International Journal of Plant & Soil Science

33(19): 100-112, 2021; Article no.IJPSS.71943
ISSN: 2320-7035

Morphological and Molecular Characterization of *Trichoderma* spp. from Rhizosphere Soil and Their Antagonistic Activity against *Fusarium spp.*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2021/v33i1930605
Editor(s):
(1) Dr. Omer Kilic, Adıyaman University, Turkey.
Reviewers:
(1) Chuanxiao Liu, Shandong Agricultural University, China.
(2) Benjapon Kunlanit, Mahasarakham University, Thailand.
(3) El-Sayed M. S. Gheith, Cairo University, Egypt.
Complete Peer review History: https://www.sdiarticle4.com/review-history/71943

Original Research Article

Received 02 June 2021
Accepted 10 August 2021
Published 14 September 2021

ABSTRACT

Trichoderma species are well known for their biocontrol activity which colonize many soil and tuber-borne and foliage plant pathogens. In this study, 12 native isolates of *Trichiderma* spp were collected from various crop rhizosphere soil samples and characterized them phenotypically based on morphological and cultural features and genotypically based on sequence analysis of internal transcribed spacer (ITS) region-PCR amplification. The results obtained from phenotypic and genotypic observation revealed that isolates were belonged to five different species namely *T. asperellum, T. harzianum, T. longibrachiatum, T. koningii* and *T. koningiopsis*. All *Trichoderma* isolates produced ~600 bp amplicon and phylogenetic analysis revealed that all isolates were grouped with respective species. Further, the antagonistic potential of all the isolates was evaluated

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against *Fusarium* spp. following in *vitro* dual culture method. The results showed that isolates of *T. harzianum* exhibited maximum growth inhibition activity. The highest rate of inhibition was recorded with *T. harzianum* isolate TBT6 (87.1%) followed by TBT7 (82.2%), while the least inhibition was observed in *T. longibrachiatum* isolate TBT10 (59.7%) after 7 days of incubation. The antagonistic *T. harzianum* isolate TBT6 can be used for development of *Trichoderma* based bio-formulation and served as bio-control agent against *Fusaium* spp. under field conditions.

Keywords: Trichoderma spp; Internal Transcribed Spacer (ITS); phylogenetic analysis; biocontrol; Fusarium spp.

1. INTRODUCTION

Filamentous fungi of the genus *Trichoderma* (teleomorph: *Hypocera*) are free living and fast growing microorganism commonly found in soil and root eco-system. *Trichoderma* species colonize a wider range of pathogenic fungi and served as bioagent becoming an effective alternative to synthetic fungicides for controlling the plant diseases. *Trichoderma* species are difficult to discriminate morphologically therefore, for accurate identification of fungal species various molecular methods including DNA sequencing have been used [1]. The technique of DNA genotyping comprises the amplification of targets that are phylogenetically informative like subunit of rRNA gene [2]. Very limited variability was observed in ITS (internal transcribed spacer) sequences in *Trichoderma* spp. isolates from various origins and thus a set of universal barcode markers were developed from the nearly conserved nature of the nuclear ribosomal internal transcribed spacer (ITS) region for imparting the individual identity to the fungus up to genus level [3].

Phytopathogens cause considerable crop losses all over the world. Framers and industries have been facing the destructive activities of various pests and pathogens, which leads not only to the reduction of yield of the crops but also the aesthetic value. Chemical control is effective for controlling the plant diseases. However, extensive use of synthetic fungicides raised a serious problem to the environment, human health and soil beneficial microorganisms such as nitrogen fixers, antagonists and mycorrhizal fungi [4]. As a biocontrol agents, *Trichoderma* spp. exercise several antagonistic mechanisms such as nutrient competition, antibiotic production, mycoparasitism and induction of systemic resistance [5,6]. *Trichoderma* is especially known for its antagonistic activities against several plant pathogens including *Rhizoctonia solani*, *Fusarium* spp., *Alternaria* spp., *Sclerotinia sclerotiorum*, *Sclerotium rolfsii* [7,8,9,10]. Mycoparasitism has been proposed as the major antagonistic mechanism displayed by *Trichoderma* spp. [11]. After host recognition, *Trichoderma* spp. attaches to the host hyphae via coiling and penetrate the cell wall by secreting cell wall-degrading enzymes [12]. Continuous production of exo-chitinases by *Trichoderma* spp. damages cell-walls of the pathogen, thereby discharging oligomers, which play a crucial part in the inhibition of phytopathogens [13]. Thus, the isolation and identification of such a valuable antagonistic fungus would help in commercialization and ultimately developing a superior strategy to eliminate pathogens that minimize the crop losses. Therefore, the objectives of this investigation were to isolate the *Trichoderma* spp. from different crop rhizospere soils and characterized them phenotypically based on cultural and morphological characteristics, and genotypically at species level using sequence analysis of the internal transcribed spacer (ITS) region and to evaluate the antagonistic potential of *Trichoderma* spp. against *Fusarium* spp. *in vitro*.

2. MATERIALS AND METHODS

2.1 Isolation and Maintenance of Native *Trichoderma* spp.

Rhizosphere soil samples from field grown crops viz., rice, tomato, potato, brinjal, sugarcane, mango and banana in the state of Uttar Pradesh, India were collected in sterile polybag and brought to laboratory (Table 1). Each sample was mixed thoroughly and native *Trichoderma* spp. were isolated on potato dextrose agar (PDA) and *Trichoderma* specific medium (TSM) following the serial dilution platting method described by Johnson and Curl [14]. Briefly, 1g air dried soil sample was added into 9 ml sterile water in a test tube to make 1:10 dilution (10^{-1}). The mixture was vigorously shaken on a vortex mixture for 5-10 minutes to obtain uniform suspension. One ml of soil suspension was

transferred into a fresh sterile test tube containing 9 ml sterile water under aseptic conditions to make 10^{-2} dilution. Further, 10^{-3} dilution was made by pipetting 1 ml suspension into another fresh test tube containing 9 ml sterile water. One ml of soil suspension was taken and uniformly spread on PDA and TSM containing Petri plates and incubated at 25±2°C for 5-7 days in the dark. After incubation as the mycelial growth were appeared, the hyphal tips from the advancing mycelium were cut and transferred into the fresh PDA medium for further purification. The purified isolates were cultured on PDA slants and maintained at 4°C for further use.

Cultural and morphological characterization of *Trichoderma* isolates: The morphological and cultural characteristics of *Trichoderma* isolates were determined on PDA medium. For cultural characterization, a 5 mm diameter mycelium disc was cut from the actively growing edge of a fresh colony (3 days old culture) of all the isolates and placed on Petri plates containing PDA medium. All the inoculated plates were incubated in BOD at 25±2°C in the dark. The colonies were observed at regular intervals for pattern of conidiation, first appearance of green conidia, formation of conidial pustules, and presence of pigmentation. Radial growth rates were recorded from the edge of the inoculum disc at 24 h intervals. Three replications were maintained for each experiment.

For morphological characterization, microscopic slides were prepared in 3% KOH followed by lactophenol-cotton blue staining from pustules where conidia were still white, generally after 5-7 days of incubation. After placing the cover slip, the slides were examined under the optical microscope for morphological features like conidiophores, branching pattern, phialide numbers and their arrangement, conidial shape and colour and formation of chlamydospores and their position. Species identification was based on the morphological and taxonomic keys provided by Bissett [15-16].

2.2 Ribosomal DNA (rDNA) Based Molecular Characterization of *Trichoderma* isolates

2.2.1 Extraction of total genomic DNA

All isolates were grown in 100 ml conical flask containing 60 ml of potato dextrose broth (PDB) medium. Two agar plugs from actively growing colony of all the isolates were inoculated to each flask aseptically in a laminar flow. The inoculated flasks were incubated for 3-5 days at 25°C with rotary shaking at 130 rpm in the dark. After incubation, mycelia were harvested by filtration through Whatman No. 1, washed thrice with 0.9% NaCl solution; blot dried and stored at -80°C for genomic DNA extraction. The fungal cell wall was disrupted by grinding in liquid nitrogen using pestle and mortar. The total genomic DNA (gDNA) was extracted following the CTAB method described by Doyle and Doyle [17] with slight modifications. The quantity and quality of purified DNA were determined using a NanoDrop instrument (Thermo Scientific, USA) and stored at -80°C for further use. Working concentration of DNA was adjusted to 50 ng μl^{-1} and stored at -20°C.

Ribosomal DNA (rDNA) - Internal Transcribed Spacer (ITS) PCR amplification and sequencing: Amplification of DNA region encoding ITS1–5.8S–ITS4 of the gDNA was done using the universal ITS1 and ITS4 primers pair [18]. The PCR amplification was performed in a total volume of 25 µl with 2.5 µl of 10x PCR buffer, 2 µl of dNTPs mix, 1.5 µl of each primer (10 pmol), 3 U Taq DNA polymerase, 1.5 µl of genomic DNA template (50 ng) and molecular grade water. The thermal cycler program included the following temperature profile: initial denaturation at 95°C for 5 min, followed by 38 cycles of 94°C for1 min, 56°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 7 min. The amplified PCR products were electrophoresed on 1.2% agarose gel in 1x TAE buffer and documented under gel documentation system.

The PCR amplified ITS-rDNA region from all the isolates were purified using GeneJET PCR purification kit (Thermo Scientific, USA) and Sanger sequencing was performed commercially at Bioserve Pvt. Ltd., Hyderabad, India. The nucleotide sequences were analyzed at NCBI GenBank database (http://www.ncbi.nlm .nih.gov/) using BLAST analysis tool. The sequences showed maximum homology and highest score with 0.0 E-value were marked for further analysis. All the generated ITS sequences were deposited and accessioned at GenBank NCBI database. Multiple sequence alignment was performed using ClustalW program of the Bioedit sequence alignment editor [19]. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA version 7.0) using Neighbour-Joining (NJ) method.

Table 1. Morphological and molecular identified isolates of *Trichoderma* spp. used in this study

Isolate Code	Place of collection soil samples	State	Crop	Year	Morphological identification	GenBank Acc. No. (ITS sequence)	Molecular identification
TBT1	SVPUAT (Campus Farm), Meerut	Uttar Pradesh	Banana	2019	*T. asperellum*	MW776752	*T. asperellum*
TBT2	SVPUA&T Meerut (HRC)	Uttar Pradesh	Mango	2019	*T. asperellum*	MW776753	*T. asperellum*
TBT3	Muzaffarnagar	Uttar Pradesh	Brinjal	2019	*T. asperellum*	MW776754	*T. asperellum*
TBT4	Hapur	Uttar Pradesh	Rice	2019	*T. asperellum*	MW776755	*T. asperellum*
TBT5	Bijnor	Uttar Pradesh	Tomato	2019	*T. longibrachiatum*	MW776756	*T. longibrachiatum*
TBT6	Faizabad	Uttar Pradesh	Rice	2019	*T. harzianum*	MW776757	*T. harzianum*
TBT7	Ghaziabad	Uttar Pradesh	Rice	2019	*T. harzianum*	MW776758	*T. harzianum*
TBT8	Bulandshahr	Uttar Pradesh	Rice	2019	*T. harzianum*	MW776759	*T. harzianum*
TBT9	Bareilly	Uttar Pradesh	Rice	2019	*T. longibrachiatum*	MW776760	*T. longibrachiatum*
TBT10	Pilibhit	Uttar Pradesh	Potato	2019	*T. longibrachiatum*	MW776761	*T. longibrachiatum*
TBT11	Saharanpur	Uttar Pradesh	Tomato	2019	*Trichoderma* spp.	-	*T. koningii*
TBT12	Bagpat	Uttar Pradesh	Sugarcane	2019	*Trichoderma* spp.	-	*T. koningiopsis*

Antagonistic activity of different isolates of *Trichoderma* spp. against *Fusarium* spp.: The antagonistic potential of *Trichoderma* spp. isolates against *Fusarium* spp. was determined by dual culture technique [20]. The test pathogen i.e. *Fusarium oxysporum* f. sp. *lycopersici* (ITCC No. 1322) was procured from division of Plant Pathology, IARI, New Delhi. A mycelium disc of 5 mm diameter was cut from 5 days old culture of different isolates of *Trichoderma* spp. and placed at one point leaving 1 cm distance from the periphery of one side of petri plate and on the opposite site; disc (5 mm diameter) of *Fusarium oxysporum* f. sp. *lycopersici* was inoculated. Plates were kept without antagonist to serve as control. The plates were incubated at 25±2°C for 7 days in the dark. Three replications were maintained for each treatment. Observations on colony growth were recorded and % inhibition was calculated using the following formula:

$$I = (X-Y) / (X) \times 100$$

Where: I= percent inhibition in pathogen mycelium growth
X = Growth of pathogen alone without antagonist (control)
Y = Growth of pathogen along with the antagonist

The antagonistic ability of all the isolates was recorded by following the rating system described by Bell et al. [21] on a scale of 1-5: Class 1: *Trichoderma* completely overgrew the *Fusarium* and covered the entire medium surface, Class 2: *Trichoderma* overgrew at least two third of the medium surface, Class 3: *Trichodema* and *Fusarium* each colonized 50 % of the medium surface and neither organism appeared to dominate the other, Class 4: *Fusarium* colonized at least two-third of the medium surface and appeared to withstand encroachment by *Trichoderma*, and Class 5: *Fusarium* completely overgrew the entire medium surface. *Trichoderma* isolates were considered to be antagonistic to the *Fusarium* if the mean score for a given comparison was class ≤ 2, but not highly antagonistic if the number was class ≥ 3.

For mycoparasitism test, the clean glass slide assay was used. Molten water agar (2%) was poured and evenly spread over the sterile glass slide to make a thin agar film. The mycelium disks of *Fusarium* and *Trichoderma* isolates were placed on the slide 1 cm apart from each other and incubate at 27±2°C for 2-3 days in the dark. After incubation, the contact/inhibition zone mounted under lectophenol-cotton-blue and observations were made under an optical microscope for the presence of coiling structures and wall disintegration in the hyphae of *Trichoderma* and *Fusarium,* respectively. The frequencies of coiling or wall disintegration were recorded by observing the coils or areas of disintegration in five different microscopic fields [22].

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of *Trichoderma* spp.

A total of 12 isolates of *Trichoderma* species were isolated from 20 rhizosphere soil samples from crops in the Uttar Pradesh state of India. Based on cultural, morphological and molecular characterization, all isolates were divided into five groups. According to descriptions described by Gams and Bissett [23] each group was identified as *Trichoderma asperellum* (33.33%), *T. harzianum* (25.0%), *T. longobrachiatum* (25.0%), *T. koningii* (8.33%) and *T. koningiopsis* (8.33%)) (Fig. 1). *T. harzianum* were mainly isolated from soil samples collected from rice rhizosphere, whereas other *Trichoderma* species were isolated from various crop rhizosphere soils.

Cultural and morphological characterization of *Trichoderma* isolates: Variability in cultural and morphological features of *Trichoderma* isolates on PDA medium is summarized in Table 2 and Fig. 2. Cultural characteristics were observed in all the *Trichoderma* isolates and revealed colonies with rapid growth rate, concentric halos and floccose or compact surface that looked like tufts on the medium. Initially, the mycelium appeared whitish in colour, acquired green, yellow shades, or remained white, due to the abundant formation of conidia. A perusal of growth data revealed that there was a significant difference in growth rate among the isolates. Isolates TBT6 and TBT8 grew faster (17.2 and 19.3 mm/day, respectively) than other isolates. Minimum growth rates (9.5 and 10.4 mm/day) were recorded in case of isolates TBT12 and TBT5, respectively (Table 2).

Table 2. Morphological descriptors used for characterization of native isolates of *Trichoderma* spp.

Isolate	Growth rate (mm/day)	Colony colour	Colony reverse colour	Colony edge	Conidiophores character	Phialide character	Conidia shape	Conidia wall	Chlamydospore formation
TBT1	15.1	Pale green	Light yellow	Smooth	Long infrequently branching and verticillate	Lageniform and convergent	Globose to ellipsoidal	Rough	Infrequent, terminal, and intercalary
TBT2	12.3	Green	Light yellow	Smooth	Broad, verticillate, and frequently branching	Lageniform, divergent, terminal philaid more elongated	Sub cylindrical to narrow ellipsoidal	Rough	Frequent, intercalary, and terminal
TBT3	14.2	Watery white	Colourless	Wavy	Rarely branched and verticillate	Cylindrical or slightly inflated and divergent	Ellipsoidal	Rough	Abundant, terminal, and intercalary
TBT4	12.3	Dark green	Colourless	Smooth	Broad frequently branching and verticillate	Ampulliform and divergent	Sub cylindrical	Smooth	Frequently intercalary and terminal
TBT5	10.4	Green to dark green	Yellowish	Smooth	Infrequently branching and verticillate	Frequently paired, lageniform, and divergent	Subglobose to obovoid	Smooth	Infrequent, intercalary, and terminal
TBT6	17.2	Light green	Dark brownish	Smooth	Frequently branching and verticillate	Ampulliform and convergent	Globose to ellipsoidal	Smooth	Frequently intercalary and terminal
TBT7	15.4	Green	Light yellow	Smooth	Narrow verticillate and frequently branching	Ampulliform and divergent	Globose to ellipsoidal	Smooth	Infrequent, internally and terminally
TBT8	19.3	Whitish green	Light yellow	Smooth	Infrequently branching and verticillate	Ampulliform and divergent	Globose to ellipsoidal	Smooth	Infrequent, intercalary, and terminal
TBT9	13.4	Green	Yellowish	Smooth	Broad, verticillate, and frequently branching	Ampulliform and divergent	Globose to subglobose	Smooth	Infrequent, intercalary, and terminally
TBT10	13.4	Dark green	Light yellow	Smooth	Broad frequently branching and verticillate	Lageniform, divergent, terminal philaid more elongated	Globose to ellipsoidal	Smooth	Frequently, intercalary, and terminal
TBT11	11.3	Greenish yellow	Yellowish	Smooth	Frequently branching and pyramidal structure	Cylindrical to sharply constrict at the tip	Subcylindrical to ellipsoidal	Smooth	Frequently, intercalary, and terminal
TBT12	9.5	Light Yellow	Yellowish	Smooth	Frequently branching and pyramidal structure	Lageniform, terminal philaid more elongated	Ellipsoidal	Rough	Abundant, terminal, and intercalary

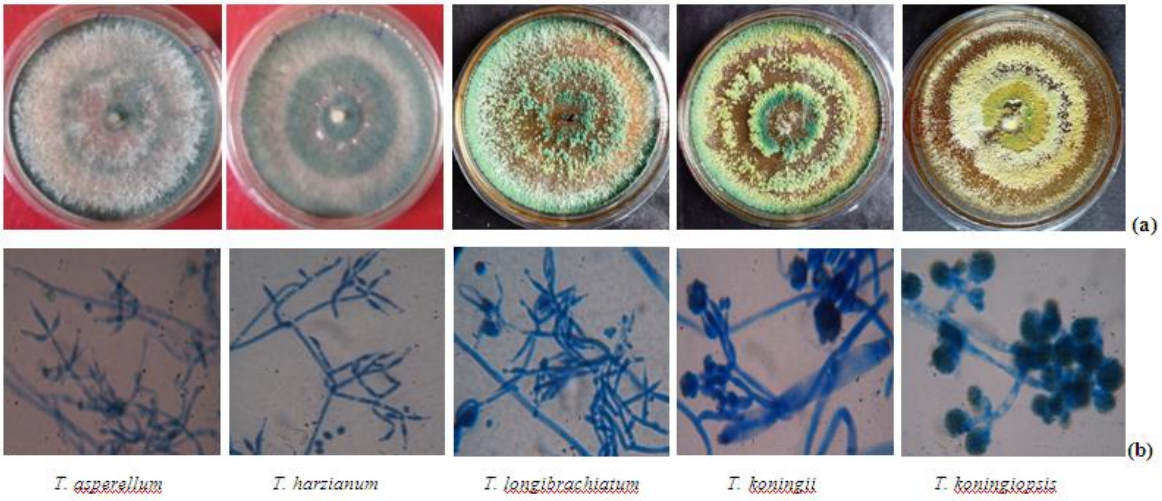

Fig. 1. (a) Characterization of different *Trichoderma* isolates by macroscopic morphology of the growth pattern of colonies in PDA medium after 7 days of incubation, (b) Microscopic observation of conidiophores and conidia of representative isolates

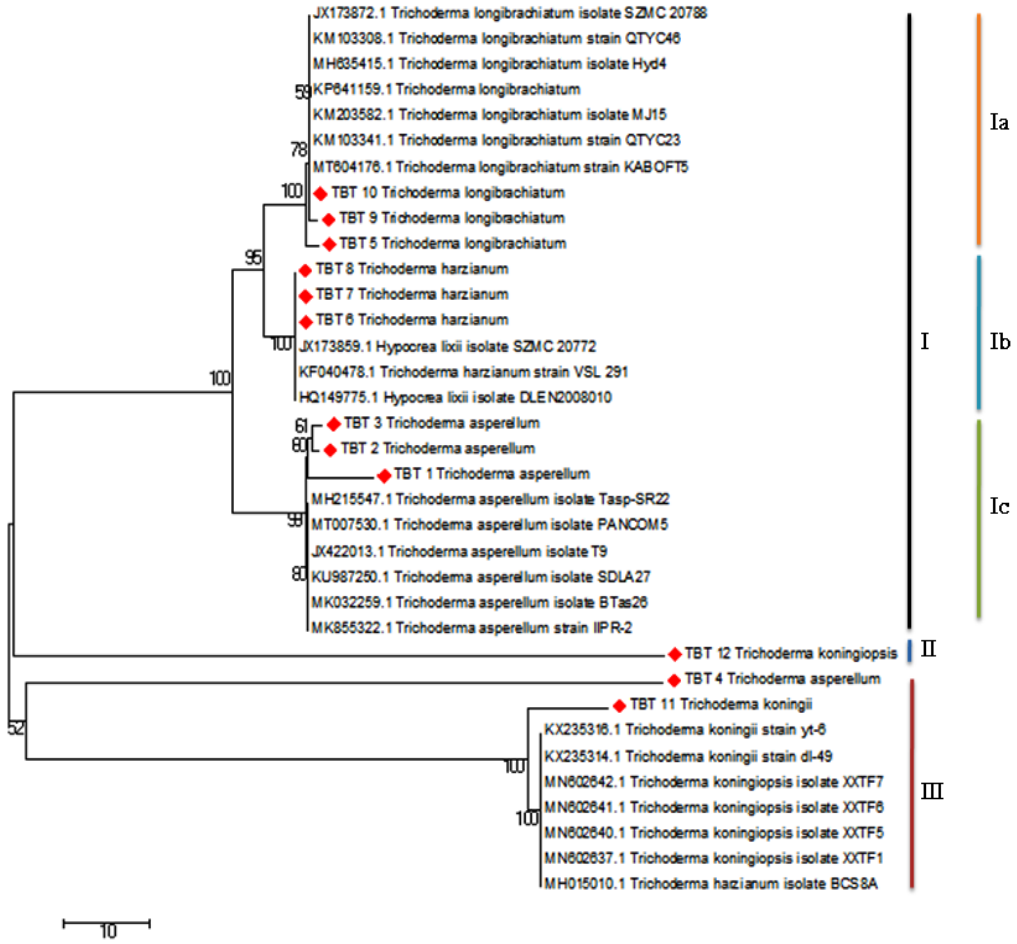

Fig. 2. Phylogenetic analysis of *Trichoderma* isolates based on rDNA-ITS sequences by Neighbor-Joining (NJ) method. The numbers given over branches indicate bootstrap coefficient

Microscopic observation revealed abundant sporulation of smooth or rough-appearance conidia which originated from branched and irregularly verticillated conidiophores, and presented conidiogenic (phialides) cells; generally ampliform or fusiform which were arranged in clusters, according to Gams and Bissett [24]. Isolates of *Trichoderma* spp. showed morphological variable colonies, where the green color of the conidia is interleaved with the white of the mycelium, which is consistent with the typical characteristics described as identification key for this fungus [25] were obtained. Isolates of *T. asperellum* showed globous, subglobous to ellipsoid, and some ovoid conidia, ampuliform phialides, and presence of chlamydospores as described Samuels et al. [26]. The results obtained with morphological characteristics of *T. harzianum* isolates are corroborated with Samuels et al. [27] which species presents subglobous to ellipsoid conidia, ampuliform phialides, and globous and subglobous chlamydospores. The results are corroborated with the findings of other workers [28,29]. Isolates of *Trichoderma longibrachiatum* possesses subglobous to ovoid conidia and lageniform phialides as described by Rifai [30] and Samuels et al. [26]. Isolates of *T. koningii* showed oblong to slightly ellipsoidal conidia, cylindrical and narrowly pyriform phialides, and globose, terminal, globose to subglobose clamydospores as described by Lieckfeldt et al. [31] *T. koningiopsis* possesses ellipsoidal conidia, straight, sinuous and narrowly lageniform phialides, terminal to intercalary and globose to subglobose chlamydospores. All isolates in this study, except TBT3 produced yellowish to brownish pigmentation as Bissett [32], reports the presence of yellowish-green pigment in the reverse of some cultures of these species. The identification of the isolates in this study yielded three species namely *T. asperellum, T. harzianum* and *T. longibrachiatum* were the most frequently sampled. The presence of these species had already been reported in various crops rhizospere soil from Uttar Pradesh, India [8]. However, the cultural and morphological characteristics used to identify fungi of the genus *Trichoderma,* it is insufficient to distinguish the species, and is necessary to confirm those species through molecular methods [33,34].

Ribosomal DNA (rDNA) based molecular characterization of *Trichoderma* isolates: Fungal specific ITS1-ITS4 universal primers pair was used for amplifying the internal transcribed spacer (ITS) region from the gDNA of all the 12 *Trichoderma* spp. isolates collected from different crop rhizosphere soil samples. The amplicon sizes and purity were measured using agarose gel electrophoresis and found approximately 600 bp in size (Fig. 3). These results are in accordance with Prasad et al. [35] who estimated the amplified rDNA fragment of around 500-600 bp in *Trichoderma* spp. using ITS-PCR amplification. The resulting rDNA sequence data was analysed by using NCBI BLAST tool and results connoted the morphological variability and supported their individual existence. Further, at NCBI GenBank database the 97–98% closest match was found with *T. asperellum, T. harzianum, T. longibractiatum, T. koningii* and *T. koningiopsis*. The obtained sequence information for the ITS rDNA region of 10 *Trichoderma* isolates were submitted in the NCBI GenBank database under the accession numbers MW776752-MW776761 (Table 1) as a new report.

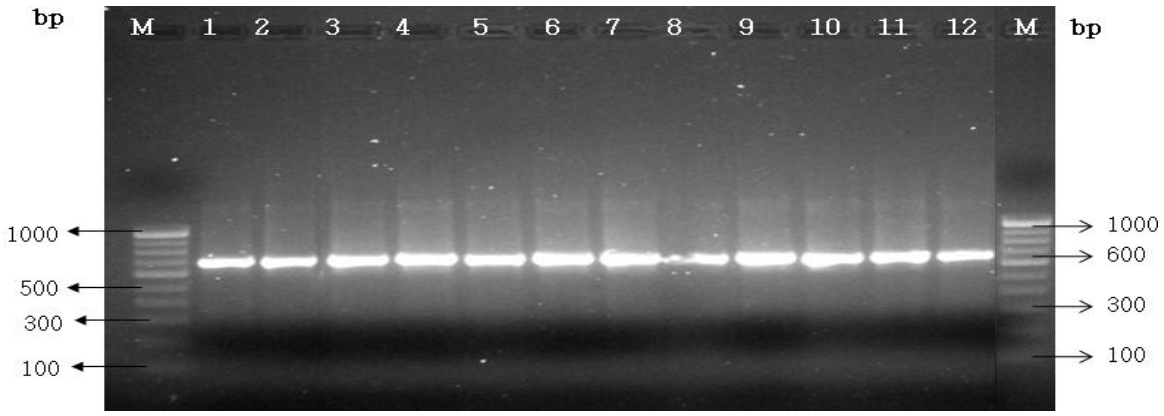

Fig. 3. Amplified PCR products of ITS region of 12 native *Trichoderma* spp. isolates from soil using ITS universal primers. Lane M: 100 bp ladder (Promega, USA), Lane 1-12: TBT1-TBT12

Table 3. Growth inhibition of *Fusarium* spp. in dual culture by different isolates of *Trichoderma* spp.

Trichoderma isolates	Growth inhibition efficiency of *Trichoderma* spp. in dual culture						Type of Inhibition	Bell's rating
	5th days after inoculation		6th days after inoculation		7th days after inoculation			
	Radial Growth (cm)*	Growth Inhibition (%)#	Radial Growth (cm)*	Growth Inhibition (%)#	Radial Growth (cm)*	Growth Inhibition (%)#		
TBT1	2.33	43.6 (41.3)	2.00	68.8 (56.1)	1.76	80.4 (63.7)	Antibiosis	(1)
TBT2	3.66	11.4 (19.7)	3.40	48.2 (43.9)	2.83	68.6 (55.9)	Mycoparasitism	(2)
TBT3	2.93	29.1 (32.7)	3.03	53.6 (47.1)	3.03	66.3 (54.5)	Antibiosis	(2)
TBT4	3.03	26.6 (31.1)	2.86	56.1 (48.5)	2.63	70.8 (57.3)	Mycoparasitism	(2)
TBT5	3.00	27.4 (31.6)	2.46	62.0 (51.9)	2.10	76.7 (61.1)	Antibiosis	(1)
TBT6	2.63	36.3 (37.1)	2.33	64.0 (53.1)	1.16	87.1 (68.9)	Mycoparasitism	(1)
TBT7	2.66	35.6 (36.6)	2.43	62.5 (52.2)	1.60	82.2 (65.1)	Mycoparasitism	(1)
TBT8	2.63	36.3 (37.1)	2.53	61.0 (51.4)	2.16	76.0 (60.7)	Antibiosis	(1)
TBT9	3.83	7.3 (15.7)	3.66	44.3 (41.7)	3.36	62.7 (52.4)	Lysis	(2)
TBT10	4.03	2.4 (8.9)	3.86	41.4 (40.1)	3.63	59.7 (50.6)	Mycoparasitism	(3)
TBT11	3.26	21.1 (27.3)	2.63	59.5 (50.5)	2.36	73.8 (59.8)	Antibiosis	(1)
TBT12	3.66	11.4 (19.7)	3.5	46.7 (43.1)	3.13	65.2 (53.8)	Lysis	(3)
Control	4.13	-	6.66	-	9.00	-	-	-
C.D. (p=0.05)	0.20	-	0.19	-	0.176	-	-	-
C.V. (p=0.05)	3.13	-	3.41	-	3.55	-	-	-

**Values are the mean of three replications, #Values are the mean percentage inhibition (angular transformed data) of three replicates*

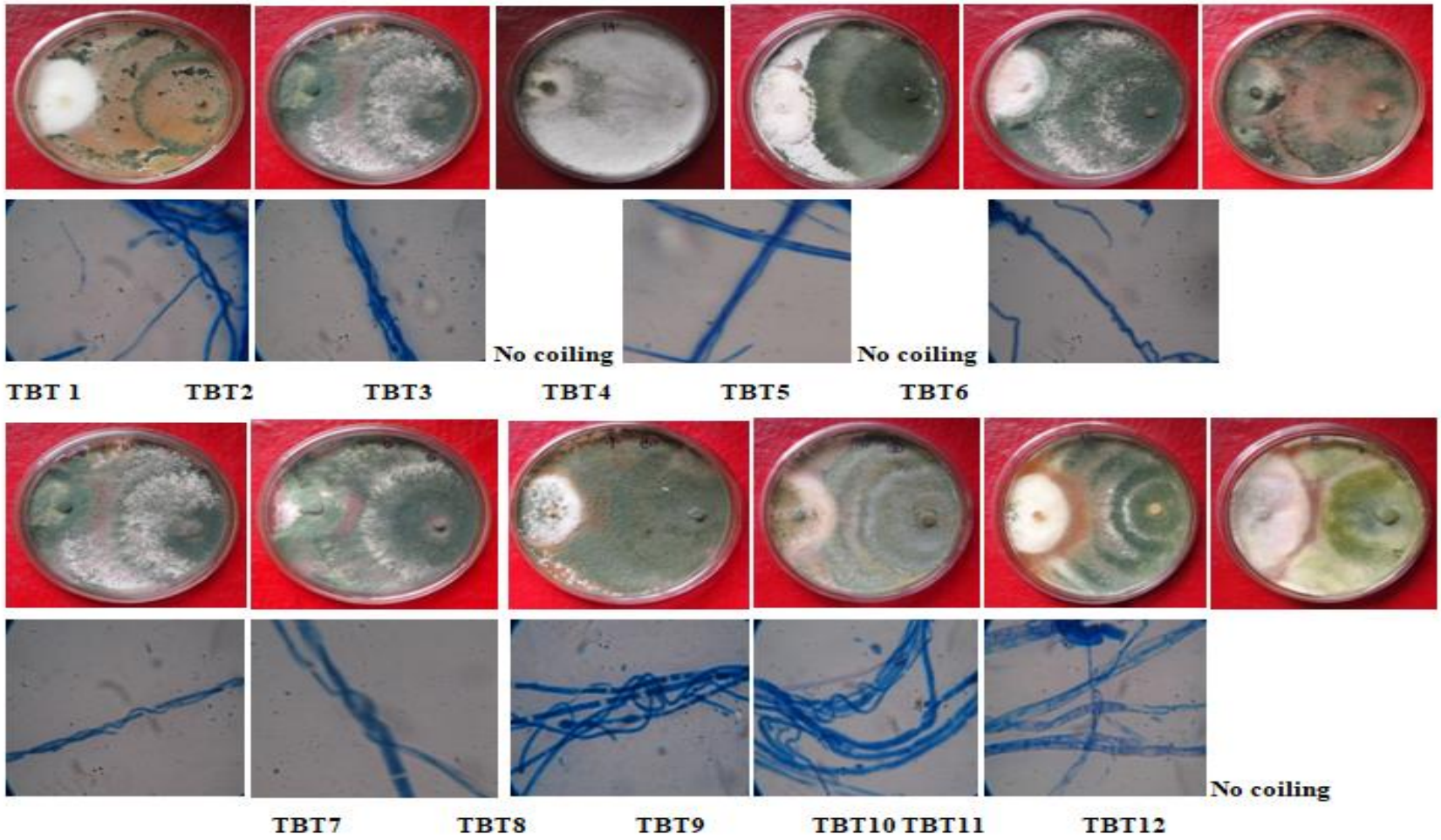

Fig. 4. Dual plate assay for antagonistic activity and photomicrographs showing hyphal interaction between of *Trichoderma* spp. isolates and *Fusarium spp.*

Phylogenetic relationship among the *Trichoderma* species was determined using the ITS sequence information of 12 *Trichoderma* spp. and 23 other *Trichoderma* sequences, including *T. asperellum, T. harzianum, T. longibrachiatum, T. koningii* and *T. koningiopsis* available in the NCBI database. A dendrogram was constructed by using the ClustalW and Mega7.0 software with the Neighbor-Joining (NJ) method. According to Saitou and Nei [36], NJ can be applied to all types of data that have evolutionary differences and use the Kimura-2 parameter formula to determine evolutionary distances or nucleotide changes that occur so that they can form phylogenetic trees between species. The bootstrap analysis was performed with a 1000 resampling to increase the value of confidence [37].

The resulted phylogenetic tree clustered all the isolates into two major groups (I and III) and one group (II) with a single isolate namely *T. konigiopsis.* The group I was divided into three clades: Ia, Ib and Ic. The clade Ia supported by a 100% bootstrap value occupied all isolates of *Thichoderma longibrachiatum* with other *longibrachiatum* species isolates. The clade Ib consisting all *T. harzianum* isolates supported by 100% bootstrap value, while clade Ic contained three *T. asperellum* isolates with other *asperellum* species isolates supported by 98% bootstrap value. Group III contained one *T. asperellum* isolate and one *T. koningii* isolate with 100% bootstrap value. Bootstrap values between 70 and 100 indicated that branching and phylogenetic trees will not change. Ubaidillah and Sutrisno [38] explained that bootstraping is a method of reshaping data for the reconstruction of phylogenetic trees.

Antagonistic activity of different isolates of *Trichoderma* spp. against *Fusarium* spp.: The antagonistic activity of all *Trichoderma* isolates was examined against *Fusarium* spp. and the results revealed that all isolates were able to suppress the mycelial growth of *Fusarium* up to certain extent (Table 3). All *T. harzianum* isolates exhibited higher growth inhibition activity of *Fusarium* compared to the other species isolates. Among the *T. harzianum* isolates highest growth inhibition percentage was recorded in TBT6 (87.1%) followed by TBT7 (82.2%), whereas the minimum growth inhibition (59.7%) was recorded in *T. longbrachiatum* isolates TBT10 after 7 DAI (Fig. 4). *T. harzianum* isolate TBT6 showed initial faster growth, overgrew on *Fusarium* and destroyed it completely and sporulated after 5 days of inoculation. All *T. harzianum* isolates showed Bell's rating of 1, which indicates that these isolates have superior antagonistic activities against *Fusarium* in-vitro as compared to other isolates of *Trichoderma* spp. The overgrowth of the antagonist is achieved when a fungus exhibits higher growth rate, tolerance against metabolites produced by the pathogens [39] and capacity to produce antibiotics. In dual cultures assay several morphological changes were observed when inhibition zone were analyzed under optical microscope. Microscopic observation of the interaction zone between *Fusarium* and *T. harzianum* showed that hyphae of *T. harzianum* coiled around the *Fusarium* hyphae (Fig. 4). Lysis of hyphae of *Fusarium* with close contact of *T. harzianum* hyphae was observed. Isolates of *T. harzianum* were found effective against various phytopathogens like *R. solani* [13], *Sclerotinia sclerotiorum* [40] *Fusarium oxysporum* [8] etc. in dual culture assay.

4. CONCLUSION

In this study, different species of *Trichoderma* viz. *T. asperellum, T. harzianum, T. longibrachiatum, T. koningii* and *T. koningiopsis* have been isolated from different cop rhizosphere soils. These species have been identified based on cultural, morphological and molecular characterization using Internal Transcribed Spacer (ITS)-PCR amplification. Further, it may be concluded that *Trichoderma* spp. have the antagonistic potentials against *Fusarium spp*. The present study also showed that most of the *Trichoderma* isolates that found effective against *Fusarium* in *in vitro* assay were belonged to *T. harzianum.* Among the *T. harzianum* isolates, TBT6 was found effective having great potential to inhibit *Fusarium* mycelial growth compared to other isolates tested. Therefore, this isolates might be used as biofertilizers-cum biopesticide for managing the *Fusarium* diseases. Furthermore, studies are needed on this promising *T. harzianum* isolate to identify potential secondary metabolites produced and evaluate possible modes of actions related to biocontrol.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not

intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ACKNOWLEDGEMENTS

The authors are highly grateful to the Head, Department of Recombination Techniques, College of Biotechnology, SVP University of Agriculture and Technology, Meerut for providing laboratory facilities to carry out this investigation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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