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Isolation, Molecular and *In-silico* characterization of *Trichoderma* spp. from Rhizospheric Soil Sample

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ABSTRACT: *Trichoderma*, soil-born filamentous fungi are capable of parasitizing several plant pathogenic fungi, it is known as biocontrol agents. Five isolates of *Trichoderma* spp. isolated from various crop rhizospheric soil in Uttar Pradesh districts were characterized by their cultural, morphological, and molecular level. The isolates differed significantly in terms of colony traits, sporulation, branching of conidiophores, and the colour and shape of phialospores. Molecular analysis of the isolates was done by sequencing the ITS region of ribosomal DNA using specific universal primers ITS 1 and ITS 4. Multiple nucleotide alignment of ITS 1, ITS 4 and 5.8s region depicted intra-specific and inter-specific variations in the ITS sequences among the different *Trichoderma* species. The result of this research obtained at morphological and ITS-based rDNA region sequencing. The sequencing of these five isolates revealed that the *Trichoderma* were characterized into *Trichoderma lixii* (Acc. No. OP031646), three *Trichoderma harzianum* (Acc. No. OP104445, OP104449, OP104451) and *Trichoderma* sp. (Acc. No. OP104454). However, significant percentage identity between the known isolate of *T. lixii* from the database and *T. lixii* (TBT-13) isolates from wheat is 97% whereas the percentage between the known isolate of *T. harzianum* and three species of *T. harzianum* (TBT-14, TBT-15, TBT-16) from potato, wheat, and mustard was 97-98% respectively. Similarly, *Trichoderma* spp. (TBT-17) are 98% identical to *Trichoderma* spp. result after NCBI-BLAST. As a result, the study was found to be helpful in identifying *Trichoderma* spp. from the rhizospheric soil of various crops.

Keywords: *Trichoderma*, Biocontrol, Glucanase, Antagonistic, Pathogen, ITS- region.

INTRODUCTION

The *Trichoderma* is a soil-born species belonging to the family *Hypocreaceae*. Persoon (1794) was the first to characterize *Trichoderma*, an ascomycete soil-dwelling fungus of the family Hypocreaceae with Hypocrea as its teleomorphic form. *Trichoderma* is a genus of multifunctional fungi that may be found in a wide range of environments, and it contributes to a significant number of its capabilities across various strains. They are typically found in rhizospheric soils from forests or farms. It is fast growing at 25–30°C, but some species have shown growth even at 45°C**.**

These fungi produce enzymes or proteins *viz.*, glucanase, chitinase, tubulins, proteinase, xylanase, monooxygenase, galacturonase etc., that help in the degradation of the pathogens and act as a bio-control. The challenges experienced while identifying *Trichoderma* isolates at the species level are made more severe by the rarity and difficulty of observing physical variations. Rifai established the idea of "species aggregation" in 1969 and divided the nine *Trichoderma* strains into aggregates based on morphological characteristics (Rifai, 1969). Unfortunately, part of the "species aggregate" has two or more non-differentiable morphologies. Later research analysed Rifai's findings, and Bissett (1991) made an effort to incorporate related forms into the species concept based on morphology, including the features of the conidiophore branching system. *Trichoderma* species have been recognized as capable of harming other fungi for more than 60 years. Additionally, researchers are aware of them as possible biological control agents (El Komy *et al.*, 2015; Naher *et al.*, 2014; Sundaramoorthy and Balabaskar 2013). *Trichoderma* spp. have been widely used in agricultural applications due to its well-known biological control mechanism. The usage of this microbial inoculant in

Trichoderma-based products attracts the attention of researchers to discover more on other potential benefits of *Trichoderma* spp (Zin and Badaluddin 2020). The major bio-control process involves antibiosis, providing plant nutrition and mycoparasitism. The *Trichoderma* helps in plant growth promotion and increasing nutrient uptake from the soil. They decrease the activity of soil borne pathogens which affects growth of the plants. Among various species *Trichoderma harzianum* is considered to be the most effective bio-control agent (Harman *et al*., 2004). *Trichoderma* spp. are among the most frequently isolates soil fungi and present root system. However, there is still considerable interest in finding more efficient mycoparasitic fungi especially within *Trichoderma* spp. having potential higher antagonistic efficiency by the selection of isolates with high potential to secret extra cellular lytic enzymes chitinase and β-1,3-glucanase. The lytic enzymes break down cell wall polysaccharides into short oligomers and by the way facilitates the hyperparasite to penetrate into the cytoplasm of the target fungi. The aim of this research paper top screen out the best *Trichoderma* isolates for their antagonistic capacity by dual culture as well as their combination of enzyme activities against pathogen tests.

MATERIALS AND METHODS

Collection of Soil samples. The Rhizospheric soil samples were collected from Muzaffarnagar, Varanasi, Ballia, Prayagraj and Ghazipur districts of Uttar Pradesh (India) having different ecological habitats of crops for the isolation of *Trichoderma*. The sample collected from the crop's rhizosphere such as tomato, wheat, mustard, and pea field. The sample was brought to the departmental laboratory of Ag. Biotechnology in SVPUAT, Meerut and sample were stored at 4 ºC. The experiment was conducted in the departmental laboratory and Plant Pathology laboratory of College of Agriculture, SVPUAT, Meerut. The extraction and purification of fungal DNA was performed according to slight modification of the methods given by Sambrook *et al*. (1989). The primer sequence used in present investigation are ITS universal primer, the forward and reverse primer for amplification of ITS region are-

ITS 1- 5' TCC GTA GGT GAA CCT TGC GG 3'
ITS 4- 3'TCC TCC GC T TAT TGA TAT GC 5'

The sequencing of *Trichoderma* isolates ITS region was done at Biokart laboratory, Bengaluru. The *in-silico* characterization was analysed by open-source software such as Mega 11.0, Bio-edit, NCBI BLAST.

RESULTS AND DISCUSSION

Isolation and maintenance of native *Trichoderma* isolates. For isolation of *Trichoderma* species take 1 gm of air-dried soil sample was added into 9 ml of sterilized distilled water in a test tube to make up volume 1:10 (10^{-1}). Six-fold serially diluted samples were prepared and 0.5 ml diluted sample was poured on the surface of TSM (Trichoderma Selective Medium) and PDA (Potato Dextrose Agar). The petri plates were incubated at 28±2 ºC for 5-6 days in incubator. Morphologically different colonies have appeared on plates that were purified in PDA medium. Due to its low glucose level, the fungus may swiftly multiply and sporulate. On PDA slant, the pure *Trichoderma* isolates were sub-cultured and maintained at 4 ºC used for their characterization. Similar result was obtained by Varsha *et al.* (2019) after 7 days of incubation, a total of 10 fungal isolates were collected from all three types of soil samples; however, only three of these strains were grown on two types media, PDA and TSM. PDA media was shown to be ideal for large-scale *Trichoderma* growth. Similar result was reported by Kale *et al.* (2018) where sixteen rhizospheric soil samples were taken from the tomato crops in eight Marathwada districts. On the PDA medium, 16 isolates were recovered from these soil samples. Upon visual observation, *Trichoderma* spp. was identified as *Trichoderma viride* isolates, *T. harzianum* isolates, and *T. hamatum* isolates.

Morphological characterization of *Trichoderma* isolates. Morphological characterization of *Trichoderma* spp. is based on their characters like mycelial growth, colour of mycelium and other microscopic observations. The culture was obtained from different rhizospheric soil samples. The large colonies and microbes appear on PDA medium of different colour, but only green to light yellow colonies appear on TSM. The *Trichoderma* species with a light green to yellowish colour colony appearance are shown in Fig. 1. The pure culture of *Trichoderma* spp. was cultured on PDA medium for mycelium growth, the initial growth was slow with white colour mycelium but 48 hrs. after inoculation, the mycelium become yellowish-green to green in colour. The petri plates were completely filled with green mycelium within a week (Fig. 1). Morphological characterization of *Trichoderma* isolates was also reported by Muthu and Pratibha (2011). For three to seven days, the isolates were grown as pure cultures on PDA, and the various mycelial and conidial characteristics of each isolate were noted. Colony growth rate (cm) after seven days at 25°C, colony colour, reverse colony colour, colony edge, culture fragrance, condition, mycelial form, mycelial colour, conidiophores branching, phialide disposition, phialide shape, conidial shape, and conidial wall were the morphological features utilized for characterization (Elsoky *et al*., 2019).

Microscopic slides were prepared of each isolate in 3 % KOH followed by lactophenicol-cotton blue staining from pustules where conidia were still white, generally after 5-6 days of inoculation. The microscopic characteristics like branching pattern, conidiophores, conidial shape, phialide numbers, formation of chlamydospores and their position were observed.

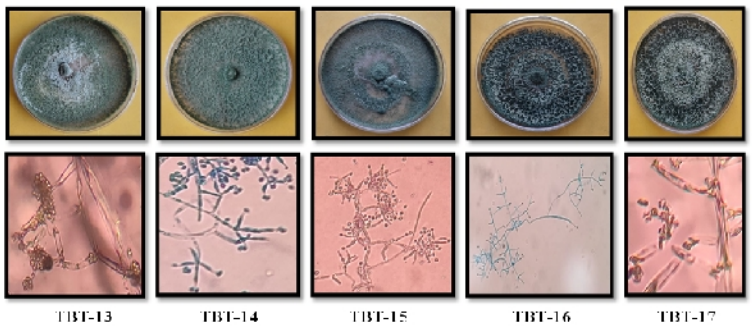
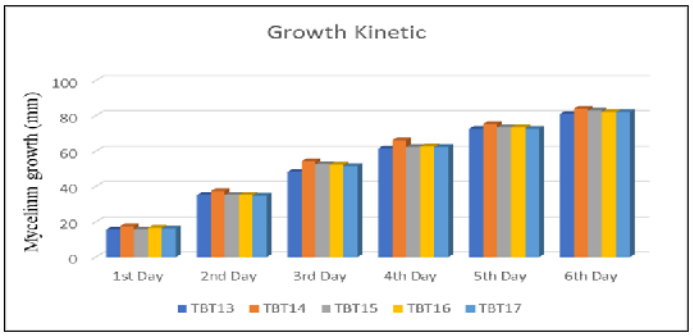


Fig. 1. Morphological and microscopic characterization of *Trichoderma* isolates isolated from different rhizospheric soils.

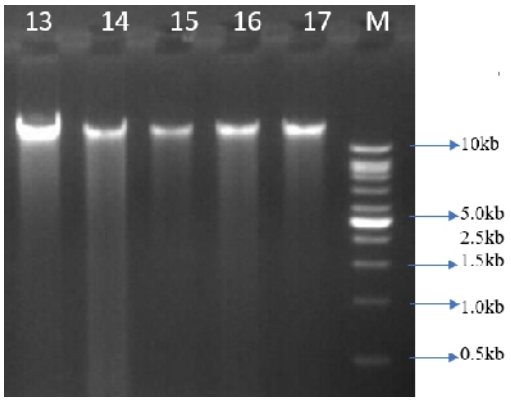
Table 1: Mean value of growth kinetics data of different *Trichoderma* isolates (mm).

Sr. No.	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day
TBT13	15.6 ± 0.12	35.0 ± 0.05	48.3 ± 0.03	61.6 ± 0.03	72.6 ± 0.03	81.0 ± 0.05
TBT14	**17.6 ± 0.03**	**37.3 ± 0.03**	**54.3 ± 0.05**	**66.3 ± 0.06**	**75.3 ± 0.03**	**84.0 ± 0.00**
TBT15	15.6 ± 0.03	35.0 ± 0.05	52.6 ± 0.03	62.3 ± 0.03	73.3 ± 0.08	83.0 ± 0.00
TBT16	16.6 ± 0.03	35.0 ± 0.05	52.3 ± 0.08	62.6 ± 0.06	73.3 ± 0.03	82.0 ± 0.05
TBT17	16.3 ± 0.08	34.6 ± 0.12	51.6 ± 0.12	62.3 ± 0.03	72.6 ± 0.06	82.0 ± 0.00
C.D.	0.167	0.187	0.253	0.274	0.373	0.208
SE (d)	0.082	0.091	0.124	0.134	0.183	0.072
C.V.	6.787	3.364	3.093	2.663	3.130	1.536



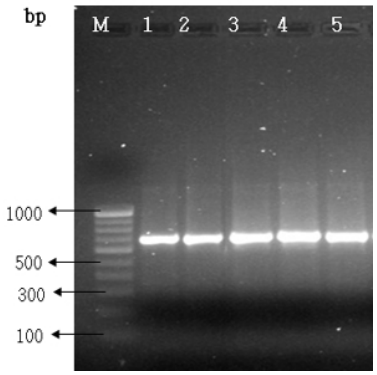
Graph 1. Mycelium growth (mm) patterns of different *Trichoderma* isolates.

Extraction of gDNA and molecular characterization of *Trichoderma* isolates. For molecular characterization, the pure culture of five *Trichoderma* isolates was grown in a conical flask filled with 50 ml potato dextrose broth containing chloramphenicol at 25-30 ppm final concentration and inoculated flasks were incubated for 5-7 days at 28±2°C with rotary shaking at 150 rpm in the orbiter incubator. The mycelium mats were harvested after incubation periods through Whatman filter paper no.1 and dried in aseptic conditions. The dried mycelium was disrupted by grinding in liquid nitrogen using a mortar and pestle. The genomic DNA (gDNA) was extracted by the CTAB method with slight modifications. The quality and quantity of gDNA were determined by measuring the absorbance at 260 nm through NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and stored at -20°C for further use (Fig. 2).



M- 1kb Marker; 13,14,15,16 and 17- TBT-13, TBT-14, TBT-15, TBT-16 and TBT-17.

Fig. 2. Agarose gel electrophoresis of total genomic DNA of *Trichoderma* isolates.



M- 100 bp Marker; 1,2,3,4 and 5- TBT-13, TBT-14, TBT-15, TBT-16 and TBT-17

Fig. 3. Amplified PCR products of ITS region of *Trichoderma* spp. isolated from soil using ITS universal primers.

The gDNA of *Trichoderma* isolates was used as a template for PCR amplification using specific ITS universal primers. The amplification was carried out in conjunction with 35 cycles of initial denaturation at 94 °C for 3 minutes, then 55 seconds at 94 °C, 50 seconds at 57 °C, and one minute at 72 °C with a final extension for 10 minutes at 72 °C. The PCR product was examined in 1.5% agarose gel. The ITS region primers used for amplification yielded an expected amplicon of 650 bp using agarose gel electrophoresis shown in Fig. 3. These findings are in accordance with Prasad *et al.* (2016) who calculated that *Trichoderma* spp. amplified rDNA fragments ranged in size from 500 to 600 bp.

The PCR amplicons were compared and amplified products were sequenced at Biokart India Pvt. Ltd. Laboratory, Bengaluru. The sequence obtained after sequencing was subjected to sequence-based identification through NCBI - BLAST tool. The pairwise sequence identification comprising the TBT-13 sequence showed 97-98% sequence *Trichoderma lixii*, TBT-14, TBT-15 and TBT-16 are similar to *Trichoderma harzianum* and TBT-17 showed 98% similarity with *Trichoderma* spp. result of NCBI BLAST tools supported morphological variability of *Trichoderma isolates*. These obtained sequences were submitted to NCBI GeneBank with accession numbers OP031646, OP104445, OP104449, OP104451 and OP104454 (Table 2).

To better understand the genetic variability of the *Trichoderma* isolates, a phylogenetic tree was constructed by using the Clustal *W* and Mega 11.0 software with the Neighbor-Joining (NJ) method (Fig. 4) based on ITS region differences of *Trichoderma* isolates and other *T.* species reported from globally. In order to create phylogenetic trees across species, Saitou and Nei (1987) claim that NJ may be used for any sort of data that contains evolutionary differences and uses the Kimura-2 parameter method to calculate evolutionary distances or nucleotide changes that are present.

Table 2: Molecular characterization of *Trichoderma* isolates.

Isolate Code	*Trichoderma* species identification	District of soil sample collection	Gen Bank Acc. No. (ITS sequence)
TBT 13	*Trichoderma lixii*	Muzaffarnagar	OP031646.1
TBT 14	*Trichoderma harzianum*	Varanasi	OP104445.1
TBT 15	*Trichoderma harzianum*	Ballia	OP104449.1
TBT 16	*Trichoderma harzianum*	Prayagraj	OP104451.1
TBT 17	*Trichoderma* spp.	Ghazipur	OP104451.1

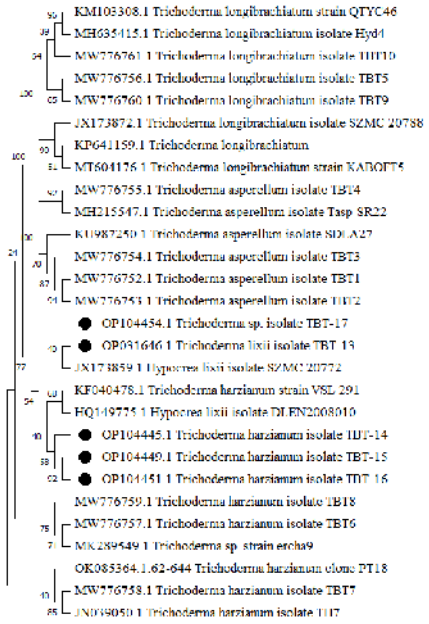


Fig. 4. Phylogenetic analysis of *Trichoderma* isolates based on rDNA -ITS sequencing by Neighbor -Joining (NJ) method.

CONCLUSION

In this study, different species of *Trichoderma* have been isolated from rhizospheric soil of various crops of different districts in Uttar Pradesh, such as *Trichoderma lixii*, three species of *Trichoderma harzianum* and one of *Trichoderma* sp. The isolated species of *Trichoderma* were identified on the basis of cultural, morphological and molecular approaches using Internal Transcribed Spacer (ITS-PCR) region amplification. Further, it may be concluded that the *Trichoderma* isolates are classified into different species after sequencing of ITS region. The obtained sequenced are *in-silico* characterized through bioinformatics tools such as NCBI- BLAST, MEGA 11.0, Bioedit, Phyre^2 etc. Furthermore, it is beneficial that the *Trichoderma* species acts as biocontrol agents against several pathogenic fungi and produces some lytic enzymes such as glucanase, chitinase, proteases, tubulises etc. These lytic enzymes helped in the management of fungal diseases in crops pand lants. So, *Trichoderma* is used as a bio-fertilizer and for seed treatment in various crops.

FUTURE SCOPE

The future aspect of *Trichoderma* isolates such as improper management of agricultural waste also pollutes the environment when it has been burned or disposed into water bodies. The best solution to overcome these problems is the application of *Trichoderma* spp. which act as biological control agents and used as biofertilizer in seed treatment. The usage of *Trichoderma* is generally advantageous because it has increased crop output and minimize pesticide and fertilizer use in the field.

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Conflict of Interest. None.

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