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Abiotic Stress Tolerant *Trichoderma asperellum* Tvb1 from Hot Spring and its Antagonistic Potential Against Soil Borne Phytopathogens

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ABSTRACT

Trichoderma is widely used for plant disease management. However, its bio-control efficacy is affected by abiotic environmental parameters. The objective of this study was to indentify Trichoderma strains that could be stable at adverse environmental conditions including high temperature, pH and high salinity. For this, several Trichoderma isolates were collected from agroecological zone IIIA of Bihar, India. In vitro antagonistic potential of Trichoderma isolates against soil borne pathogens (Sclerotium rolfsii, Rhizoctonia solani and Fusarium oxysporum) were evaluated. In all in vitro tests, Trichoderma isolate Tvb1 (isolated from hot spring soil) was found to be the most efficient tolerating high temperature up to 45 °C for four days, 1750 mM salt (NaCl) concentration, and pH up to 11. In molecularly characterization, by 18S ribosomal RNA gene sequencing, the fungus was identified as Trichoderma asperellum. The study recognized a thermo, saline and high pH tolerant strain of T. asperellum Tvb1 that could be used as a potential biocontrol agent against soil borne plant pathogens in abiotic stress conditions.

Keywords: *Biological control, abiotic stress tolerant, soil borne pathogens, extremophilic fungi.*

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INTRODUCTION

Trichoderma are outstanding among filamentous ascomycetes fungi due to their high adaptability to various ecological conditions. They live in soil and grow saprophytically on various substrates such as wood, bark or leaf and interact with plants. These fungi have the ancestral life style with ability to parasitize and kill other fungi called mycoparasitism [41,9,33]. *Trichoderma* outstanding ability is not only to parasitize plant pathogenic fungi but also to boost plant defence against invading pathogens even at sites away from the point of application, *Trichoderma* species are widely used commercial biofungicides as of today's agriculture [58,53,1,74,84]. Apart from this, several *Trichoderma* strains are known to promote vigor and plant growth as well as tolerance to abiotic stresses like drought [57]. Extreme abiotic environmental parameters such as high temperature, high alkalinity and high pH may have negative influence on the bio-control efficacy of *Trichoderma* strains [60]. However, some genes of *Trichoderma* species can provide resistance to the biotic and abiotic stresses such as salt, heat and drought [42] by adopting some mechanism to alter the

drought response includes drought tolerance through physiological and biochemical adaptations, and enhanced drought recovery [38].

Among several soil borne pathogens, *Sclerotium rolfsii* Sacc. *Rhizoctonia solani* and *Fusarium oxysporum* are major one of various crops in all growing areas of India[15]. The pathogens are polyphagus fungi cause substantial losses in quality and productivity of yield. These fungi are non-specialized pathogen of worldwide importance and have a wide host range[62].

Sclerotium diseases occur primarily in warm climates. They cause damping-off of seedlings, stem canker, crown blight, root, crown, bulb, and tuber rot, and fruit rots. *Sclerotium* frequently causes severe losses of fleshy fruits and vegetables during shipment and storage. In the United States, they are often called southern wilts or southern blights and affect a wide variety of plants, including most vegetables, flowers, legumes, cereals, forage plants, and weeds. *Rhizoctonia* diseases occur throughout the world. They cause losses on almost all vegetables and flowers, several field crops, turf grasses, and even perennial ornamentals, shrubs, and trees. Symptoms may vary somewhat on the different crops, with the stage of growth at which the plant becomes infected, and with the prevailing environmental conditions. The most common symptoms on most plants are damping-off of seedlings and root rot, stem rot, or stem canker of growing and grown plants. On some hosts, however, *Rhizoctonia* also causes rotting of storage organs and foliage blights or spots, especially of foliage near the ground. *Fusarium* causes vascular wilts of vegetables and flowers, herbaceous perennial ornamentals, several field crops, such as cotton and tobacco; plantation crops, such as banana, coffee, and sugarcane; and a few shade trees. Fusarial wilts are most severe under warm soil conditions and in greenhouses. *Fusarium* fungi belong to the species *Fusarium oxysporum*. Different host plants are attacked by special forms or races of the fungus. The fungus that attacks tomato is designated *F. oxysporum* f. sp. *lycopersici*; cucurbits, *F. oxysporum* f. sp. *conglutinans*; banana, *F. oxysporum* f.sp. *cubense*; cotton, *F. oxysporum* f. sp. *vasinfectum*; carnation, *F. oxysporum* f. sp. *dianthii*; and so on [2].

Diseases caused by soil borne pathogens are being managed by chemical methods for a long time. The hazardous nature of chemical fungicides and extreme adverse abiotic factors such as high temperature, salinity and high soil pH warrant to switch towards biological control or pathogens antagonists having abiotic tolerance character in true sense. To mitigate adverse environmental conditions, several *Trichoderma* spp. have been identified and tested as efficient biocontrol agent [70,57]. Some *Trichoderma* spp. have been identified as heat tolerant [55, 32, 75,14, , 60,] , some as salt tolerant [46] and high pH [10,19]. As far as antagonistic activity is concerned, some abiotic stress tolerant *Trichoderma* spp. show highly antagonistic to soil borne pathogens [38,60]. In all the findings, *Trichoderma* spp. are being identified as heat tolerant after the exposure of high temperature for few hours. However, hot environmental condition prevails for few days in natural condition. Till now, none of the *Trichoderma* spp. had been evaluated *in vitro* for pathogen antagonism at high temperature, and high salinity and pH. Therefore it is very important to identify abiotic stress tolerant *Trichoderma* strain that could not only sustain adverse environmental conditions for long time but also be equally effective suppressing plant pathogens to mitigate extreme adverse environmental conditions in the field. Keeping all in mind the present investigation was conducted to identify thermo, saline and pH tolerant *Trichoderma* strain to mitigate extreme adverse environmental conditions and evaluated against soil borne pathogens at high temperature (35 °C).

MATERIAL AND METHODS

Isolation, purification and characterisation of *Trichoderma*

Extensive collection of soil samples was done from different arable, non arable and hot spring of agro ecological Zone IIIA of Bihar. Isolation was done by employing serial dilution technique (Waksman, 1922)[83]. *Trichoderma* selective medium (TSM) (Glucose 3.0g, KCL 0.15g, $MgSO_4.7H_2O$ 0.2g, water 1L, NH_4NO_3 1.0g, K_2HPO_4 0.9g, Agar 15g) [25, 8] was used for isolation of *Trichoderma*. One ml soil suspension was taken with the help of micropipette and poured on the Petri plate seeded with TSM. The entire process was carried out in Laminar air flow under aseptic conditions. The plates were incubated at 28 ± 1 °C for five days.

Trichoderma isolates were purified by single spore culture. The spores of the isolates were inoculated onto a Petri dish seeded with potato dextrose agar (PDA) medium (Agar 20g, Dextrose 20g, peeled potato 200 g, and distilled water 1L). Sub-culturing was done from the growing front of the single new colony. Small amount of spores were taken on the tip of a sterilized inoculating needle and streaked on potato dextrose agar poured Petri dishes. Colony arisen from single spore was picked up and inoculated on a fresh plate.

Morphological characterization of *Trichoderma* isolates was done following the key of Rifai [66] and/or by observing radial mycelial growth of the isolates or followed the method of Sultana *et al.* [79]. Spore density was also determined by observing ten days old conidial suspension of *Trichoderma* isolates taken in a beaker from the media and stirring. The volume of the beaker with conidial suspension was made about 500ml with sterile water and 1 drop Tween-20 was added to it and stirred to disperse well. From this solution 1 drop of suspension was taken on the center of haemocytometer and a cover slip was placed on it. Finally, spores were counted under microscopic power of 40X.

Putting the average number of spore per unit cell in the following formula, the number of spore per 1 ml was determined.

$$\text{Number of spores per cubic mm Sporulation} = \frac{\text{Number of spores counted X dilution}}{\text{Number of smallest square counted}}\ x\ 4000$$

Molecular Characterization

DNA isolation

Genomic DNA of *Trichoderma* isolates was isolated from mycelium mat by using CTAB method. Mycelium mat was cultured on the potato dextrose broth (PDB) from pure culture of the isolate. Culture of the isolate was purified on the PDA after inoculation of the mycelium tip of the isolated *Trichoderma*. Harvested mycelium mat samples (1gm) were ground in liquid N_2 using mortar and pestle. Approximately 350 mg of the Mycelium mat samples were quickly transferred into 1.5 ml of microcentrifuge tube and equal volume (W/V) (350 µl) of hot (65°C) 2X CTAB buffer [2% CTAB (w/v): 100 mM Tris-Cl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4M NaCl, 1% PVP (Mr: 40,000)] was added into the microcentrifuge tube and mixed thoroughly by vigorous shaking for 2 min. Approximately, 700µl of ice cold chloroform: isoamyl alcohol (24:1) (for 50 ml; Chloroform 48ml, Isoamyl alcohol 2ml) was added into the microcentrifuge tube, mixed well by inversion and centrifuged at 12,000 rpm for 5 min. The top aqueous phase was collected using cut tips into a new microcentrifuge tube and $1/5^{th}$ volume of 5% CTAB solution (5% CTAB: 0.35 M NaCl) was added in the microcentrifuge tube and mixed well by gentle inversion. Further, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed well by inversion and centrifuged at 12,000 rpm for 5 min. The top aqueous phase was collected using cut tips into a new microcentrifuge tube and equal volume of CTAB precipitation buffer (1% CTAB: 50 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0) was added, mixed and incubated on ice for 5 min. After incubation, microcentrifuge tube was centrifuged at 12,000 rpm for 5 min and supernatant was discarded. Fifty microliters of high salt buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0; 1M NaCl) was added into the microcentrifuge to dissolve the pellet. Further, DNA was precipitated by addition of ice cold ethanol (2.5 volumes of the supernant) and mix gently by inversion. Microcentrifuge tubes were centrifuged at 12,000 rpm for 15 min and supernatant was discarded. Finally, DNA pellet was washed with 70% ethanol, air dried and dissolved in 30 µl of TE buffer (0.1x TE buffer:1mM Tris-Cl, pH 8.0; 0.1 mM EDTA, pH 8.0) ($T_{10}E_1$).

18S rRNA genes amplification

18S rRNA genes were amplified by polymerase chain reaction (PCR) with forward primer A (5'-AACCTGGTTGATCCTGCCAG-3') and reverse primer SSU-inR1 (5'-CACCAGACTTGCCCTCCA-3') based on the conserved domain region of 18S rDNA. Amplification of the 18S rDNA region was conducted in a reaction mixture with a final volume of 25 µl volume containing 1X XT-5 DNA Polymerase buffer, 200 µM dNTPs mixture, 0.5 µM primer, 25 ng of template DNA and 1 U of XT-5 DNA polymerase (Bangalore Genei, Bangalore, India) in a thermal cycler. The reaction consisted of initial denaturation at 94°C for 3 min followed by 25 cycles of denaturation at 94°C for 30s, annealing at 51°C for 30s, and extension at 72°C for 1 min, with a final extension at 72°C for 3 min. Amplified products were then subjected to electrophoresis in 1.2% agarose gel

using 1 X TBE and detected by ethidium bromide staining, viewed under UV light and photographed with Gel-documentation system.

Sequencing and phylogenetic analysis

The amplified PCR products were purified using PCR purification kit (XcelGen, India) and send for direct sequencing. The determined 18S rDNA sequences were analyzed by a similarity search on the NCBI GenBank database using BLAST to compare with the related sequences.

A number of *Trichoderma* sequences were selected on the basis of a similarity score of 90% with database sequences. Multiple sequence alignment of these selected homologous sequences and 18S rRNA gene sequence of test strain was performed using Clustal W (Thompson *et al.*, 1994)[81]. Subsequently, an evolutionary distance matrix was then generated from these nucleotide sequences in the dataset. A phylogenetic tree was then drawn using the Neighbour Joining Method. Phylogenetic and molecular evolutionary analysis were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura *et al.*, 2007)[80].

Construction of phylogenetic tree

MEGA 7 was downloaded from http://www.megasoftware.net and following steps were followed. In mega click on the align drop down menu and select edit/build alignment; select create new alignment; select isolates. Either paste in two all *Trichoderma* isolates, and the moss sequence or use Edit> insert sequence from file. Select ALL sequence; edit menu. Align by Muscle from the alignment drop down menu (or click on the arm icon), click compute, click export alignment as MEGA format, save file. From the phylogeny drop down menu, select construct/ test neighbour joining tree. Select the meg file saved above as your source of data; use the scroll down menu under test of phylogeny to select bootstrap (500); click compute (do not change other defaults). To root your tree using the moss homologue, go to the sub tree menu, click on root, then click on the phpat. branch of tree. In the image menu, export your tree as both a pdf file and an EMF file (the latter for publication). Evaluate your tree – if the boot strap value are high most > 50 many > 80 then you have a nice tree if not then you may have to do the following; try both separately.

a. Check your *Trichoderma* isolates file for apparent incomplete sequence; remove these and resave your file with a new file name (don't delete them permanently – we can't totally ignore). redo the tree with just full length sequence.
b. Create two tree; one with CBP60a, g, h like proteins and another with CBP60 bcdef like proteins.
c. Save the original tree and any others.

Screening of *Trichoderma* isolates for heat stress tolerance

The optimum temperature requirement and temperature tolerance limit for *Trichoderma* isolates were studied under *in vitro* conditions. All *Trichoderma* isolates were incubated at different temperatures (20, 25, 30, 35, 40 and 45 °C). The monosporidial disc of fungus was inoculated on potato dextrose agar medium separately and tolerance limit was studied by maintaining the prescribed temperature inside the BOD incubator. *Trichoderma* isolated from commercial formulation (TC) was taken as control and after 4 days of incubation radial growth of the fungus was measured.

Screening of *Trichoderma* isolates for salt tolerance

Effect of salt (NaCl) on growth of *Trichoderma* isolates was studied by exposing isolates to different concentrations of NaCl viz., 250 mM (14.61 gL^{-1}), 500 mM (29.22 gL^{-1}), 750 mM (43.83 gL^{-1}), 1000 mM (58.44 gL^{-1}), 1250 mM (73.05 gL^{-1}), 1500 mM (87.66 gL^{-1}), 1750 mM (102.27 gL^{-1}) and 2000 mM (116.88 gL^{-1}) in PDA. PDA without salt was taken as control. PDA was prepared by adjusting the pH to 6.0. After that, sterilized medium was poured into Petri plate and then inoculated with 5mm culture disc of *Trichoderma* isolate. Plates were incubated for five days at 28 ± 1 °C. Each treatment was replicated thrice. The radial growth of mycelia was recorded and results were analyzed statistically.

Effect of different pH on radial growth, sporulation and dry mycelia weight of *Trichoderma* isolates

Effect of pH on the growth of the *Trichoderma* isolates was tested in the laboratory using liquid cultures containing different pH levels (pH 3, pH 5, pH 7, pH 9, pH 11). Potato dextrose broth medium was used to study the effect of pH medium on the growth of

different isolates of *Trichoderma,* pH 7 was considered as control. Thirty ml of liquid medium was poured into a 150 ml conical flask under aseptic conditions. The reaction of the medium was adjusted to the desired pH by adding 0.1N NaOH or 0.1N HCl (Naik *et al.*, 1988)[56]. The medium was buffered with Disodium hydrogen phosphate citric acid buffer according to the schedule of Vogel (1951)[82]. Flasks were sterilized at 121°C at 15 psi for 20 minutes. Each flask was inoculated with each isolate using 5 mm diameter mycelial disc of 5 days old culture in sterilized conditions. Inoculated flasks were incubated at 28 ± 1°C for ten days and the dry mycelial weight was obtained. The cultures were filtered through Whatman No. 42 filter paper and the dry mycelial weight was measured by subtracting the initial weight of the filter paper from the weight of the filter paper along with the mycelial mat. Before harvest of mycelial mat, growth and sporulation of *Trichoderma* isolates were recorded as: (-) No growth; (+) growth but no sporulation; (++) growth with sporulation and (+++) growth with profused sporulation.

Evaluation of *Trichoderma* isolates for antagonism against soil borne pathogens at high temperature

The dual culture technique described by Dennis and Webster (1972)[21] was used to test the antagonistic ability of *Trichoderma* isolates against soil borne pathogen viz., *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizoctonia solani*. The pathogen and *Trichoderma* was grown on PDA for four days at 25 ± 1ºC, 30 ± 1ºC and 35 ± 1ºC in BOD incubator separately. From these plates small block (5 mm) of the target fungi cut from the periphery was transferred to the Petri dish previously poured with PDA. At the same time pathogen and *Trichoderma* isolate were transferred aseptically in the same plate of opposite end and incubated at different temperatures of 25 ± 1 ºC, 30 ± 1 ºC and 35 ± 1ºC for five days and radial growth was observed. The medium inoculated with *Trichoderma* and pathogen alone and incubated at 25 ± 1 ºC, 30 ± 1 ºC or 35 ± 1ºC was served as respective control. The experiment was replicated thrice and percent growth inhibition was calculated by the formula

$I = (C-T)/C \times 100$

Where C is mycelial growth in control plate, T is mycelial growth in treated plate, and I is percent inhibition of mycelial growth.

Statistical analysis

Least significance difference (LSD) was calculated at $P \leq 0.01$ for in vitro tests for all variables to compare individual treatment. According to Gomez and Gomez [30]. Duncan's new multiple range test (DMRT) was applied to identify efficient isolates of *Trichoderma*. The data has been presented in tabulated form and entire statistical analysis was done using minitab-15, statistical software.

RESULTS AND DISCUSSION

Collection, isolation and identification of indigenous *Trichoderma* isolates

Extensive collection of 1005 soil samples was done from different arable, non arable lands and hot spring of Agro ecological Zone IIIA of Bihar. A total number of 311 isolates were successfully isolated from all samples. Among 311 isolates, eleven isolates were selected on the basis of mycelial growth rate (data not presented) and coded as Thg1, Thg2, Thg3, Thg13, T14, TB, Tvb1, T3.1, T3.2, Thb21 and Tvpp. One isolate, TC (*Trichoderma viride*) was isolated from commercial formulation (Bioderma, Biotech International Ltd.) after procurement from the local market of Bhagalpur. These eleven isolates were isolated from rhizosphere soil samples of ten different crop fields or orchards such as cucumber, cowpea, guava, mustard, tomato, mango, wheat, bean, potato, pigeonpea and one isolated from hot spring soil (Fig.1).

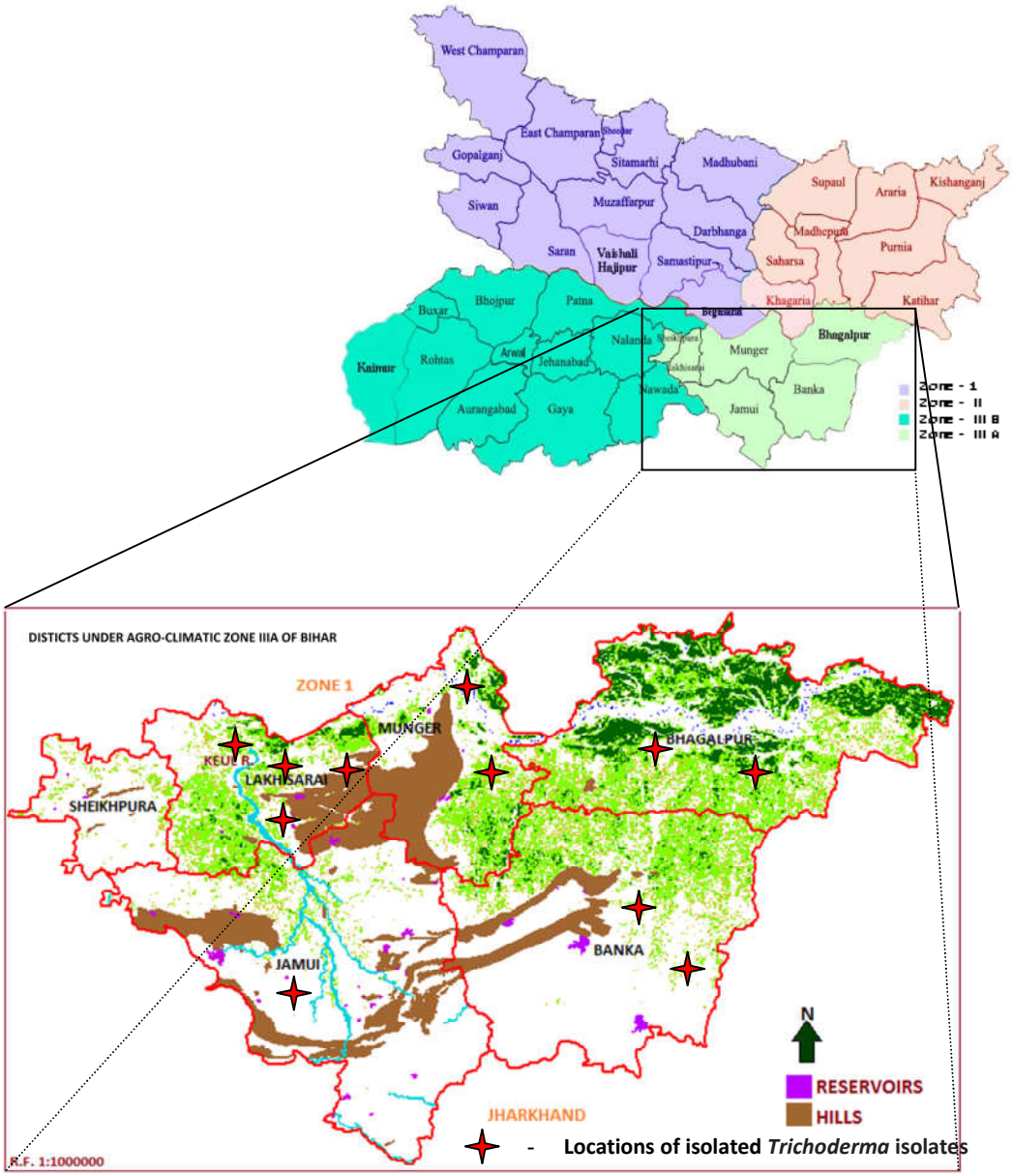

Fig.1 Red star sign indicates the different locations at Zone III A of Bihar from *Trichoderma* isolates were isolated.

Trichoderma spp. are free-living fungi that are common in soil, root and foliar ecosystems throughout the world and are highly interactive. They are present in nearly all types of soils and other natural habitats especially those containing high organic matter. Being secondary colonizers, they are often isolated from well decomposed organic matter like decaying barks and from sclerotia and fruiting bodies of other fungi. *Trichoderma* generally exhibit a preference for wet soils but individual species exhibit different preferences for soil temperature, moisture and pH. While species like *T. viride* and *T. polysporum* are generally found in areas with low temperature, *T. harzianum* most commonly occurs in warm climatic regions. *T. hamatum* and *T. koningii* can occur in diverse climatic conditions. Many workers isolated *Trichoderma* spp. from different habitats and they were quantified and characterized. Rahman *et al.* [64] collected five isolates of *Trichoderma* spp. form different habitats. Sinha *et al.* [78] isolated seven *Trichoderma* spp. from different groundnut growing areas of Manipur and identified as *T. harzianum* (five isolates) and two as *T. viride.* Devi and Sinha (2014)[22] also collected *Trichoderma* spp. from French bean from different locations of Manipur. Chakraborty *et al.* (2010)[16] studied the nineteen isolates of *T. viride* and *T. harzianum* obtained from rhizosphere soil of plantation crops, forest soil and

agricultural fields of North Bengal region. Kumar *et al*. [45] isolated twelve isolates of *Trichoderma* spp. from different location of South Andaman. Sharma and Singh [73] isolated *Trichoderma* spp. from rhizosphere soil of different plants at different locations of Nainital, Almora, Udham Singh Nagar, Derhadum, Haridwar and Tehri Garhwal districts of Uttarakhand, India. Sinha *et al*. [78] isolated *Trichoderma asperellum* from soil sample collected from CSA Farm, Kanpur district of Uttar Pradesh, India. Shahid *et al.* [69] collected different isolates of *Trichoderma* from rhizosphere of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh. Gherbawy *et al*. [29] isolated *Trichoderma* spp. from soil samples collected from different locations in Taif, Kingdom of Saudi Arabia.

Morphological characterization of *Trichoderma* spp.

Different isolates of *Trichoderma* spp. distinctly differed on their cultural and morphological properties. Mycelial growth rate of different isolates varied considerably up to 72 hours (3 days). Linear/apical growth of mycelia on PDA plates at 28 ± 1°C ranged from 29.91 mm to 90.00 mm. Based on color, growth habit and colony consistency on PDA medium were divided into several groups. Based on color, isolates were divided into five groups. Results indicated that five isolates Tvpp, Thg2, Thg3, Thg13, and TC had dark green color which was distinctly different from other isolates. Tvb1 and Thg1 showed green colony. TB, and T3.2 isolates were light green color. Out of 12 isolates, only T14 was yellowish green. Rest of the *Trichoderma* isolates T3.1 and Thb21 were whitish green. On the basis of growth, isolates were divided into three categories such as fast, medium and slow. Among these all isolates had fast growth, some had medium growth and T3.1 performed slow growth. Similarly, on the basis of consistency, the isolates were categorized into three groups such as very compact, compact and loose. Few isolates were very compact such as Tvb1, Thg1 and T14; most of the isolates showed compact appearance and only few T3.1, TC was loose (Table 1).

A similar study was conducted in Hebron University, Palestine by Radwan *et al.* [63]. They also measured radial mycelial growth above 80 mm as the mean of two perpendicular diameters and the present results agree with this report. Spores density among different *Trichoderma* isolates were determined. Highest spore density was recorded among isolates of dark green and yellowish green color. Whereas lowest was found among the isolates of light green, green and whitish green color. From this result, it is evident that *Trichoderma* isolates showed differences in spore production (Table 1) which agree with Singh *et al*. [77] who showed significant differences in sporulation capacity of *Trichoderma* isolates and found highly variable sporulation pattern within the species. According to Shamsuzzaman and Hossain [72] the number of conidia varied from 1.44×10^8 to 7.12×10^8/plate (90 mm dia.).

Molecular characterization

Rapid identification of bioagents is very necessary and important in the pathological laboratory to take decision for installment of commercialized bioformulation. The rRNA based analysis is a central method in pathology used not only to explore microbial diversity but also to identify new strains (Tvb1 and Thg1). Ribosomal RNA (rRNA) sequence analysis has been well-documented as a means of determining phylogenetic relationships in all of the major organism domains. The genomic DNA was extracted from two potential isolates. In the present study, 18S ribosomal RNA gene sequences were used for the species identification of *Trichoderma*. The genomic DNA was extracted using CTAB method. The extracted genomic DNA was used for the amplification of 18S rRNA gene. An amplicon of ~ 650 bp long were obtained in both the isolates for the identification of isolated fungal strain. The purified DNA amplicon were sent for sequencing of 18S rRNA gene fragment (Fig. 2). For identification of strain the 18S rRNA gene sequence of the two isolates were used for BLASTN searches against (nr/nt) database using NCBI web interface separately. The top ten hits for both the isolates were selected and sequences were obtained. The duplicate entries were removed manually. Subsequently, an evolutionary distance matrix was then generated from these nucleotide sequences in the dataset. A phylogenetic tree was then drawn using the Neighbour Joining method. Phylogenetic tree was constructed using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0. The 18S rDNA gene sequence based phylogenetic tree showing the relationships between the isolates and selected representatives of the genus *Trichoderma* is given in (Fig. 3). Neighbor joining tree using

Jukes-Cantor model [36] has widely used to study genetic diversity among different species of *Trichoderma* [29]. Both the isolates were clustered together. The nucleotide sequence of two isolates were aligned using Pairwise Sequence Alignment tool from EMBOSS Needle software (http://www.ebi.ac.uk). The nucleotide sequences of two isolates showed 97.8% similarity (Fig. 4). Both isolates showed highest sequence similarity with KM458788.1 *Trichoderma asperellum* strain NFML CH10 (Fig. 4). Further, Pairwise Sequence Alignment tool from EMBOSS Needle software (http://www.ebi.ac.uk) showed 41.7% similarity between Tvb1 and KM458788.1 *Trichoderma asperellum* strain NFML CH10 and 42.0% similarity between Thg1 and KM458788.1 *Trichoderma asperellum* strain NFML CH10.

Table 1. Colony characters of *Trichoderma* isolates with sporulation capacity

Group	Color	Trichoderma isolates	Shape	Growth habit	Colony consistency	Sporulation capacity (Number of spores/ml)
1	Dark green	Thg3	Regular	Medium	Compact	0.84×10^{10}
		Thg2	Regular	Fast	Compact	0.91×10^{10}
		Thg13	Regular	Medium	Compact	0.59×10^{10}
		Tvpp	Regular	Medium	Compact	0.78×10^{10}
		TC	Regular	Medium	Loose	0.52×10^{10}
2	Green	Thg1	Regular	Fast	Very compact	1.05×10^{10}
		Tvb1	Regular	Fast	Very compact	1.11×10^{10}
3	Light green	TB	Regular	Fast	Compact	0.78×10^{10}
		T3.2	Regular	Fast	Compact	0.93×10^{10}
4	Whitish green	T3.1	Regular	Slow	Loose	0.75×10^{10}
		Thb21	Regular	Medium	Compact	0.98×10^{10}
5	Yellowish green	T14	Regular	Fast	Very compact	1.01×10^{10}

Many reports have been published on isolation and identification of *Trichoderma* in India and abroad [29, 76]. These reports suffer from the fact that species identification had been performed morphologically following the key of Rifai [66]. The studies on molecular characterization by sequencing 18S RNA for the collected isolates are limited. In recent years, efforts involving molecular methods have been made for the identification and taxonomic investigation of different *Trichoderma* spp., including members of the section Longibrachiatum [23]. Shahid *et al*. [68] reported the genetic variability within 69 bio-control isolates of *Trichoderma* collected from different geographic locations and culture collections and their phylogenetic analysis were done with the help of the sequence data obtained from the Inter Transcribed Spacer 1(ITS1) region of ribosomal DNA.

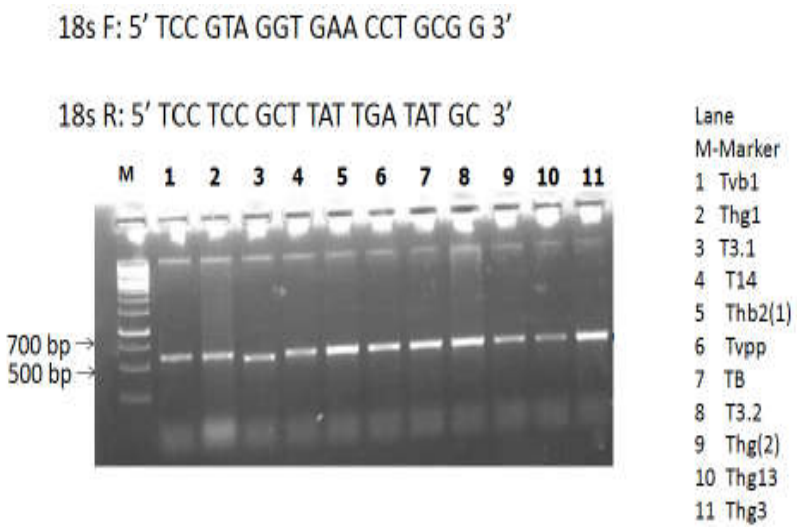

Fig. 2 18S rDNA amplification of *Trichoderma* isolates

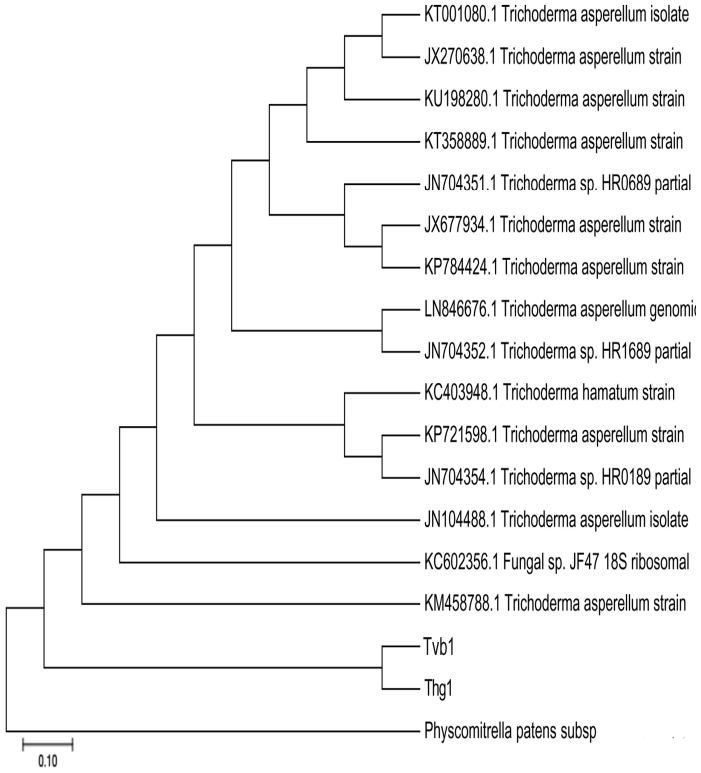

Fig. 3 Molecular Phylogenetic analysis of *Trichoderma* 18S rDNA sequences by Maximum Likelihood method by MEGA 7.

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Tvb1          1 GTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCA     50
                ||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1          1 --GCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCGGCGAGACCGCCA     48

Tvb1         51 CTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCG    100
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1         49 CTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCAACGCCG     98

Tvb1        101 ATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCC    150
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1         99 ATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCC    148

Tvb1        151 GCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT    200
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1        149 GCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACT    198

Tvb1        201 GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGA    250
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1        199 GAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGA    248

Tvb1        251 TGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTGAATTT    300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1        249 TGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTGAATTT    298

Tvb1        301 TTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACTACAGAAAGAGTT    350
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1        299 TTGCTCAGAGCTGTAAGAAATACGTCCGCGAGGGGACTACAGAAAGAGTT    348

Tvb1        351 TGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCGG    400
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1        349 TGGTTGGTTCCTCCGGCGGGCGCCTGGTTCCGGGGCTGCGACGCACCCGG    398

Tvb1        401 GGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGG    450
                ||||||||||||||||||||||||||||||||||||||||||||||||||||
Thg1        399 GGCGTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGGTTTGG    448

Tvb1        451 GAGTTGTAAACTCGGTAATGATCCCTCCGCAGG-TCCCCCTA-ACGGGAA    498
                |||||||||||||||||||||||||||||||| ||.||||| |   |||
Thg1        449 GAGTTGTAAACTCGGTAATGATCCCTCCGCAGGTTCACCCTACA---GAA    495

Tvb1        499 G---    499
                |
Thg1        496 GCAG    499
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Fig. 4 The global alignment of two 18S rDNA sequence of two isolates (Tvb1 and Thg1) using the Needleman-Wunsch algorithm (http://www.ebi.ac.uk/Tools/psa/). The nucleotide sequences of two isolates showed 97.8% similarity.

Screening of *Trichoderma* isolates for heat tolerance

The radial growth of twelve isolates of *Trichoderma* in mm at different temperatures (20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45°C) were recorded after three days of incubation. At all tested temperatures, variation in radial growth was seen by all *Trichoderma* isolates. At a particular temperature, variant radial growth was seen by different isolates at $P \leq 0.01$ level. All the isolates showed maximum radial growth of 70 mm at 30^0C, and at increased temperature of 35 °C maximum growth shown by Thg1, TB, T3.2, Thb21, Tvb1, T14 with 70 mm (Full Petri plate growth) followed by Tvpp (66 mm), T3.1 (60 mm), Thg3 (47 mm), Thg13 (30 mm), and least by Thg2 (21 mm) growth. At 40 °C radial growth drastically decreased in all isolates except Tvb1 with 70 mm. At 45 °C minimum radial growth was seen in all isolates with 6-8 mm except Tvb1 with 45 mm growth. After 45 °C heat shock, all isolates were grown at 28 ± 1 °C and some morphological change of their colonies were seen especially in T14 with yellowish colony. At lower temperature of 20 °C and 25 °C all isolates shown decreasing trends of radial growth with decrease in temperature in comparison to 30 °C (Table 3). Grand mean radial growth at all temperatures indicates that Tvb1 isolate is statistically superior to all isolates followed by Thg1, Thb21, and rest of the isolates at $P \leq 0.01$ level.

Trichoderma spp. have been widely used as potential biological control agents in commercial agriculture over the past 2 to 3 decades. Interestingly, the extracellular enzyme system of

Trichoderma important for competition and mycoparasitism remains active even under environmental conditions unfavourable for mycelial growth and hence there is a possibility to improve the strains for better stress tolerance [38]. Abiotic stresses are often interrelated and either individually or in combination, they cause morphological, physiological and molecular changes that adversely affect the growth of the organism. The efficient use of these bioagents across different agricultural soils and climatic conditions was highly limited by the soil hydrological factors [48, 51], with soil temperature being an important parameter affecting the radial growth and competitive colonization of the bioagents in the soil [37]. In a study, higher thermotolerant isolate *T. asperellum* TaDOR673 was attributed to its ability to accumulate stress protectant as evident from the HPLC study. Under stress conditions there was an increase in accumulation of trehalose, mannose and raffinose. These sugars were the most abundant polyols in the cells exposed to various stress conditions. Studies have shown that mannose constitutes 10–15% of dry weight of filamentous fungi and aids in abiotic stress tolerance [37]. Several authors attempted to examine the influence of environmental parameters (water activity, temperature and pH) on the growth of *Trichoderma* and other fungal strains.

Most naturally existing ascomycetes fungi have growth maximum at 30°C [50, 12], the present investigation has identified a thermotolerant *Trichoderma asperellum* Tvb1 with highest growth rate up to 45 °C. Among the all isolates tested for improved thermotolerance, *T. asperellum* Tvb1 was also identified as the superior stress tolerant isolate that survived prolonged exposures for four days to heat stress up to 45 °C. This was clearly indicated by its colony diameter and biomass (dry weight) at 35 °C and at high salt concentration up to 1750 mM. However, in a study when the hyphal cultures of thermotolerant isolates were exposed to heat stress at 52 °C for 4 h, only *T. asperellum*, TaDOR673 was able to survive and found superior mycoparasitic fungi that are able to grow at 40-45 °C [52].

Table 3. Radial growth (mm) of *Trichoderma* isolates at different temperatures after four days of incubation.

Isolates	Radial growth (mm) at different temperatures after three days of incubation						
	20 ºC	25 ºC	30 ºC	35 ºC	40 ºC	45 ºC	Grand mean
Thg3	41e	44g	70	47d	22e	6b	38g
Thg 2	32f	49f	70	48d	15f	7b	37h
Thg1	61a	65a	70	70a	45b	7b	53b
Thg13	48d	55d	70	30e	25d	7b	39g
TB	51c	60c	70	70a	32c	8b	49c
T3.1	42e	51f	70	60c	20e	7b	42f
T3.2	49d	60c	70	70a	26d	8b	47d
Thb21	58b	63b	70	70a	32c	7b	50c
Tvb1	62a	68a	70	70a	70a	45a	64a
T14	53c	62b	70	70a	24d	8b	48d
Tvpp	42e	61b	70	66b	20e	7b	44e
TC	41e	53e	70	62c	22e	7b	43e
LSD (P ≤ 0.01)	2.3	2.0		3.0	2.9	2.1	1.8
CV (%)	2.0	1.5		2.1	4.2	8.4	1.6

Each value is a mean of three replicates. Means followed by same letter within a column are not significantly different at the *P ≤ 0.01* level according to Duncan's New Multiple Range Test. TC = *Trichoderma viride* isolated from commercial formulation.

Screening of *Trichoderma* isolates for salt tolerance

Twelve *Trichoderma* isolates were screened for high salt (NaCl) tolerance at different concentrations of 750 mM, 1000 mM, 1250 mM and 1500 mM, 1750 mM and 2000 mM at 28 ± 1°C for five days incubation. Increase in radial growth reduction of all *Trichoderma* isolates was observed with increased salt concentrations. Variation in percent reduction was recorded from 18.6 to 71.4%, 45.7 to 82.9%, 58.6 to 100%, 62.9 to 100%, 92.9 to 100% and cent percent at 750 mM, 1000 mM, 1250 mM and 1500 mM, 1750 mM and 2000 mM salt concentrations, respectively. At 1500 mM, salt concentration almost all isolates of thermotolerant *Trichoderma* could not survived whereas Tvb1 survived with 62.9% growth reduction and Thg survived with 92.9% growth reduction. At 1750 mM only Tvb1 showed growth with 90% reduction whereas rest of the isolates did not show any radial growth. At

2000 mM none of the thermotolerant *Trichoderma* isolates including Tvb1 shown any growth (Fig. 5 & Fig. 6).

At soil temperature greater than 37 °C the growth of fungi depends greatly on moisture levels and water activity acts as a crucial environmental factor that influences the radial growth of *Trichoderma* strains [12]. Moreover, low osmotolerance levels of *Trichoderma* isolates limits its boundaries for use as biofungicides [48, 51, 38]. Thus, the present study also focused on the salinity tolerance of all twelve isolates. The statistical analysis revealed that *T. asperellum* Tvb1 has highest tolerant to salt stress.

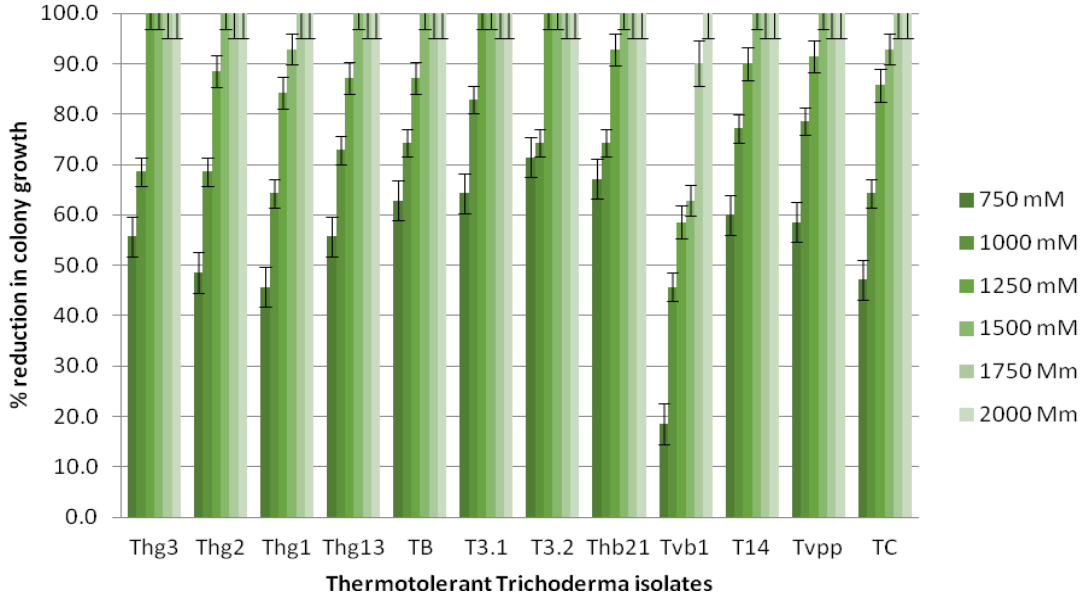

Fig.5 Effect of high salt (NaCl) concentrations on radial mycelia growth of *Trichoderma* isolates. Vertical bar indicates the standard error of three replicates.

Effect of different pH on radial growth, sporulation and dry mycelia weight of *Trichoderma* isolates

All twelve *Trichoderma* isolates were grown on PDB at different pH viz., 3 pH, 5 pH, 7 pH, 9 pH, and 11 pH for ten days of incubation at 28 ± 1 °C in BOD incubator. Variation in sporulation was seen at different pH by different isolates of *Trichoderma*. All isolates shown highest profused sporulation at 7 pH followed by 5 pH, 3 pH, 9 pH and least with 11pH (Table 5). At 3 pH, all isolates viz, Thg3, Thg2, Thg1, Thg13, TB, Thb21, T3.2, Tvb1, T 14, and Tvpp were shown radial growth with sporulation but at same pH two isolates T3.1 and TC were shown radial growth without sporulation. At 5 pH, four isolates viz, Thg13, T3.2, Thb21, Tvpp shown radial growth with profused sporulation, followed by isolate

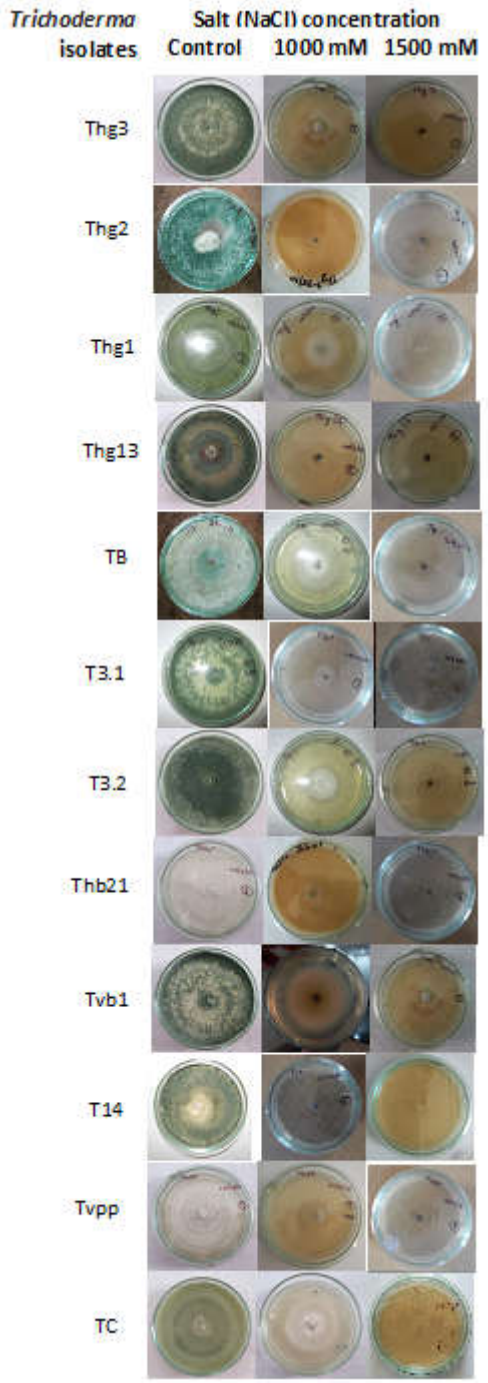

Fig. 6. Effect of different salt concentrations on radial mycelia growth of *Trichoderma* isolates.

Thg3, Thg2, Thg1, TB, T3.1, Tvb1 and T14 with radial growth and sporulation. TC shown radial growth but no sporulation at 5 pH, after ten days of incubation. At 7 pH, only one isolates T3.1 shown growth with sporulation, and rest eleven isolates shown radial growth with profused sporulation after ten days of incubation. At alkaline pH 9, two isolates Thg1 and Tvb1 were shown radial growth with profused sporulation and seven isolates shown radial growth with sporulation but isolates Thg3, T3.1 and T3.2 shown growth with no sporulation after ten days of incubation. At highly alkaline pH 11, only one isolates Tvb1 shown growth with sporulation followed by Thg3, Thg2, Thg1, Thg13, TB, Thb21, T14, Tvpp and TC shown radial growth with no sporulation. Isolates T3.1 and T3.2 were recorded no growth after ten days of incubations. (Table 5, Table 6).

T**able 5.** Effect of different pH on growth and sporulation of *Trichoderma* isolates after 10 days of incubation

pH levels	Sporulation pattern											
	Trichoderma isolates											
	Thg3	**Thg2**	**Thg1**	**Thg13**	**TB**	**T3.1**	**T3.2**	**Thb21**	**Tvb1**	**T14**	**Tvpp**	**TC**
pH- 3	++	++	++	++	++	+	++	++	++	++	++	+
pH- 5	++	++	++	+++	++	++	+++	+++	++	++	+++	+
pH- 7	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
pH- 9	+	++	+++	++	++	+	+	++	+++	++	++	++
pH- 11	+	+	+	+	+	-	-	+	++	+	+	+

Each value is a mean of three replicates. TC = *Trichoderma viride* isolated from commercial formulation. Sporulation: (-) No growth; (+) growth but no sporulation; (++) growth with sporulation; (+++) growth with profused sporulation.

T**able 6.** Effect of different pH on radial growth of *Trichoderma* isolates after 10 days of incubation

pH levels	Radial growth (mm)												
	Trichoderma isolates												
	Thg3	**Thg2**	**Thg1**	**Thg13**	**TB**	**T3.1**	**T3.2**	**Thb21**	**Tvb1**	**T14**	**Tvpp**	**TC**	**Grand mean**
pH- 3	48c	55c	52c	53c	48c	32c	48c	41c	65c	53c	49c	54c	50c
pH- 5	62b	65b	64b	64b	63b	58b	62b	60b	68b	64b	63b	64b	63b
pH- 7	70a	70a	70a	70a	70a	70a	70a	70a	70a	70a	70a	70a	70a
pH- 9	24d	35d	52c	39d	38d	21d	18d	37d	52d	41d	49c	36d	37d
pH- 11	17e	23e	36d	22e	23e	5e	5e	22e	39e	35e	32d	34e	24e
LSD ($P \leq 0.01$)	3.10	2.70	1.73	2.65	2.57	3.67	3.87	2.61	1.79	2.03	1.99	2.22	2.57
CV (%)	1.71	1.41	0.89	1.38	1.35	2.20	2.22	1.41	0.86	1.02	1.00	1.13	1.34

Each value is a mean of three replicates. Means followed by same letter within a column are not significantly different at the $P \leq 0.01$ level according to Duncan's New Multiple Range Test. TC = *Trichoderma viride* isolated from commercial formulation.

Variation in the dry mycelium weight was seen same as variation in the radial growth and sporulation at different pH. Among the all isolates of *Trichoderma*, Tvb1 was found to be the most significant ($P \leq 0.01$) with highest mean dry mycelial weight of 0.515 mg followed by Thg1 with 0.456 mg; T14 with 0.360 mg; Thg2 with 0.305 mg and Thg13 with 0.310 mg; Tvpp with 0.277 mg and T3.2 with 0.268 mg; TB with 0.242 mg and TC with 0.238 mg; and least by Thg3 with 0.202 mg (Table 7). To be a great biocontrol agent, *Trichoderma* strain should have better stress tolerance level than phytopathogens against which they are going to be used. The abiotic factors deteriorate the antagonistic properties of *Trichoderma* spp. against the phytopathogenic fungi. Besides the effect of temperature, heavy metals, water relations, even the pesticides and pH influence on mycelial growth of phytopathogenic fungi as well as biocontrol agents. As in all micro-organisms even in *Trichoderma*, the external factors modify its morphological characteristics as well as physiological functions. Longa *et al.* [47] observed that *Trichoderma* strains were able to grow in a wide range of pH from 2 to 6. Jackson [35] found that optimum biomass production of three *Trichoderma* isolates

occurred at pH range between 4.6 to 6.8 as in this study optimum biomass was found at pH 7 followed by pH 5. In another study by Kredics *et al*. [40], *Trichoderma* strains were found to be able to display their activities under wide range of pH levels. Bhattiprolu (2008)[13] studied the growth of *T. virens* at pH range of 4-9 but optimum pH varied from 5-6. The different strains of *Trichoderma* could grow at pH range between 5-9. Strain M-7 of *Trichoderma* showed best growth and sporulation at pH 8. This strain may be the nature of alkaline soil and this because adaptive to high pH condition and grow well in the alkaline pH (Jackson, 1973 and Bandyopadhyaya *et al*., 2002)[35,11]. In this study, all *Trichoderma* isolates shown better mycelial weight at pH 9 than pH 3 except two (Thg2 and TB). Recently Prabhavathi *et al*, (2014)[61] studied the radial growth of four isolates at different pH and found maximum at pH 6 in *T. harzianum, T. viride, T. hamatum* and T. *virens* with 90, 88, 92 and 91 mm, respectively followed by at neutral pH with 89 mm by *T. harzianum*, 91 mm by *T. viride*, 89 mm by *T. hamatum* and 87 mm by *T. virens.* She also found maximum dry mycelium weight of *T. harzianum* (729 mg), *T. viride* (1639.67 mg), *T. hamatum* (798 mg) and *T. virens* (583.67 mg) was at pH 8 and minimum at pH 4.

Table 7. Dry mycelium weight of *Trichoderma* isolates after 10 days of incubation at different pH levels

	Dry mycelium weight (mg)					
Trichoderma	pH levels					
Isolates	pH-3	pH-5	pH-7	pH-9	pH-11	Mean
Thg3	0.121f	0.148h	0.488g	0.142g	0.109f	0.202h
Thg2	0.238b	0.475d	0.542f	0.158e	0.112e	0.305e
Thg1	0.252b	0.723b	0.841a	0.255c	0.209b	0.456b
Thg13	0.221c	0.245f	0.714b	0.245c	0.125d	0.310e
TB	0.152d	0.235g	0.544f	0.147f	0.132c	0.242g
T3.1	0.147d	0.236g	0.256h	0.187d	0.142c	0.194h
T3.2	0.158d	0.417e	0.473g	0.167e	0.125d	0.268f
Thb21	0.137e	0.574c	0.585e	0.255c	0.129d	0.336d
Tvb1	0.288a	0.814a	0.845a	0.345b	0.284a	0.515a
T14	0.212c	0.456d	0.587d	0.412a	0.131d	0.360c
Tvpp	0.134e	0.247f	0.636c	0.247c	0.122e	0.277f
TC	0.140e	0.280f	0.475g	0.158e	0.136c	0.238g
LSD (P ≤ 0.01)	0.013	0.039	0.031	0.015	0.010	0.016
CV (%)	3.027	4.213	2.279	0.139	0.117	0.127

Each value is a mean of three replicates. Means followed by same letter within a column are not significantly different at the *P*≤0.01 level according to Duncan's New Multiple Range Test. TC = *Trichoderma viride* isolated from commercial formulation

Evaluation of *Trichoderma* isolates for antagonism against soil borne pathogens

In vitro evaluation of all 12 *Trichoderma* isolates against *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lentis* for antagonism at different temperatures viz., 25 °C, 30 °C and 35 °C were done in dual inoculation test and evaluated after 5 days of incubation. All isolates of *Trichoderma* suppressed all three pathogens maximum at 30 °C (from 64.5 to 92.3%) followed by at 25 °C (from 38.7 to 64.8%) and least at 35 °C (from 25.16 to 42.12%).

At 30 °C all *Trichoderma* isolates overgrew on all three pathogens, after 5 days of incubation. At 25 °C, all *Trichoderma* isolates overgrew *F. oxysporum* f sp. *lentis* but at the same temperature *S. rolfsii* and *R. solani* overgrew *Trichoderma* isolates except Tvb1, Thg1, Thg2, TC, T14 and Tvpp. At 35 °C, all *Trichoderma* isolates suppressed all three pathogens but variations in suppression by the isolates were seen among them. Maximum suppression of all three pathogens were seen with highest efficacy (*P ≤ 0.01*) by Tvb1

followed by Thg1, Thg2, TC and rest of the isolates at 35 °C (Fig. 7). Irrespective of temperature, the mean suppression of pathogen was significantly ($P \leq 0.01$) maximum with Tvb1 (64.99%) followed by Thg1 (60.49%), and Thg2 (55.99%), TC (54.52%) and rest of the isolates (Table 8). In general, *Trichoderma* spp., are favoured by acidic soil conditions [18,59] and also classified as mesophilic organism not extremophilic [49,39,38,43]

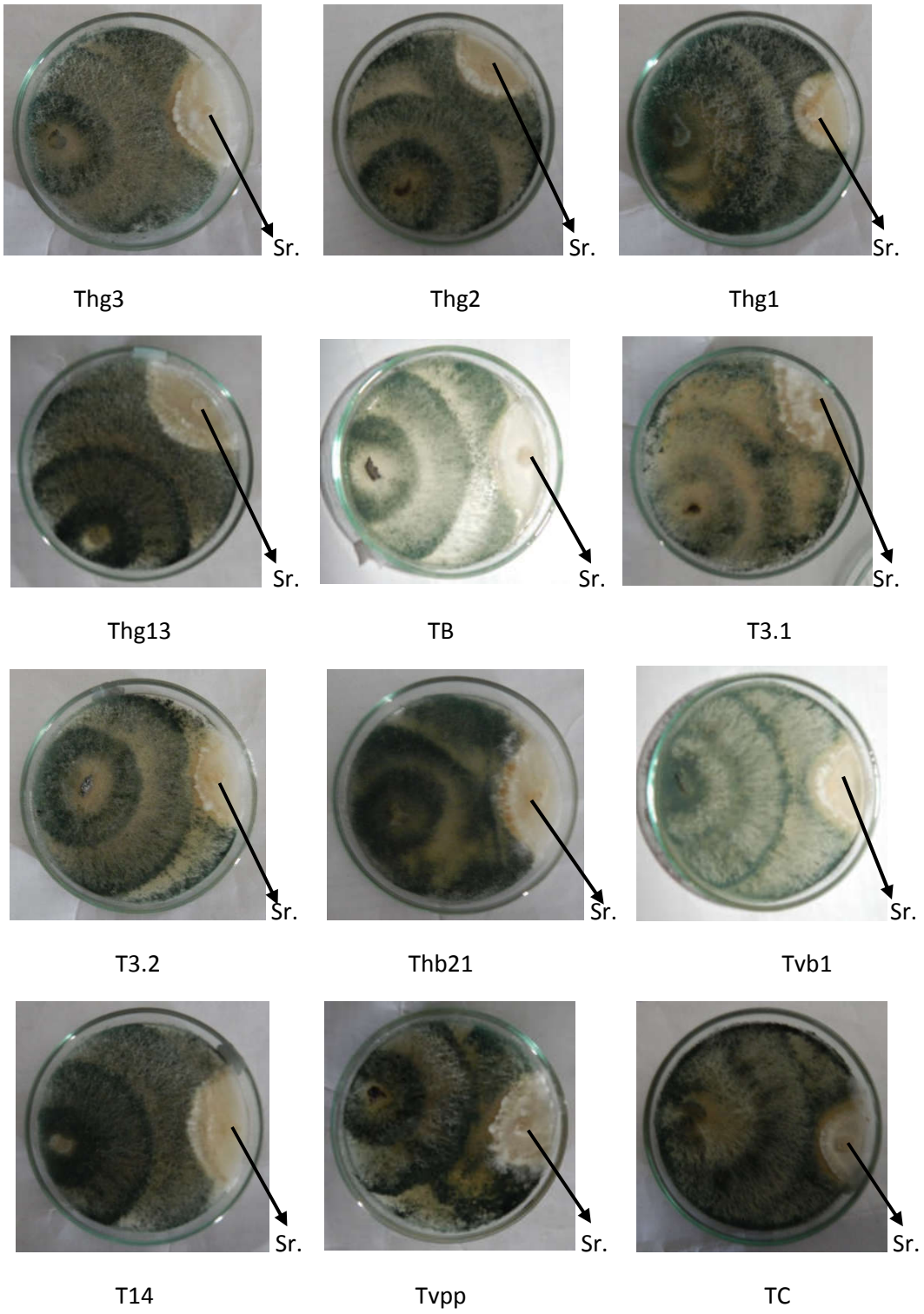

Fig. 7. Suppression of *Sclerotium rolfsii* with different *Trichoderma* isolates in dual culture assay at 30±1°C for five days of incubation. Sr.= *S. rolfsii.*

Table 8. *In vitro* evaluation of *Trichoderma* isolates against *Sclerotium rolfsii, Rhizoctonia solani* and *Fusarium oxysporum lentis* for antagonism at different temperatures (25 °C, 30 °C and 35 °C) after 5 days of incubation.

***Trichoderma* isolates**	**% inhibition of pathogens**									**Mean**
	Sclerotium rolfsii			***Rhizoctonia solani***			***Fusarium o.* f. sp. *lentis***			
	Temperature (°C)			**Temperature (°C)**			**Temperature (°C)**			
	25	**30**	**35**	**25**	**30**	**35**	**25**	**30**	**35**	
Thg3	44.20f	65.10f	28.73f	48.20f	69.50g	31.33f	56.70d	70.20f	36.86d	50.09g
Thg2	51.20c	74.50c	33.28c	55.60c	78.80c	36.14c	58.10c	78.50c	37.77c	55.99c
Thg1	59.00b	78.30b	38.35b	59.20b	85.50b	38.48b	59.40b	87.60b	38.61b	60.49b
Thg13	44.20f	70.20e	28.73f	45.20g	73.90e	29.38g	58.10c	75.20d	37.77c	51.41f
TB	43.30f	72.80d	28.15f	42.60i	79.80c	27.69h	58.00c	78.30c	37.70c	52.04f
T3.1	38.70h	70.00e	25.16h	39.80j	72.70f	25.87i	48.60g	71.10f	31.59g	47.06i
T3.2	46.50e	77.50b	30.23e	44.50h	75.80d	28.93g	45.90i	73.20e	29.84i	50.27g
Thb21	40.80g	74.50c	26.52g	42.40i	74.00e	27.56h	47.20h	72.40e	30.68h	48.45h
Tvb1	62.20a	89.20a	40.43a	62.80a	90.20a	40.82a	64.80a	92.30a	42.12a	64.99a
T14	49.90d	72.20d	32.44d	51.40e	74.20e	33.41e	54.00e	75.40d	35.10e	53.12e
Tvpp	50.60c	65.70f	32.89c	50.80e	64.50h	33.02e	52.10f	69.50g	33.87f	50.33g
TC	51.30c	74.30c	33.35c	54.30d	71.40f	35.30d	58.80b	73.70e	38.22b	54.52d
LSD ($P \leq 0.01$)	1.054	1.173	0.650	1.127	1.301	0.763	1.110	1.322	0.705	0.984
CV (%)	0.946	0.692	0.898	0.986	0.746	1.027	0.878	0.752	0.856	0.804

Each value is a mean of three replicates. Means followed by same letter within a column are not significantly different at the $P \leq 0.01$ level according to Duncan's New Multiple Range Test. TC = *Trichoderma viride* isolated from commercial formulation.

CONCLUSION

Most of the leguminous and other field crops undergo huge loss with three main soil borne pathogens, *Sclerotium rolfsii* (causes collar rot), *Fusarium oxysporum* (causes wilt diseases) and *Rhizoctonia solani* (causes root rot). Biological control in recent times have been accepted as more natural and environmentally acceptable alternative to the existing chemical treatments [26,34,27,71,4,6,3]. In this experiment, indigenous *Trichoderma* isolates have been investigated as an important antagonistic to soil pathogen especially fungal, having the ability to reduce disease incidence. Antagonistic properties of *Trichoderma* spp. against soil borne pathogens have been also observed by many researchers [28,7,24]. Rhizosphere competence of antagonists is a prerequisite for the biological control of soil borne plant pathogens. Several research papers reveal the fact that success of bioagent introduced in soil does not guarantee the control of target pathogen(s) because plants, physicochemical and biological factors affect establishment and antagonism of introduced bioagents. In this context, it is presumed that to ensure success of introduced bioagents, they should be isolated from the local area where they exist. Similar efforts have been tried here forth [44,17]. The present study is focused on this theme that after isolation and identification of local *Trichoderma* isolates from different fields and hot spring soil and tested their antagonistic potential under *in vitro* conditions they can be prescribed for application in fields under abiotic stress conditions such as high temperature and saline soil with high pH. Another important information can be reported from the present investigation that identified *T. asperellum* Tvb1 check *F. oxysporum* f. sp. *lentis*, *R. solani* and *S. rolfsii* at high temperature of 35°C.

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