of salt glands appeared to have increased due to NaCl stress (Fig. 2c-d). TEM study revealed the increase in vacuolarization in the NaCl treated plants (200 mM NaCl for 3 days) in comparison to the control plants. However there was no visible impact up on the integrity of chloroplast in the treated samples (Fig. 2e-f).

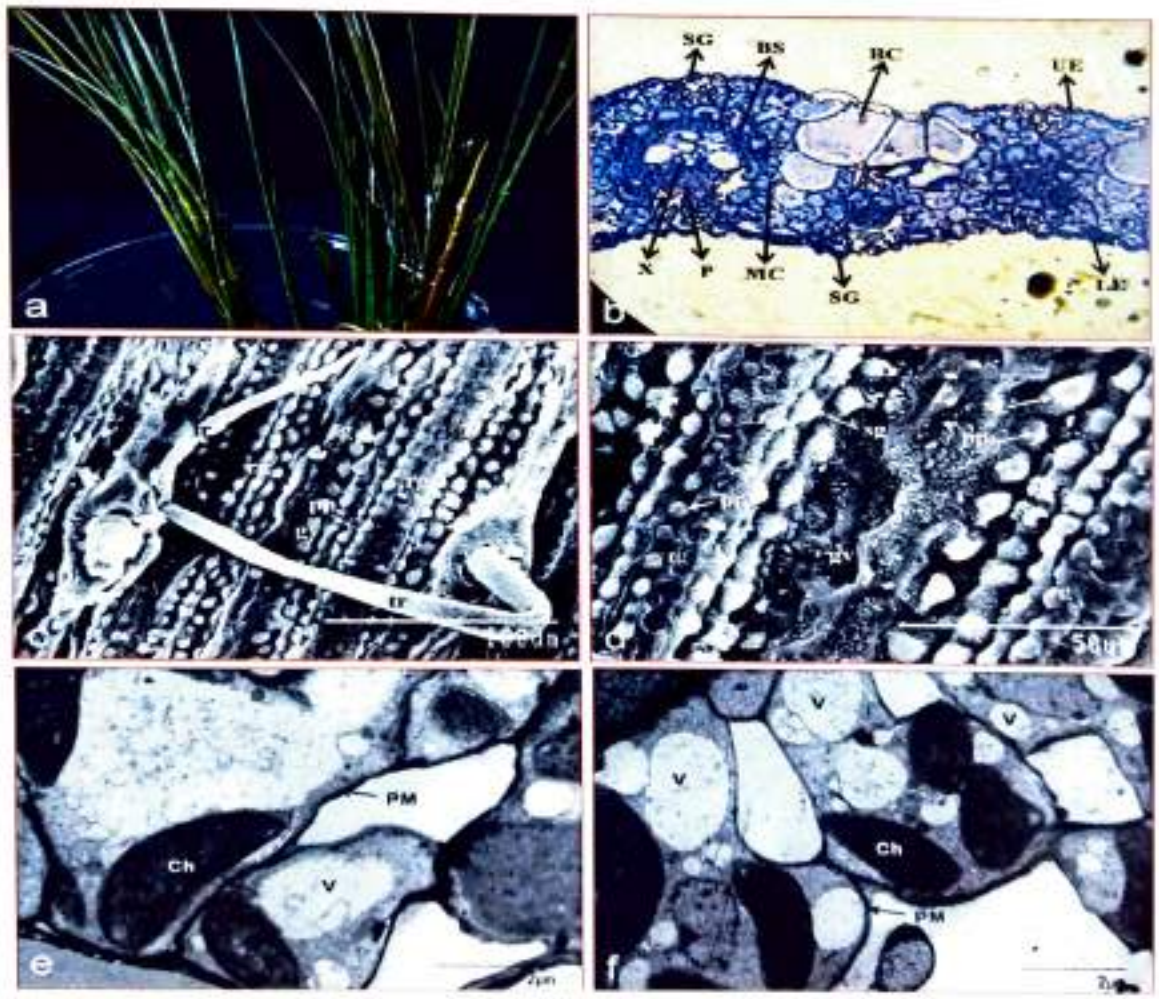

Figure 2 (a) Salt crystals on the leaf surface of plants kept 200 mM NaCl sol. for 7 days; (b) Light microscopy of T.S. of leaf showing salt gland; (c) SEM of leaf surface of control plants; (d) SEM of leaf surface of plants kept 200 mM NaCl sol. for 3 days; (e) TEM showing the ultrastructure of mesophyll cells of control plants; and (f) TEM showing the ultrastructure of mesophyll cells of plants kept 200 mM NaCl sol. for 3 days.
[**BS** - bundle sheath, **MC** - mesophyll cell; **UE** - upper epidermis, **LE** - lower epidermis; **X** - xylem; **P** - phloem; **BC** - bulliform cell; **sg** - salt gland; **pp** - papilla, **tr** - trichome; **rg** - ridges; **gr** - grooves; **PM** - plasma membrane; **V** - vacuoles; **Ch** - chloroplast]

Effect of NaCl on ion accumulation and exclusion

The leaf Na^+ content was not found to increase significantly up to a concentration of 200 mM NaCl treated plants. At 500 mM NaCl, the accumulation of Na^+ was comparatively much higher at the end of 7 days (Fig. 3a). However, the leaf K^+ content remained more or less constant at every treatment (Fig. 3b). Similarly in the roots, no significant time dependent increase in Na^+ content was observed for any treatments (Fig. 3c). But the K^+ content was observed to decline slightly in case of plants kept at 200 and 500 mM NaCl (Fig. 3d).

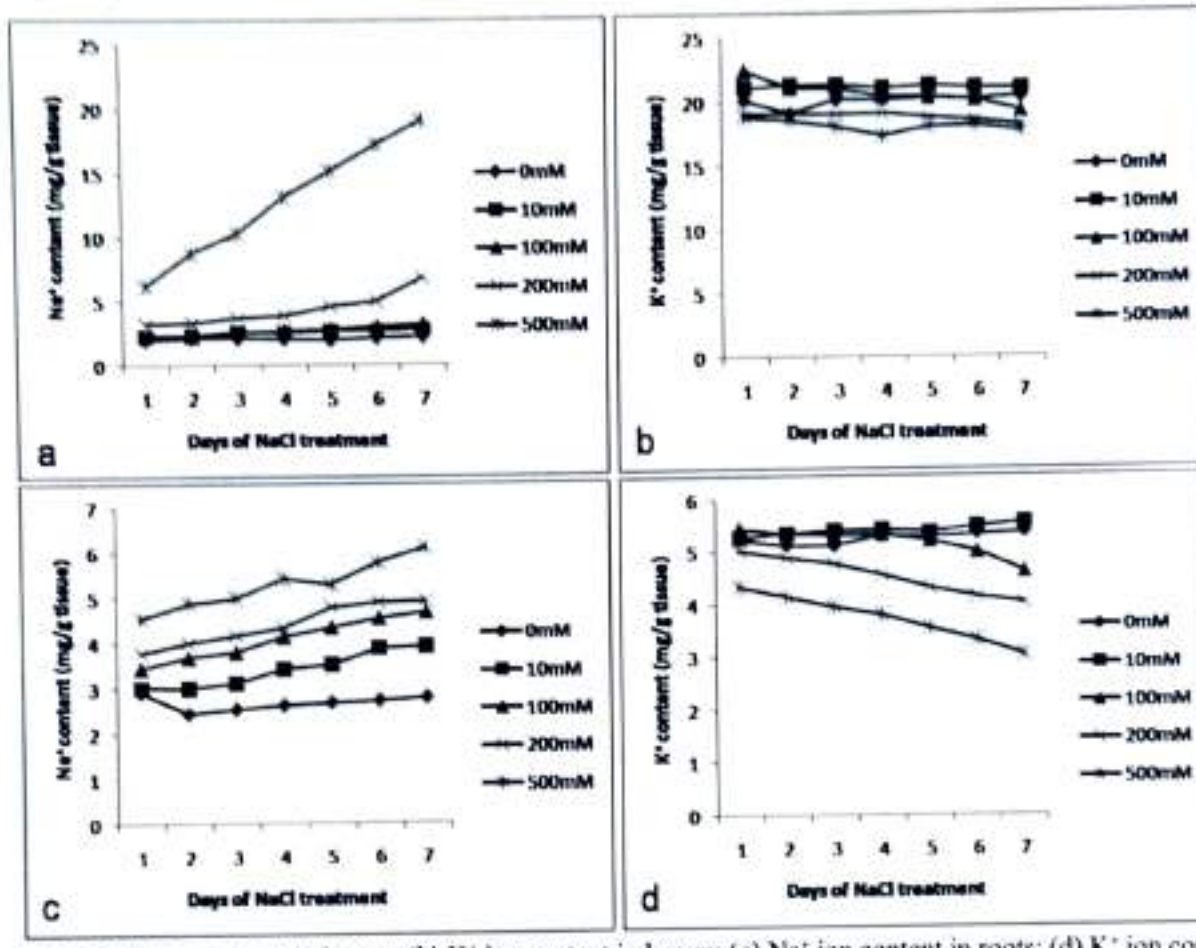

Figure 3 (a) Na^+ ion content in leaves; (b) K^+ ion content in leaves; (c) Na^+ ion content in roots; (d) K^+ ion content in roots up to 7 days of NaCl treatments.

Na^+ exclusion was observed in the plants kept at NaCl concentrations e" 200 mM. At 200 mM and 500 mM NaCl, Na^+ exclusion was observed after 3 days and 2 days respectively (Fig. 4). Time dependent Na^+ exclusion was observed to increase significantly in plants kept at 200 and 500 mM NaCl conc. However, no detectable K^+ exclusion was observed in any plants.

Effect of NaCl on H_2O_2 and O_2^- localization

After 24 hours of NaCl treatment, H_2O_2 localization was found to increase with the increase in the

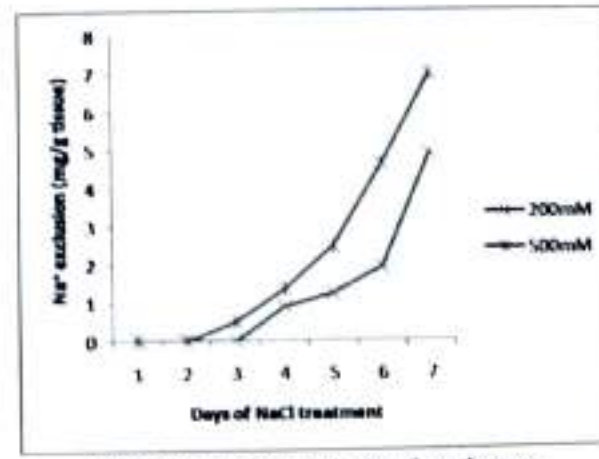

Figure 4 Na^+ ion exclusion from leaves

concentration of NaCl as indicated by the DAB staining. The proportion of H_2O_2 in the tissue was maximum in the leaves of plants kept at 500 mM NaCl. However, after 72 hours of treatment, the stained regions in the leaf tissues were found to decrease at all the NaCl treatments.

Similar results were observed in case of O_2^- localization. The blue formazan regions were found to increase with the increase in NaCl concentration after 24 hours of treatment and after 72 hours of treatment, the blue regions decreased indicating the lowering of O_2^- radicals.

Effect of NaCl on electrolyte leakage, membrane lipid peroxidation and proline content

The electrolyte leakage was observed to increase with increasing NaCl concentration both after 24 and 72 hours of treatment, though the increase was not significant (Table 1). Remarkably there was no increase in electrolyte leakage after 72 hours as compared to the values after 24 hours treatment. MDA content also gradually increased with the increase in NaCl concentration both after 24 and 72 hours of treatment (Table 1). However, the time dependent accumulation of MDA content was found to be more or less stable. Leaf proline content was also found to increase with increasing NaCl concentration (Table 1). Maximum proline accumulation was observed in the plants kept at 200 mM NaCl. This increase was about 2 times from that of the control plants. Also the proline content remained stable with time.

Discussion

RWC values determine the changes in the water level inside the plant cells, thus providing an insight in to the health status of a plant (Kramer and Boyer, 1995). In our study, it was found that the RWC values in all the plants decreased with the increase in NaCl concentrations. However, this decrease in RWC was not pronounced and negated by the increased accumulation of proline. Proline accumulation was found to increase and approximately 2 fold increase was noticed in the plants kept at 200 mM NaCl solution, which is considered to be sufficiently high salt concentration for plant growth (Flowers and Colmer, 2008).

The presence of salt glands was confirmed by both light microscopy and SEM. The functionality of the salt glands were confirmed by the Na^+ ion exclusion from the leaf surface of *Imperata* at high salt concentrations (e"200 mM NaCl), which helped in lowering the Na^+ ion content inside the leaves. This control of Na^+ inside the leaf tissue accounts for better salt tolerance. This is in agreement with the works of Davenport *et al.* (2005). Though the K^+ content in leaves and roots remained more or less stable, the Na^+ ion content was found to increase with an increase in NaCl concentration. The sequestration of excess Na^+ ion in to the vacuoles is another mechanism by which the plants cope up

Table 1 Electrolyte leakage, membrane lipid peroxidation and proline content of leaves under different NaCl concentration after 24 and 72 hours of treatment

NaCl conc. (in mM)	Electrolyte leakage (in %)		Membrane lipid peroxidation (in mM MDA/g tissue)		Proline (µg/g tissue)	
	24 hours	72 hours	24 hours	72 hours	24 hours	72 hours
0	15.1 ± 0.7	14.6 ± 0.6	6.11 ± 0.63[a]	6.27 ± 0.5	65.2 ± 2.1	63.5 ± 1.7
10	16.1 + 1.3	15.7 + 0.8	6.92 + 0.7	6.72 + 0.54	71.5 + 1.8	74.3 + 2.7
100	18.2 ± 0.6	18.5 ± 0.7	7.11 ± 0.6	7.23 ± 0.72	80.8 ± 3.2	85.1 ± 2.8
200	19.1 ± 1.1	18.2 ± 1.3	8.17 ± 0.82	8.05 ± 0.8	130.1 ± 3.8	124.6 ± 4.6
500	21.2 + 0.9	21.9 + 1.1	9.34 + 0.56	10.15 + 0.85	95.1 + 2.9	98.2 ± 2.7

Values denote mean + SE (n = 3).

with the adverse effects of NaCl stress (Horie and Schroeder, 2004). TEM studies reveal the increase in vacuolarization in plants kept at 200 mM NaCl for 3 days without any detrimental changes in the chloroplasts. This increase in vacuolarization may account for the secretion of excess Na^+ ion, thus keeping the water balance of the cytoplasm optimum for the biochemical processes.

The health status of the plants under stress was analyzed by *in situ* localization of H_2O_2 and $O_2^{\cdot-}$ radical. These are the reactive oxygen species (ROS) that are formed due to the adverse effects of toxic Na^+ ion. The concentration of these ROS increased initially but after 72 hours of treatment, the accumulation of these ROS was lowered and controlled. This could be attributed to the activity of the salt glands in lowering the Na^+ ion from the cytoplasm. This result is also in accordance with the previous workof Sobhanian *et al.* (2012) on the halophytic grass *Aeluropus*. The results on electrolyte leakage and MDA content revealed that the stability of plasma membrane was not severely hampered in *Imperata* under salinity stress and thus had little impact up on the proper functioning of the salt glands.

In summary, it can be concluded that the salinity stress tolerance mechanism in *Imperata* is the inherent property of the structural and functional mechanism of salt glands present on the leaf surface. The efficient functioning of salt glands is responsible for maintaining ion equilibrium and is an important asset for the salt tolerance of this plant.

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References

Barr H.D., Weatherley P.E. (1962) A re-examination of the relative turgidity technique for estimating water deficit in leaves. *Aust J Biol Sci* 15: 413-428.

Bates H.S., Waldren R.P., Treare I.D. (1973) Rapid estimation of free proline for drought stress determination. *Plant Soil* 39:205-208.

Campbell R.J., Grayson R.L., Marini R.P. (1990) Surface and ultrastructural feeding injury to strawberry leaves by the two spotted spider mite. *Hort Sci* 2.5(8): 948-951.

Davenport R., James R.A., Zakrisson-Plogander A., Tester M., Munns R. (2005) Control of sodium transport in durum wheat. *Plant Physiol* 137(3): 807-818.

Flowers T.J. (1985) Physiology of halophytes. *Plant and Soil* 89: 41-56.

Flowers T.J., Colmer T.D. (2008) Salinity tolerance in halophytes. *New Phytol* 179:945-963.

Haberlandt G. (1914) Physiological Plant Anatomy. Macmillan and Co. Ltd., London, UK.

Hameed M., Ashraf M., Naz N. (2009) Anatomical adaptations to salinity in cogon grass [*Imperata cylindrica* (L.) Raeuschel] from the Salt Range, Pakistan. *Plant and Soil* 322(1-2): 229-238.

Health R.L., Packer L. (1968) Photoperoxidation in isolated chloroplasts I: Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys.* 125: 189-198.

Horie T., Schroeder J.I. (2004) Sodium transporter in plants. Diverse gene and physiological functions. *Plant Physiol* 136(1): 2457-2462.

King S.E., Grace J.B. (2000)The effect of soil flooding on the establishment of cogon grass (*Imperata cylindrica*), a non indigenous invader of the southeastern united states. Wetlands 20: 300-306.

Kramer P.J., Boyer J.S. (1995) Water relations of plants and soils. San Diego, Academic Press, pp. 495.

Lutts S., Kinet J.M., Bouharmont J. (1996) NaCl-induced senescence in leaves of rice (*Oryza*

sativa L.) cultivars differing in salinity resistance. *Ann Bot.* 78: 389-398.

Marcum K.B. (1999) Salinity tolerance mechanisms of grasses in the subfamily Chloridoideae. *Crop Sci.* 39:1153-1160.

Marcum K.B., Murdoch C.L. (1990) Salt Glands in the Zoysieae. *Ann Bot.* 66: 1-7.

Marcum K.B., Anderson S.J., Engelke M.C. (1998) Salt gland ion secretion: A salinity tolerance mechanism among five zoysia grass species. *Crop Sci.* 38: 806-810.

Naidoo Y., Naidoo G. (1999) Cytochemical localization of adenosine triphosphatase activity in salt glands of *Sporobolus virginicus* (L.) Kunth. *S. Afr. J. Bot.* 65:370-373.

Oross J.W., Thomson W.W. (1982) The ultrastructure of salt glands of *Cynodon* and *Distichlis* (Poaceae). *Am. J. Bot.* 69(6): 939-949.

Pathan A.K., Bond J., Gaskin R.E. (2008) Sample preparation for scanning electron microscopy of plant surfaces - horses for courses. Micron 39: 1049-1061.

Romero-Puertas M.C., Rodriguez-Serrano M., Corpas F.J., Gomez M., DelRio L.A., Sandalio L.M. (2004) Cadmium-induced subcellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in pea leaves. *Plant Cell Environ.* 27(9): 1122-1134.

Roy S., Chakraborty U. (2014) Salt tolerance mechanisms in salt tolerant grasses (STGs) and their prospects in cereal crop improvement. *Bot. Studies.* 55:31.

Sobhanian H., Motamed N., Jazii F.R., Razavi K., Niknam V., Komatsu S. (2010) Salt stress responses of a halophytic grass *Aeluropus lagopoides* and subsequent recovery. *Russian J. Plant Physiol* 57(6): 784-791.

Tester M., Bacic A. (2005) Abiotic stress tolerance in grasses - From model plants to crop plants. *Plant Physiol.* 137: 791-793.

Thordal-Christensen H., Zhang Z., Wei Y., Collinge D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.*11(6): 1187-1194.