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**ABSTRACT**

Large proportions of agricultural land in Goa are low lying saline fields and are called Khazan soils. Jaya (hybrid salinity tolerant), Jyoti (hybrid salinity sensitive) and Korgut (non-hybrid salinity tolerant) are the major rice varieties grown in these Khazan soils. Experiments were carried out with 50, 100 & 150 mM concentrations of sodium chloride (NaCl) to determine the changes in photosynthetic pigments sugar content, proline & Protein content. Damage due to membrane lipids were also studied as TBA-MDA adduct formation. The study showed that salinity stress resulted in physiological and biochemical changes in rice varieties compared to their control. Jyoti rice variety shows better adaptation to salt stress in comparison to Jaya rice variety. The level of Proline content was significantly higher in Jyoti at 150mM salt stress than in Jaya variety. Korgut however is much more tolerant in comparison to hybrid rice varieties.

INTRODUCTION

Salinity is one of the major abiotic stress affecting seedling growth and crop productivity (Kumar et al., 2000). The ability of plants to cope with salinity stress is an important determinant of crop distribution and productivity and hence it is important to understand the mechanisms that confer tolerance to saline environment. Salinity influences osmotic inhibition and ion toxicity. Osmotic inhibition reduces the ability of the plant to take up water leading to slow growth (Mansour et al., 2005) & reduction in overall growth (Gama, 2007). The metabolic imbalances due to toxic inhibition, osmotic stress, and nutritional deficiency by salinity may lead to oxidative stress by generation of reactive oxygen species (Zhu, 2002). Osmotic adjustment of plants is shown by accumulation of compatible solutes like proline, soluble sugars, sugar alcohols and glycine betaine (Gama, 2008; Hasegawa et al., 2000). Flower & Yeo (1986) have shown that injury due to salt stress (Na⁺ and Cl⁻) or both lead to accumulation of these excess ions that inhibit enzyme. There are reports showing salt effects on photosynthetic enzymes, chlorophylls and carotenoids (Stepien and Klobus; 2006). Munns (2002) reported that salt-tolerant species, with low rate of salt uptake and efficient salt compartmentation show metabolic and developmental processes similar to that of water stress plants. Salt stress induced ROS can damage membrane lipids, proteins and nucleic acids (Mittler, 2002; Grant and Louke, 2000). Lipid peroxidation, induced by free radicals, is also important in membrane deterioration (Khan and Panda, 2008; Mandhania et al., 2006; Demiral and Turkan, 2005). In coastal belt of India, salinity stress is a major stress reducing crop productivity (kumar et al., 2008). Salinity may affect the crop either at germination stage, seedlings growth, or during reproductive growth with loss of economic yield and poor quality (Sairam & Tyagi 2004). However, response to salt stress in plant is dependent on salt type, its concentration and genotypes. In Goa, rice is a major agricultural crop and is grown in Khazan or morod fields. Khazan fields are well-drained, low sandy saline soils with a lateritic substratum called kher. They are spread over about 17,000 ha of land. Jaya, Jyoti (salinity tolerant and salinity sensitive hybrid rice varieties resp.) & Korgut (non-hybrid tolerant variety) are the major rice varieties grown in these khazan fields. The varieties are most preferred by locals for their high yielding capacity. It is hence, important to understand salinity tolerance capacity of these genotypes. The objective of this study was to evaluate the physiological & biochemical responses of these three rice varieties for their tolerance level to salinity stress. Our results showed that Jyoti although a salinity sensitive hybrid rice variety is better adapted to salinity stress compared to Jaya and Korgut.

**Methodology**

**Plant material:** Seeds of Jaya (hybrid salinity tolerant), Jyoti (sensitive hybrid) & Korgut (salinity tolerant non-hybrid) rice varieties were obtained from ICAR old Goa.

**Growth conditions & salinity treatment:** Seeds were surface sterilized with 0.1% mercuric chloride, thoroughly washed in distilled water, soaked overnight and allowed to sprout for 2 days in a muslin cloth to facilitate sprouting. Seeds were then potted in plastic pots containing soil: sand: peat (1:1:2 v/v) and grown in the laboratory condition under illumination at 250C relative humidity (RH) of 70% and 200µmol m-2 s-1 light intensity for 2 weeks. Two week old seedlings from respective cultivars were then treated separately with 50, 100, and 150 mM NaCl solution & allowed to grow for next 12 days. Control plants were grown in distilled water alone. 12 days old seedlings were randomly sampled (2nd leaf) and leaf tissue was immediately used for studying various parameters.

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Physiological and Biochemical Analyses

Relative water content

Relative water content (RWC) was determined according to the method of Turner (1981). Leaf sample (0.1g) was cut into smaller pieces and weighed to determine Fresh weight (FW). They were then soaked overnight in distilled water, reweighed to determine turgid weight (TW) and were then dried in the oven at 80°C for 1 day to get dry weight (DW). The relative water content (RWC) was determined using the following formula:

\[ RWC = \left(\frac{FW - DW}{TW - DW}\right) \times 100 \]

Root shoot ratio

Salinity induced changes in root shoot ratio were determined according to the method of Mane et al., (2011). Ten plants from each treatment (50-150mM salt stress along with the control) were uprooted carefully washed with water and root & shoot length in cm was noted to calculate root: shoot ratio.

Photosynthetic Pigment analysis

Photosynthetic pigments (Chl a, b, & carotenoids) were studied spectrophotometrically according to the method of Arnon (1949). Plant tissue (0.1g) was homogenized with 10 ml of 80% acetone. Homogenate was centrifuged for 1 min at 1000 rpm. Absorbance of the supernatant was read at 663nm, 645nm and 445nm for Chl a, b, and carotenoids using Thermoscientific UV-Visual spectrophotometer. Amount of total chlorophyll was expressed as mg/ml of the sample. Photosynthetic pigments were also separated by HPLC according to the method of Sharma and Hall, (1996). Fresh plant tissue (0.1) was homogenized using liquid nitrogen in 1.5ml of 100% acetone. The extract was centrifuged using Eltek RC 4100F at 4°C for 10 min at 6500rpm. The supernatant was collected in 1ml eppendorf tube and filtered through 0.2µm filters and used for loading in HPLC. Pigments were detected using reverse phase C-18 HPLC column (Et250/4 Nucleosil 100-5 C-18 ODS) with Acetetonitrile & water as mobile phase. Quantification of the pigments was done using β carotene as standard.

Soluble sugars

Total & reducing sugars were determined spectrophotometrically at 490 & 510 nm resp. as described by Dubois et al., (1956). Sugars were extracted twice using 0.1 g of leaf sample in 5 ml 90% hot alcohol. The extract was centrifuged in a Remi R-24 centrifuge for 10 min at 5000 rpm. Supernatant was evaporated to complete dryness & residues were re-dissolved completely in 5 ml of distilled water. For reducing sugars extract in a final volume of 3ml with distilled water was mixed with 3ml DNSA (Dinitrosalicylic acid) and warmed in a water bath for 5min. 1ml 40% Rochelle salt was mixed to the incubate and absorbance was read at 510nm. Total sugars were assayed using 5% phenol added to the extract in final volume of 1ml with distilled water. 5ml Conc. H2SO4 was added to this mixture and mixed well. Absorbance was read at 490nm. Reducing and total sugars (µg/g F.w.) were calculated using glucose as standard.

Protein assay

Protein content was studied according to the method of Lowry et al., (1951) with BSA as standard. Plant tissue (0.5g) was homogenized in 10ml 50 mM phosphate buffer pH 7.8. Extract was centrifuged at low speed and supernatant was used for the assay. An aliquot in known concentration was mixed with 5ml alkaline copper sulphate solution consisting of 2.5% sodium carbonate in 0.1 N sodium hydroxide and 0.5% copper sulphate in 1% potassium sodium tartrate. The contents were mixed and allowed to stand for 10 min at room temperature. 0.5 ml of Folin ciocalateau reagent diluted 1:1 was added to this incubate, mixed well and incubated in dark at room temperature for 30 min. Absorbance was read at 660nm.

Estimation of proline

Free proline was estimated at 520 nm according to the method of Bates et al., (1973) with L-proline as standard. Plant tissue (0.1 g) was homogenized in 5ml of 3% sulphosalicylic acid using mortar and pestle. The extract was centrifuged for 7 min at 2000rpm. 1ml of the supernatant was mixed with 1ml each of glacial acetic acid and acidic Ninhydrin. The tube contents were vortexed for 5min and then incubated in a boiling water bath for an hour. The test tubes were then cooled to room temperature and placed on ice. 4ml of toluene was added to each test tube and mixed by vigorous shaking. The pink red chrophore developed in the toluene layer was read at 520nm in a spectrophotometer. Amount of proline (µmol/g. Fw) in the sample was calculated based on the standard curve.

Lipid peroxidation

Peroxidation of membrane lipids was studied as TBA-MDA adduct formation according to the method of Sharma & Singhal (1992). Tissue (0.1g) was homogenized using mortar and pestle in 1% TCA solution to a final volume of 5ml. The homogenate was centrifuged at 2000 rpm for 5 min. Known Concentration of the supernatant was then mixed with 2.5 ml of 0.5% TBA in 20% TCA and 2.5ml of incubation buffer composed of 50mM Tris-HCl and 175mM NaCl, pH 8.0. The contents of the tubes were then incubated at 95°C for 30 min, cooled and absorbance was read at 600 and 532 nm. MDA was expressed as µmol/g F.w.

Statistical analysis

All experiments were repeated thrice independently with similar results. Data shown are expressed as mean ± standard deviation (S.D) with reading of three samples per treatment. Analysis of stress intensity was based on 1-way analysis of variance (ANOVA). All statistical analysis was performed by Microsoft Excel version 7.

RESULTS AND DISCUSSION

Our study showed reduction in overall growth of these seedlings due to increased salinity level.

Effect of salinity stress on RWC and root shoot ratio

Our results showed decline in relative water content with salinity stress content in all the three rice varieties compared to their respective controls. Jaya showed maximum relative water content at 150mM salinity treatment compared to Jyoti and Korgut (Table 1). Relative water content (RWC) in stress plants is associated with a decrease in plant vigour and is observed in many plants (Hilder and Burrage 2003, Lopez et al. 2002). The decrease in moisture content in the leaves under salinity stress may be also due to osmotic stress induced by NaCl (Mane et al., 2011). Results of root shoot ratio showed decline with increasing salinity stress in all three rice varieties studied (Fig. 1A). Jaya, Jyoti and Korgut rice varieties showed 19%, 9.8%, 19% decline in root shoot ratio at 150mM NaCl treatment compared to their respective controls.
This decline in root shoot ratio may be due to decrease in leaf water status under salinity stress causing osmotic imbalance and overall reduction in growth of the seedlings (Amirjani et al., 2010).

**Total Chlorophyll content**

Total chlorophyll content did not show much difference with increasing salinity stress in all the three rice varieties studied (Fig.1B). Jaya, Jyoti & Korgut rice varieties showed 18.93%, 16.82% & 8.4% decrease in total Chl content at 150mM salinity treatment. However, when compared to Jaya & Jyoti, Korgut showed least amount of total chlorophyll under control and treated conditions. Pigment analysis by HPLC showed higher Chl a content than Chl b in all the three rice varieties studied (Table 2).

Sugar content

Results of reducing & total sugar content are shown in (Fig. 2C). Reducing sugars showed a significant difference with increasing salinity stress in all the three rice varieties studied (*P*<0.001). Total sugars showed significant increase with increasing salinity stress in Jaya (*P*<0.05) and highly significant difference in Jyoti & Korgut (*P*<0.001) compared to control. When both these varieties were compared to each other Jaya rice variety showed higher total sugars than Jyoti at all the salinity treatments (50-150mM). Non hybrid Korgut rice variety accumulated 3 times higher sugars than hybrid rice varieties (Jaya & Jyoti) suggesting that sugars play important role in osmoregulation and increase in sugars are correlated with net photosynthetic rate necessary for maintaining photosynthetic efficiency under stress conditions.

Amarjani et al., (2011), Cha-um et al., (2009b) & Pattangul and Thitisaksakul (2008) have reported increase in soluble sugars in HJ and PT1 rice varieties under high NaCl salt treatment and our results are also similar to these reports.

**Protein content**

Effect of salt stress (NaCl) on total protein content are shown in (Fig 2D). Our study showed that protein content increases with increasing salinity treatment (50-150mM) in all the three rice varieties studied. Jaya (salinity tolerant) rice variety showed 83% (*P*<0.001) increase in protein content at higher salinity stress (150mM) compared to its control. Korgut, rice also showed 31.80% increase compared to its respective control. However when compared to Jaya & Jyoti, Korgut had the least protein content at 150mM salinity stress.

Jaya & Jyoti rice varieties did not show much variation in Chl a & b content compared to its control. However when compared to Korgut they had more Chl a & b content. β-carotene declined with increasing salinity stress and Jyoti showed least amount of β-carotene amongst the three rice varieties studied. The decline in chlorophyll content is a common phenomenon under salinity stress (Djanaguiraman et al., 2006) and this decline could be due to increase in the activity of chlorophyll degrading enzyme chlorophyllase (Yang et al., 2009; Xu, Xinwen et al., 2008) or might be associated with disruption in cellular functions and damage to photosynthetic electron transport chain due to accumulation of ions (Mane et al., 2011). β-carotenes are directly associated with photoprotection under stress conditions and are known to influence antioxidant system (Sankhalkar & Sharma 2005).
Generally salt stress results in reduction of soluble protein content (Azooz et al., 2004 and Dager et al., 2004; Khan, 2003) indicating inhibition of its synthesis or increase in its hydrolysis (Irigoyen et al., 1992). Study by Gandonou et al., (2011a) has shown significant increase in soluble proteins in the salt sensitive sugarcane cultivar (CP65-357). The possible increase in protein under salinity stress may be due to expression of several osmo-protective proteins or synthesis of existing proteins. Our results are thus in conformity to the reports of Gandonou et al., (2011a). In our study the non-significant increase of protein content in Jyoti & Korgut (ns $P>0.05$) indicate accumulation of several proteins under stress and this needs further investigation.

**Proline content**

Effect of salt stress on proline accumulation are shown in (Fig. 3E). Our study showed that proline content increased in all three rice varieties. Highest level of proline was observed in Jyoti rice variety (349%) than Jaya (294%) while Korgut rice showed 159% increase in proline at highest salinity level when compared to its control. Our observation show that Jyoti & Korgut rice varieties are significantly tolerant to salinity ($P<0.001$) compared to Jaya ($P<0.01$). Our results are in correlation with number of reports showing proline accumulation in salt stressed plants suggesting a possible correlation with adaptation to salinity (Shafi et al., 2011, Misra and Saxena, 2009).

### Table 1: Effect of salinity stress (NaCl) alone on, Relative water content (%) in vitro. The values are mean of 3 experiments ± S.D (n=3).

<table>
<thead>
<tr>
<th>Variety</th>
<th>Control</th>
<th>30mM NaCl</th>
<th>100mM NaCl</th>
<th>150mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jaya</td>
<td>90.5±0.47</td>
<td>87.79±0.82</td>
<td>85.25±0.66</td>
<td>83.27±1.91</td>
</tr>
<tr>
<td></td>
<td>(-3.08%)</td>
<td>(-5.85%)</td>
<td>(-8.07%)</td>
<td></td>
</tr>
<tr>
<td>Jyoti</td>
<td>88.67±1.22</td>
<td>86.05±1.64</td>
<td>79.8±2.05</td>
<td>76.32±5.20</td>
</tr>
<tr>
<td></td>
<td>(-2.96%)</td>
<td>(-10.0%)</td>
<td>(-13.93%)</td>
<td></td>
</tr>
<tr>
<td>Korgut</td>
<td>88.7±8.42</td>
<td>85.86±6.52</td>
<td>78.7±2.15</td>
<td>76.21±5.05</td>
</tr>
<tr>
<td></td>
<td>(-3.21%)</td>
<td>(-11.29%)</td>
<td>(-14.08%)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Effect of salinity stress (NaCl) alone on, Plant pigments by HPLC in vitro. The values are mean of 3 experiments ± S.D (n=3).

<table>
<thead>
<tr>
<th>Variety</th>
<th>Concentration</th>
<th>Pigments (µmoles/gm F.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chl b</td>
</tr>
<tr>
<td>Jaya</td>
<td>Control</td>
<td>16.49</td>
</tr>
<tr>
<td></td>
<td>30mM NaCl</td>
<td>25.11</td>
</tr>
<tr>
<td></td>
<td>100mM NaCl</td>
<td>22.13</td>
</tr>
<tr>
<td></td>
<td>150mM NaCl</td>
<td>28.43</td>
</tr>
<tr>
<td>Jyoti</td>
<td>Control</td>
<td>13.97</td>
</tr>
<tr>
<td></td>
<td>30mM NaCl</td>
<td>12.81</td>
</tr>
<tr>
<td></td>
<td>100mM NaCl</td>
<td>16.21</td>
</tr>
<tr>
<td></td>
<td>150mM NaCl</td>
<td>28.04</td>
</tr>
<tr>
<td>Korgut</td>
<td>Control</td>
<td>17.76</td>
</tr>
<tr>
<td></td>
<td>30mM NaCl</td>
<td>19.98</td>
</tr>
<tr>
<td></td>
<td>100mM NaCl</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>150mM NaCl</td>
<td>27.10</td>
</tr>
</tbody>
</table>
Lipid peroxidation

The damage to the cellular membranes was studied by the accumulation of the malondialdehyde (MDA) levels (Fig.3F). Our results showed that MDA level significantly increases with increasing salinity stress in all the three rice varieties studied. Jaya & Jyoti rice varieties showed 107% & 150% increase in MDA content respectively. While, Korgut rice variety showed 246% increase in MDA at 150mM salinity in comparison to their controls. Malondialdehyde (MDA) is produced when polyunsaturated fatty acids in the membrane undergo oxidation by the accumulation of free oxygen radicals (Khan & Panda, 2008; Hernandez et al., 2000). The increase in lipid peroxidation may be due to the poor or inability of antioxidants to scavenge reactive oxygen species resulting from salt stress (Ben Amor et al., 2005; Chaparzade et al., 2004). Our result are in comparison to the above study and indicates that all the three varieties are significantly tolerant to the salinity stress (P< 0.001).

CONCLUSION

Salinity stress has affected physiological and biochemical growth processes in the rice seedlings by decline in relative water content leading to turgor loss. Compatible solute accumulation like proline, sugars and increasing MDA suggested better protection in hybrid salinity sensitive & tolerant rice varieties Jyoti & Jaya respectively. While, Korgut rice variety shows better tolerance to salinity stress. Increase in proline content under salinity stress in Jyoti rice variety suggests its adaptation to Khazan fields and these could be the possible reasons for growing these rice varieties in the Khazan fields of Goa.

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References


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