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Morphomolecular Identification of *Trichoderma* sp. and their Mycoparasitic Activity Against Soil Borne Pathogens

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Abstract

This study was carried out to identify and characterize *Trichoderma* species isolated from rhizospheric soil of Uttar Pradesh, India, by using single spore technique. Morphological, cultural and molecular characterization were done with sequence analysis of the internal transcribed spacer (ITS) region. The classes were compared with morphological identification and rDNA sequence data for every class of all *Trichoderma* strains were of the same identity. These strains belonged to *T. harzianum* (*Th azad*), *T. viride* (01PP), *T. asperellum* (T_{asp}/CSAU), *T. Koningii* [T_K (CSAU)], *T. atroviride* (71L), *T. longibrachiatum* (21PP), *T. virens* [T_{vi} (CSAU)], *T. reesei* [*Tr* (CSAU)], *T. aggressivum* [T.agg(CSAU)], *T. aureoviride* [T. avi (CSAU)], *T. citrinoviride* [T. cvi (CSAU)], *T. erinaceum* [T. eri (CSAU)], *T. koningiopsis* [T. kop (CSAU)], *T. tomentosum* [T. tos (CSAU)], *T. mintisporum* [T. mip (CSAU)], *T. pubscenes* [T. sce (CSAU)], *T. saturnisporum* [T. ssp (CSAU)], *T. spirale* [T. sp. (CSAU)]. Morphological studies were based on the colony appearance, growth rate and microscopic features such as branching patterns of conidiophores, the arrangement of phialospores and their shape, size and color. The 5.8S-ITS regions of the *Trichoderma* strains were amplified using ITS1 and ITS4 primers. The rRNA based analysis is a central method used not only to explore microbial diversity but also to identify new strains. Validations of ITS marker with 18 *Trichoderma* sp. were done and their sequences were deposited at NCBI GenBank their permanent accession no. were allotted.

Keywords: Morphology, genetic identification, trichokey, mycoparasitic activity

1. Introduction

The export of agricultural commodities like vegetables and fruits has been banned or restricted from developed countries due to pesticide residue. In the last few years, integrated pest management strategies and avoidance or regulation of pesticides by using more fungal biocontrol agents especially *Trichoderma* spp. reduced the use of pesticides against economically important crops. *Trichoderma* spp. are the most widely used fungal biocontrol agents against fungal diseases of pulses, grapes, cotton, onion, carrot, peas, plums, maize, apple, etc. Trichoderma spp. grow very fast and can produce polysaccharide degrading enzymes, so it

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can be grown on a large number of substrates. They can also tolerate different kinds of environmental condition (Papavizas 1985; Elad et al. 1993).

Trichoderma spp. known as eco-friendly biocontrol agent because of their ability to antagonize and parasitize plant-pathogenic fungi and to stimulate plant growth and defense responses. The fungal genus *Trichoderma* includes important species for production of antibiotics and enzymes (Howell 2003; Viesturs et al. 1996) and biocontrol activity against fungi and nematodes (Brunner et al. 2005; Sahebani and Hadavi 2008). It also helps in induction of systemic acquired resistance in plants by endophytism (Brunner et al. 2005; Hanson and Howell 2004; Kubicek et al. 2001). Trichoderma species can also enhance plant growth and development (Chang et al. 1986; De Souza et al. 2008; Gravel et al. 2007). Insertion or resident living organisms allude to purposeful utilisation of biological control other than disease-resistant host plants to suppress the activities of plant pathogens (Pal and Gardener 2006).

For the first time biocontrol laboratory of CSAUA&T Kanpur created *Trichoderma* library for the storage and preservation of biocontrol agents in which more than 100 isolates of *Trichoderma* were collected from different rhizospheric soil of leguminous plants especially chickpea, pigeonpea and lentil wilt infected crops are target. After screening pathological and antagonistic activity eight species of *Trichoderma* were found potential and effective against different phytopathogens. Their morphological, cultural and molecular data's and information completely compiled within the style of Trichoderma library for future references.

2. Materials and Methods

2.1. Isolation and identification of Trichoderma species

A total 18 isolates were isolated from soil samples taken from different rhizospheric soils of legume crop of various district of Uttar Pradesh, India. Collected strains of *Trichoderma* were isolated and identified on PDA medium by following serial dilution plate technique as delineated by Johnson and Curl, 1972. The pure culture was obtained by adopting single spore technique. The growth characters of culture and sporulation patterns varied noticeably within and between the species (Table 1). The identity of the *Trichoderma isolates* was confirmed both by morphological and molecular characters and also confirmed by the ITCC, Division of Plant Pathology, IARI, New Delhi. Their identification is important in developing a potential strain for further analysis. Identified cultures were finally deposited to culture bank NBAIM, Mau and allotted with a unique NBAIM Accession number.

Validations of ITS marker with 18 *Trichoderma* sp. were done and their sequences were deposited at NCBI GenBank their permanent accession no. were allotted. These *Trichoderma* sp. listed below were also submitted at ITCC- IARI, New Delhi NBAIM, Mau for future reference.

2.2. Morphological characterization and microscopic study of Trichoderma isolates

Morphological characterizations including mycelial color, colony texture and shape) and microscopic observations (conidia shape, conidia color, conidiophore–branching, phialides width and phialides length were conducted according to Sharma and Singh (2014). Considering all the morphological characters, isolates of *Trichoderma* were placed under suitable group according to an interactive key provided by Samuels et al. (2002).

The nucleotide sequences (submitted and retrieved from NCBI) of all ten *Trichoderma* species are analyzed through *Trich*OKEY 2 program for their validation post molecular identification. This has confirmed the selected sequences as specific strains of *Trichoderma* species. A set of 5 oligonucleotide sequences, which are present in all known *Hypocrea/Trichoderma* ITS1 - 5.8S RNA - ITS2 sequences, is used in combinations to identify the species at generic level.

*Tricho*MARK v. 1.0 was used for the detection of multiloci phylogenetic markers. It detects the presence of Internal Transcribed Spacer (ITS) regions in the entered sequences.

2.3. Genomic DNA isolation from selected Trichoderma species

Pure culture of the target fungus was grown overnight in liquid Potato Dextrose Broth medium for the isolation of genomic DNA using a method described by White et al., 1990. The total genomic DNA was extracted from isolate of *Trichoderma* sp. based on Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method of Crowhurst et al., 1995 with minor modification.

2.4. Molecular characterization

The Internal Transcribed Spacer (ITS) regions of the rDNA repeat from the 3'-end of the 18S and the 5'-end of the 18S gene were amplified using the two primers, ITS-1 and ITS-4, respectively, which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene. The PCR amplification reactions were performed in a 50 ml mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM $MgCl_2$, 200 mM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 µmM of each primer, 40 mg ml^{-1} of template and 2.5U of Taq polymerase. The cycle parameters included an initial denaturation for 5 minutes at 94°C; followed by 40 cycles of denaturation for 1 minute at 94°C; primer annealing at 55°C for 2 minutes; primer extension for 3 minutes at 72°C, and, a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1 mg ml^{-1}) and the complete electrophoresis gel setup was carried out for 3 hours at 60 volts in TAE buffer. A marker of 1 Kb ladder (MBI, Fermentas) was used in the gel. The gel was observed in a trans-illuminator over ultraviolet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit for purification (Singh et al., 2014 and Shahid et al., 2014).

Table 1: Bio-agents deposited in National/ International collection /culture/ GenBank

Sl. No.	Name of strains	Strain code	GPS location	ITCC Accession No.	NBAIM, Mau (UP) accession No.	NCBI GenBank accession No.
T_1	*T. harzianum*	Th azad	Longitude: 81° 59' 2.979" Latitude: 25° 8' 34.821"	6796	NAIMCC-F-03109	KC800922
T_2	*T. viride*	01PP	Longitude: 80° 7' 47.751" Latitude: 27° 23' 40.729"	8315	NAIMCC-F-03110	JX119211
T_3	*T. asperellum*	Tasp/CSAU	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	8940	NAIMCC-F-03108	KC800921
T_4	*T. Koningii*	TK (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	5201	NAIMCC-F-03112	KC800923
T_5	*T. atroviride*	71L	Longitude: 80° 18' 26.361" Latitude: 26°29' 28.323"	7445	NAIMCC-F-03107	KC008065
T_6	*T. longibrachiatum*	21PP	Longitude: 79° 18' 24.623" Latitude: 26°34' 27.61"	7437	NAIMCC-F-03111	JX978542
T_7	*T. virens*	Tvi (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	4177	NAIMCC-F-03106	KC800924
T_8	*T. reesei*	Tr (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7284	NAIMCC-F-03185	KM999966
T_9	*T. aggressivum*	T. agg (CSAU)	Longitude: 81° 59' E Latitude: 25° 35' N	7277	NAIMCC-F-03193	KT315919
T_{10}	*T. aureoviride*	T. avi (CSAU)	Longitude: 80° 12' E Latitude: 25° 58' N	6131	NAIMCC-F-03194	KT337463
T_{11}	*T. citrinoviride*	T. cvi (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7283	NAIMCC-F-03195	KT315921
T_{12}	*T. erinaceum*	T. eri (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7287	NAIMCC-F-03192	KT315922
T_{13}	*T. koningiopsis*	T. kop (CSAU)	Longitude: 81°16' E Latitude: 26° 14' N	7291	NAIMCC-F-03191	KT337462
T_{14}	*T. tomentosum*	T. tos (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7269	NAIMCC-F-03186	KT315920
T_{15}	*T. mintisporum*	T. mip (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7280	NAIMCC-F-03187	KT626565
T_{16}	*T. pubscenes*	T. sce (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7268	NAIMCC-F-03188	KT337461
T_{17}	*T. saturnisporum*	T. ssp (CSAU)	Longitude: 81° 54' E Latitude: 25° 25' N	7274	NAIMCC-F-03189	KT626566
T_{18}	*T. spirale*	T. sp. (CSAU)	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7276	NAIMCC-F-03190	KT626567

2.5. Purification of PCR product

The PCR product was purified by QIAGEN gel extraction kit using the protocol as described here. The DNA fragment was excised from the agarose gel with a clean sharp scalpel. The gel slice was then weighed in an eppendorf and 3 volumes of buffer QG was added to 1 volume of gel l). The mixture was then incubated at 50°C for 10 min. The gel (100 m mg ~ 100 was dissolved in a vortex mixer until the mixture color is uniformly yellow. Further, 1 volume of isopropanol was added to the sample and mixed. A QIA quick spin column is then placed in a 2 ml collection tube provided. The sample is applied to the QIA quick column followed by centrifugation for 1 minute so that DNA binds to the column. The supernatant is then discarded and the QIA quick column is placed back in the collection tube. A volume of 0.75 ml of PE was added to QIAquick column and centrifuged for 1 minute to wash. The supernatant is again discarded and the QIA quick column centrifuged for an additional 1 minute at 10000x g. The

QIAquick column is now placed into a clean 1.5 ml eppendorf. We then added 50 ml of Eluent Buffer (EB) (10 mM Tris-Cl, pH 8.5) to the center of the QIA quick membrane and centrifuged the column for 1 minute to elute the DNA.

2.6. DNA sequencing of the 18S rDNA fragment

The 18S rDNA amplified PCR product (100 mg concentration) was used for sequencing with the single 18S rDNA forward primer and reverse primer: 5'- synthesized by DNA Sequencer at Merck laboratory (Bangalore, India). The genomic DNA was extracted from isolated fungal strain *Trichoderma* sp. and universal primers ITS primers were used for the amplification and sequencing of the 18S rRNA gene (LoBuglio et al., 1993, Kimura, 1980) fragment listed in Table.

Seq No.	Name	Sequences 5'-3'	Length	Tm
T_1	ITS1	TCCGTAGGTGAACCTGCGG	19	58.2
T_2	ITS2	TCCTCCGCTTATTGATATGC	20	61.5
T_3	ITS3	TCTGTAGGTGAACCTGCGG	19	63.9
T_4	ITS4	GGAAGTAAAAGTCGTAA-CAAGG	22	59.9

2.7. A Multi detector of phylogentetic DNA marker DNA synthesis

2.7.1. ITS DNA synthesis

2.8. Genomic analysis of the important genes/ nucleotides involved in biocontrol mechanism in Trichoderma spp. by bioinformatics tools

2.8.1. Sequence analysis

Sequence analysis of the sequenced gene was initiated with the use of a similarity searching algorithm such as BLAST (Basic Local Alignment Search Tool). The gene of interest, 18S rRNA of the test strain, was searched for similar gene sequences using nucleotide BLAST program against a non-redundant nucleotide (nr/nt) database. The database sequences that were found to be ~90% similar to the test sequence were selected as the best matching homologs and were then subjected to a multiple sequence alignment in the ClustalW program (Thompson et al., 1994, Tamura et al., 2011).

Based on the multiple sequence alignment of the selected sequence set, an evolutionary distance matrix and a phylogenetic tree were then computed using the Neighbor-Joining method. MEGA (Molecular Evolutionary Genetics analysis) version 4.0 was used for phylogenetic and molecular evolutionary analyses (Saitou and Nei, 1987).

The 18S rRNA gene sequence of the test strain was again compared with a different set of sequence databases such as small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA) using Ribosomal RNA BLAST program (Altschul et al., 1957). 18S rRNA gene sequence of test strain is also compared against those sequences in Ribosomal Database Project (Kusaba and Tsuge, 1995) by using the RDP Classifier check Program. The annotated information for the sequence in the database to which 18S rRNA aligns is used for the fungal identification.

2.9. Mycoparasitism

2.9.1. Dual culture plate assay and ultramicroscopic studies

Hyphal interactions between *T. harzianum* (*Th* Azad/6796) and *Fusarium oxysporum f.* sp. *ciceri* were studied. Mycelial bit (5 mm) cut from the actively growing edge of a 5 day old culture of a single antagonist and the pathogen were placed opposite each other on a 90 mm diameter Petri dish containing PD agar medium. Each bioassay was replicated three times and was incubated for 5 days at 28°C temperature. After 72h of incubation, the culture plates were observed under a light microscope to verify the early stage of interaction. The interaction site was marked and an agar plate was send for SEM preparation.SEM analysis was done by Electron Microscopy Unit, CSIR-CDRI, Lucknow. According to the standard preparation protocol described previously Kathuria et al 2010. with minor modifications, samples were fixed in 2.5% Glutaraldehyde in cacodylate buffer, post-fixed in OsO4 and subsequently dehydrated through an ascending ethanol series. Sample were dried using a critical point dryer and sputter coated with Au-Pd (80:20) before analysis under a FEI Quanta 250 SEM.

3. Results and Discussion

Trichoderma spp. collected from different soil samples and location were having variation in morphology, growth kinetics and antagonistic activity. This showed that the location specific native *Trichoderma* have adaptability according to environmental as well as vegetation niche of particular locality. Therefore, variation in growth kinetics and antagonistic potentialities may be more as per local strains. Their identification is important in developing a potential strain for further analysis. The method evolved in identification is summarized and concluded as following:

1. Morphology of *Trichoderma* spp. is a key identification characteristic such as colony morphology, colony colour, growth pattern and speed along with morphology of conidia, reverse colour, colony edge and phialides, conidia colour, shape and size of conidia and phialides etc Table 2).

2. Production of pigmentation is another characteristic feature of particular strain, because different *Trichoderma* strains produce varied pigmentation on media (Table 3).

3. Kinetic growth and sporulation are important characters of bio-control and survival activity of *Trichoderma* spp.

The results of the cultural and morphological observations of Trichoderma are given in the Figure 1.

ISTH (International Sub-commission on *Trichoderma* and *Hypocrea* Taxonomy), a Sub-commission of ICTF (International Commission on the Taxonomy of Fungi), hosts an online method for the quick molecular identification of *Hypocrea/ Trichoderma* species based on an oligonucleotide barcode: a

diagnostic combination of several oligonucleotides (hallmarks) specifically allocated within the internal transcribed spacer 1 and 2 (ITS1 and 2) sequences of rDNA repeat. It helps in identifying specific strains of *Trichoderma* by comparing the sequence with the database by locating genus specific hallmarks (GSH).

The nucleotide sequences (submitted and retrieved from NCBI) of all eighteen *Trichoderma* species are analyzed through *Trich*OKEY 2 program for their validation post molecular identification (Table 4). This has confirmed the selected sequences as specific strains of *Trichoderma* species. A set of 5 oligonucleotide sequences, which are present in all known *Hypocrea/Trichoderma* ITS1 - 5.8S RNA - ITS2 sequences, is used in combinations to identify the species at

Table 2: Morphological descriptors used in characterization of *Trichoderma* sp.

Name of Strains	Conidiation	Conidiophore branching	Phialide shape	Phialide size	Conidial shape	Conidial size
T. harzianum	Ring like	Highly branched	Short skittle shaped	7.2-11.2×2.5-3.1 µm	Ellipsoidal to ampulliform	2.8-3.1×2.4-3.1 µm
T. viride	Ring like	Highly branched	Sigmoid or hooked shaped	7.5-11.6×2.5-2.8 µm	Ellipsoidal to ampulliform	7.5-11.6×2.5-2.8 µm
T. asperellum	Ring like zones	Branched, regular	Cyllindrical	6.2-10.6×3.1-4.0 µm	Globose to sub-globose	2.8-3.1×2.4-3.1 µm
T. Koningii	Irregular	Branched, regular	Nin-pin shaped	4.9-7.8×2.1-2.8 µm	2.8-3.1 x 2.4-3.1 µm	Ovoid
T. atroviride	Irregular	Irregular, fertile	Ampulliform	6.2-11.2×2.4-3.1 µm	Subglobose	2.8-3.1×2.4-3.1 µm
T. longibrachiatum	Irregular	Rarely rebranched	Lageniform	5.3-11.6×2.0-3.2 µm	Lageniform	3.0-3.2×2.3-3.0 µm
T. virens	Irregular	Rarely rebranched	Lageniform	5.3-11.6×2.0-3.2 µm	Ellipsoidal, ob-void	3.0-3.2×2.3-3.0 µm
T. ressei	Irregular	Rarely rebranched	Lageniform	5.3-11.6×2.0-3.2 µm	Ellipsoidal, ob-void	3.0-3.2×2.3-3.0 µm
T. aggressivum	Irregular	Regular branched	Cylindrical	4.3-5.9×2.4-3.1 µm	Ellipsoidal	3.1-3.8×2.8-3.1 µm
T. aureoviride	Irregular	Highly branched	Short skittle shaped	6.5-9.0×2.4-3.1 µm	Ovoid	3.1-4.6×2.4-3.4 µm
T. citrinoviride	Concentric, regular	Arise single, cylindrical	Lageniform to ampulliform	6.2-6.8×3.1-3.4 µm	Some like ellip-soidal	2.8-3.1×2.1-2.8 µm
T. erinaceum	Regular ring like zones	Highly branched	Cylindrical	4.3-6.5×2.4-3.1 µm	Ellipsoidal to ampulliform	3.1-3.4×2.1-2.4 µm
T. koningiopsis	Irregular	regular branching	Oblong shape	4.3-6.5×2.8-3.1 µm	Ellipsoidal	3.1-3.4×2.8-3.1 µm
T. tomentosum	Irregular	Highly branched	Ampulliform	4.0-5.9×2.1-3.4 µm	Ellipsoidal to ampulliform	3.1-3.7×2.8-3.4 µm
T. minitisporum	Ring like zones	Short, highly branched	lageniform	4.6-7.8×5.6-6.2 µm	Ellipsoidal to ampulliform	3.1-4.6×2.8-3.1 µm
T. pubscenes	Concentric regular	Highly branched	lageniform	4.6-7.8×5.6-6.2 µm	ampulliform	4.3 -6.2×2.8-3.7 µm
T. saturnisporum	Irregular	regular branching	lageniform	4.3-6.5×2.8-3.1 µm	Oblong shape	3.1-3.4×2.8-3.1 µm
T. spirale	Ring like	Highly branched	dolliform	5.3-6.8×2.1-3.7 µm	Ellipsoidal	2.8-3.1×2.4-3.1 µm

Table 2: Continue...

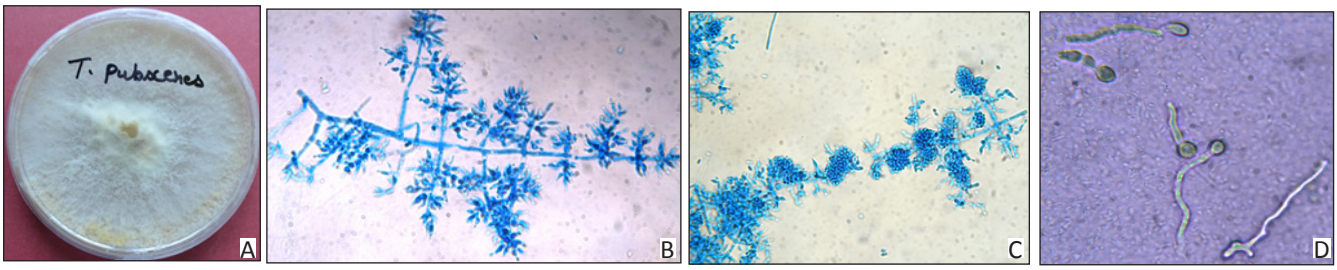
Name of Strains	Conidial wall	Conidial colour	Chlamydo-spores	Spore germination Time
T. harzianum	Smooth	Dark green	present	12 Hours
T. viride	Smooth	Light green	present	12 Hours
T. asperellum	Smooth	Light green	Present	12 -13 Hours
T. Koningii	Rough	Grayish green	Not seen	14 Hours
T. atroviride	Rough	Yellowish green	Not seen	12 Hours
T. longibrachiatum	Smooth	Grayish green	present	12 Hours
T. virens	Smooth	Grayish green	present	12 Hours
T. ressei	Smooth	Grayish green	No	12 Hours
T. aggressivum	Smooth	Dark green	Not seen	12 Hours
T. aureoviride	Rough	Dark green	Not seen	12-13 Hours
T. citrinoviride	Smooth	Green	Not seen	12 Hours
T. erinaceum	Smooth	Grey	Not seen	12 Hours
T. koningiopsis	Smooth	light green	Abundently present	12 Hours
T. tomentosum	Smooth	Gray	Rarely present	12 Hours
T. minitisporum	Smooth	Light green	Rarely present	After 12 Hours
T. pubscenes	Smooth	Bright green	present	12 -13Hours
T. saturnisporum	Smooth	light green	Abundently present	12 Hours
T. spirale	Smooth	Light green	present	11-12 Hours

Table 3: Cultural observations of the selected *Trichoderma* isolates collected from Kanpur and different locations of Uttar Pradesh, India

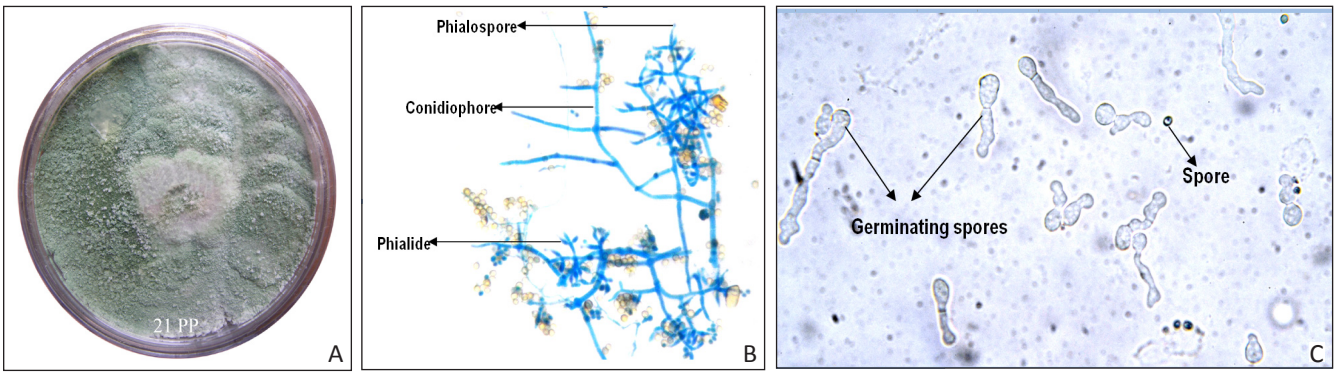
Sl. No.	*Trichoderma* Isolates	Overall appearance					Mycelium	
		Colony growth rate (cm)	Colony colour	Reverse colony colour	Colony edge	Culture smell	Mycelial form	Mycelial Colour
T_1	*T. harzianum*	7-8	Light bice green	Uncoloured ring like zones	Smooth	Sour coco-nut-like	Floccose to arachnoid	Cottony white
T_2	*T. viride*	7-8	Cress green	Uncoloured ring like zones	Smooth	Sour coco-nut-like	Floccose to arachnoid	Cottony white
T_3	*T. asperellum*	7-8	Dark yellowish green	Colourless	Smooth	No character-istic odour	Smooth	Cottony white
T_4	*T. koningii*	6-7	Terreverte	Light-yellowish	Smooth	No character-istic odour	Floccose to arachnoid	Watery white
T_5	*T. atroviride*	6-7	Light celandine green	Colourless	Effuse	No character-istic odour	Floccose to arachnoid	Watery white
T_6	*T. longibrachia-tum*	7-8	Lily green	Dirty yellowish	Effuse	No character-istic odour	Effuse	Water white
T_7	*T. virens*	7-8	Bice green	Uncoloured	Smooth	No character-istic odour	Floccose to arachnoid	Watery white
T_8	*T. reesei*	6-7	Pale green yellow	Light lemon yellow	Smooth reguler surface	No character-istic smell	Tufted, crysty	Lihgt yel-lowish

Table 3: Continue...

Sl. No.	*Trichoderma* Isolates	Overall appearance					Mycelium	
		Colony growth rate (cm)	Colony colour	Reverse colony colour	Colony edge	Culture smell	Mycelial form	Mycelial Colour
T_9	*T. aggressivum*	7-8	Light lettuse green	Light	Smooth	No characteristic odour	Irregular, arachnoid	Watery white
T_{10}	*T. aureoviride*	6-7 cm	Watery white	colourless	Smooth	Coconut like smell	Floccose to arachnoid	Watery white
T_{11}	*T. citrinoviride*	8-9	parrot green	Bright yellow	Smooth	No characteristic odour		Floccose to arachnoid
T_{12}	*T. erinaceum*	6-7	Mustard yellow with green granules	Uncoloured	Smooth	No characteristic odour	Floccose to arachnoid	Watery white
T_{13}	*T. koningiopsis*	7-8 cm	Milky white	Naples yellow	Smooth	No smell	Tuft abundant, effusion	cottony white
T_{14}	*T. tomentosum*	7-8 cm	cossack green	Light yellow	Smooth	Sweet corn like smell	Luxuriant,	durty white
T_{15}	*T. minutisporum*	7-8 cm	citron green	Dark antimony yellow	Smooth	No characteristic odour	Compact, whorls, crowded	Watery white
T_{16}	*T. pubscenes*	5-6	Cottony white	Uncoloured	Smooth	No characteristic odour	Luxuriant, arachnoid	Watery white
T_{17}	*T.saturnisporum*	5-6 cm	lettuce green	Ilm green	Smooth	No characteristic odour	Irregular, crysty, effussion	White
T_{18}	*T. spirale*	7-8	Deep dull yellow green	Pale fluorite green	Smooth	No characteristic odour	Floccose to arachnoid	Cottony white

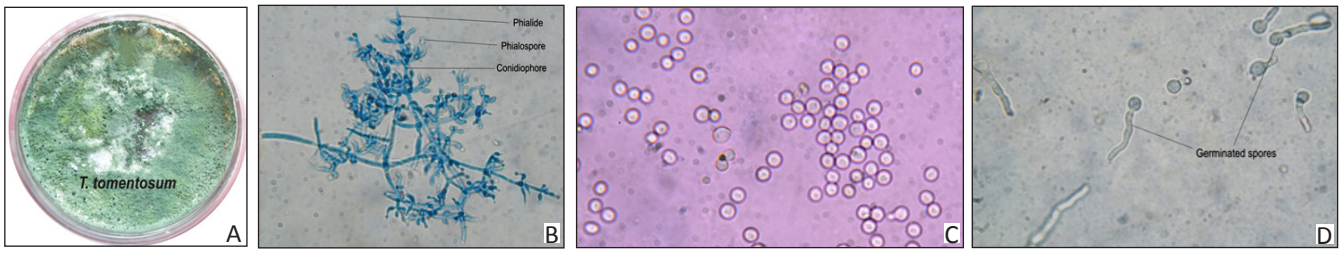


(A) Growth on PDA Media (B) Light micrographs of *T. pubscenes* at 40x (C) Mass of spores (D) Spore germination

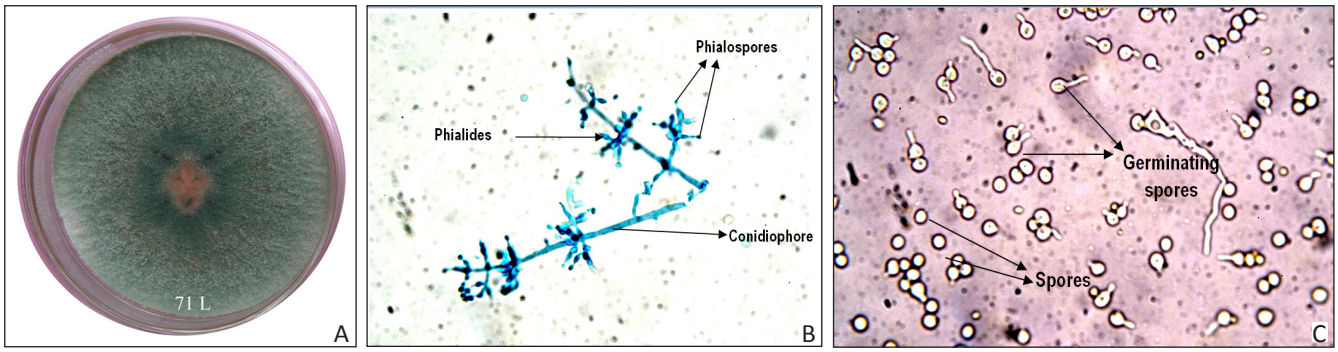


(A) Growth on PDA Media (B) Light micrographs of *T. longibrachiatum* at 40x (C) Spore germination

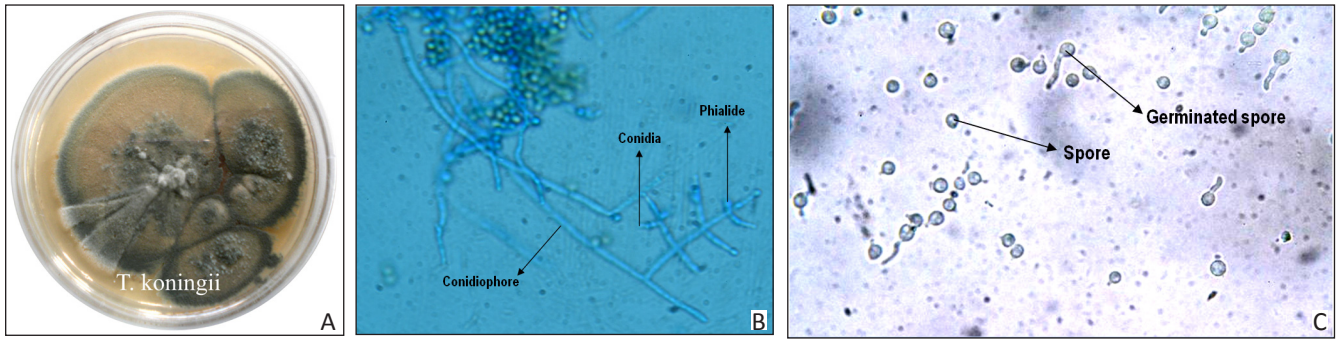
Figure 1: Continue...



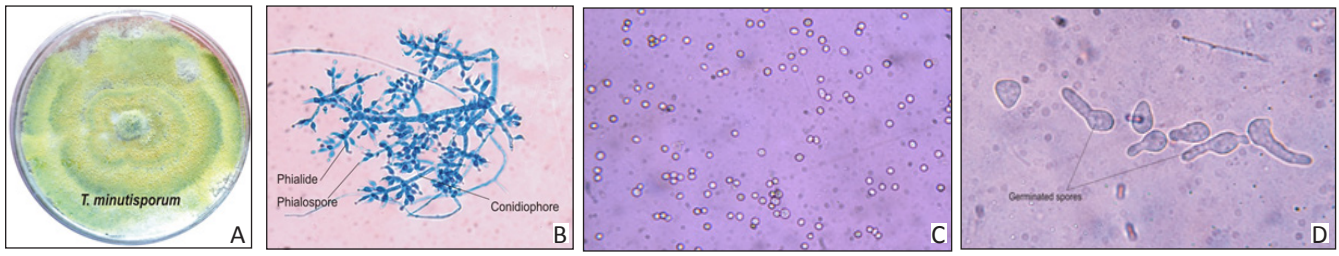
(A) Growth on PDA Media (B) Light micrograph of *T. tomentosum* at 40x (C) Spore mass (D) Spore germination



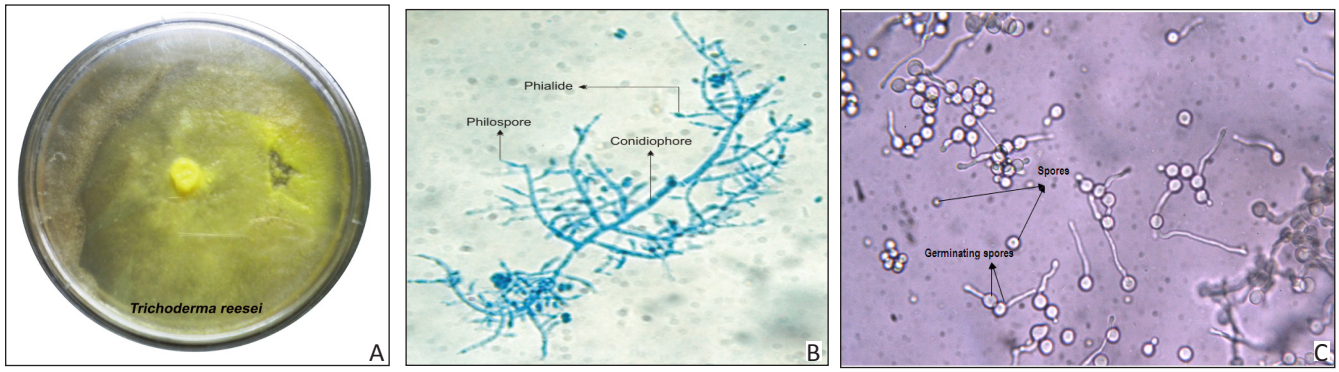
(A) Growth on PDA Media (B) Light micrograph of *T. viride* at 40x (C) Spore germination



(A) Growth on PDA Media (B) Light micrographs of *T. koningii* (C) Spore mass (D) Spore germination

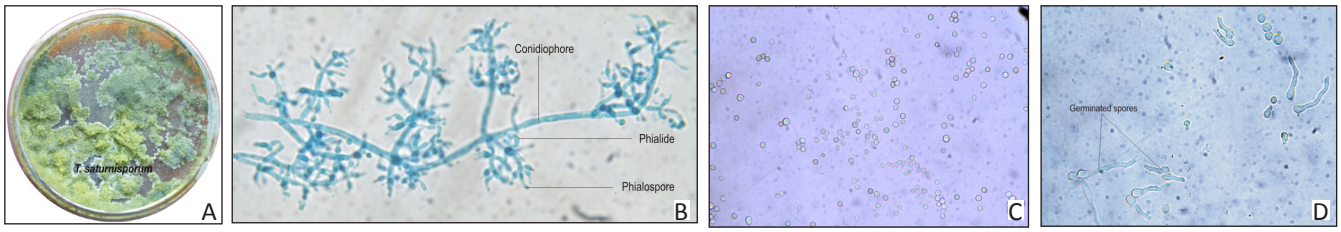


(A) Growth on PDA Media (B) Light micrographs of *T. minutisporum* at 40x (C) Spore mass (D) Spore germination

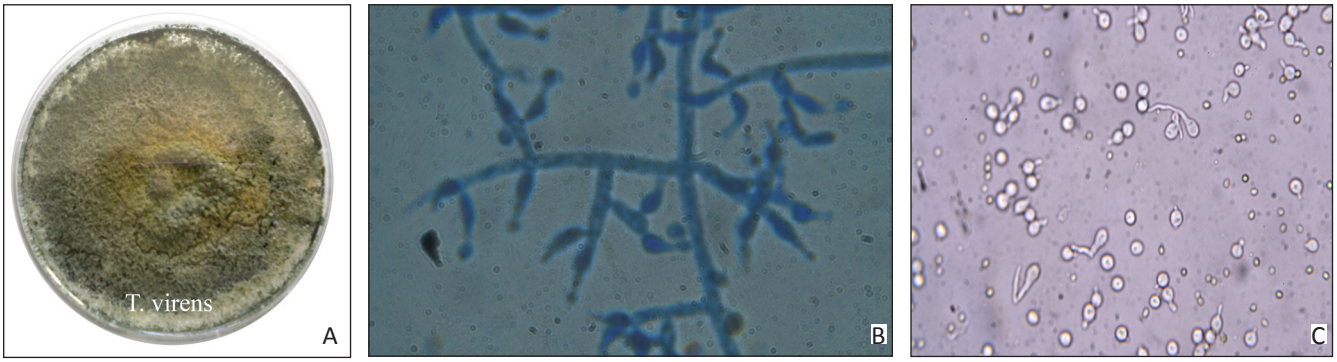


(A) Growth on PDA Media (B) Light micrograph of *T. reesei* at 40x (C) Spore germination

Figure 1: Continue...



(A) Growth on PDA Media (B) Light micrographs of *T. saturnisporum* at 40x (C) Spore mass (D) Spore germination



(A) Growth on PDA Media (B) Light micrograph of *T. virens* at 40x (C) Spore germination

Figure 1: Cultural and morphological observations of *Trichoderma* spp used

generic level (Nei and Li, 1979).

*Trichos*MARK v. 1.0 was used for the detection of multiloci phylogenetic markers. It detects the presence of Internal Transcribed Spacer (ITS) regions in the entered sequences.

Once the strains are isolated in wet lab and their morphology is studied based on which the strain identification is done, the identification of isolated strains is done and validated at the ISTH website. As ISTH is solely dedicated for the identification of different strains of *Trichoderma* and *Hypocrea* species based on ITS sequences and other taxonomical data, the strains under this study are also validated through ISTH database.

3.1. Phylogeny of the Genus Trichoderma based on sequence analysis of the Internal transcribed spacer region 1 of the rDNA cluster

Bio-control agent *Trichoderma* has attained importance for substitute of chemical pesticides and hence an attempt was intended to corroborate the positive relatedness of molecular and morphological characters. The fungal strains of *Trichoderma* spp. was isolated from the different location and collected from rhizosphere soil of different district of Uttar Pradesh, India. The universal ITS primers were used for amplification of the 18S rRNA gene fragment and strain characterized by using 18S rRNA gene sequence with the help of ITS marker (Anderson and Stasovski, 1992, Dubey and Singh, 2013). Integrated management of major diseases of mungbean by seed treatment and foliar application of insecticide, fungicides and bioagent. Crop Prot. 47: 55-60.).

The sequence was deposited in GenBank with the accession number JX119211, KC800922, KC800921, KC800923, KC008065, JX978542, KC800924, KM999966, KT315919, KT337463, KT315921, KT315922, KT337462, KT315920, KT626565, KT337461, KT626566 and KT626567.

The primers (ITS1 to ITS4) were used for amplifying ITS regions, followed by sequencing, for all the 18*Trichoderma* isolates. The resulting amplicons of approximately 600bp were obtained in all the *Trichoderma* isolates. The sequences of these amplified products showed 90-100% identity with other documented sequences of *Trichoderma* strains in the BLASTN search. The 600 nucleotide long ITS sequences obtained with ITS primers were used for the construction of phylogenetic trees (Felsenstein, 1985 and 1991). All the ITS sequences of *Trichoderma* isolates as well as taken from the NCBI data base fall into four clusters in the NJ tree (Rehner and Samuels, 1994, Shanmugam et al., 2008). From Figure 2 it is clear that cluster I is divided into 2 subgroups in first subgroup *T. koningii and T. viride* and in second subgroups *T.* reesei and *T. aggressivum* occurs. In cluster II is further sub-divided into 2 groups and these two groups were again divided into 3 subgroups. In first subgroup, *T. aureoviride* and *T. minitisporum;* in second subgroup *T. atroviride* and *T. virens* and in third subgroup *T. tomentosum* occurred. In second group of cluster II, which is divided into two subgroups in first subgroup *T. citrinoviride* and *T. saturnisporum* and in second subgroups *T. longibrachiatum* occurred. Similarly, III cluster *Trichoderma harzianum* alone occurred. Finally, in cluster IV is divided into two groups and they are further divided into two subgroups. In first subgroups *T. pubescens* and *T. spirale* occurs. In second subgroup *T. erinaceum* and *T. koningiopsis* occurred. In second group of cluster IV only *T. asperellum* occurred.

Table 4: Describing the ISTH *TrichOKEY* and *TrichoMARK* results for all eighteen *Trichoderma* species

Input (Nucleotide Sequence)	*TrichOKEY* results		*TrichoMARK* results
T. viride (01PP/8315)	Found 1 GSH (Anchors)	Barcode identification of the query sequence is not possible because only one genus specific hallmark (Anchor 2) is found.	Found 2 ITS anchors (ITS1 and ITS4) from 4
T. harzianum (Th azad/6796)	First anchor (GSH) was found in position 95; Second anchor (GSH) was found in position 116	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, *Hypocreales*, *Ascomycota*.	Found 2 ITS anchors (ITS1 and ITS2) from 4
T. asperellum (T. asp/CSAU/8940)	First anchor (GSH) was found in position 96; Second anchor (GSH) was found in position 117; Third anchor (GSH) was found in position 262; Fourth anchor (GSH) was found in position 420; Fifth anchor (GSH) was found in position 519	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota. Other closely related species are *Trichoderma koningiopsis*, *Trichoderma ovalisporum* and *Trichoderma asperellum*	Found 4 ITS anchors (ITS1, ITS2, ITS4 and ITS5) from 4
T. koningii (Tk (CSAU)/ 5201	Found 3 genus-specific hallmarks (Anchors)	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota.	Found 0 ITS anchors from 4
T. atroviride (71L/7445)	First anchor (GSH) was found in position 93; Second anchor (GSH) was found in position 115; Third anchor (GSH) was found in position 277; Fourth anchor (GSH) was found in position 435; Fifth anchor (GSH) was found in position 536	Genus Identification: *Hypocrea*/ Trichoderma, Hypocreaceae, Hypocreales, Ascomycota. The sequence is similar to *Hypocrea lixii/ Trichoderma harzianum*	Found 4 ITS anchors (ITS1, ITS2, ITS4 and ITS5) from 4
T. longibrachiatum (21PP/7437)	First anchor (GSH) was found in position 90; Second anchor (GSH) was found in position 114; Third anchor (GSH) was found in position 296; Fourth anchor (GSH) was found in position 454; Fifth anchor (GSH) was found in position 548	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota. The sequence is similar to *T. longibrachiatum* and *Hypocrea orientalis*	Found 4 ITS anchors (ITS1, ITS2, ITS4 and ITS5) from 4
T. virens (Tvi (CSAU)/ 4177)	First anchor (GSH) was found in position 87; Second anchor (GSH) was found in position 109; Third anchor (GSH) was found in position 270; Fourth anchor (GSH) was found in position 428; Fifth anchor (GSH) was found in position 529	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota. The sequence is similar to *Hypocrea lixii/Trichoderma harzianum*	Found 4 ITS anchors (ITS1, ITS2, ITS4 and ITS5) from 4

Table 4: Continue...

Input (Nucleotide Sequence)	*TrichOKEY* results		*TrichoMARK* results
T. reesei (Tr (CSAU)/ 7284)	Second anchor (GSH) was found in position 360	Barcode identification of the query sequence is not possible because only one genus specific hallmark (Anchor 2) is found.	Found 2 ITS anchors (ITS1 and ITS4) from 4
Trichoderma citrinoviride (T. cvi/7283)	First anchor (GSH) was found in position 54 .... Second anchor (GSH) was found in position 77 ... Third anchor (GSH) was found in position 257 .... Fourth anchor (GSH) was found in position 415 ... Fifth anchor (GSH) was found in position 509	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota region 1 (10nt) was detected; region 2 (174nt) was detected; region 3 (183nt) was detected; 5.8S RNA gene was removed	Identification reliability: high barcode was developed on the basis of 42 vouchered sequences which showed 1 ITS 1 and 2 alleles
Trichoderma aggressivum (T. agg/7277)	First anchor was not found .... Second anchor was not found .... Third anchor was not found .... Fourth anchor was not found .... Fifth anchor was not found	Found 0 genus-specific hallmarks (Anchors):	No Anchors were found.
Trichoderma erinaceum isolate T. eri/7287	First anchor (GSH) was found in position 56 .... Second anchor (GSH) was found in position 77 .... Third anchor (GSH) was found in position 225 .... Fourth anchor (GSH) was found in position 383 .... Fifth anchor (GSH) was found in position 485	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota region 1 (8nt) was detected; region 2 (142nt) was detected; region 3 (177nt) was detected; 5.8S RNA gene was removed	Identification reliability: standard barcode was developed on the basis of 7 vouchered sequences which showed 1 ITS 1 and 2 alleles
Trichoderma tomentosum (T. tos/ 7269)	First anchor (GSH) was found in position 67 .... Second anchor (GSH) was found in position 90 .... Third anchor (GSH) was found in position 251 .... Fourth anchor (GSH) was found in position 409 .... Fifth anchor (GSH) was found in position 508	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota region 1 (10nt) was detected; region 2 (155nt) was detected; region 3 (197nt) was detected; 5.8S RNA gene was removed	Identification reliability: standard barcode was developed on the basis of 15 vouchered sequences which showed 1 ITS 1 and 2 alleles
Trichoderma minitisporum (T. mip/7280)	First anchor (GSH) was found in position 187 .... Second anchor (GSH) was found in position 209 . Third anchor (GSH) was found in position 370 .... Fourth anchor (GSH) was found in position 528 ... Fifth anchor (GSH) was found in position 630 ...	Genus Identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota region 1 (9nt) was detected; region 2 (155nt) was detected; region 3 (238nt) was detected; 5.8S RNA gene was removed	Identification reliability: high barcode was developed on the basis of 285vouchered sequences which showed 33ITS 1 and 2 alleles

Table 4: Continue...

Input (Nucleotide Sequence)	*TrichOKEY* results		*TrichoMARK* results
Trichoderma saturnisporum (T. ssp/7274)	First anchor (GSH) was found in position 55 .... Second anchor (GSH) was found in position 77 ... Third anchor (GSH) was found in position 256 .... Fourth anchor (GSH) was found in position 414 ... Fifth anchor (GSH) was found in position 510	Genus identification: *Hypocrea/ Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota region 1 (9nt) was detected; region 2 (173nt) was detected; region 3 (185nt) was detected; 5.8S RNA gene was removed	Identification reliability: low barcode was developed on the basis of 1 vouchered sequences which showed 1 ITS 1 and 2 alleles
Trichoderma spirale (T. sp/7276)	First anchor (GSH) was found in position 194 .... Second anchor (GSH) was found in position 215 . Third anchor (GSH) was found in position 361 .... Fourth anchor (GSH) was found in position 519 ... Fifth anchor (GSH) was found in position 618	Genus Identification: *Hypocrea/* Trichoderma, Hypocreaceae, Hypocreales, Ascomycota region 1 (8nt) was detected; region 2 (140nt) was detected; region 3 (197nt) was detected; 5.8S RNA gene was removed	Identification reliability: high barcode was developed on the basis of 38 vouchered sequences which showed 1 ITS 1 and 2 alleles

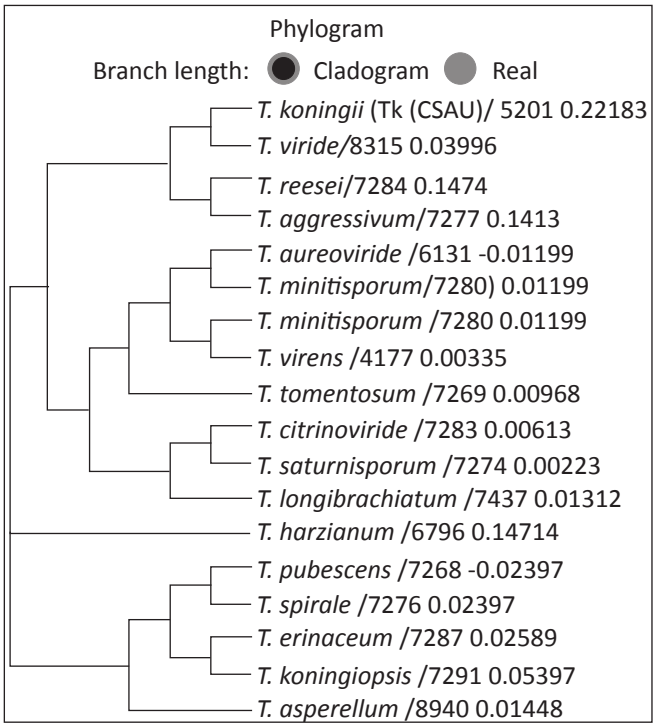


Figure 2: The evolutionary history (Phylogram) was inferred using nearly complete ITS sequences (~600 bp) using ITS 1 and 4 primers from 18 *Trichoderma* spp. and available NCBI sequences, constructed by Neighbour Joining method (Saitu and Nei, 1987)

Trichoderma being cosmopolitan bio-agent has been gaining maximum popularity and acceptance as bio-control. Identification of the potential species and molecular characterization of *Trichoderma* strain available in a particular environment become essential for effective management of the disease and developing a potential bio-agent for a particular region. a, Rifai, 1969, Bisset, 1991 b and c and Sammuls, 1996 made detailed study on morphological characters to characterize and differentiate species of *Trichoderma.* Castle et al., 1998 reported that green mold disease (causal agent, *Trichoderma*) has resulted in severe crop losses on mushroom farms worldwide in recent years. They analyzed 160 isolates of *Trichoderma* from mushroom farms for morphological, cultural, and molecular characteristics and classified these isolates into phenotypic groups. Sheila and Odhiambo (2009) made genus identification of green fungus isolated from 120 soil samples. Colony characteristics, growth rate in culture and morphological characters used for identification. Microscopic examination was carried out by mounting the culture and lacto phenol cotton blue but for size measurements KOH and water was used as the mounting fluid. A small amount of material was placed in a drop of 3% KOH on a slide and then replaced with water.

Devi et al., 2012 studied the positive relatedness of molecular and morphological characters with antagonistic ability of *Trichoderma* species. This result was in concordance with the result obtained from the DNA sequence data analysis of internal transcribed spacer 1 and 2 region (ITS1 and ITS2) and the elongation factor 1-alpha gene (*tef1*). The phylogenetic analyses of the above two marker loci sequences done. Maymon et al. (2004) collected 76 isolates and representative isolates were further characterized into three main groups by internal transcribed spacer (ITS) sequence analysis. Consequently, a reliable phylogenetic tree was constructed containing isolates belonging to the *T. harzianum* group

(comprising *T. aureoviride, T. inhamatum,* and *T. virens*), the *T. longibrachiatum* and *T. saturnisporum* cluster, and that including the species *T. asperellum*, *T. atroviride*, *T. koningii* and *T. viride*. Hermosa et al., 1999 done sequencing of internal transcribed spacers 1 and 2 (ITS1 and ITS2) revealed three different ITS lengths and four different sequence types. ITS2 sequences were also useful for locating the biocontrol strains in *T. atroviride* within the complex *T. atroviride* and *T. koningii*.

4. Conclusion

Rapid identification of microorganisms is necessary for taking decision for preparation of bioformulation. The rRNA based analysis is a central method used not only to explore microbial diversity but also to identify new strains. Thus, an integrated approach of morphological and molecular markers can be employed to identify a superior strain of *Trichoderma* for its commercial exploitation.

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