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Characterization and Identification of Different Strains of *Trichoderma* Species using Bio-molecular Techniques

Mohammad Shahid, Sonika Pandey, Mukesh Srivastava, Vipul Kumar,Anuradha Singh, Shubha Trivedi, Y.K. Srivastava and Shivram

Biocontrol Laboratory, Department of Plant Pathology,
CSA University of Agriculture & Technology, Kanpur-208002, Uttar Pradesh, India.

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Ten species of *Trichoderma* were isolated from the rhizospheric soil, collected from the different locations of U.P. Both morphological and molecular characterization of the isolated species was done. All the ten isolated species were screened for chitinase enzyme production on solid agar medium using bromocresol purple for developing the clear zone around colonies, and characterized due to its antagonistic effect against mycelia growth of pathogenic fungi. The nucleotide sequences (submitted and retrieved from NCBI) of all ten *Trichoderma* species are analyzed through TrichOKEY 2 program for their validation post molecular identification. This has confirmed the selected sequences as specific strains of *Trichoderma* species.

Keywords: *Trichoderma,* TrichOKEY, chitinase.

Soil borne pathogens have a broad host range and persist for longer periods in soil by resistant resting structures. Chemical control of soil borne pathogens provides certain degree of control but at the same time have adverse effects on environment affecting the beneficial soil microorganisms. Therefore, biological control of plant pathogens has been considered as a potential control strategy in recent years and search for these biological agents is increasing. *Trichoderma* is the most commonly used fungal biological control agent and have long been known as effective antagonists against plant pathogenic fungi [Margolles-Clark *et al*., 1996 , Harman, *et al* 2004 and Chet, *et al* 1981]. In which, *Trichoderma harzianum* has been accepted as one of the most potent biocontrol agents against plant diseases and used as an antagonist against many soilborne phytopathogenic fungi over the past few years (Samuels *ET AL*., 2004). Various strategies of biocontrol have been proposed. They include the creation of competition for nutrients or space; the production of antibiotics and lytic enzymes; the inactivation of the enzymes of phytopathogenic fungi; and parasitism (Viterbo *et al* 2002). The cell wall-degrading enzymes (CWDEs), mostly chitinases, glucanases, and proteases, are major lytic enzymes that are secreted by biocontrol agents (Benitez T., 2004). CWDEs attack the cell wall of phytopathogenic fungi to cause cell lysis and subsequent death. Although the mechanism of mycoparasitism is not completely understood, this process has been assumed to involve the expression of extracellular CWDEs (Pandey S *et al* 2014).

* To whom all correspondence should be addressed.
E-mail: biocontrol.csa@gmail.com

The ability of *Trichoderma* to parasitize and kill other fungi has been the major driving force behind the commercial success of these fungi as biofungicides. The genome size of mycoparasites of *Trichoderma* species ranged from 36.1Mb to 40.98 Mb. The genome size of *T. asperellum* is 37.4 Mb (Druzhinina *et al*. 2011). Harman *et al*. (2004) summarized the results of many previous studies and they documented that *T. asperellum* and *T. asperelloides* are highly rhizosphere competent and are able to stimulate growth and immune defense of plants. *Trichoderma viride* as biological control agent was inoculated into the soil, to suppress the activity of the pathogenic fungi *Fusarium oxysporum* and *Rhizoctonia solani* on tomato. Hafez *et al*., 2013, examined the up and down regulated genes in both treated and non-treated plants, using differential display technique. Samuels *et al*. (2002) characterized and distinguished *Trichoderma* species by morphological characteristics and provided detailed observations on the morphological characters of defined species in *Trichoderma*. Taxonomy of *Trichoderma* is based on morphological characteristics alone for detecting *Trichoderma* has not lead to a satisfactorily taxonomy between *Trichoderma* species. In contrast, molecular techniques such as PCR and DNA sequencing are very sensitive, reliable and rapid methods for species detection. Molecular markers demonstrate the variation in DNA sequences within and between the species and provide the basis for precise identification. Polymerase chain reaction (PCR) methods have found widespread use for pathogen identification, and a number of PCR-based assays have been developed for use in the diagnosis and characterization of *Trichoderma* species (Jaklitsch 2009). rRNA is essential for the survival of all cells and the genes encoding the rRNA are highly conserved in the fungal and other kingdoms. The universal primers were used for amplification of the 18S rRNA gene fragment and strain was characterized by using 18S rRNA gene sequence. The identified strain *Trichoderma atroviride* TAU8 based on phylogenetic tree analysis together with the 18S rRNA gene sequence. The sequences of the rRNA and proteins comprising the ribosome are highly conserved throughout evolution, because they require complex inter and intra-molecular interactions to maintain the protein synthesizing machinery (Singh *et al*. 2014). There are several molecular methods to characterize fungi species and revealed the intra-generic relationships amongst species of *Trichoderma*, including ITS and 5.8S rDNA sequences and fingerprinting techniques (Kim *et al*. 2000). Sequence analysis of internal transcribed spacer (ITS) region is the most widely sequenced DNA region in fungi and it useful for molecular systematic study at species levels and even within species. In eukaryotic cells, rRNA cistrons made up of 18S, 5.8S and 28S rRNA genes are transcribed by RNA polymerase I. Then, RNA splicing of the cistrons will remove the two internal transcribed spacers flanking the 5.8S gene. The two spacers, together with the 5.8S gene, are normally referred to as the ITS region (Schoch *et al*. 2012).The rRNA genes are universally conserved, while the ITS region and intergenic spacer (IGS) are highly variable. The ITS region and IGS region are the fastest evolving regions and they may vary among species within a genus. Thus, the sequences of these regions can be used for identification of closely related species (Lieckfeldt *et al*. 2002; White *et al*. 1990). However, Consolo *et al*. (2012) showed the important of study the genetic variation within *Trichoderma* strains and their biological activities to improve the selection of potential isolates as biocontrol agents. The main aim of this study was to isolate and charactrize new bioagents from the rhizospheric soils and to assess their antagonistic activity.

MATERIALS AND METHODS

Isolation of *Trichoderma species* from collected soil samples

Ten *Trichoderma species* used in this study was isolated from rhizosperic soils collected from the differnt locations of U.P.and maintained on potato dextrose agar (PDA) (Himedia, USA) at 28±2°C for 5 days. Ten *Trichoderma* species were isolated from rhizosphere samples according to soil dilution plate method described by Kucuk and Kivanc (2003) with some modification. One ml of each appropriate dilution (10^{-3} to 10^{-5}) was pipetted in petri dishes, then the sterilized and cooled at 45°C Rose Bengal Agar medium (RBA) was poured

and left to solidified. All plates were incubated at 28°C for 7 days. The culture plates were examined daily and individual colonies were isolated and purified, then transferred to fresh potato dextrose Agar medium (PDA). Distinct morphological characteristics were observed for identification and the plates were stored at 4°C for further experiments.

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, phialide width and phialide length were determined according to Sharma and Singh 2014, Singh A, *et al* 2014.

Screening of *Trichoderma isolates* for chitinase production

Induction of chitinase

For chitinases activity, two different insoluble chitin sources (colloidal chitin derived from commercial chitin and Seashells) were used. *Trichoderma* isolate were screened for chitinase activity and to assess the *Trichoderma* isolates spectrophotometrically for N-acetyl-â- D-glucosamine (NAGA) (for total chitinolytic activity) and p-nitrophenol (pNP) (for exochitinase activity).

Agar medium for detection of chitinase-positive microorganisms

Chitinase detection medium consisted of a basal medium comprising (per liter) 0.3g $MgSO_4.7H_2O$, 3g $(NH_4)_2SO_4$, 2g of KH_2PO_4, 1g of citric acid monohydrate, 15g agar, 200ìl Tween-80, 4.5g chitin source and 0.15g bromocresol purple; pH was adjusted to 4.7 and then autoclaved at 121°C for 15min. Lukewarm medium was poured in Petri plates and allowed to solidify. Fresh culture plugs of the isolate to be tested for chitinase activity were inoculated on the medium and incubated at 25±2°C and were observed for colored zone formation (Pandey S *et al* 2014).

Total Chitinolytic activity

Chitinolytic activity was assayed by measuring the release of reducing saccharides from colloidal chitin. A reaction mixture containing 1ml of culture supernatant, 0.3ml of 1 M sodium acetate buffer (SA-buffer), pH 4.6 and 0.2ml of colloidal chitin was incubated at 40°C for 20h and then centrifuged at 13,000 rpm for 5 min at 6°C. After centrifugation, an aliquot of 0.75ml of the supernatant, 0.25ml of 1% solution of dinitrosalycilic acid in 0.7 M NaOH and 0.1ml of 10M NaOH were mixed in 1.5ml microcentrifuge tubes and heated at 100°C for 5min. Absorbance of the reaction mixture at A_{582} was measured after cooling to room temperature. Calibration curve with N-acetyl-â–D-glucosamine (NAGA) was used as a standard to determine reducing saccharide concentration. Chitinolytic activity was estimated in terms of the concentration (mg/ml) of NAGA released.

Exochitinase activity

N-acetyl-â-D-glucosaminidase (exochitinase) activity was measured and monitored spectrophotometrically as the release of p-nitrophenol (pNP) from p-nitrophenyl-Nacetyl- â-D-glucosaminide (pNPg). A mixture of 25ìl of culture filtrate, 0.2ml of pNPg solution (1mg pNPg ml-1), and 1ml of 0.1 M SA-buffer (pH 4.6) was incubated at 40^0C for 20h and then centrifuged at 13,000 rpm. An aliquot of 0.3ml of 0.125 M Sodium tetraborate–NaOH buffer (pH 10.7) was added to 0.6ml of supernatant, absorbance at 400nm (A_{400}) was measured immediately after mixing and pNP concentration.

***In vitro* antagonistic effect of *Trichoderma species* against plant pathogen fungi**

Antagonistic potential of *Trichoderma* sp was examined by dual culture technique as described by Morton and Stroube. A 5mm disc were taken from the edge of actively growing colonies of fresh fungal cultures and placed on the surface of a fresh PDA plate 4 cm apart. The plates were incubated at 28 ± 2°C for 7 days. Control plates were maintained without *Trichoderma*. The experiment was replicated thrice and percent growth inhibition was calculated by the following formula $I = (C"T)/C \times 100$,

where C is mycelial growth in control plate, T is mycelial growth in test organisms inoculated plate and I is inhibition of mycelial growth.

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 is used for the detection of multiloci phylogenetic markers. It detects the presence of Internal Transcribed Spacer (ITS) regions in the entered sequences.

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 Hiney. The total genomic DNA was extracted from isolate of *Trichoderma harzianum Th azad*/6796 based on Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method of Crowhurst *et al* with minor modification.

Molecular characterization

The Internal Transcribed Spacer (ITS) regions of the rDNA repeat from the 32 -end of the 18S and the 52 -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), 0.2µmM of each primer, 40ng/ml 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 (1mg/ml) 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 (Shahid M *et al* 2014).

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 (100 mg ~ 100 ?l). The mixture was then incubated at 50°C for 10 min. The gel 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 QIAquick 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 QIAquick 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 QIAquick 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 50ml of Eluent Buffer (EB) (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuged the column for 1 minute to elute the DNA.

DNA sequencing of the 18S rDNA fragment

The 18S rDNA amplified PCR product (100 ng concentration) was used for sequencing with the single 18S rDNA 20F ITS-1 forward primer: 5'-TCCTCCGCTTATTGATATGC-3' and 22R ITS-4 reverse primer: 5'-GGAAGTAAAAGTCGTA ACAAGG-3' synthesized by DNA Sequencer at Merck laboratory (Bangalore, India).

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 [15]. 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 JD *et al* 1994, Tamura K *et al*).

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 N *et al*,1987, Tamura K *et al* 2007).

The18S 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 SF et 1957 and Cole JR *et al* 2009). 18S rRNA gene sequence of test strain is also compared against those sequences in Ribosomal Database Project (Kusaba M, Tsuge T 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.

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

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 *Trichoderma* species were 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 is used for the detection of multiloci phylogenetic markers. It detects the presence of Internal Transcribed Spacer (ITS) regions in the entered sequences.

RESULTS

Isolation and morphological identification of *Trichoderma* isolates

Genus and species level identification of *Trichooderma* species isolated from the rhizosphere soil of different locations of U.P.were done based on morphological and microscopic observation. The identification of *Trichoderma* isolates were confirmed both by morphological and molecular characters (ITS) and deposited in Indian Type Culture Collection (ITCC), IARI, Pusa, New Delhi. The size of conidia and some morphological characteristics of the isolated *Trichoderma* species are summarized in Table (1).

Screening of selected *Trichoderma species* for chitinase production and their activity

Colloidal chitin media containing bromocresol purple (pH 4.7) when inoculated with chitinolytic *Trichoderma*, resulted in breakdown of chitin into N-acetyl glucosamine causing a corresponding shift in pH towards alkalinity and change in colour of pH indicator dye (BCP) from yellow to purple zone surrounding the inoculated fresh culture plugs in the region of chitin utilization. Chitinase activity exhibited by *Trichoderma* was determined by the diameter of the purple colored zone after 3-7 days of incubation in the colloidal chitin supplemented agar medium.

Chitinolytic activity

Chitinolytic activity was assayed by measuring the release of reducing saccharides from colloidal chitin. A reaction mixture containing 1ml of culture supernatant, 0.3ml of 1 M sodium acetate buffer (SA-buffer), pH 4.6 and 0.2ml of colloidal chitin was incubated at 40°C for 20h and then centrifuged at 13,000 rpm for 5 min at 6°C. After centrifugation, an aliquot of 0.75ml of the supernatant, 0.25ml of 1% solution of dinitrosalycilic acid in 0.7 M NaOH and 0.1ml of 10M NaOH were mixed in 1.5ml microcentrifuge tubes and heated at 100°C for 5min. Absorbance of the reaction mixture at A_{582} was measured after cooling to room temperature. Calibration curve with N-acetyl-â–D-glucosamine (NAGA) was used as a standard to determine reducing saccharide concentration. Chitinolytic activity was estimated in terms of the concentration (mg/ml) of NAGA released.

Table 1. Sequences of potential strains of *Trichoderma* deposited at NCBI

Strain No.	Name of Bioagent	Strain code	Source	GPS location	ITCC Accession No.	NCBI GenBank. Accession No with ITS marker	EMBL Data base Accession No with *tef* marker	NBAIM, Mau Accession No.
T_1	*T. aggressivum*	T.agg (CSAU)	Pratapgarh, UP	Longitude: 81° 59' ELatitude: 25° 35' N	7277	KT315919	LN897318	NAIMCC-F-03193
T_2	*T. aureoviride*	T.avi (CSAU)	Hamirpur, UP	Longitude: 80° 12' ELatitude: 25° 58' N	6131	KT337463	LN897319	NAIMCC-F-03194
T_3	*T.citrinoviride*	T.cvi (CSAU)	New Dairy Farm, CSA Kanpur	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7283	KT315921	LN897320	NAIMCC-F-03195
T_4	*T. erinaceum*	T. eri (CSAU)	New Dairy Farm, CSA Kanpur	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7287	KT315922	LN897321	NAIMCC-F-03192
T_5	*T. koningiopsis*	T. kop (CSAU)	Raibareilly, UP	Longitude: 81°16' ELatitude: 26° 14' N	7291	KT337462	LN897322	NAIMCC-F-03191
T_6	*T. tomentosum*	T. tos (CSAU)	Legume Research Farm, CSAU Kanpur	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7269	KT315920	LN897323	NAIMCC-F-03186
T_7	*T.mintisporum*	T. mip (CSAU)	Vegetable Farm, CSAU Kanpur	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7280	KT626565	LN897312	NAIMCC-F-03187
T_8	*T. pubscenes*	T. sce (CSAU)	Student Instruction Farm, CSAU Kanpur	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7268	KT337461	LN897315	NAIMCC-F-03188
T_9	*T. saturnisporum*	T. ssp (CSAU)	Allahabad, UP	Longitude: 81° 54' ELatitude: 25° 25' N	7274	KT626566	LN897313	NAIMCC-F-03189
T_{10}	*T. spirale*	T. sp. (CSAU)	Nawabganj Farm, CSAU Kanpur	Longitude: 81° 24' 11.414" Latitude: 25° 21' 39.794"	7276	KT626567	LN897314	NAIMCC-F-03190

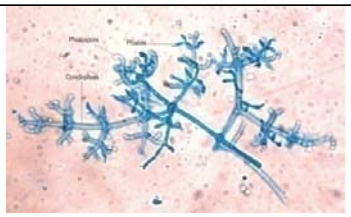
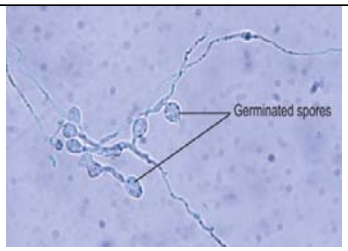
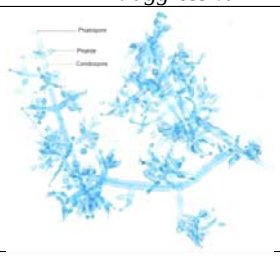
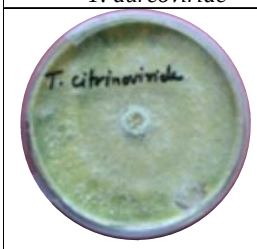
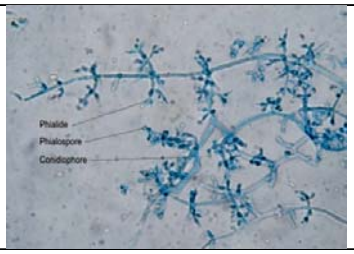
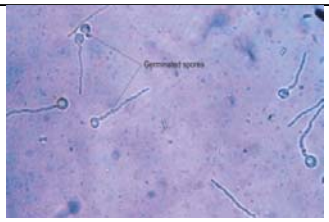
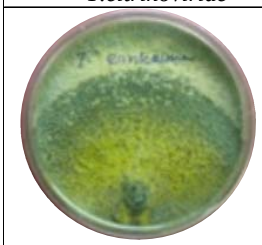
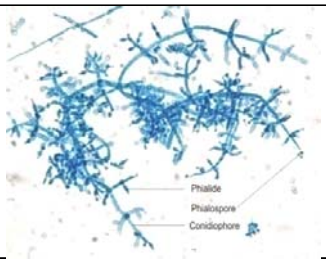
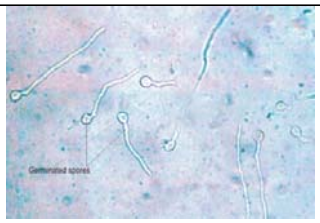
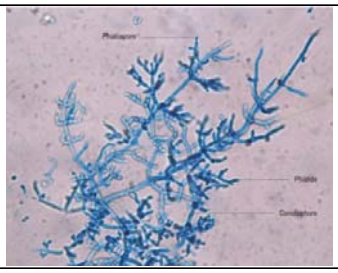
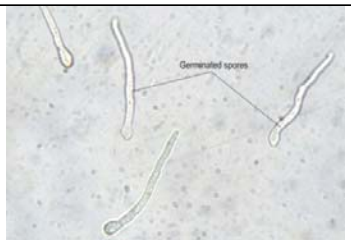
Table 2. Morphological descriptors used in characterization of isolates of *Trichoderma* sp.

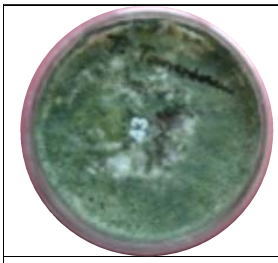
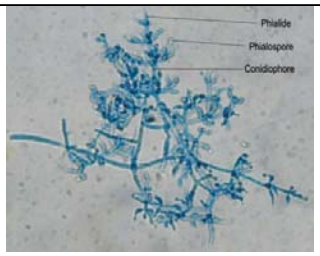
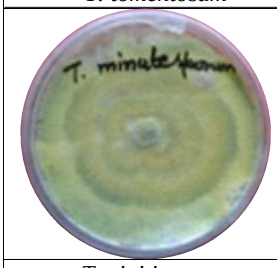
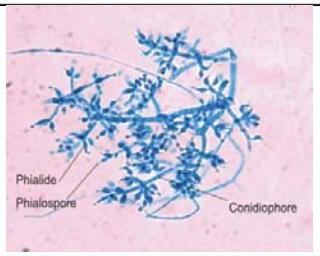
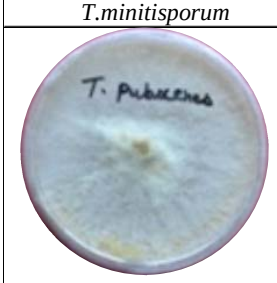
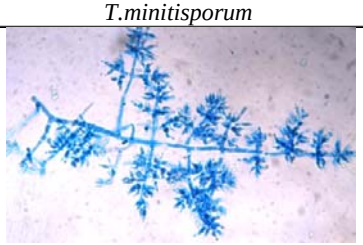
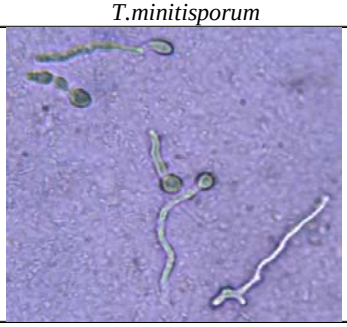
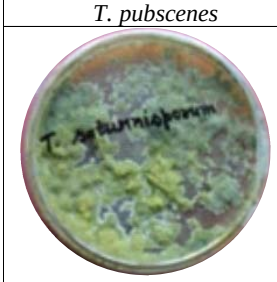
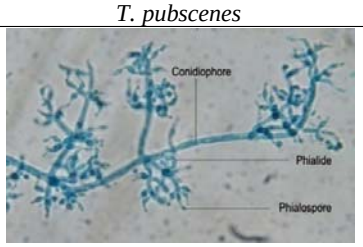
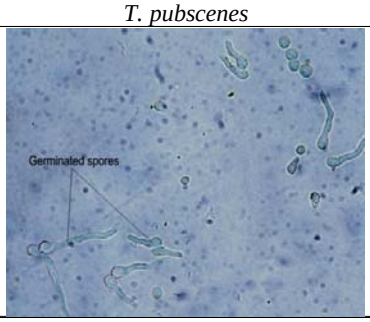
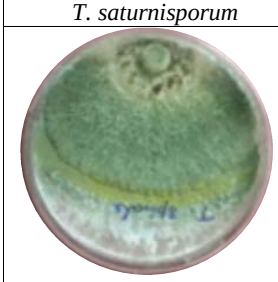
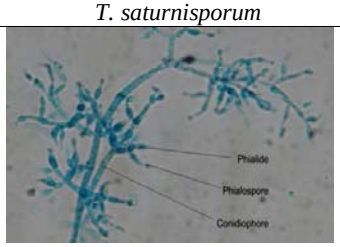
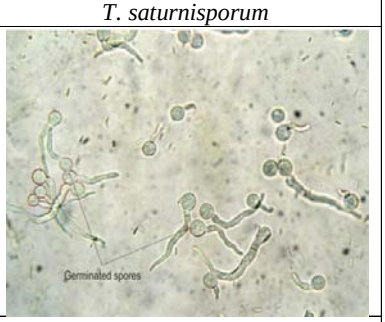
Name of Strains	Colony Growth rate (cm/day)	Colony colour	Reverse colour	Colony edge	Mycelial form	Mycelial colour	Conidi-ation	Conidiophore branching	Conidial wall	Conidial colour	Chlamydo-spores
T. aggressivum	7- 8	Light lettuse green	Light	Smooth	Irregular, arachnoid	Watery white	irregular	regular branched	Smooth	Dark green	No
T. aureoviride	6-7	Watery white	Colourless	Smooth	Floccose to arachnoid	Watery white	irregular	Highly branched	Smooth	Dark green	present
T.citrinoviride	8-9	parrot green	Bright yellow	Smooth	Floccose to arachnoid	Watery white	Concentric, regular	Arise single, slendrical	Smooth	green	Abundently present
T. erinaceum	6-7	Mustard yellow with green granules	Uncoloured	Smooth	Floccose to arachnoid	Watery white	Regular ring like zones	Highly branched	Smooth	grey	present
T. koningiopsis	7-8	Milky white	Naples yellow	Smooth	Tuft.a bundant, effusion	cottony white	Concentric regular	Highly branched	Smooth	Light gray	present
T. tomentosum	7-8	cossack green	Light yellow	Smooth	Sweet corn like smell	Luxuriant, compact, irregular	Irregular	Highly branched	Smooth	gray	No
T.minitisporum	7-8	citron green	Dark antimony yellow	Smooth	Compact, whorls, crowded	Watery white	Ring like zones	Short, highly branched	Smooth	Light green	Rarely present
T. pubscenes	5-6	Cottony white	Uncoloured	Smooth	Luxuriant, arachnoid	Watery white	Concentric regular	Highly	Smooth	Bright green	present
T. saturnisporum	5-6	lettuce green	Ilm green	Smooth	Irregular, crysty, effussion	white	Irregular	regular branching	Smooth	light green	Abundently present
T. spirale	7-8	Deep dull yellow green	Pale fluorite green	Smooth	Floccose to arachnoid	Cottony white	Ring like	Highly branched	Smooth	Light green	present

Table 3a. 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
1.	*T. aggressivum*	7-8	Light lettuse green	Light	Smooth	No characteristic odour	Irregular arachnoid	Watery white
2.	*T. aureoviride*	6-7 cm	Watery white	colourless	Smooth	Coconut like smell	Floccose to arachnoid	Watery white
3.	*T. citrinoviride*	8-9	parrot green	Bright yellow Watery white	Smooth	No characteristic odour		Floccose to arachnoid
4.	*T. erinaceum*	6-7	Mustard yellow with green granules	Uncoloured	Smooth	No characteristic odour	Floccose to arachnoid	Watery white
5.	*T. koningiopsis*	7-8 cm	Milky white	Naples yellow	Smooth	No smell	Tuft.abundant, effusion	cottony white
6.	*T. minutisporum:*	7-8 cm	citron green	Dark antimony Watery white yellow	Smooth	No characteristic odour	Compact, whorls,crowded	
7.	*T. pubscenes*	5-6	Cottony white	Uncoloured	Smooth	No characteristic odour	Luxuriant, arachnoid	Watery white
8.	*T.saturnisporum*	5-6 cm	lettuce green	Ilm green	Smooth	No characteristic odour	Irregular, crysty, effussion	white
9.	*T. spirale*	7-8	Deep dull yellow green	Pale fluorite green	Smooth	No characteristic odour	Floccose to arachnoid	Cottony white
10.	*T. tomentosum*	7-8 cm	cossack green	Light yellow	Smooth	Sweet corn like smell	Luxuriant, compact, irregular	durty white

Table 3b. Morphological observations of the selected *Trichoderma* isolates collected from Kanpur and different locations of Uttar Pradesh

		
T. aggressivum	*T. aggressivum*	*T. aggressivum*
		
T. aureoviride	*T. aureoviride*	*T. aureoviride*
		
T.citrinoviride	*T.citrinoviride*	*T.citrinoviride*
		
T. erinaceum	*T. erinaceum*	*T. erinaceum*
		
T. koningiopsis	*T. koningiopsis*	*T. koningiopsis*

		
T. tomentosum	*T. tomentosum*	*T. tomentosum*
		
T.minitisporum	*T.minitisporum*	*T.minitisporum*
		
T. pubscenes	*T. pubscenes*	*T. pubscenes*
		
T. saturnisporum	*T. saturnisporum*	*T. saturnisporum*
		
T. spirale	*T. spirale*	*T. spirale*

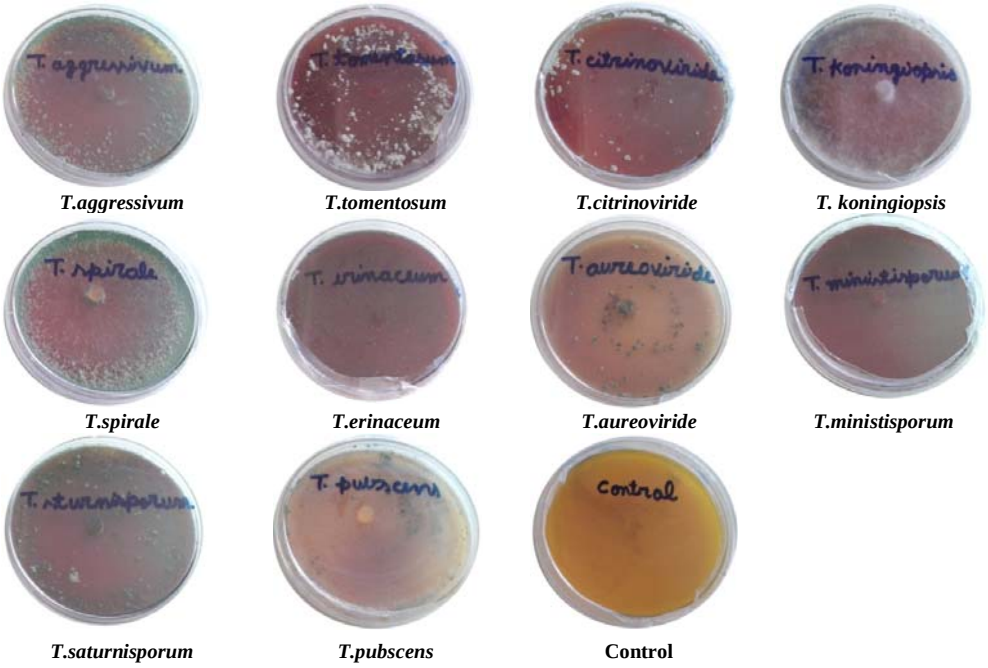


Fig. Chitinase activity observed on Chitinase Detection Media supplemented with colloidal chitin

Table 5. Spectrophotometric determination of chitinolytic and exochitinase activity of *Trichoderma* isolates in media supplemented with colloidal chitin

Isolates	Strain code	Chitinolytic activity mg/ml		Exochitinase Activity(U/ml × 10^{-3}	
		Colloidal	Seashell	Colloidal	Seashell
T.aggressivum	T.agg(CSAU)	7.76	6.6	0.0167	0.00709
T.aureoviride	T.avi(CSAU)	6.06	5.32	0.0145	0.00605
T.citrinoviride	T.cvi (CSAU)	5.9	4.07	0.0120	0.00607
T.erinaceum	T. eri(CSAU)	5.4	4.12	0.0132	0.00508
T.koningiopsis	T. kop(CSAU)	6.1	5.08	0.0142	0.00603
T.tomentosum	T. tos(CSAU)	6.38	4.34	0.0153	0.00703
T.ministisporum	T. mip(CSAU)	5.89	3.6	0.0132	0.00408
T.pubscens	T. sce (CSAU)	4.76	3.2	0.0090	0.00504
T.saturnisporum	T. ssp (CSAU)	5.27	4.0	0.0130	0.00607
T.spirale	T. sp. (CSAU)	6.09	6.04	0.0165	0.00701

Exochitinase activity

N-acetyl-â-D-glucosaminidase (exochitinase) activity was measured and monitored spectrophotometrically as the release of p-nitrophenol (pNP) from p-nitrophenyl-Nacetyl- â-D-glucosaminide (pNPg). A mixture of 25ìl of culture filtrate, 0.2ml of pNPg solution (1mg pNPg ml-1), and 1ml of 0.1 M SA-buffer (pH 4.6) was incubated at 40^0C for 20h and then centrifuged at 13,000 rpm. An aliquot of 0.3ml of 0.125 M Sodium tetraborate–NaOH buffer (pH 10.7) was added to 0.6ml of supernatant, absorbance at 400nm (A_{400}) was measured immediately after mixing and pNP concentration.

The results of this analysis clearly shows that all the tested species shows chitinolytic activity.The highest chitinolytic activity was achieved by *T.aggressivum*. Chitinolytic enzymes play a very important role in mycoparasitic activity as the cell wall of most the phytopathogens is made up of chitin.

Table 6. *In vitro* antagonistic efficacy of *Trichoderma* isolates

Sl No.	Name of bioagent	Strain code	Source	ID No.	Average growth in (mm)	% inhibition growth (mm)	Average growth in (mm)	% inhibition growth (mm)
					Fusarium oxysporum		*Sclerotium rolfsii*	
1.	*T. aggressivum*	T.agg (CSAU)	Pratapgarh, UP	7277	15.0	50.0	33.3	63.0
2.	*T. aureoviride*	T.avi (CSAU)	Hamirpur, UP	6131	14.5	51.6	36.6	59.3
3.	*T. citrienoviride*	T.cvi (CSAU)	New Dairy Farm, CSAUA&T, Kanpur	7283	18.0	40.0	35.5	60.5
4.	*T. erinaceum*	T. eri (CSAU)	New Dairy Farm, CSAUA&T, Kanpur	7287	14.2	52.6	38.3	57.4
5.	*T. koningiopsis*	T. kop (CSAU)	Raibareilly, UP	7291	13.5	55.0	32.5	60.8
6.	*T. tomentosum*	T. tos (CSAU)	Legume Research Farm, CSAU, Kanpur	7269	20.5	31.6	45.0	50.0
7.	*T. minutisporum*	T. mip (CSAU)	Vegetable Farm, CSAUA&T, Kanpur	7280	23.0	23.3	50.8	43.5
8.	*T. pubscenes*	T. sce (CSAU)	Student Instruction Farm, CSAU, Kanpur	7268	22.5	25.0	39.1	46.5
9.	*T. saturnisporum*	T. ssp (CSAU)	Allahabad, UP	7274	17.0	43.3	47.5	47.8
10.	*T. spirale*	T. sp. (CSAU)	Nawabganj Farm, SAUA&T, Kanpur	7276	18.5	38.3	32.5	63.8

In vitro antagonistic efficacy of *Trichoderma* isolates against plant pathogenic fungi

The inhibitions of mycelial growth of pathogenic fungi were showed in Table (6). In all the dual culture plates tested, the contact zone was a curve, with concavity oriented towards the pathogenic fungi. In the negative control plates, only pathogenic fungi were inoculated.

The results were represented in table (6) showed that, all selected *T. species* exhibited inhibition on the mycelial growth of *F. oxysporum* and *Sclerotium rolfsii*. The highest inhibition percentage against *F. oxysporum* was recorded for *T. koningiopsis* (55.0%) , followed by *T. erincaceum T. aureoviride* and *T. aggressivum* respectively, while, the highest inhibition percentage against *Sclerotium rolfsii* was recorded for *T. spirale* and *T. aggressivum* (63.8) followed by *T. koningiopsis* and *T. citrienoviride* respectively.

PCR amplification of ITS region of rDNA of *Trichoderma* species

Genomic DNA of the ten selected *Trichoderma species* was analysed by PCR amplification of rDNA gene including 5.8S gene and the flanking intergeneic transcribed spacer ITS region of rDNA. Amplification of the ITS with primers ITS1 and ITS4 yielded a single product estimated by gel electrophoresis of approximately 600 bp was obtained from all the PCR amplification for biocontrol isolates of *Trichoderma*.

Sequencing of ITS region of *Trichoderma* species

The sequence of the ten selected *Trichoderma species* was done to confirm species identified previously according their morphological and microscopic observation. PCR products amplified from the four *Trichoderma asperellum* isolates were sequenced. They could be aligned and a consensus sequence was generated from each alignment made. Then, BLAST was used to determine the species identity of *Trichoderma* isolates. The sequences of ITS region of the ten *Trichoderma species* were submitted to GenBank under the following accession numbers are awaiting respectively.

Phylogenetic analysis

The Phylogenetic tree obtained by sequence analysis of ITS1 and ITS4 of *T. species* isolates and the sequences of 31 *Trichoderma* spp

Table 7. Describing the ISTH *Trich*OKEY and *Tricho*MARK results for all seven *Trichoderma* species

Input (Nucleotide Sequence)	*Trich*OKEY Results		*Tricho*MARK Results
Trichoderma harzianum strain 6796	First anchor (GSH) was found in position 95 Second anchor (GSH) was found in position 116 Third anchor was not found Fourth anchor was not found Fifth anchor was not found		The query sequence is diagnosed as ITS1 and 2 region of Hypocrea/Trichoderma.
Hypocrea rufa isolate 01PP8315/ 11	First anchor was not found Second anchor (GSH) was found in position 2 Third anchor was not found Fourth anchor was not found Fifth anchor was not found	Barcode identification of the query sequence is not possible because only one genus specific hallmark (Anchor 2) is found.	
Trichoderma asperellum strain 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	region 1 (8nt) was detected; region 2 (139nt) was detected; region 3 (213nt) was detected
Hypocrea koningii strain 5201	First anchor was not found Second anchor (GSH) was found in position 50 Third anchor was not found Fourth anchor was not found Fifth anchor was not found	Barcode identification of the query sequence is not possible because only one genus specific hallmark (Anchor 2) is found.	
Trichoderma atroviride strain TAU8	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	region 1 (9nt) was detected; region 2 (156nt) was detected; region 3 (184nt) was detected
Trichoderma longibrachiatum strain 21PP	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	region 1 (11nt) was detected; region 2 (176nt) was detected; region 3 (201nt) was detected
Trichoderma virens strain 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	region 1 (9nt) was detected; region 2 (155nt) was detected; region 3 (199nt) was detected

Trichoderma reesei strain Tr(CSAU)	First anchor was not found Second anchor (GSH) was found in position 487 Third anchor was not found Fourth anchor was not found Fifth anchor was not found	Barcode identification of the qu ery sequence is not possible be cause only one genus specific h allmark (Anchor 2) is found.	
Trichoderma aggressivum strain T.agg/7277	First anchor (GSH) was found in position 60 Second anchor (GSH) was found in position 81 Third anchor was not found Fourth anchor was not found Fifth anchor was not found		The query sequence is diagnosed as ITS1 and 2 regio n of Hypocrea/ Trichoderma
Trichoderma aureoviride strain T.aur/6131	First anchor (GSH) was found in position 68 Second anchor (GSH) was found i n 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 511	**Genus Identification:** Hypocrea/*Trichoderma*, Hypoc reaceae, Hypocreales, Ascomycota	region 1 (9nt) was detected; region 2 (155nt) was detected; region 3 (200nt) was detected
Trichoderma citrinoviride	First anchor (GSH) was found in position 54 Second anchor (GSH) was found i n 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*, Hypoc reaceae, Hypocreales, Ascomycota	region 1 (10nt) was detected; region 2 (174nt) was detected; region 3 (177nt) was detected;
Trichoderma erinaceus isolate T.eri/7287	First anchor (GSH) was found in position 56 Second anchor (GSH) was found i n 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*, Hypoc reaceae, Hypocreales, Ascomycota	region 1 (8nt) was detected; region 2 (142nt) was detected; region 3 (174nt) was detected
Trichoderma koningiopsis strain T.kop/7291	First anchor (GSH) was found in position 62 Second anchor (GSH) was found in position 83 Third anchor (GSH) was found in position 227 Fourth anchor (GSH) was found in position 385 Fifth anchor was not found	The query sequence belongs to an unidentified species of Hypocrea/*Trichoderma*	
Trichoderma tomentosum strain T.tos/ 7269	First anchor (GSH) was found in position 67 Second anchor (GSH) was found i n 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*, Hypoc reaceae, Hypocreales, Ascomycota	region 1 (10nt) was detected; region 2 (155nt) was detected; region 3 (197nt) was detected

Trichoderma minitisporum strain T.mip/7280	First anchor was not found Second anchor (GSH) was found in position 66 Third anchor was not found Fourth anchor was not found Fifth anchor was not found	Barcode identification of the qu ery sequence is not possible be cause only one genus specific h allmark (Anchor 2) is found.	
Trichoderma pubescens isolate T.pub/7268	First anchor (GSH) was found in position 93 Second anchor (GSH) was found i n position 114 Third anchor (GSH) was found in position 260 Fourth anchor (GSH) was found in position 418 Fifth anchor (GSH) was found in position 517	**Genus Identification:** Hypocrea/*Trichoderma*, Hypoc reaceae, Hypocreales, Ascomycota	region 1 (8nt) was detected; region 2 (140nt) was detected; region 3 (161nt) was detected
Trichoderma saturnisporum strain T.ssp/7274	First anchor (GSH) was found in position 16 Second anchor (GSH) was found i n position 38 Third anchor (GSH) was found in position 217 Fourth anchor (GSH) was found in position 375 Fifth anchor (GSH) was found in position 471	**Genus Identification:** Hypocrea/*Trichoderma*, Hypoc reaceae, Hypocreales, Ascomycota	region 1 (9nt) was detected; region 2 (173nt) was detected; region 3 (177nt) was detected
Trichoderma spirale isolate T.sp/7276	First anchor (GSH) was found in position 78 Second anchor (GSH) was found i n position 100 Third anchor (GSH) was found in position 259 Fourth anchor (GSH) was found in position 417 Fifth anchor (GSH) was found in position 511	**Genus Identification:** Hypocrea/*Trichoderma*, Hypocreaceae, Hypocreales, Ascomycota	region 1 (9nt) was detected; region 2 (153nt) was detected; region 3 (118nt) was detected;

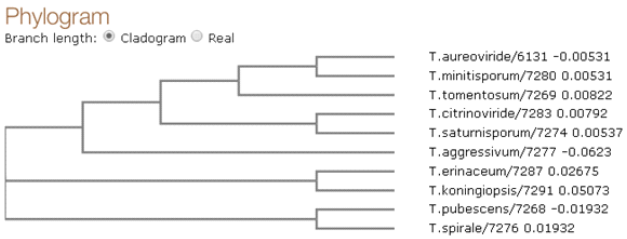


Fig. ISTH *Trich*OKEY and *Tricho*MARK results for all ten *Trichoderma* species

obtained from NCBI, GenBank is represented in Figure (2). According to the NCBI BLAST search of the sequence of our *Trichoderma species* against the sequences of 31 other *Trichoderma* spp. *Trichoderma* were identified as (*T. aggressivum, T. aureoviride T. citrienoviride, T. erinaceum, T. koningiopsis, T. tomentosum, T. minutisporum, T. pubscenes, T. saturnisporum & T. spirale*) (table 6).

Bioinformatics analyses of the strains of *Trichoderma* species, starting from obtaining the nucleotide sequences and then performing the complete genomic analysis of the same is carried out.

DISCUSSION

Morphological characterization is a potential tool for the characterization of *Trichoderma* species *Trichoderma* species (Anees *et al*. 2010). The few morphological characters with limited variation may lead to an overlap and

misidentification of the strains and showing the necessity of DNA based characters to complete identification evident from the present study. In the present study, the morphological identification of *Trichoderma* isolates isolated from Indian rhizosphere soils was complemented by a molecular identification based on internal transcript spacers (ITS region) of rDNA sequences. Based on the phenotypic characteristics (colony texture, conidia, phialides, chlamydospore and conidiophore) and genotypic characteristics (ITS region of 18s rRNA), were identified as *Trichoderma*. These results indicate that molecular systematic study based on the sequence of ITS region is important for confirmation of phenotypic characterization of *Trichoderma* isolates. The rRNA is essential for the survival of all the cells and the genes encoding rRNA is highly conserved in the fungal. In eukaryotes, the genes encoding ribosomal RNA are organized in arrays which contain repetitive transcriptional units involving 16-18S, 5.8S and 23-18S rRNA, two transcribed intergenic spacers ITS1 and ITS2. These units are transcribed by RNA polymerase 1 and separated by non transcribed intergenic spacer (IGS). The product of RNA polymerase 1 is processed in the nucleolus, where the ITS 1 and ITS2 are excised and three types of rRNA are produced (Shahid *et al.* 2014). The ITS sequence was chosen for this analysis because it has been shown to be more informative with various sections of the genus *Trichoderma* (Kuhls *et al*. 1997, Ospina-Giraldo *et al*. 1998).

There are many mechanisms which are employed by *Trichoderma* against pathogens. During antibiosis the anatgonistic fungus produce antibiotics, compete for nutrients, while in case of mycoparasitism *Trichoderma* directly attacks the pathogen by secreting CWDEs such as chitinase xylanase, glucanase protease etc. CWDEs play a crucial role in the anatagonistic mechanism as they degrade the cell wall of pathogens. These enzymes lysesed the pathogen cell wall. Present findings showed higher specific activity of enzymes-chitinase and â-1, 3 glucanase in *Trichoderma* spp. Activity of these enzymes varied among the *Trichoderma* species. This may be due to the expression of certain gene in *Trichoderma* spp. Marco *et al* showed that Two isolates of *T.harzianum* secretes chitinolytic enzymes and it increased within 72 h.

The potentiality of *Trichoderma* spp. as biocontrol agents of phytopathogenic fungi in several crops is well known especially to *Fusarium* spp. and *Rhizoctonia* spp. (Poddar *et al.* 2004 ; Rojo *et al*. 2007). *Trichoderma* suppressed the growth of *F. semitectum* through the over growth. In second case *Trichoderma* was observed to cluster around *F. semitectum* by the formation of small tufts thus limiting the growth of the pathogen of sheath blight. In both the cases formation of sclerotial bodies of *F. semitectum* was suppressed (Shalini and Kotasthane, 2007). Tondje *et al.* (2007) reported that direct mycoparasitism is considered to be the main mechanism of action for *T.asperellum*. Petrescu *et al.* (2012) reported the curvature of the contact area between the colony of antagonistic fungi and the colony of pathogenic fungi in the same PDA medium depends on the growth rate of the colonies. If one colony has a faster growth rate than the other, a curve in the contact zone will most probably be observed. However, the average inhibition percentage of mycelial growth of pathogenic fungi was frequently used and shown to be a useful way in assessing the antagonistic potential of the antagonistic fungi. Overall the four *T. asperelum* isolated in this study have the ability to inhibition the mycelial growth of *Alternaria alternate* and *Fusarium semitectum*. This result was in agreement with the results found by (Ommati and Zaker 2012), they found that *T.asperellum* had the highest inhibition on the growth of *Fusarium oxysporum*. Segarra *et al*. 2010 reported that *T.asperellum* was used as an efficient biological control agent in controlling *Fusarium* wilt in tomato. Rapid growth of *Trichoderma* is an important advantage in competition with plant pathogenic fungi for space and nutrients (Deacon and Berry 1992).

Scanning electron microscopy analysis of hyphal interaction between *Trichoderma harzianum* and phytopathogens clearly indicates that *Trichoderma* coils around the phytopathogen and parasitized the phytopathogen mycelium. Elad *et al* 1983 observed the hyphal interaction between *Trichoderma* and *Sclerotium rolfsii* through SEM and showed that *Trichoderma* coils around the phytopathogen cells.

In conclusion, our results suggest for the identification of *Trichoderma* species molecular identification is very important and it must be used

to confirm morphological approaches in the identification of *Trichoderma* isolates. Accordingly we must use combing morphological and molecular methods for success identification of *Trichoderma* isolates. Worth mentioning that, the ten species of *Trichoderma* isolates (Tas1, Tas2, Tas3 and Tas4) were highly producer for chitinase and showing high antagonistic activity against tested pathogenic fungi. However, further study must be done for developing these isolate as new bio fungicides at large scales production.

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