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Physiological responses of crop plants against *Trichoderma harzianum* in saline environment

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Abstract – The physiological response of crop plants against *Trichoderma harzianum* (*Th*-6) in a saline habitat was studied. *Trichoderma harzianum* (*Th*-6) is an endophytic fungus that shows salt tolerance and establishes a symbiotic relationship with a host plant. To evaluate the role of *Trichoderma harzianum* (*Th*-6) in mitigating the consequences of salinity stress on crop plants, seeds of maize and rice were coated with *Trichoderma* before sowing and salt treatment. Later, after germination, twenty-one day old seedlings were subjected to NaCl concentrations (50, 100 and 150 mM). Salinity negatively affected all investigated physiological parameters in both crops. Treatment of seeds with *Trichoderma* improved plant growth and *Th*-treated plants exhibited substantial physiological adjustment in a saline environment compared to *Th*-untreated plants. The *Th*-treated plants under salt stress showed higher relative water content and stomatal conductance, better photosynthetic performance and higher pigment concentrations, as well as higher catalase and superoxide dismutase activities. Moreover, proline content in salt stress environment was higher in *Th*-treated plants, while H_2O_2 content declined. The physiological role of *Trichoderma harzianum* in mitigating the salt related consequences of both crop plants is discussed.

Keywords: antioxidant enzymes activity, maize, physiological performance, rice, salinity, *Trichoderma harzianum*

Introduction

Plants are often subjected to unfavorable changes in their environment. Abiotic stresses play a major role in reducing crop production around the globe (Bybordi 2012). Among them, salinity is one of the most important abiotic stresses that are widely distributed in both irrigated and non-irrigated areas of the world.

Research on salinity in plants has produced a vast literature showing its negative influence on crop plant productivity (Mahajan and Tuteja 2005, Oliveira et al. 2013). Most salt sensitive crops cannot tolerate a high concentration of NaCl, especially in the soil (Prasad et al. 2000). This results in poor germination, growth and biomass allocation (Neumann 2008, Ahmad and Prasad 2012). Studies have shown that plants at vegetative and reproductive phases are more sensitive to soil salinity (Hu and Schmidhalter 2005, Lauchli and Grattan 2007, Siddiqui et al. 2014). Prolonged exposure to salinity causes specific ion toxicity, nutritional and hormonal imbalance, reduced water potential and so on (Ahmad et al. 2010, Siddiqui and Khan 2013)

Trichoderma sp are beneficial endophytic plant symbionts that are widely used as biocontrol agents against fungal diseases in crop plants (Harman 2011, Afzal et al. 2013). However, some studies have reported that *Trichoderma* induces tolerance to biotic and abiotic stresses in plants (Monnet et al. 2001, Evelin et al. 2009, Mastouri et al. 2010, Shoresh et al. 2010, Estrada et al. 2013). Treatment of seed with *Trichoderma* spp in many cereals and vegetable crops has a positive impact on plant growth, improving hormone performance (Howell 2003, Harman 2006), which could enhance tolerance to salinity stress (Gachomo and Kotchoni 2008, Rawat et al. 2011, Hashem et al. 2014). However, the role of *Trichoderma harzianum* in crop plant tolerance to abiotic stress like salinity and drought and the physiological mechanism involved needs to be closely monitored. Therefore, the present study was designed to examine the physiological responses of two crops plants, maize and rice, in a saline environment following seed treatment with *Trichoderma harzianum* (*Th*-6). Some physiological attributes of salt tolerance were selected for this study.

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Materials and methods

Seed selection

Seeds of maize (*Zea mays* L.) var. NT6621 and rice (*Oryza sativa* L.) var. Kernel were obtained from the Department of Plant Protection, Karachi, Pakistan. Seeds were surface sterilized in 10% sodium hypochlorite solution for 3 minutes and rinsed thoroughly with distilled water then air dried.

Culture collection and treatment

The pure strain of *Trichoderma harzianum* (*Th*-6) was obtained from the Plant Pathology Laboratory, Department of Botany. First, an experiment was conducted in petriplates containing potato dextrose agar at different NaCl concentrations (25, 50, 100, 150, 200 mM) together with a *Trichoderma harzianum* (*Th*-6) disc for 8 days. The salt concentration was selected according to the optimum growth achieved by *Trichoderma harzianum* in saline media (Fig. 1). Seeds of maize (*Zea mays* L.*)* var. NT6621 and rice (*Oryza sativa* L.) var. Kernel were treated with *Trichoderma harzianum* using 2% gum arabic as sticker. The colony forming unit (cfu) was 67.3 conidia 10^{-3} of *Trichoderma.* Later, seeds were sown on a pot filled with 500 g soil each (one plant per pot). Autoclaved (1 hour at 80 °C) soil was used for the experiment with the following composition: sand particles; 80.5, silt; 7.1, clay; 8.1, organic carbon; 0.20, nitrogen. pH 7.5 and EC 1.8 ds m^{-1} were recorded according to Dahnke and Whitney (1988) by a CMD 500 WPA conductivity meter, Linton Cambridge U.K). Maize and rice crops were allowed to grow at an average day-night temperature (26±4 °C and 18±3 °C). Salt stress was applied to twenty-one-day old seedlings each day by using 25 mM NaCl to achieve the desired level (Gorham et al. 1987) and moisture contents were maintained with tap water. Three NaCl concentrations (50, 100 and 150 mM NaCl) were applied. Plant treated with tap water served as control. Each treatment and control was replicated four times. Root, shoot length, biomass, physiological parameters and antioxidant enzyme activities were examined.

Fig. 1. *Trichoderma harzianum* was cultured in potatoes dextrose agar containing 100 mM NaCl.

Relative water content

Four leaf strips of 4×2 cm^2 from the mid-veins and the edge section of leaves were cut with scissors from each treatment of rice and 4 1.2 cm^2 discs of maize were excised and fresh weights (FW) were determined. For the measurement of turgid weight (TW), leaves were left in distilled water for 24 h under low irradiance condition. Samples were then dried at 80 °C for 48 h in oven and dry weight (DW) was determined. Relative water content (RWC) was calculated by the fresh leaf sample method described by Barrs and Weatherley (1962) and modified as:

$$RWC=(FW–DW/TW–DW)\times100$$

Quantum yield PSII and stomatal conductance

Measurements of chlorophyll fluorescence emission from the 20 randomly selected leaves were monitored with a fluorescence monitoring system (Handy PEA) in the pulse amplitude modulation mode. A leaf adapted to dark conditions for 30 min using leaf clips, was initially exposed to the modulated measuring beam of far-red light (LED source with typical peak at wavelength 735 nm). The original (F_0) and maximum (F_m) fluorescence yields were measured under weak modulated red light (0.5 µmol m^{-2} s^{-1}) with 1.6 s pulses of saturating light (6.8 µmol m^{-2} s^{-1} PAR). The variable fluorescence yield (F_v) was calculated by the equation $F_m–F_0$. The ratio of the variable to maximum fluorescence (F_v/F_m) was calculated as the dark-adapted quantum yield of PSII photochemistry and performance index and non-photochemical quenching were calculated as described by Maxwell and Johnson (2000). Likewise, the stomatal conductance (g_s) of 20 randomly selected leaves of each treated and control plant was examined using a leaf porometer (Model SC-1, Decagon).

Photosynthetic pigment extraction and estimation

Leaf samples (0.5 g) were ground in 10 mL of 96% methanol and then centrifuged at 4000 rpm for 10 min. Total chlorophyll [$Chl_{(a+b)}$], chlorophyll a (C_a), and chlorophyll b (C_b) contents were determined according to Lichtenthaler (1987). The supernatant was separated and the absorbances were read at 666, 653 and 470 nm on spectrophotometer. The amount of these pigments was calculated according to the following formulas:

$$C_a=15.65\times A_{666}–7.340\times A_{653}$$

$$C_b=27.05\times A_{653}–11.21\times A_{666}$$

$$C_{x+c}=1000\times A_{470}–2.860\times C_a–129.2\times C_b/245$$

where, C_a – Chlorophyll a, C_b – Chlorophyll b, C_{x+c} – total carotenoids

Chlorophyll contents of leaf tissues were expressed as µg mg^{-1} FW.

Proline content

Proline content was measured according to the procedure of Bates et al. (1973). Leaf samples (0.5 g) were homogenized with 5 mL sulphosalicylic acid (3% w/v) and the homogenate was filtered on Whatman No. 1 filter paper.

Then, 2 mL of extract in test tube was taken and 2 mL of glacial acetic acid and 2 mL of acid ninhydrin where added. The mixture was heated in a boiling water bath at 100 °C for an hour. A brick red color developed. After cooling of the reaction mixture, 4 mL toulene was added and mixed vigorously for 15 to 20 seconds. Chromophore containing toluene was separated from the aqueous phase. Then the mixture was allowed to reach room temperature. The absorbance was recorded at 520 nm against a toluene blank. Proline content in sample was estimated by referring to a standard curve made from known concentrations of proline by taking following formula:

$$\mu mol\ proline\ g^{-1}_{FW} = (\mu g\ proline/mL \times mL\ toulene)/ /115.5\ \mu g/\mu mol)/(g\ sample)/5$$

H_2O_2 production

Hydrogen peroxide content was measured according to the procedure of Velikova et al. (2000). Freshly harvested leaf samples (100 mg) were homogenized with 3 mL of 0.1% (w/v) trichloroacetic acid in an ice bath and the homogenate was centrifuged at 12,000 g for 15 min. Later, 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI) were added to 0.5 mL of the supernatant. The absorbance of the supernatant was read at 390 nm. The amount of H_2O_2 was calculated using a standard curve and expressed as μmol g^{-1} FW.

Enzyme assays

Leaf samples (500 mg) were crushed and homogenized in 10 mL protein extraction buffer containing Tris-HCl pH 6.8, 50 mg polyvinylpyrrolidone and 0.05 mM ethylenediaminetetraacetic acid (EDTA). Whole contents were centrifuged at 12,000 rpm for 10min in a Smart R-17, Hanil centrifuge. The supernatant was collected and used to determine the activities of catalase and superoxide dismutase. Total protein was estimated by the method of Bradford (1976).

Catalase (CAT; EC 1.11.1.6) activity was estimated by method of Patterson et al. (1984). The decomposition of H_2O_2 was measured at 240 nm taking Δε as 43.6 mM cm^{-1}. The reaction mixture (3.0 mL) consisted of 10.5 mM H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0) and the reaction was initiated after the addition of 0.1 mL enzyme extract at 25 °C. The decrease in absorbance at 240 nm was used to calculate the activity. One unit of CAT activity is defined as the amount of enzyme that catalyzes the conversion of 1 mM of H_2O_2 min^{-1} at 25 °C.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was recorded according to the method of Beyer and Fridovich (1987). The reaction mixture consisted of 27.0 mL of 0.05 M potassium phosphate buffer (pH 7.8), 1.5 mL of L-methionine (300 mg per 2.7 mL), 1.0 mL of nitrobluetetrazolium salt (14.4 mg per 10 mL), and 0.75 mL of Triton X-100. Aliquots (1.0 mL) of this mixture were delivered into small glass tubes, followed by the addition of 20 mL enzyme extract and 10 mL of riboflavin (4.4 mg per 100 mL). The cocktail was mixed and then illuminated for 15 minutes in an aluminum foil-lined box, containing 25 W fluorescent tubes. In a control tube the sample was substituted for by 20 mL of buffer and the absorbance was measured at 560 nm. The reaction was stopped by switching off the light and placing the tubes in the dark. Increase in absorbance due to the formation of formazan was measured at 560 nm. Under the described conditions, the increase in absorbance in the control was taken as 100% and the enzyme activity in the samples were calculated by determining the percentage inhibition per minute. One unit of SOD is the amount of enzyme that causes a 50% inhibition of the rate for reduction of nitrobluetetrazolium salt under the conditions of the assay.

Statistical analysis

Statistical analysis was carried out using the personal computer software packages SPSS version 20. All data were subjected to SPSS and two-way ANOVA was performed.

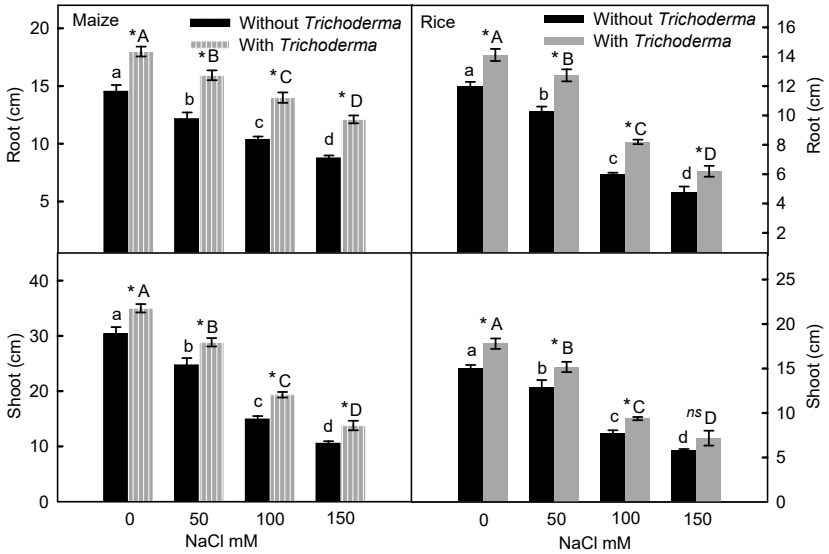

Fig. 2. Effect of *Trichoderma harzianum* seed treatment on seedling growth of maize (*Zea mays* L.) var. NT6621 and rice (*Oryza sativa* L.) var. Kernel in saline environment. Results are expressed as means±standard errors. Same alphabets show non-significant difference within each treatment, (*) stands for significant and (*ns*) for non-significant difference among the treatments with and without *T. harzianum.*

Results

The results showed that shoot and root length significantly declined with the increase in salinity concentration in the soil (Fig. 2). However, seed treated with *Trichoderma harzianum* (*Th)* has shown substantial increase in plant growth. Shoot and root length increased significantly in *Th*-treated maize and rice plants subjected to 50, 100 and 150 mM NaCl treatments as compared to those plants that were not treated with *Trichoderma* (Fig. 2).

Relative water content (RWC) of maize and rice significantly decreased at all salinity concentrations (50, 100, 150 mM) (Fig. 3). However, in *Th*-treated plants, the adverse effects of salinity were alleviated, showing a substantial increase in the RWC of both crop plants over *Th*-untreated plants.

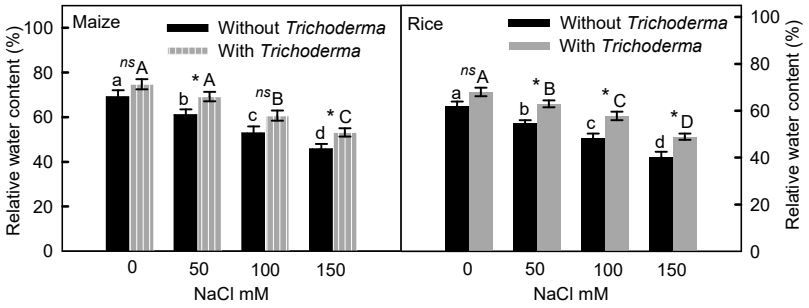

Fig. 3. Effect of *Trichoderma harzianum* seed treatment on relative water content and biomass accumulation of maize (*Zea mays* L.) var. NT6621 and rice (*Oryza sativa* L.) var. Kernel in saline environment. Results are expressed as means±standard errors. Same alphabets show non-significant difference within each treatment, (*) stands for significant and (*ns*) for non-significant difference among the treatments with and without *T. harzianum.*

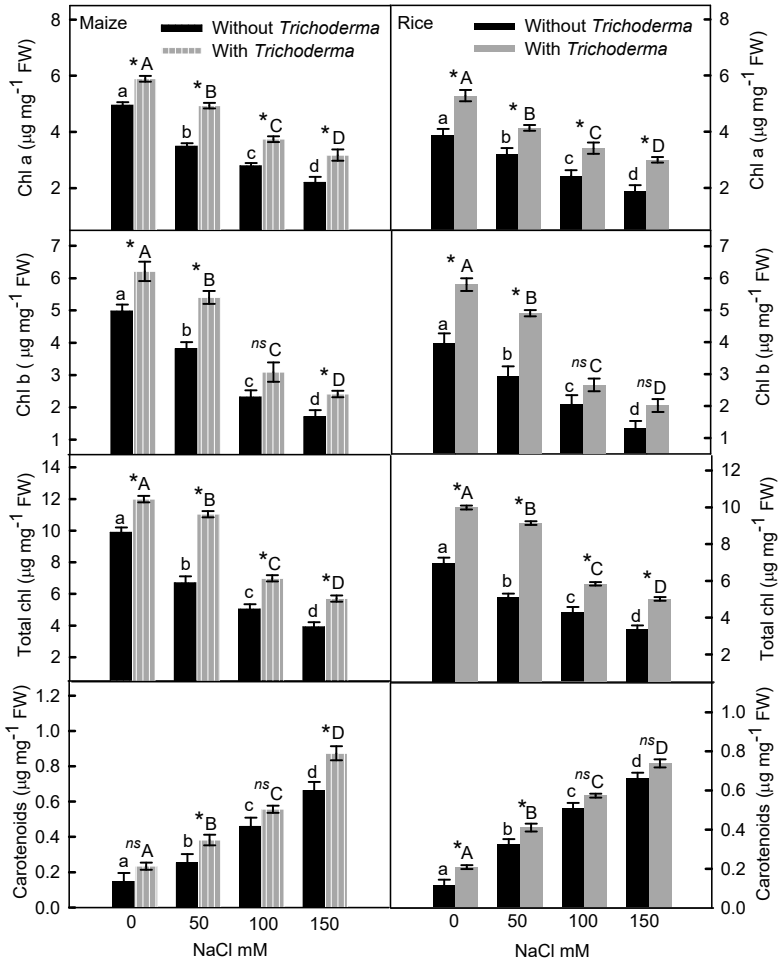

Fig. 4. Effect of *Trichoderma harzianum* seed treatments on photosynthetic pigments of maize (*Zea mays* L.) var. NT6621 and rice (*Oryza sativa* L.) var. Kernel in saline environment. Results are expressed as means±standard errors. Same alphabets show non-significant difference within each treatment, (*) stands for significant and (*ns*) for non-significant difference among the treatments with and without *T. harzianum.*

Salinity caused a considerable decrease in pigment content but *Th* treatment mitigated the salinity affect and improved pigment concentration during all NaCl treatments (Fig. 4).

The values of photosynthetic attributes like dark-adapted quantum yield (F_v/F_m ratio), performance index (PI_{abs}), photochemical quenching (qP) and stomatal conductance (g_s) were reduced in all salt treatments but *Th*-treated plants showed higher values than *Th*-untreated plants (Fig. 5).

In the present study, free proline and H_2O_2 contents of both crop plants were measured at different NaCl concentrations (50, 100, and 150 mM) with and without the *Trichoderma* inoculum (Fig. 6). Results revealed that proline and H_2O_2 contents were significantly influenced by the presence of *Trichoderma* in a saline environment. However, in comparison between the crop plants, free proline content was significantly higher in maize than in rice. Seeds that were treated with *Trichoderma* showed maximum accumulation of proline content in all saline treatments (0, 50, 100, 150 mM) as compared to those treatments without *Trichoderma.* However, maximum proline content was recorded at 150 mM NaCl (Fig. 6). H_2O_2 production was elevated at all salinity levels in plants that were not treated with *Trichoderma* (Fig. 6). However, H_2O_2 production significantly lowered with the increasing concentration of NaCl in *Th*-treated plants.

Activity of antioxidant enzymes like SOD and CAT were measured at different NaCl concentrations with or without the *Trichoderma* inoculum (Fig. 7). Observations revealed that the SOD and CAT activities of both maize and rice plants were substantially increased with increased NaCl concentration. However, in a comparison between the crop plants, a maize plant showed greater antioxidant activity than rice (Fig. 7). It was observed that the presence of *Trichoderma* in a saline environment additionally increased the activity of antioxidant enzymes as compared to plants in saline medium without *Trichoderma.*

Discussion

Salinity is a major abiotic factor that restricts plant growth and productivity, which not only causes osmotic stress but also alters physiological and biochemical mechanism in plants. Crops such as rice and maize (Poaceae) are sensitive or moderately sensitive to salinity. They are unable to tolerate a higher amount of salt in soil. Results showed that the application of *Trichoderma* to the crop plants enhances tolerance to a high concentration of NaCl. *Trichoderma* is an endophytic symbiont, as its inoculation

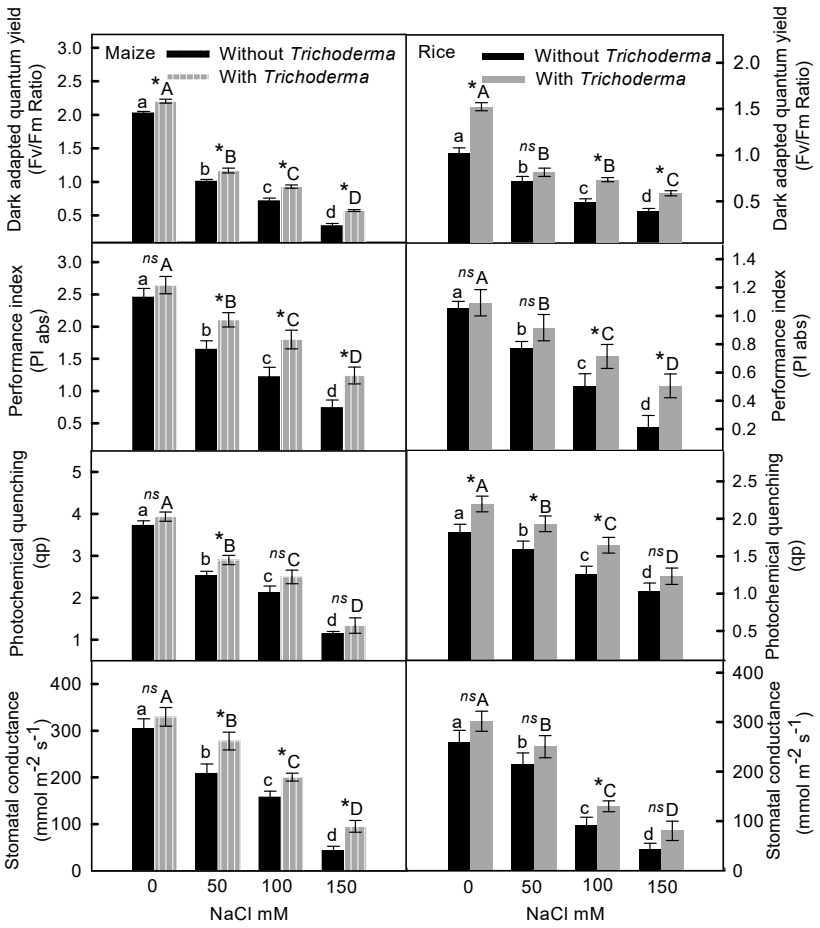

Fig. 5. Effect of *Trichoderma harzianum* seed treatments on photosynthetic attributes of maize (*Zea mays* L.) var. NT6621 and rice (*Oryza sativa* L.) var. Kernel in saline environment. Results are expressed as means±standard errors. Same alphabets show non-significant difference within each treatment, (*) stands for significant and (*ns*) for non-significant difference among the treatments with and without *T. harzianum.*

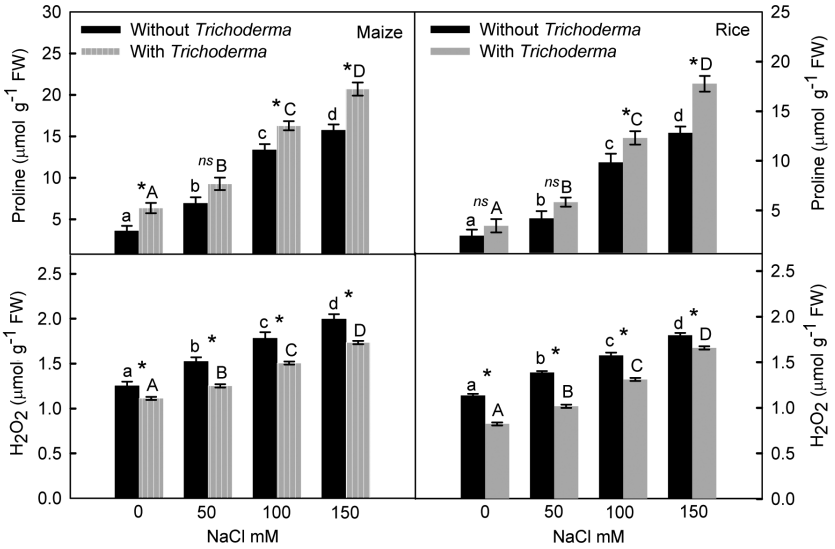

Fig. 6. Effect of *Trichoderma harzianum* seed treatments on proline and hydrogen peroxide content of maize (*Zea mays* L.) var. NT6621 and rice (*Oryza sativa* L.) var. Kernel in saline environment. Results are expressed as means±standard errors. Same alphabets show non-significant difference within each treatment, (*) stands for significant and (*ns*) for non-significant difference among the treatments with and without *T. harzianum.*

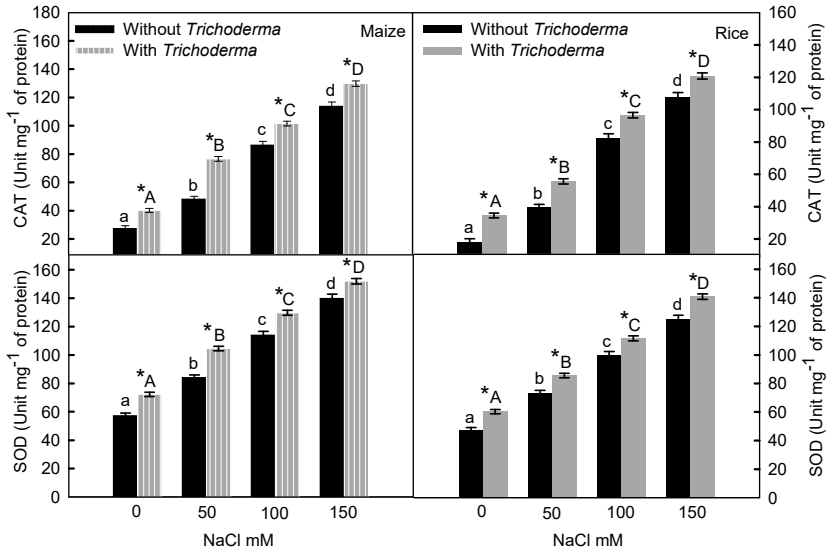

Fig. 7. Effect of *Trichoderma harzianum* seed treatments on catalase (CAT) and superoxide dismutase (SOD) antioxidant enzyme activity of maize (*Zea mays* L.) var. NT6621 and rice (*Oryza sativa* L.) var. Kernel in saline environment. Results are expressed as means±standard errors. Same alphabets show non-significant difference within each treatment, (*) stands for significant and (*ns*) for non-significant difference among the treatments with and without *T. harzianum.*

has antagonistic properties and therefore enhances the systemic tolerance to salt stress in plants (Harman et al. 2004).

It was observed that salinity caused a substantial reduction in growth and biomass of those plants without a *Th* treatment. The application of *Th* mitigates salt-related consequences in plants, which results in considerable increases in growth and biomass production. It was reported that the application of *Th* in a saline habitat improved biomass and growth parameters (Moud and Maghsoudi 2008, Rasool et al. 2013, Ahmad et al. 2014). The increase in growth relative water content and biomass production with a *Th* application may be due to its ability to produce phytohormones like gibberellins and cytokine, which may not only promote the plant growth but also increase some degree of tolerance in a saline environment (Harman 2000, Benitez et al. 2004, Iqbal and Ashraf 2013, Ahmad et al. 2015).

Relative water content decreased in maize and rice crops under NaCl stress but increased due to the application of *Th*. The maintenance of a substantial amount of relative water content in leaves is a main strategy for maintaining optimal growth of plants under salinity (Siddiqui et al. 2014). It was reported that *T. harzianum* provides better ability to regulate additional intracellular water relations due to biomass accumulation resulting from the uptake of more water under salt stress (Rawat et al. 2011, Hashem et al. 2014).

Photosynthetic attributes like F_v/F_m ratio, PI_{abs}, qP and g_s of *Th*-treated plants were increased in all NaCl treatments compared to *Th*-untreated plants. It has been demonstrated that content of plant photosynthetic pigments like chl *a*, chl *b*, total chlorophyll and carotenoids generally reduces under NaCl (Parida and Das 2005, Sairam et al. 2002). The result of the decrease in carotenoid contents under salt stress might be due to decrease in β-carotene and zeaxanthin formation (Sultana et al. 1999). Salt stress directly affects the chloroplast function, degrading enzymes which results in substantial reduction in photosynthesis of plants (Siddiqui et al. 2014). However, in the present study, the application of *Trichoderma* in a saline environment produced a considerable increase in all the tested photosynthetic attributes. It was reported that *Th* stimulates the synthesis of chlorophyll enzymes and phytohormones under different biotic stress in plants (Rawat et al. 2011, Zhang et al. 2013, Hashem et al. 2014). There was a maximum amount of photosynthetic pigments present in *Th*-treated plants in maize and rice crops as compared to untreated plants under NaCl condition. Further, these results are in accordance with the results of Mishra and Salokhe (2011) who reported that inoculation of seed by *Trichoderma* enhanced pigment system PSII performance and produced a higher rate of transpiration in plants under salt stress conditions. In plants subjected to salinity stress, photosynthetic rate and stomatal conductance might be disturbed due to the higher amount of Na^+ ions accumulation which disturb the electron transport chain during photosynthesis (Kanwal et al. 2011). From the present investigation, it was clearly observed that the dark adapted quantum yield (F_v/F_m ratio), performance index (PI_{abs}), photochemical quenching (qP) and stomatal conductance (g_s) were decreased with the increase in salinity concentration but increased by *Th* application. This could be due to an increase in chlorophyll concentration by the application of *Trichoderma* in saline environment (Sheng et al. 2008).

Results showed a substantial decrease of proline and an increase of H_2O_2 contents in untreated plants subjected to salt stress, but increased proline with decreased H_2O_2 production in *Th*-treated plants was observed in both crops. It was suggested that proline accumulation provided protection to the cell through balancing osmolyte concentration under salt stress conditions in tolerant plants (Greenway and Munns 1980). In an abiotic stress like salinity, elevated H_2O_2 production damages protein and lipid molecules (Siddiqui et al. 2014). It is presumed that the application of *Trichoderma* lowers H_2O_2 production in salt stress due to proline accumulation. Moreover, it was reported that *Trichoderma* enhances antioxidant enzymes such as glutathione S-transferases (GSTs) and peroxidase (POD) activities and lowers ROS production (Hajiboland et al. 2010, Wu et al. 2010, Alqarawi et al. 2014).

It was reported that maximum quantum yield and photosynthetic performance control the production of ROS (Siddiqui and Khan 2013, Siddiqui et al. 2014). Photosynthetic performance and quantum yield are inter-related and are often decreased in abiotic stress and under elevated ROS level. Fluctuating response with respect to quantum yield and performance index in stress environment are diversified and specific for some plant species (Behera et al. 2002). Substantial photosynthetic performance index and quantum yield in maize treated with *Trichoderma* indicate that maize could develop better symbiotic relationship with *Th* in saline environment compared to rice.

In our study, it was detected that the presence of *Trichoderma* in a saline environment increased the activity of antioxidant enzymes as compared to those saline media that did not have *Trichoderma.* Earlier, it was reported that the activities of antioxidant enzymes like CAT, SOD, POD and ascorbate peroxidase (APX) were increased in a saline environment (Siddiqui 2013). It is presumed that the presence of *Trichoderma* in a saline environment diminished H_2O_2 production due to elevated antioxidant enzyme activities as well as proline production. It was reported that antioxidant enzymes, production of osmolytes and polyols like proline, sorbitol etc. are important physiological strategy for coping with the consequences of abiotic stress and maintaining ion homeostasis (Sairam et al. 2002, Siddiqui and Khan 2011, Rasool et al. 2013). Further it was observed that a *Trichoderma* inoculation enhanced antioxidant enzyme activities and decreased salt stress in plants (Hajiboland et al. 2010, Hashem et al. 2014, Ahmad et al. 2015).

It can be concluded that application of *Trichoderma harzianum* enhances salt tolerance of maize and rice through higher antioxidant activities and high proline content. Treatment with *Trichoderma harzianum* not only enhanced some physiological parameters but also lowered the H_2O_2 concentration reducing the damaging effect of ROS within plants.

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