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Selection of Salinity Tolerant Trichoderma Isolates and Evaluating their Performance in Alleviating Salinity Stress in Rice (Oryzae Sativa L.)

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Abstract: *The impact of salt tolerant Trichoderma harzianum isolates applied through seed treatment and seedling dip at the time of transplanting on rice response to salinity was studied. Growth, physiological and biochemical parameters severely affected under salinity stress were monitored under greenhouse conditions with or without stress to explore the mechanism underlying plant abiotic stress resilience in response to Trichoderma inoculation.*

With or without exposure to salt stress, the impact of Trichoderma inoculation on rice was enhancement in seedling growth and fresh weight of shoot and root. Root colonization by Trichoderma altered many physiological and biochemical parameters like chlorophyll content, chlorophyll fluorescence and greenness of plants. Salt stress by supplementing different concentrations of NaCl viz., 0, 70, 150 and 240 mM NaCl induced an increase in the concentration of many stress induced metabolites in rice leaves of control plants, while seedlings obtained from seeds pretreated with Trichoderma caused a decrease in MDA and H_2O_2 contents while an increase in proline and phenolics concentration. Gene expression profiling for some key genes potentially involved under stress condition such as SOD, PAL, APX, and P5CS was done trough RT-PCR (reverse transcriptase) and found that stress induced changes in gene expression pattern were significantly effected in Th-14 treated seedlings. Thus, these experiments indicate that rice plants can exhibit enhanced salt stress tolerance via root colonization with endophytic fungus Trichoderma. These findings provide a novel paradigm for developing alternative, environmentally safe strategy to alleviate salt stress which may be useful in mitigating impacts of climate change and expanding agricultural production onto marginal lands.

Keywords: *Oryza sativa, Trichoderma treatments, salinity stress, root colonization.*

INTRODUCTION

Climate change and catastrophic events have contributed to rice shortages in several regions due to decreased water availability and soil salinization. During the last several decades, there have been major climatic events that decreased agricultural productivity of rice (one of the four major food crops) at locations around the world[1]. Salinity is a widespread soil problem in rice-growing countries. The area affected by salinity in the world covers about 400 million hectares, of which 54 millions are found in south and Southeast Asia[2]. Rice (Oryza sativa L.) as a paddy field crop is considered as a salt sensitive monocot[3]. The addition of salts to water lowers its osmotic potential, resulting in decreased availability of water to roots and thus exposes to plants to secondary osmotic stress. This implies that all the physiological responses associated with the drought stress can also be invoked by salt stress. High salt concentration primarily damages cell membrane and inhibits the activity of various enzymes and functioning of photosynthetic apparatus.

The need of the improvement of salt tolerance in crop plants, rice in particular, is well documented[4]. Breeding for salt tolerance in rice is difficult due to the involvement of several genes and insufficient knowledge about mechanism(s)

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controlling the character[4]. In spite of extensive literature, there is still a controversy with regard to the mechanisms of abiotic stress tolerance of plants[5]. Plant adaptation to abiotic stresses likely involves a combination of phenotypic plasticity and genetic constitution & it is thought to involve processes exclusive to the plant genome. However, the mechanisms responsible for adaptation of plants to high stress are poorly defined[1].

Attempts to reduce soil salinity using methods such as irrigation, drainage, reclamation as well as plant hormones are not always practical or economical. However, most ecological studies consider the fact that all plants in natural ecosystems are thought to be symbiotic with fungal endophytes and these endophytes can have profound effects on plant stress tolerance and fitness[1]. Fungal endophyte Trichoderma sp. can confer fitness benefits to plants including increased root and shoot biomass, increased yield, tolerance to abiotic stresses such as heat, salt, drought and to biotic stresses such as pathogens and hervibores[6]. Incorporation of Trichoderma in many cereals has resulted in induced disease resistance, plant growth promotion, improved abiotic stress tolerance and some of the mechanisms involved in the alteration of drought response by Trichoderma include drought avoidance through physiological and biochemical adapta-tions, and enhanced drought recovery[7].

Root size and archi-tecture are the factors which determine yield performance, particularly under conditions of limited water availability[8]. The root colonization by Trichoderma increases the growth of roots and of the entire cacao plant, thereby increasing plant productivity and the yields of reproductive organs[9]. However, one of the most crucial boundaries to use Trichoderma strains in saline soils is their low osmotolerance level. Soil hydrological characters are considered as restraining parameters touching Trichoderma activities, most particularly spore germination, germ tube growth, mycelial growth as well as on enzyme production[1o]. In view of this, it is necessary to study the influence of abiotic stresses on Trichoderma strains before introducing them in heterogenous environmental conditions. There is always the need to screen for stress tolerant Trichoderma isolates by testing them under lab conditions through applying artificial stress and in fields where such adverse conditions prevail.

The present study is therefore conceived with to ameliorate the adverse effects of salinity stress in rice plants with a particular reference by using endophytic fungus Trichoderma and to examine the treatments induced changes in leaf water content, chlorophyll content, proline content, membrane stability, lipid peroxidation and expression of stress related genes in rice plants.

MATERIALS AND METHODS

Selection of Salt Tolerant Trichoderma Antagonists

Forty five Trichoderma isolates, obtained from the repository of Biocontrol Laboratory of Discipline of Plant Pathology, College of Forestry, Ranichauri, V.C.S.G Uttarakhand University of Horticulture and Forestry, were tested for their tolerance against salinity stress. Those isolates were grown on PDA supplemented with different concentrations of NaCl *viz.*, 4, 6, 9 and 10 dS/m and incubated at 27 ±2°C for 4 days.

Five isolates *viz.*, Th-13, Th-14, Th-19, Th-33 and Th-50, selected on the basis of their better performance on PDA amended with different levels of salt stress, were selected for further evaluation at higher salt concentration upto 240 mM NaCl and were considered as salinity tolerant (ST). Th-25, selected randomly from the left out isolates showing adverse effect on its growth at different salt stress levels, was considered as salinity sensitive (SS) isolate and used for the comparative study.

Measure of Linear Growth and Mycelial Dry Weight of Selected Trichoderma Isolates at Different Salt Stress Levels

The growth of the Trichoderma isolates in natural (PDA) and saline media (PDA amended with different NaCl conc. *viz.*, 0, 70, 150 and 240 mM) was estimated by the measurement of linear growth after four days of incubation at 27± 2°C. After filtration on muslin of two weeks old Trichoderma liquid cultures, the collected mycelium was washed twice and put to dry at 80°C over the night. Thereafter, dry weight of the mycelium was measured.

Greenhouse Experiments

Five ST Trichoderma isolates *viz.*, Th-13, Th-14, Th-19, Th-33 and Th-50, selected on the basis of their better growth on PDA amended with different levels of salt stress were used for further evaluation against salinity stress under green house conditions. A pot experiment was conducted under greenhouse conditions to assess the efficiency of selected saline tolerant Trichoderma isolates in inducing relative salt stress tolerance of rice plants.

Preparation of Talc-based Formulation of Antagonists

Mass culture of each selected Trichoderma isolate was prepared separately on barnyard millet (Echinocloa frumentacae) grains. Grains were soaked in water for 12 h and filled in 250 ml Erlenmeyer flasks (@ 50 g/flask). The flasks were autoclaved at 15 lbs psi for 30 minutes. After cooling to room temperature, the flasks were inoculated with mycelial discs cut from three-day old cultures of Trichoderma and incubated at 27±2°C for 12 days.

Trichoderma colonized barnyard millet grains were air dried in open shade and ground with Willy Mill to a fine powder. The powder was passed through 50 and 80 mesh size sieves to obtain a pure spore powder. The talc formulation was prepared by diluting spore powder with talcum powder (350 mesh, 95% whiteness) and 1 % carboxy methyl cellulose (CMC), used as a sticker, to get desired concentration of biocontrol agents in the formulation. The final Trichoderma inoculum in the formulation was adjusted to 5×10^6 Cfu/g.

Experimental Site

The experiment was conducted during 2014-15 in the green house of Discipline of Plant Pathology, College of Forestry, Ranichauri, V.C.S.G Uttarakhand University of Horticulture and Forestry. The conditions maintained in the green house during the experiment were- minimum of 25°C during the night and 28°C during the day: automatic venting at 33±3°C with supplemental light for 12 hd^{-1}. The light flux density ranged from 400 μ mol $m^{-2}s^{-1}$ to 1000 μ mol $m^{-2}s^{-1}$.

Seed Material

Rice (PD-11) seeds were obtained from Krishi Vigyan Kendra, Ranichauri, V.C.S.G UUHF, Bharsar. 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.

Seed Bioprimimg

Seeds were separately treated with talc formulation of each selected isolate of Trichoderma spp. at the recommended dose of 10g/kg of seeds. Untreated seeds were considered as control. Seeds were then kept under warm and moist conditions at 28°C for 24 hrs until prior to radical emergence. Those seeds were shown in nursery and again root treatment with abovementioned treatments was given prior to transplanting.

Salinity Stress

Greenhouse experiment was performed with plants grown in a temperature controlled room with a 12 hr fluorescent light regime. Pots were randomly placed in the growth room for salt stress experiments. Eight seedlings of 28 days old were transplanted in plastic pots (16 cm diameter and, 22cm height) filled with 3 Kg of air dried 1:1 soil and sand mixture previously passed through 2mm sieve. Roots treatment by seedling dip in each selected talc formulation of Tricchoderma @ 10g/ liter was given prior to transplanting.

After a week, thinning was done to 5 seedlings per pot. The plants were watered regularly after transplanting for one 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 200 mM) in complete Hoagland's nutrient solution for next 21 days. The experiment was laid out in a completely randomized design (CRD) with three replications.

Observations

All the measurements on the young and fully expanded leaves were made, when plants had achieved a steady state after 21 days of salinity stress to rice plants. Leaf samples were collected from two plants per treatment per replication.

Growth Parameters

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

Measurement of Leaf Water Content

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:

$$LWC = (FW - DW)/\text{FW} \times 100$$

Where *FW* is leaf fresh weight and *DW* is leaf dry weight.

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;

$$F_v/F_m = (F_m - F_0)/F_m$$

Total Chlorophyll Content

The total chlorophyll content (CC) of fresh leaves was estimated according to Barnes *et. al.* [11] with the following formula:

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

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, he 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, [12] according to the formula:

$$MSI = 1 - C_1/C_2$$

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.* [13].

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[14].

Total Phenols

Total phenol content was estimated by the method suggested by Thimmaiah [15] 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/100g.

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[16].

Gene Expression Analysis

Treatment Th-14 was selected to evaluate the gene expression pattern of the key components involved in salt stress tolerance as the amelioration was better in Th-14 in most of the parameters studied under salt stress conditions.

Isolation of Total RNA

In a lab experiment, rice seeds were sterilized in 1% [v/v] NaClO for 20 min and thoroughly rinsed in sterile distilled water. Seeds were bioprimed with Th-14 and untreated seeds were used as control. Seeds were than grown hydroponically in tap water for a week, and then seedlings were transferred to nutrient solution and grown for two weeks. Total RNA was isolated, from leaves of 3 week old treated and untreated rice seedlings subjected to different levels of salt stress (0 mM, 70 mM, 150 and 240 mM

NaCl) for 24 hrs. The plants were selected randomly in three replicates. Total RNA was isolated from each replicate with the help of RNeasy Plant Minikit.

C-DNA Synthesis

For amplification of gene one step RT-PCR kit was used.

Programme used for RT-PCR in thermal cycler is as follows:

Steps

(i) Reverse transcription at 50°C for 30 minutes.

(ii) PCR activation for 15 minutes at 95°C.

(iii) Subsequent 35 cycles of 95°C denaturation for 1 minute.

(iv) Annealing at 58°C for 1 minute.

(v) Extension at 72°C for 1 minute.

(vi) Final extension at 72°C for 10 minutes.

(vii) Lid temperature of cycler was fixed at 104°C

(viii) PCR tubes were placed in thermal cycler as temperature reaches 50°C, taken out and stored at 4°C after completion of programme.

Analysis of Amplicons

Amplicons obtained were analyzed on 1.2 per cent agarose gel. Total 10 µl amplicon (2 µl gel loading dye + 8 µl of PCR amplicon) was loaded in wells.

Primers

List of primers used in for gene expression studies is given in Table 4. Tubulin gene was selected for use as endogenous internal standard because it is a constitutively expressed gene *i.e.* its level of expression is expected to be constant in all tissues and at all times.

RESULTS

Effect of Salinity on Colony Growth of Trichoderma Isolates

A significant difference among growth response ($p \leq 0.05$) of Trichoderma isolates was observed at different salt concentrations *viz.*, 4, 6, 9 and 10 dS/m (Table 1). All the Trichoderma isolates acquired the full growth of 9.0 cm at 4 dS/m after four days incubation period. At 6 and 9 dS/m, tested Trichoderma isolates showed reduction in radial growth. Only ten isolates *viz.*, Th-1, Th-3, Th-5, Th-6, Th-9, Th-13, Th-14, Th-19, Th-33 and Th-50 showed optimum colony growth ranging from 8.1 to 9.0 cm after four days at 9 dS/m. Five isolates, Th-13, Th-14, Th-19, Th-33 and Th-50, were found attaining a full growth of 9.0 cm even up to 10 dS/m salt concentration.

Trichoderma Growth and Mycelial Dry Weight at Different Salt Stress Levels

The Trichoderma isolates were further validated for their salt tolerance capacity by evaluating their linear growth and mycelial dry weight under salt stress conditions. Reduction in linear growth of ST isolates was not exaggerated when exposed to 70, 150 and 240 mM NaCl. The isolate Th-14 achieved a maximum linear growth (mean at salt stress = 8.8 cm) and mycelial dry weight (mean at salt stress = 190.26 mg) after 4 days of incubation at all salt levels followed by Th-19 (8.76 cm linear growth and 188.32 mg mycelial dry weight, considering mean at salt stress). Colony growth of selected Trichoderma isolates (Th-13, Th-14, Th-19, Th-33 and Th-50) on growth medium supplemented with different concentrations of NaCl *viz.*, 70, 150 and 240 mM NaCl after four days of incubation period is shown in Figure 1.

Meanwhile, substantial adverse consequences were observed towards the SS isolate Th-25, in terms of growth and mycelial dry weight under salt stress upto 240 mM. Reduction in linear growth and mycelial dry weight of the SS isolate, Th-25 increased corresponding to the increase in NaCl concentration (Table 2). Percentage of reduction in linear growth in Th-25 was 41% at 70 mM, 61% at 150 mM and 80% at 240 mM NaCl in comparison to its growth at 0 mM NaCl. Whereas, ST isolates, Th-14 and Th-19 showed a maximum delayed reduction among all the tested isolates in their linear growth reaching 2% at 70 mM, 2% at 150 mM, 5% at 240 mM and 1% at 70 mM, 1% at 150 mM, 9% at 240 mM, respectively.

Table 1
Colony growth (cm) of Trichoderma isolates as affected by salt stress at 4 days after incubation (DAI). Forty five Trichoderma isolates were grown on PDA medium supplemented with different concentrations of NaCl *viz.*, 4, 6, 9 and 10 dS/m. Plates were then incubated at 27 ± 2°C for 4 days. Statistical analysis was carried out as described in the text. Data are the mean values (n = 3).

		Colony growth (cm)				
Sl. No.	*Trichoderma isolate code*	*4 dSm^{-1}*	*6 dSm^{-1}*	*9 dSm^{-1}*	*10 dSm^{-1}*	*Mean*
1.	Th-1	9.0	8.5	8.5	6.8	8.2
2.	Th-2	9.0	5.6	5.4	4.1	6.0
3.	Th-3	9.0	8.2	8.4	6.1	7.9
4.	Th-4	9.0	8.0	7.5	5.4	7.4
5.	Th-5	9.0	8.5	8.1	6.7	8.0
6.	Th-6	9.0	8.4	8.2	6.4	8.0
7.	Th-8	9.0	8.0	5.3	4.7	6.7
8.	Th-9	9.0	8.5	8.2	6.2	7.9
9.	Th-10	9.0	8.3	5.2	4.9	6.8
10.	Th-11	9.0	8.1	7.7	5.8	7.6
11.	Th-12	9.0	8.5	7.8	6.2	7.8
12.	Th-13	9.0	9.0	9.0	9.0	9.0
13.	Th-14	9.0	9.0	9.0	9.0	9.0
14.	Th-15	9.0	5.5	4.5	4.1	5.7
15.	Th-16	9.0	7.9	7.1	6.2	7.7
16.	Th-17	9.0	7.1	5.8	4.2	6.5
17.	Th-19	9.0	9.0	9.0	9.0	9.0
18.	Th-20	9.0	7.2	6.7	3.6	6.6
19.	Th-21	9.0	7.1	5.7	5.8	6.9
20.	Th-22	9.0	7.2	6.7	4.8	6.9
21.	Th-23	9.0	7.0	4.5	3.4	5.9
22.	Th-25	9.0	8.2	5.4	4.2	6.7
23.	Th-28	9.0	7.1	6.2	3.7	6.5
24.	Th-29	9.0	6.6	5.6	5.1	6.5
25.	Th-30	9.0	7.4	6.7	3.5	6.6
26.	Th-31	9.0	7.2	6.5	4.1	6.7
27.	Th-32	9.0	8.1	7.1	4.2	7.1
28.	Th-33	9.0	9.0	9.0	9.0	9.0
29.	Th-34	9.0	8.0	5.4	5.6	7.0
30.	Th-35	9.0	7.1	6.5	5.2	6.9
31.	Th-36	9.0	7.0	5.7	4.8	6.6
32.	Th-37	9.0	7.0	6.3	6.2	7.1
33.	Th-38	9.0	6.7	6.5	5.1	6.8
34.	Th-39	9.0	6.8	6.6	5.1	6.8
35.	Th-40	9.0	5.3	4.6	3.7	5.7
36.	Th-41	9.0	7.2	5.1	7.8	7.2
37.	Th-42	9.0	7.7	6.8	6.2	7.4
38.	Th-43	9.0	7.3	6.4	5.7	7.1
39.	Th-44	9.0	7.6	7.1	6.2	7.4
40.	Th-45	9.0	7.5	7.2	6.5	7.5
41.	Th-46	9.0	7.2	5.7	4.9	6.7
42.	Th-47	9.0	7.1	5.4	4.9	6.6
43.	Th-48	9.0	7.2	5.6	5.4	6.8
44.	Th-49	9.0	6.6	4.9	3.4	5.9
45.	Th-50	9.0	9.0	9.0	9.0	9.0

	Isolate (a)	Salt stress (b)	$a \times b$
SEM ± CD	0.058	0.010	0.025
($p \leq 0.05$)	0.161	0.029	0.072

Effect of Selected Trichoderma Isolates on the Growth, Physiological and Biochemical Parameters of Rice Under Different Levels of Salt Stress.

The growth, physiological and biochemical consequences of different levels of salt stress in rice pretreated with different treatments were assessed in relation to length and fresh weight of shoot and root, number of leaves, LWC, chlorophyll fluorescence, CC, SPAD value, MSI, proline content, MDA content and phenolics. The effect of salt injury at 240 Mm NaCl in treated and untreated plants is shown in Figure 1. Generally, the effect of different treatments and salinity levels were statistically significant (p = 0.05) as revealed by the analysis of variances of the characters investigated. The interaction between treatment and salinity was also significant on most characters like chlorophyll fluorescence, MSI, proline content, MDA content and phenolics (Table 3). The data are depicted in the form of line and bar diagrams (Figures 3, 4 and 5) for the selection of treatments for different parameters.

Length and Fresh Weight of Shoot and Root

Mean square from analysis of variance of data of rice when subjected to 21 days salinity treatment (0, 70, 150, 240 mM NaCl) indicated that both length and fresh weight of shoot and root decreased with

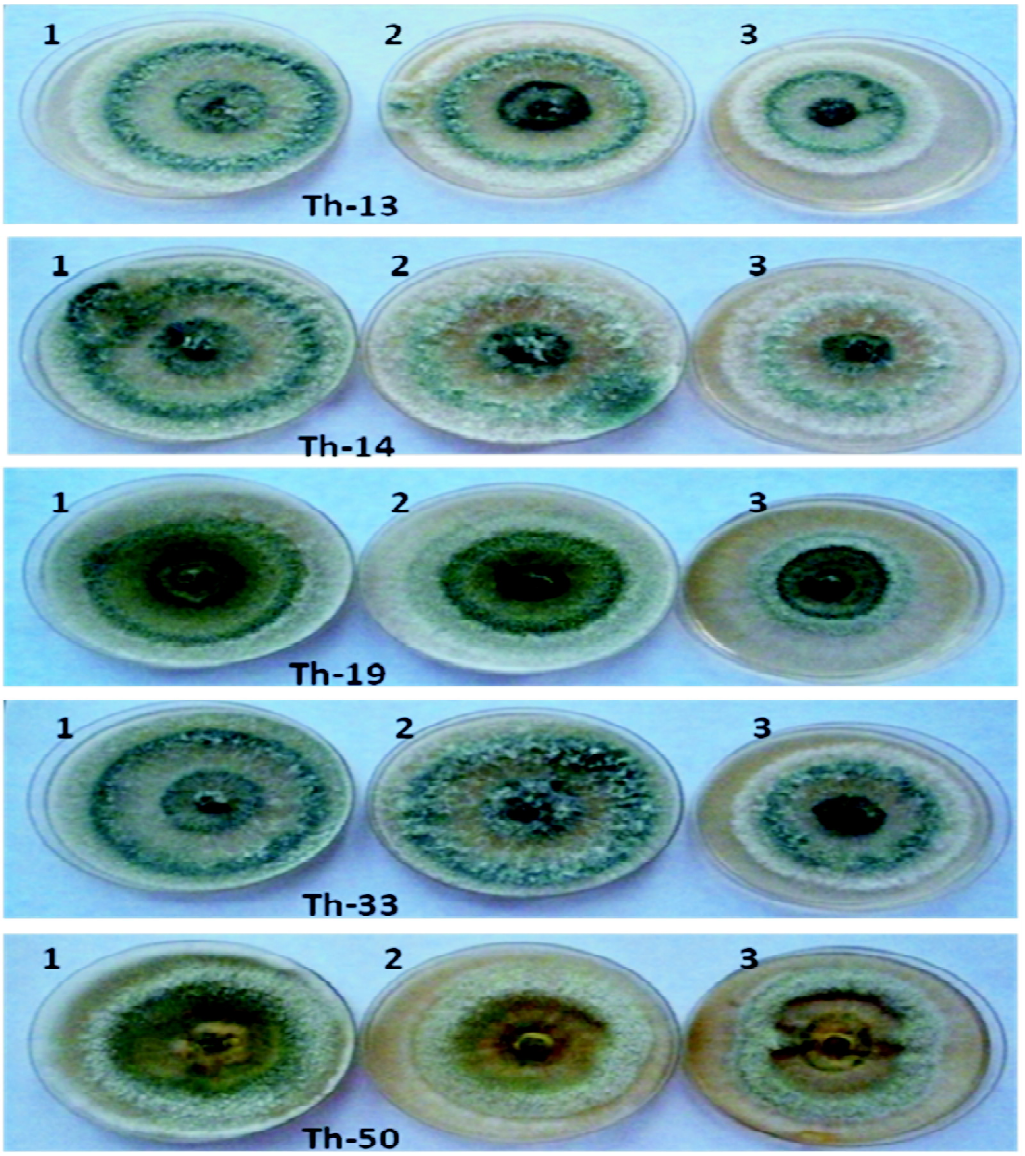

Figure 1: Colony growth of selected Trichoderma isolates (Th-13, Th-14, Th-19, Th-33 and Th-50) on growth medium supplemented with different concentrations of NaCl after four days of incubation period.
1 = 70 mM NaCl, 2 = 150 mM NaCl, 3 = 240 mM NaCl

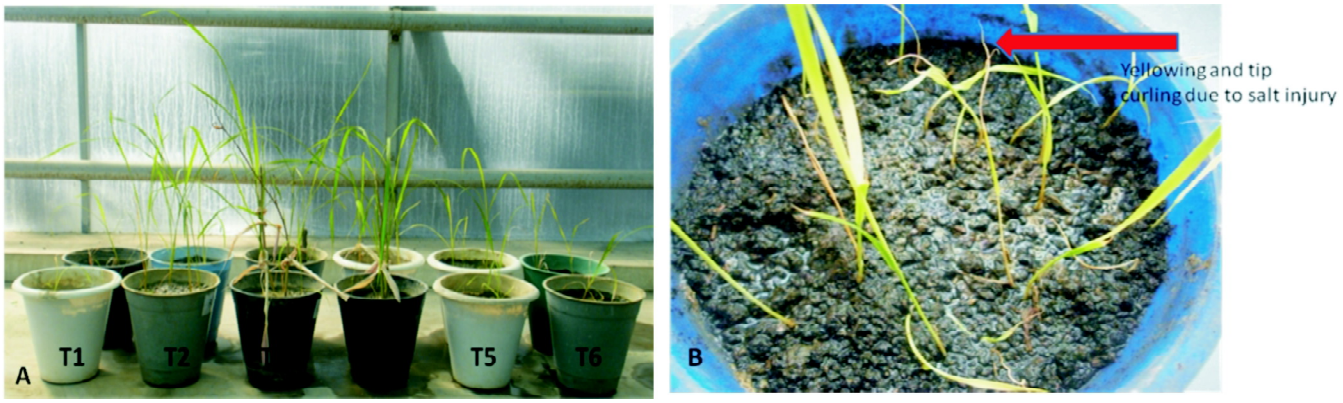

Figure 2: (A) Effect of salt injury in rice plants when subjected to 21 days salinity stress at 240 mM NaCl.
(B) Yellowing and tip curling due to salt injury in control (untreated plants) at240 mM NaCl.