SUMMARY

A greenhouse experiment was conducted to examine the effect of seed biopriming with five salinity tolerant isolates of *Trichoderma harzianum* on the response of rice (*Oryza sativa* L.) to different salt stress levels. One factor was different *Trichoderma* treatments (Th-13, Th-14, Th-19, Th-33 and Th-50) and the second factor was four levels of salt stress viz., 0, 70, 150 and 240 mM NaCl. Growth, physiological and biochemical parameters were determined to characterize salt tolerance. Salt stress adversely affected the studied parameters. However, the data revealed that *Trichoderma* treatments alleviated the stress condition and significantly increased length and fresh weight of shoot and root, number of leaves, leaf area, photosynthetic rate, chlorophyll fluorescence, chlorophyll content and soil plant analysis development (SPAD) value in comparison to control at all stress levels. *Trichoderma* treatments also resulted in the alleviation of oxidative damage, as indicated by the decrease of malondealdehyde content in comparison to control. Seedlings raised from seeds bioprimed with Th-14 had significantly higher proline, membrane stability index and phenol content than other treated or untreated seeds under both non-saline and saline conditions. Besides, linear regression showed that the photosynthetic rate had a significant positive relationship with the number of leaves, leaf area, chlorophyll fluorescence, chlorophyll content and SPAD value, but no relation with leaf water content. Results indicate the potential of using salinity tolerant *Trichoderma* isolates through seed biopriming for reducing the deteriorating effects of salinity, with Th-14 giving the most consistent effect under the present experimental material and conditions.

Key words: *Oryza sativa*, *Trichoderma* treatments, salinity, oxidative damage.

INTRODUCTION

Salinity is one of the most important abiotic stress factors limiting plant growth and productivity (Khan and Panda, 2008). For centuries, agriculture in arid and semi-arid environments has faced an increase in soil salinity. Salinity affects almost every aspect of the physiology and biochemistry of plants, which are responsible for reduction of plant growth.

Among cereal crops, rice (*Oryza sativa* L.) is a major source of food after wheat for more than 2.7 billion people on a daily basis. Rice is considered as a salt sensitive monocot (Mass and Hoffman, 1997). Qayyum and Malik (1998) have reported 64% reduction in yield of rice even on moderately salt-affected soils. Rice is relatively salt-tolerant at germination but becomes very sensitive at the young seedling stage, which impacts the stand density in salt-affected fields (Lutts *et al*., 1995).

Salinity causes decrease both in growth and net photosynthesis of higher plants. The decline in productivity observed for many plant species subjected to excess salinity is often associated with the reduction in photosynthesis capacity (Long and Baker, 1986). The reduction in photosynthesis under salinity can also be attributed to a decrease in chlorophyll content (Delfine *et al*., 1999) and the growth reduction at higher salinity could be attributed to a decrease in leaf area expansion (Marcelis and Hooijdonk, 1999). Chlorophyll fluorescence, a tool that monitors the function of the photosynthetic apparatus, was also shown to change in response to water stress and salinity (Jamil *et al*., 2007). Characters like leaf damage, plant height and survival are the most commonly used criteria for identifying salinity tolerance (Gamma *et al*., 2007). Other indices of tolerance have also been proposed that are based on specific physiological characteristics. Accumulation of compatible solutes in shoots or leaves may play a role in combating salinity stress (Ashraf and Harris, 2004). Salt stress induces cellular accumulation of damaging reactive oxygen species (ROS), which can damage membrane lipids, proteins and nucleic acids (Mittler, 2002). Lipid peroxidation, induced by free radicals, is also important in membrane deterioration (Khan and Panda, 2008).
Soil salinity causes an imbalance of the cellular ions resulting in osmotic stress, which makes water uptake by roots difficult. However, incorporation of *Trichoderma* enhances deep root growth, which helps in more water acquisition and nutrient uptake (Harman, 2006; Azarmi et al., 2011). *Trichoderma* application through seed biopriming helps seeds to germinate even under adverse soil conditions (Singh et al., 2003). Seed biopriming is a process of biological seed treatment that refers to a combination of seed hydration and seed inoculation with beneficial organisms to protect seed. Beneficial activities attributed to *Trichoderma*-plant interactions include induced disease resistance, plant growth promotion, and tolerance to abiotic stresses (Shoresh and Harman, 2008; Mastouri et al., 2010).

As a consequence of primary effects (hyper osmotic stress, ion imbalance) of high salt concentrations, secondary stresses, such as oxidative stresses due to overproduction of ROS often occur. However, root colonization by *Trichoderma harzianum* results in increased level of plant enzymes, including various peroxidases, chitinases, α-1,3-glucanases, lipoxygenase-pathway hydroperoxide lyase and such changes in plant metabolism can lead to accumulation of compounds like phytoalexins and phenols to provide durable resistance against any biotic and abiotic stress (Mohiddin et al., 2010).

The present research was, therefore, carried out to elucidate in detail the usefulness of seed biopriming with salinity tolerant (ST) isolates of *T. harzianum* in alleviation of the adverse effects of salt stress on growth, physiological and biochemical parameters in rice and also to find the relationship between photosynthetic rate and other photosynthetic parameters, like number of leaves, leaf water content, leaf area, chlorophyll content, soil plant analysis development (SPAD) value and biochemical efficiency of PS2, under salt stress. In this study, we have examined and compared the proline, malondialdehyde and phenol accumulation in *Trichoderma*-treated and untreated rice seedlings grown under different degrees of salt stress in order to exploit the salinity tolerant *Trichoderma* strains in relation to salt stress tolerance in rice. This research is important as it reveals the role of *Trichoderma* that imparts stress resistance and provide insight in to the potential for rice plants to adapt to saline conditions.

**Materials and Methods**

**Experimental site.** The experiment was conducted during 2009-2010 in the greenhouse of the Department of Plant Pathology, College of Agriculture, G.B.P.U.A and T, Pantnagar (India), under the following conditions: minimum of 25°C during the night and 28°C during the day, automatic venting at 33±3°C with supplemental light for 12 h d-1. The light flux density ranged from 400 µmol m-2 s-1 to 1000 µmol m-2 s-1.

**Seed material.** Rice (Kalanamak-3131) seeds were obtained from Breeders Seed Processing Centre, Pantnagar (India). Before the start of the experiment, seeds were surface-sterilized in 1% sodium hypochlorite solution for 3 min, then rinsed with sterilized water and air-dried.

**Preparation of *Trichoderma* inoculum and seed biopriming.** Five previously tested ST isolates of *Trichoderma harzianum* (Th-13, Th-14, Th-19, Th-33 and Th-50) were obtained from the repository of Biocontrol Laboratory of the Department of Plant Pathology, G.B.P.U.A and T, Pantnagar, and grown in *Trichoderma* selective medium (TSM) at 25±2°C for five days without light. Two agar plugs (0.5 cm in diameter) of actively growing *Trichoderma* isolates were added to 250 ml Erlenmeyer flasks containing autoclaved barnyard millet (*Echinochloa frumentacea*) grains (local name “Jhanghora”). Flasks were incubated at 25±2°C for two weeks when the *Trichoderma*-colonized *Jhanghora* grains were air-dried and ground to fine powder with Willy Mill (Acmas Technography, India). Fine pure powder of *Trichoderma* was then mixed with talcum powder and the final *Trichoderma* isolates at 10 g/kg of seeds. Seeds were then kept under warm and moist conditions for 24 h to facilitate *Trichoderma* colonization on spermosphere during incubation. Seeds without *Trichoderma* treatment were used as control. The list of different *Trichoderma* treatments (at a dose of 10 g/kg seeds) taken for seed biopriming of rice seeds is given in Table 1.

**Salinity treatment.** Eight seedlings (28-day-old) obtained from seeds bioprimed with ST *Trichoderma* isolates were transplanted in plastic pots (22 × 16 cm in size) filled with 3 kg of air-dried 1:1 soil and sand mixture previously passed through 2 mm sieve. The electrical conductivity (EC) and pH of soil:water suspension were

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Control (untreated)</td>
</tr>
<tr>
<td>T2</td>
<td><em>Trichoderma harzianum</em> (Th-13)</td>
</tr>
<tr>
<td>T3</td>
<td><em>Trichoderma harzianum</em> (Th-14)</td>
</tr>
<tr>
<td>T4</td>
<td><em>Trichoderma harzianum</em> (Th-19)</td>
</tr>
<tr>
<td>T5</td>
<td><em>Trichoderma harzianum</em> (Th-33)</td>
</tr>
<tr>
<td>T6</td>
<td><em>Trichoderma harzianum</em> (Th-50)</td>
</tr>
</tbody>
</table>
of original soil used before application of salinity treatment were 0.39 dS m\(^{-1}\) and 7.6, respectively. After a week, thinning was done to maintain 5 seedlings per pot. The plants were watered regularly after transplanting for two weeks near to field capacity to stabilize them and then salinity treatment was applied by irrigating pots twice per day with different concentrations of NaCl (0, 70, 150 and 240 mM) in complete Hoagland's nutrient solution for the next 21 days. Saline solution used in this experiment was prepared artificially by dissolving calculated or weighed amount of commercial grade NaCl pellets to make 0, 70, 150 and 240 mM NaCl solution. The EC (dS m\(^{-1}\) at 25°C) of 1:2 soil water suspension of the soil in plastic pots was tested at regular intervals and final salt stress level was recorded to be 0.39, 2.69, 5.57 and 6.68 dS m\(^{-1}\) in pots with 0, 70, 150 and 240 mM NaCl salt stress levels, respectively, at the end of the experiment. The experiment was laid out in a completely randomized design with three replications. Factor one was different Trichoderma treatments (Th-13, Th-14, Th-19, Th-33 and Th-50) and factor two was different levels of salinity stress viz., 0, 70, 150 and 240 mM NaCl.

**Observations.** All observations were recorded after 21 days of salinity stress to rice plants. Leaf samples were collected from two plants per treatment per replicate.

**Growth parameters.** Plants were uprooted carefully and washed with distilled water. The length, fresh weight of roots and shoot of the plants and the number of leaves per plant were measured after 21 days of salinity treatment.

**Measurement of leaf area and leaf water content.** The leaf area was measured with an area meter (AM200, ADC Bio Scientific, UK). The fresh weight of the leaves was determined and, thereafter, leaves were oven-dried at 80°C for 48 h to obtain the dry weight. The leaf water content (LWC) was calculated as follows:

\[
\text{LWC} = \frac{\text{FW}-\text{DW}}{\text{FW}} \times 100
\]

where FW is leaf fresh weight and DW is leaf dry weight.

**Measurement of leaf gas exchange.** Net photosynthesis (\(\mu\text{mol CO}_2\text{ m}^2\text{ s}^{-1}\)) was measured using a CO\(_2\) gas analyzer (CID, USA) on intact leaves under full sunlight.

**Chlorophyll fluorescence (\(F_v/F_m\) ratio).** Chlorophyll ‘a’ fluorescence emitted by green plants reflects photosynthetic ability of PS II. A handy plant efficiency analyzer (Handy PEA, UK) was used to monitor chlorophyll fluorescence ratio (\(F_v/F_m\)) according to the equation:

\[
\frac{F_v}{F_m} = \frac{(F_m-F_o)}{F_m}
\]

**Total chlorophyll content.** The total chlorophyll content (CC) of fresh leaves was estimated according to Barnes et al. (1992) with the following formula:

\[
\text{Total CC (mg/g)} = (2.02 \times A_{645}) + (8.02 \times A_{663}) / (\text{weight in g} \times 1000)
\]

**Measurement of leaf greenness.** Leaf greenness was estimated using SPAD value. The SPAD value that is quite well correlated with chlorophyll content was recorded by a portable SPAD meter (Opti Science, CMM-200, USA). At each evaluation, the content was measured six times from leaf to base and the average was used for analysis.

**Membrane stability index.** The membrane stability index (MSI) of fresh leaves was determined as suggested by Sairam et al. (1997) according to the formula:

\[
\text{MSI} = 1 - \frac{C_1}{C_2}
\]

where \(C_1\) = conductivity at 40°C, \(C_2\) = conductivity at 100°C.

**Proline analysis.** Proline content in the tissue was estimated by colorimetric method as described by Bates et al. (1973).

**Malondialdehyde content.** Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction as described by Heath and Packer (1968).

**Total phenols.** Total phenol content was estimated by the method suggested by Thimmaiah (1999) using Folin-Ciocalteu reagent and the absorbance was measured at 650 nm against each blank. The content of phenol was obtained from different concentration of catechol and expressed as mg/100 g.

**Statistical analysis.** Experimental data were analyzed as per the procedure of two factor CRD and standard error of each mean was calculated and represented in the bar diagram. The critical dimension (CD) values were computed by multiplying the standard error of difference (SED) with table t value at error degrees of freedom (Gomez and Gomez, 1984). Linear regression was made by the Minitab statistical package (Mayer and Krueger, 2004).

**RESULTS**

The growth, physiological and biochemical consequences of different levels of salt stress in rice pretreated with different isolates of *T. harzianum* were assessed
in relation to length and fresh weight of shoot and root, number of leaves, leaf area, LWC, PR, chlorophyll fluorescence, CC, SPAD value, MSI, proline content, MDA content and phenolics. Generally, the effect of different treatments and salinity levels were statistically significant (p=0.05) as revealed by the analysis of variance of the characters investigated. The interaction between treatment and salinity was also significant for most characters like root fresh weight, number of leaves, leaf area, chlorophyll fluorescence, PR, MSI, proline content, MDA content and phenolics (Table 2). The effect of salt injury to rice plants when subjected to 21 day salinity treatments is shown in Fig. 1 A and B. It was found that no plant could survive at a salt stress level higher than 240 mM NaCl in T1 (control) when given long term (28 days) salinity stress treatment (Fig. 1C). The data are depicted in the form of bar diagrams (Fig. 2 and 4) for the selection of treatments for different parameters. The relationship between PR and other photosynthetic parameters under salt stress is shown in Table 3.

**Table 2.** Mean squares from analysis of variance of data for growth, physiological and biochemical parameters of rice grown under different levels of salt stress after seed biopriming with salinity tolerant isolates of *Trichoderma harzianum.*

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Shoot length (cm)</th>
<th>Root length (cm)</th>
<th>Shoot fresh weight (g)</th>
<th>Root fresh weight (g)</th>
<th>Number of leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments¹ (a)</td>
<td>5</td>
<td>42.28**</td>
<td>19.31**</td>
<td>111.2**</td>
<td>51.63**</td>
<td>10.00**</td>
</tr>
<tr>
<td>Salinity² (b)</td>
<td>3</td>
<td>922.7**</td>
<td>180.5**</td>
<td>969.9**</td>
<td>522.2**</td>
<td>199.3**</td>
</tr>
<tr>
<td>a x b</td>
<td>15</td>
<td>1.847 ns</td>
<td>0.450 ns</td>
<td>2.155 ns</td>
<td>2.368**</td>
<td>1.969*</td>
</tr>
<tr>
<td>Error</td>
<td>48</td>
<td>2.742</td>
<td>0.625</td>
<td>4.865</td>
<td>0.873</td>
<td>0.807</td>
</tr>
<tr>
<td>Sem</td>
<td></td>
<td>0.956</td>
<td>0.456</td>
<td>1.273</td>
<td>0.539</td>
<td>0.518</td>
</tr>
<tr>
<td>CD at 5%</td>
<td></td>
<td>2.71</td>
<td>1.29</td>
<td>3.621</td>
<td>1.534</td>
<td>1.475</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Leaf area (cm²)</th>
<th>Leaf water content (%)</th>
<th>Photosynthetic rate (µmol CO₂ m⁻² s⁻¹)</th>
<th>Chlorophyll fluorescence (Fv/Fm)</th>
<th>Total chlorophyll (mg/g fr.wt.)</th>
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</thead>
<tbody>
<tr>
<td>Treatments¹ (a)</td>
<td>5</td>
<td>174.79**</td>
<td>27.97**</td>
<td>8.402**</td>
<td>0.0664**</td>
<td>0.6069**</td>
</tr>
<tr>
<td>Salinity² (b)</td>
<td>3</td>
<td>6428.8**</td>
<td>102.1**</td>
<td>28.601**</td>
<td>0.4590**</td>
<td>3.8438**</td>
</tr>
<tr>
<td>a x b</td>
<td>15</td>
<td>13.963**</td>
<td>0.770 ns</td>
<td>1.596**</td>
<td>0.00468**</td>
<td>0.3473 ns</td>
</tr>
<tr>
<td>Error</td>
<td>48</td>
<td>1.9505</td>
<td>5.097</td>
<td>0.247</td>
<td>0.00058</td>
<td>0.3639</td>
</tr>
<tr>
<td>Sem</td>
<td></td>
<td>0.806</td>
<td>1.303</td>
<td>0.287</td>
<td>0.0139</td>
<td>0.11</td>
</tr>
<tr>
<td>CD at 5%</td>
<td></td>
<td>2.292</td>
<td>3.706</td>
<td>0.8162</td>
<td>0.0397</td>
<td>0.313</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SPAD value</th>
<th>Membrane stability index (%)</th>
<th>Proline content (µmol/g fr.wt.)</th>
<th>MDA content (µmol/g fr.wt.)</th>
<th>Phenol content (mg/100 g fr.wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments¹ (a)</td>
<td>5</td>
<td>112.07**</td>
<td>175.98**</td>
<td>179.76**</td>
<td>11.608**</td>
<td>21.690**</td>
</tr>
<tr>
<td>Salinity² (b)</td>
<td>3</td>
<td>965.43**</td>
<td>669.96**</td>
<td>749.20**</td>
<td>35.807**</td>
<td>45.125**</td>
</tr>
<tr>
<td>a x b</td>
<td>15</td>
<td>1.9736 ns</td>
<td>13.711**</td>
<td>21.422**</td>
<td>1.2021**</td>
<td>0.8493**</td>
</tr>
<tr>
<td>Error</td>
<td>48</td>
<td>3.3351</td>
<td>4.7176</td>
<td>1.6151</td>
<td>0.1626</td>
<td>0.2304</td>
</tr>
<tr>
<td>Sem</td>
<td></td>
<td>1.054</td>
<td>1.254</td>
<td>0.733</td>
<td>0.232</td>
<td>0.277</td>
</tr>
<tr>
<td>CD at 5%</td>
<td></td>
<td>2.998</td>
<td>3.565</td>
<td>2.086</td>
<td>0.662</td>
<td>0.788</td>
</tr>
</tbody>
</table>

¹T1(T1(control), T2(Th-13), T3(Th-14), T4(Th-19), T5(Th-33) and T6(Th-50).
²0, 70, 150 and 240 mM NaCl.
** Significant (p = 0.05); ns= non significant; fr.wt. = fresh weight.

Effect of seed biopriming with ST isolates of *T. harzianum* on the growth parameters of rice under different levels of salt stress.

**Length and fresh weight of shoot and root.** Mean square from analysis of variance of data of rice when subjected to 21 day salinity treatment (0, 70, 150, 240 mM NaCl) indicated that both length and fresh weight of shoot and root decreased with increase in salt concentration. The maximum reduction in length and fresh weight of shoot and root (33.26 cm, 11.33 g and 11.53 cm, 2.62 g, respectively) under highest level of salinity i.e. 240 mM of NaCl, was detected in control (Fig. 2a-d). The data on average length and fresh weight of shoots and roots showed a strong inhibition with increasing level of salt solution in control as compared to Th-14, which proved to be effective both under normal (0 mM NaCl) and saline conditions. Th-14 was found effective among all the treatments in increasing length and fresh weight of shoots and roots (45.18 cm, 29.11 g and 19.50 cm, 14.97 g, respectively) closely followed by...
Th-19 (44.44 cm, 27.17 g and 18.35 cm, 13.95 g, respectively), considering mean at salt stress.

**Number of leaves.** Salinity concentration caused considerable reduction in the number of leaves. The effect of 150 mM and 240 mM NaCl was far more evident than that for 70 mM NaCl. The total number of leaves averaged over treatments indicated that it decreased (from 11.51 at 0 mM NaCl to 3.95 at 240 mM NaCl) significantly with increase in salt concentrations. Treatments differed significantly under both normal (0 mM NaCl) and different levels of salt concentrations (Fig. 2e). Salinity significantly affected the mean number of leaves. Minimum number of leaves (mean at salt stress = 6.9) was recorded in T1 (control) showing significant effect of salinity on leaf numbers by inhibiting their formation or increase, while maximum number of leaves was observed in Th-14 (mean at salt stress = 9.69) among all the treatments followed by Th-13 (8.53) and Th-19 (8.49), which showed 40, 24 and 23% increment, respectively, over T1 (control).

**Leaf area.** Salinity stress caused considerable reduction of the leaf area in all the treatments (Fig. 2f). However, a marked decrease in leaf area from 63.36 cm² at 0 mM NaCl to 15.24 cm² at 240 mM NaCl due to salt stress was observed in T1 (control) compared with other treatments. Maximum leaf area was recorded in Th-14 (mean at salt stress = 47.23 cm²) followed by Th-19 (46.02 cm²) and Th-13 (45.04 cm²) treated plants with substantial reduction under salt stress.

**Effect of seed biopriming with ST isolates of T. harzianum on the physiological parameters of rice un-**
der different levels of salt stress.

Leaf water content. The interaction of different treatments and salinity levels had non-significant effect on leaf water content (LWC) (Table 2). Maximal LWC (mean at salt stress = 86.57%) was achieved due to seed biopriming with Th-14 closely followed by Th-33 (85.82%) under normal (0 mM NaCl) conditions. Under saline conditions all the seed biopriming treatments slightly improved LWC as compared to control (Fig. 3a) but did not produce any significant change under saline conditions. Minimum LWC was recorded in T1 (82.13%) followed by Th-50 (84.87%), considering mean at salt stress.

Photosynthetic rate. Photosynthetic rate (PR) was significantly reduced in all treatments when plants were grown under salt stress. However, application of different Trichoderma treatments through seed biopriming resulted in significant increase in PR (Fig. 3b) in comparison to control, under both normal and saline conditions. Maximum PR was shown by plants bioprimed with Th-14 (mean at salt stress = 16.80 µmol CO$_2$/m$^2$/s) followed by Th-19 (mean at salt stress = 16.58 µmol CO$_2$/m$^2$/s), which were statistically at par, and minimum PR was recorded by control (14.45 µmol CO$_2$/m$^2$/s, considering mean at salt stress).

Chlorophyll fluorescence. Treatments exerted significant effect on chlorophyll fluorescence (CF) under salt stress. CF was almost equivalent in all the treatments
under normal condition (0 mM NaCl) but significant differences were noted amongst the treatments at higher salt stress levels (Fig. 3c). CF averaged over treatments decreased up to 49% from 0.71 at 0 mM NaCl to 0.36 at 240 mM NaCl. The lowest mean CF over salt stress treatments was observed in T1 (control). Plants obtained from seeds bioprimed with Th-14 showed relatively higher CF (mean at salt stress = 0.61) among all the treatments followed by Th-19 (mean at salt stress = 0.58), Th-13 (mean at salt stress = 0.57), Th-33 (mean at salt stress = 0.56) and Th-50 (mean at salt stress = 0.54), which were statistically at par.

Chlorophyll content and SPAD value. The chlorophyll content (CC) and SPAD value of leaves decreased at higher salt stress levels of 240 mM NaCl (1.62 mg/g fresh weight and 23.94, respectively) compared with 0 mM NaCl (2.65 mg/g fresh weight and 40.90, respectively) in all treatments. Treatment Th-14 accumulated maximum chlorophyll content and SPAD value (2.45 mg/g fresh weight and 36.11, respectively) closely followed by Th-19 (2.31 mg/g fresh weight and 35.43, respectively), while maximum decrease in CC (mean at
salt stress = 1.83 mg/g fresh weight) and SPAD value (mean at salt stress = 27.60) was detected in T1 (Fig. 3d and e). However, the interaction of both Trichoderma and salt application had non-significant effect on chlorophyll content and SPAD value (Table 2).

Linear regression was used to find the relationship between PR and maximum quantum yield of PS II (Fv/Fm), number of leaves, leaf area, LWC, CC, and SPAD value (Table 3). Positive relationships were found between PR and Fv/Fm, number of leaves, leaf area, CC and SPAD value. A strong significant positive ($R^2 = 0.815, P= 0.903$) relationship was observed between PR and Fv/Fm followed by that between PR and SPAD value ($R^2 = 0.799, P=0.894$), PR and CC ($R^2 = 0.788, P = 0.888$) and PR and leaf area ($R^2 = 0.732, P = 0.856$). There was also a non significant relationship between PR and LWC (Table 3).

**Effect of seed biopriming with ST isolates of T. harzianum on biochemical parameters of rice under different levels of salt stress.**

**Proline content.** Proline content increased under salt stress in all the treatments (Fig. 4a). The results demonstrated that the proline content of leaves significantly increased at higher salt stress level of 240 mM NaCl (17.57 µmol/g fresh weight) compared with normal condition of 0 mM NaCl (3.01 µmol/g fresh weight) in all treatments. Treatment Th-14 had accumulated maximum proline content (25.28 µmol/g fresh weight) followed by Th-19 (21.45 µmol/g fresh weight) and Th-13 (18.54 µmol/g fresh weight) at higher salt stress of 240 mM NaCl. Considering the mean value at salt stress, Th-13-treated plants accumulated approximately threefold maximum proline content (15.09 µmol/g fresh weight) as compared to the control (4.01 µmol/g fresh weight). However, this fluctuation was less dramatic among the Trichoderma treatments.

**Malondialdehyde content.** The malondialdehyde (MDA) content was higher in T1 (control) at all stress levels (Fig. 4b) from 0 mM NaCl (2.14 µmol/g fresh weight) to 240 mM NaCl (7.75 µmol/g fresh weight), followed by Th-33 (1.81 µmol/g fresh weight at 0 mM NaCl to 5.40 µmol/g fresh weight at 240 mM NaCl), indicating higher rate of lipid peroxidation. The accumulation of MDA content was lowest in treatment Th-14 (mean at all stress level = 2.10 µmol/g fresh weight) revealing reduced accumulation of lipid peroxides in seedlings raised from Th-14 bioprimed seeds under salt stress.

**Membrane stability index.** The membrane stability index (MSI) was high for all treatments under normal (0 mM NaCl) condition. The MSI decreased as salt stress level increased. The MSI was reduced to more than 50% under 240 mM NaCl (40.12) from normal (0 mM NaCl) condition (85.14), considering the mean MSI of all the treatments. Under saline conditions, all the Trichoderma pretreated treatments (T2=Th-13, T3=Th-14, T4=Th-19, T5=Th-33 and T6=Th-50) showed higher MSI (Fig. 4c) as compared to T1 (untreated). Among Trichoderma isolates, MSI was significantly high in Th-14 (63.22) followed by Th-19 (62.24) and Th-13 (60.51), considering mean at salt stress.

**Phenol content.** The phenol content increased substantially in all treatments with increase in salt stress level. The maximum phenol content under salt stress was recorded in Th-14 (6.55 mg/100 g fresh weight) followed by Th-19 (5.71 mg/100 g fresh weight) and Th-13 (5.29 mg/100 g fresh weight), which showed a steep increase under salt stress. The magnitude of increase in Th-14 from 70 mM to 240 mM NaCl was higher than that in control at 240 mM NaCl salt stress. Minimum phenol content (Fig. 4d) was observed in T1 (control) at all stress levels.

![Fig. 4](image-url)
DISCUSSION

The main objective of this study was to evaluate the possibility of reducing the negative effects of salinity stress in rice by the application of salinity tolerant isolates of Trichoderma harzianum through seed biopriming. Salt stress leads to various negative effects on different physiological and biochemical mechanisms related to plant growth and development. Salt stress has both osmotic (cell dehydration) and toxic (ion accumulation) effects on plants. Among several strategies used to improve crop growth under salt stress, use of salinity tolerant Trichoderma strains could be an effective and easily adaptive strategy. To improve the efficiency of such strategies and to develop new options, a better understanding of the physiological and molecular bases of salt tolerance in plants is required. Although a wide range of genetic adaptations to saline conditions has been observed, underlying mechanisms of salt tolerance in plants are still poorly understood. With respect to physiological and biochemical aspects of salt tolerance in crop plants, a number of mechanisms have been studied that operate at the whole plant level (Blum, 1998). In various plants, Trichoderma species are primarily being studied for their ability to control diseases, promotion of plant growth, and tolerance to abiotic stresses (Bae et al., 2009; Mastouri et al., 2010). Among several mechanisms employed by Trichoderma to enhance salt tolerance, the most studied mechanism appeared to be Trichoderma-induced significant changes in the plant metabolic machinery, which in most cases, benefits the plant (Segarra et al., 2007; Tijerino et al., 2011). In the present investigation, different salt-induced physiological and biochemical parameters that are supposed to be severely influenced under salt stress were taken into consideration to investigate the effect of Trichoderma on plant response under salt stress. Our study presents a novel insight into a possible role of T. harzianum in inducing relative salt tolerance in rice plants.

The root and shoot lengths are the most important traits for salt stress because roots are in direct contact with the soil and absorb water for shoot supply. For this reason, root and shoot lengths provide an important clue to the response of plants to salt stress (Jamil and Rha, 2004). Salinity reduced root and shoot lengths as the level of salt increased. Our results are in conformity with the findings of Jamil et al. (2007) who demonstrated that the reduction of plant growth under saline condition is a common phenomenon. According to Erhenhi et al. (2008), at higher salinities a significant reduction in growth occurred because of the inability of the plant to adjust osmotically. High salinity may inhibit root and shoot elongation due to lower water uptake by the plant (Werner and Finkelstein, 1995). In the present study, higher shoot and root length were observed in plants treated with Th-14 followed by Th-19 as compared to untreated plants under salt stress. These results are in agreement with those of other workers (Cornejo et al., 2009; Mohiddin et al., 2010) who reported that Trichoderma strains produce plant growth hormones like auxin, cytokinin-like molecules, gibberellins GA3 or GA3-related. Trichoderma strain T-22 has been shown to increase shoot and root development in maize and numerous other plants (Shoresh and Harman, 2008). Enhanced root growth helps with more water acquisition, thereby, increasing the ability of plants to resist abiotic stresses (drought, salt, etc) and uptake of nutrients.

Fresh weight of root and shoot was reduced by increasing salt levels. The results are in agreement with those of Jeannette et al. (2002) who reported that total fresh weight of root and shoot of cultivated plants was reduced with increased salt stress. However, maximum fresh weight of root and shoot was observed again in plants treated with Th-14 followed by Th-19. Root colonization by Trichoderma strains has been proved to enhance the total biomass of plants (Cornejo et al., 2009). Plant growth vigor, e.g., plant height or shoot biomass, is reported to have dilution effect on sodium accumula-

Table 3. Relation between photosynthetic rate (PR) and number of leaves, leaf area, LWC, CC, SPAD value and Fv/Fm of rice treated with different salinity tolerant Trichoderma isolates under different levels of salinity stress.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Regression equation</th>
<th>Regression coefficient (R²)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of leaves</td>
<td>Y = 0.418x+12.622</td>
<td>0.782</td>
<td>0.884**</td>
</tr>
<tr>
<td>Leaf area</td>
<td>Y = 0.219x+2.567</td>
<td>0.732</td>
<td>0.856**</td>
</tr>
<tr>
<td>LWC</td>
<td>Y = 0.738x+12.856</td>
<td>0.145</td>
<td>0.381ns</td>
</tr>
<tr>
<td>CC</td>
<td>Y = 2.822x+10.012</td>
<td>0.788</td>
<td>0.888**</td>
</tr>
<tr>
<td>SPAD value</td>
<td>Y = 0.186x+9.853</td>
<td>0.799</td>
<td>0.894**</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>Y = 8.317x+11.55</td>
<td>0.815</td>
<td>0.903**</td>
</tr>
</tbody>
</table>

Note: ² leaf area in cm², ² leaf water content in per cent, ³ chlorophyll content in mg/g fresh weight, ⁴ soil plant analysis development value, ⁵ maximal quantum yield of PS II.

x- denotes the parameters in the linear equation.

**significant at 1%; ns= non significant.
tion in leaves of rice (Yeo et al., 1990), which contributes in combating salt stress.

Plant exposed to saline environment generally have a reduced leaf area. In the present study, leaf area, CC and SPAD value were reduced as the salinity level increased with no significant effect on LWC (Fig. 2f; 3A, D and E). Earlier reports in radish, cabbage and sugar-beet (Jamil et al., 2007) support our results which show that leaf area, CC and SPAD value were significantly reduced by increasing salinity levels and the magnitude of the reduction varied among treatments. Salt stress reduced the leaf growth rate by shortening the length of the leaf elongating zone and decreasing growth intensity in its central and distal portions (Bernstein et al., 1995). At higher salinities, growth reduction could be attributed to a decrease in leaf area expansion (Marcelis and Hooijdonk, 1999). NaCl stress decreased the total CC of the plant by increasing the activity of the chlorophyll degrading enzyme chlorophyllase, inducing destruction of the chloroplast structure and destabilizing pigment protein complexes (Singh and Dubey, 1995). It has been reported that CC decreases in salt-susceptible plants (Hamada and El-Enany, 1994). In the present investigation, maximum CC, SPAD value and leaf area expansion were recorded in Th-14 followed by Th-19-treated plants while the values were minimum in the control (untreated) under both normal and salt stress conditions. However, the decrease in the above parameters with increased salinity was observed in all treatments.

Maximal quantum yield of PS II (Fv/Fm) was reduced consistently as the salt level increased, especially at higher salt concentrations, and the rate of decrease was higher in T1 (control) among all treatments. The reduction of CF is associated with the increase of Na accumulation (Dionisio-Sese and Tobita, 1998). Salt stress decreases the efficiency of photosynthesis (Ashraf and Shahbaz, 2003). Our results indicate that Trichoderma application through seed biopriming enhanced CF at all stress levels in comparison to the untreated control, with substantial reduction under salt stress.

It has been reported earlier that with salt stress PR decreases (Abdeshahian et al., 2010), which might be due to the fact that salt stress severely impairs photosynthetic activities as well as the photosynthetic apparatus. Reduction in photosynthesis could also be attributed to a decrease in CC (Delfine et al., 1999) or it might be due to reduction in leaf area per plant (Munns and Termaat, 1986). In the present investigation, reduction in PR was highest in the control while Th-14-treated plants showed maximum PR among all treatments at all stress levels. Photosynthesis is directly correlated with the number of leaves and leaf area per plant (Reich et al., 1999). It is well documented that Trichoderma increases the total biomass of plants, which might contribute to higher photosynthesis.

The data on MSI showed a decreasing trend with increase in salt concentration. The presence of NaCl in the rooting medium caused disturbance to membrane permeability expressed by an increase in solute leakage (Ghoulam et al., 2002). The leakage was higher in untreated plants than in Trichoderma-treated plants, indicating severe membrane damage in the former under salt stress. The higher leakage of solutes was probably due to enhanced H2O2 accumulation and lipid peroxidation under salt stress (Dionisio-Sese and Tobita, 1998). An important consequence of salt stress is generation of ROS. These oxidants, formed under salt stress, cause membrane disorganization and metabolic toxicity, resulting in higher leakage of solutes. Minimum MSI was recorded in the control, which was probably due to loss of ability to reorganize cellular membranes rapidly and completely. The leakage was lowest in Th-14-treated plants revealing reduced accumulation of lipid peroxides under osmotic stress. The reduced leakage in Trichoderma-treated plants might be related to induction of antioxidant responses that protect the plant from oxidative damage. Root colonization by T. harzianum T22 resulted in enhanced concentration of antioxidant plant enzymes like peroxidases, chitinases, etc. (Shoresh and Harman, 2008). These antioxidant enzymes act as scavengers of ROS, and thus, result in membrane stability. In the present study, a direct negative correlation was found between MSI and MDA content (Fig. 4C and B) as the former decreased with increase in level of MDA content.

A positive relationship was observed between PR and maximal quantum yield (Fv/Fm), number of leaves, leaf area, CC and SPAD value but PR did not correlate with LWC (Table 3). A similar relationship was observed by Seemann and Critchely (1985) and Jamil et al. (2007). Our data clearly support the general correlation between the photosynthetic capacity and leaf area, in agreement with the hypothesis of Reich et al. (1999) that no species can improve photosynthetic capacity without increasing leaf area due to biophysical limitations. Similar results were also observed by Schulze et al. (1994). In the present investigation, it was found that Trichoderma has a role in increasing the number of leaves and leaf area, which might have contributed to higher PR in Trichoderma-treated plants under salt stress in comparison to untreated (control) plants.

Proline accumulation is supposedly correlated with adaptation to salinity (Ashraf and Harris, 2004). The increased level of proline under salt stress has been reported in rice by Thach and Pant (1999). Our results implicate that proline accumulation in rice seedlings obtained from Trichoderma treated seeds was comparatively higher (2.49-4.62 fold, considering mean at salt stress) as compared to the control (untreated seeds). The higher concentration of proline under salt stress is favourable to plants as proline participates in the osmotic potential of the leaf and, thus, in the osmotic ad-
justment. The higher proline content in Th-14-treated plants under salt stress helped maintaining structure and function of cellular macromolecules. Besides the role of osmolyte, proline can also confer enzyme protection and increased membrane stability under various conditions. Proline accumulations may also help in non-enzymatic free radical detoxification caused by salinity (Khan et al., 2002).

Lipid peroxidation measured as the amount of thio- barbituric acid reactive substance or MDA is produced when polyunsaturated fatty acids in the membrane undergo oxidation by the accumulation of free oxygen radicals. As lipid peroxidation is the symptom mostly ascribed to oxidative damage, it is often used as an indicator of increased damage (Khan and Panda, 2008). Our results show that under both normal and salt stress levels, the degree of accumulation of MDA was higher in T1 (control) whereas the smallest accumulation was recorded in treatment T3 (Th-14) followed by T4 (Th-19), indicating reduction in the rate of lipid peroxidation as a result of seed biopriming with the Trichoderma strains. Lowest MDA content in Th-14 might be due to an increase in expression of stress-related proteins such as glutathione S-transferase (GST), glutathione-dependent formaldehyde dehydrogenase (FALDH), and peroxidase. A similar effect was observed by Trichoderma strain T-22 in maize (Shoresh and Harman, 2008). Under stress conditions, when activated free radicals are produced, these detoxifying proteins triggered by Trichoderma inoculation act as scavenging enzymes and play a central role in protecting the cell from oxidative damage.

Total phenolics increased significantly under different treatments with increase in salt stress. Phenolics are reported to play very important role to cope up with abiotic stress. Maximum phenol content was observed in plants treated with Th-14 (6.55 mg/100 g fresh weight), which was 2.5 fold more than that in T1 (control). Our results are supported by previous findings, which showed that root colonization by T. harzianum results in increased levels of plant enzymes, including peroxidases, chitinases and the resulting changes in plant metabolism could lead to the accumulation of compounds like phytoalexins and phenolics (Shoresh et al., 2010; Bae et al., 2011). Phenolic compounds, besides having antifungal, antibacterial and antiviral activities, possess also antioxidant properties, and, thus, act as scavengers of ROS.

The present research, thus, confirms the potential of Trichoderma to alleviate NaCl-induced growth reduction and other salt injuries in rice plants which might be due to the fact that Trichoderma colonizes and penetrates root tissues and initiates a series of morphological and biochemical changes in the plant. The resulting plant-mediated mechanism enhances natural defences against any biotic and abiotic stress. The overall positive effect of seed biopriming with Trichoderma resulted in improved growth, physiological and biochemical parameters under salt stress. Biopriming technique facilitates Trichoderma to enhance its colonization and number by almost hundred times on the spermosphere during the period of incubation in warm and moist conditions and also results in rapid and uniform seedling emergence (Singh et al., 2003). Roots obtained by these bioprimed seeds are colonized by Trichoderma, the resulting root-fungus association stimulates plant defense mechanisms. At the molecular level, resistance results in an increase in the concentration of metabolites and enzymes such as phenyl-alanine ammonia-lyase (PAL) and chalcone synthase (CHS), involved in the biosynthesis of phytoalexins, chitinases and glucanases (Mohiddin et al., 2010; Druzhinina et al., 2011). However, determining physiological and biochemical differences still leaves a long way from understanding genetic differences as the links between genes and physiology generally remain tenuous in plants. It is still necessary to make that jump to discover the genetic basis of phenotypic differences in resistance. Understanding of the mechanisms which regulate gene expression in response to salt stress into plants will expand the way in which plants can be utilized (Joseph and Jini, 2010).

In our results, seed biopriming with T. harzianum Th-14 was found best in terms of reducing the detrimental effects of salinity on growth, photosynthetic and biochemical parameters in rice. Plants pretreated with Trichoderma responded to salinity stress by modulating physiological and biochemical parameters, which lead to the restoration of cellular homeostasis, detoxification of toxins and recovery of growth. This area of research merits further attention and could, additionally, open the avenue for the use of Trichoderma application through seed biopriming in plants for enhanced salt tolerance. Research on new isolates of Trichoderma more tolerant to salt will help selecting more performing strains to be used through seed biopriming to alleviate salt stress in crops where salinity of soils constitute a factor that reduces crop productivity. The results of the seed biopriming technique with salinity tolerant isolates of T. harzianum recorded in different salt concentrations constitute a first phase for the potential use of these isolates in natural saline areas. It would be necessary to study the adaptation of these strains to saline soil conditions before introducing them in to different environments. Knowing the mechanism that underlies the plant response under salt stress to Trichoderma inoculation could be useful in designing new generations of more efficient biocontrol agents and growth enhancement strategies using Trichoderma in saline soils. In conclusion, Trichoderma treatments significantly improved the parameters affected by high salinity. Finally, in the future, these seed biopriming treatments may be used for improving plant growth and yield of rice in saline area.
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