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Identification of *Trichoderma* Isolates from Tobacco Growing Regions of West Godavari District, Based on Sequence Analysis of ITS Region of rDNA and Morphological Variations in the Strains

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The aim of this study was to determine the phylogenetic relationships of *Trichoderma* isolates obtained from NLS Tobacco region. Utilizing the sequence analysis of internal transcribed spacer-1 (ITS-1) region of the ribosomal DNA the rDNA of Five *Trichoderma* isolates was amplified by polymerase chain reaction (PCR) using universal primers (ITS-1 and ITS-4). PCR products were purified and these purified products were used to amplify the ITS-4 region of the five *Trichoderma* isolates. The amplified DNA was sequenced and aligned against ex-type strain sequences from TrichoBLAST and established *Trichoderma* taxonomy. Two isolates were positively identified as *Trichoderma harzianum* and three isolates were identified as *Trichoderma aesperulum* which were used as an outgroup in these analyses. The ITS-1 region sequences have been used as the reference's sequence and this could be further used for future study involving the identification and taxonomy of *Trichoderma* Amplification of ITS-1 region of the rDNA. This method proved as a rapid and reliable technique for identifying *Trichoderma* strains successfully.

Introduction

The genus *Trichoderma* contains species that are of great economic importance due to their ability to act as biological control agents against a large variety of fungal plant pathogens (Sharma and Singh 2014). The largest distribution of *Trichoderma* bio-products is found in Asia, succeeded by Europe, South-Central America and North America (Sheridan *et al.*, 2014). Biological control using microbial antagonists is considered as a good alternative of management of root diseases in many crops (Dileep *et al.*, 1999; Mukhopadhyay *et al.*, 1992; Sheela *et al.*, 1998; Khot *et al.*, 1996; Gholve and Kurundkar 2002). Trichoderma, commonly available in soil and root ecosystem, has gained immense importance since last few decades due to its biological

control ability against several plant pathogens (Shahid *et al.,* 2014). Accurate and definitive fungal identification is essential for correct disease diagnosis and treatment of associated fungal infections. Characterization of fungal species using classical methods is not as specific as the genotyping methods. Genotypic techniques involve the amplification of a phylogenetically informative target, such as the small-subunit (18S) rRNA gene (Woese *et al.,* 1977). The Internal Transcribed Spacer (ITS) regions of the rDNA are perhaps the most widely sequenced DNA regions in fungi. It has typically been most useful for molecular systematic study at the species level, and even within species (Ospina-Giraldo *et al.,* 1998; Kubicek *et al.,* 2000; Kulling *et al.,* 2002; Lee and Hseu, 2002). Kindermann *et al.,* (1998) attempted a first phylogenetic analysis of the whole genus of *Trichoderma* using sequence analysis of the ITS-1 region of rDNA. The sequences of 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 (Sacchi *et al.,* 2002; Hillis *et al.,* 1991; Woese, 1987).

Materials and Methods

Isolation of *Trichoderma* from soil sample

The rhizosphere soil sample was collected from various Tobacco growing fields of West Godavari District of Andhra Pradesh, India. The strains were isolated and identified in Potato Dextrose Agar (PDA) with low sugar medium by serial dilution method (Johnson and Crul, 1972; Nirenberg, 1976).

Conidial morphology– spore size

The variation in spore sizes of the five isolates of *Trichoderma* were identified through Phase contrast microscopy. Carl Ziess, Axioskop 2 Plus, microscope was used and the spore shape was determined using Axioscope 4.0 software.

Genomic DNA extraction from isolates of Trichoderma

Isolation of fungal genomic DNA was done following standard technique (Raeder, 1985). Fungal mycelia from 3-4 days old culture was crushed with liquid nitrogen and incubated with lysis buffer containing 250 m MTris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 650C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min,and further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and chloroform (in the ratioof 1:4 v/v) was added followed by 0.5M Na-acetate (inthe ratio of 1:10 v/v). Next Isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12000 rpm for 15 min followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in TE buffer pH 8.

Molecular characterization

The strains were then identified at the molecular level using PCR amplification of the specific gene sequence with universal primer set ITS-1 (forward) and ITS-4 (reverse) sequenced and identified using NCBI BLAST. Ten microlitres of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0% agarose with Ethidium Bromide (EtBr) at 8 V/cm and the reaction product was visualized under a UV

transilluminator. The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3′ end of the 28S and the 5' end of the 28S gene were amplified using the two primers, ITS-1 and ITS-4 which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White *et al.,* 1990). The PCR-amplification reactions were performed in a 50 µl mixture containing 50 mMKCl, 20 mM, TrisHCl (pH 8.4), 2.0 mM MgCl2, 200 µM of each of the four deoxynucleotide triphosphates (dNTPs), 0.2 µmM of each primer, 40 ng/µl of template and 2.5 U of Taq polymerase (Fermentas). The cycle parameters included an initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, primer extension for 3 min 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 µg/ml) and electrophoresis was carried out at 60 V for 3 h in TAE buffer. One Kb ladder (MBI, Fementas) was used as a marker. The gel was observed in a transilluminator over ultraviolet light. The desired bands were cut from the gel with a minimum quantity of gel portion using the QIAGEN gel extraction kit. The PCR product was purified by QIAGEN gel extraction kit using the protocol described in the manufacturer's manual. A pair of universal ITS primers ITS-1 (forward) and ITS-4 (reverse) were used for sequencing of the amplified product and this step was carried out at the Merck Laboratory (Bangalore, India). The details of the primers are mentioned in Table 1.

Sequence analysis and phylogeny

A comparison of the 28S rRNA gene sequence of the test strain against nucleotide collection (nr/nt) database was done using BLAST program (Zhang *et al.,* 2000). Sequences that shared about 90% similarity with the test sequence were selected for a multiple sequence alignment that was carried out using ClustalW (Thompson *et al.,* 1994). Subsequently, an evolutionary distance matrix was generated from these nucleotide sequences in the dataset. A phylogenetic tree was then constructed using the Neighbour-joining method (Saitou and Nei, 1987). Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4.0 (Tamura *et al.,* 2007).

The 16S rRNA gene sequence of test strain was 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 (Altschul *et al.,* 1997). 28S rRNA gene sequence of the test strain was also compared against those sequences, in Ribosomal Database Project (Cole *et al.,* 2009) by using the RDP Classifier check program (Wang *et al.,* 2007). The annotated information for the sequence in the database to which 28S rRNA aligns is used for fungal identification. (Soumya P *et al.,* 2016)

Results and Discussions

The 5 isolates of Trichoderma were isolated from the rhizosphere of tobacco growing soils of West Godavari District of Andhra Pradesh, India. The strains were isolated and identified in Potato Dextrose Agar (PDA) with low sugar medium by serial dilution method. The morphological differences of the Trichoderma isolates were observed and are tabulated in Table 2. Among the strains strain ITC-04 was distinct from the other four and produced flourocent yellowish green pigmentation, remaining other four strains were found to be green to greenish yellow in colour (Figure 2). Strain ITC-01, ITC-02 and ITC-05 were very

fast growers and produced luxuriant mycelia, compared to ITC-03 and ITC-04 were slow growers. When spore shape was compared among the strains, ITC-01 and ITC-02 produced round spores while the ITC-03 and ITC-04 produced Oblong spores while the spores of ITC-05 were round to oblong (Figure 3).

Fig.1 Phylogenetic tree of the *Trichoderma* strains

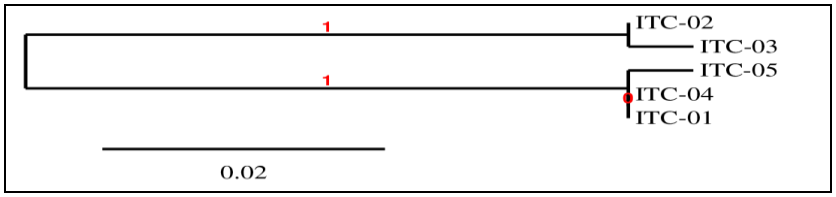

Fig.2 *Trichoderma* strains on PDA plates

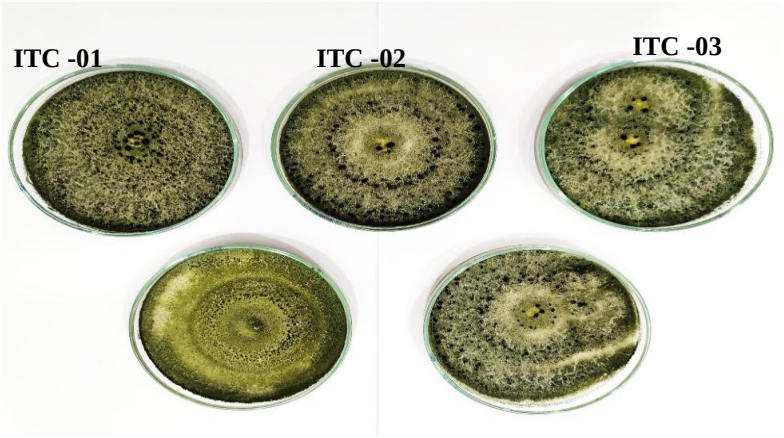

Fig.3 *Trichoderma* mycelia and spores under microscope

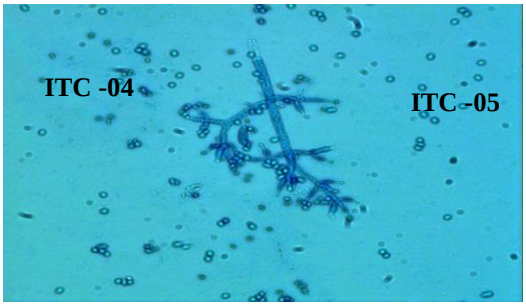

Table.1 Gene sequence with universal primers

Primer Name	Sequence (5'-3')	Length	Tm (˚C)	GC content (%)
ITS-1	TCTGTAGGTGAACCTGCGG	19	53	58
ITS-4	TCCTCCGCTTATTGATATGC	20	50	45

Table.2 Morphological characters of *Trichoderma* strains

Morphological characters			
Isolate	**Colony characteristics**		
	Colour	**Mycelial growth**	**Spore shape**
***Trichoderma asperellum* (ITC-1)**	Green	+++	Round
***Trichoderma harzianum* (ITC-2)**	Green	+++	Round
***Trichoderma harzianum* (ITC-3)**	Greenish Yellow pigmented	++	Oblong
***Trichoderma asperellum* (ITC-4)**	Florescent Green pigmented	++	Oblong
***Trichoderma asperellum* (ITC-5)**	Green	+++	Oblong to Round

Table.3 Gene Sequence of the *Trichoderma* strains

Isolate	Gene Sequence
***Trichoderma asperellum*(ITC-1)**	AAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAG TTTACAACTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTCACG CCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACTC TTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGA ATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACA TTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAA CCCCTCCGGGGGGATCGGCGTTGGGGATCGGGACCCCTCACACGGGTGCCGGCCCCGA AATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACC GGGAGCGCGGCGCGTCCACGTCCGTAAAACACCCAACTTTCTGAAATGTTG
***Trichoderma harzianum*(ITC-2)**	TTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCGCC GGAGGACCAACCAAAACTCTTATTGTATACCCCCTCGCGGGTTTTTTTATAATCTGAG CCTTCTCGGCGCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGAT CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC GGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTG GGGATCGGCCCTGCCTTGGCGGTGGCCGTCTCCGAAATACAGYGGCGGTCTCGCCGC AGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGASCGCGGCGCGTCCACAGCC GTTAAACACCCMACTTCTG
***Trichodermaharzianum*(ITC-3)**	CTGTCGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCC GCCGGAGGACCAACCAAAACTCTTATTGTATACCCCCTCGCGGGTTTTTTTATAATCT GAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCGATAATGAATCAAAACTTTCAACAACG GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCT GGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGTCGGCG TTGGGGATCGGCCCTGCCTTGGCGGTGGCCGTCTCCGAAATACAGYGGCGGTCTCGCC GCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGASCGCGGCGCGTCCACAG CCGTTAAACACCCMACTC
***Trichoderma asperellum*(ITC-4)**	TGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCG CCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCT CTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCA TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCG AGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGATCGGGACCCCT CACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCA GTAGTTYGCACAACTCGCACCGGGAGCSCGGCGCGTCCACGTCCGTAAAACACCCA
***Trichoderma asperellum*(ITC-5)**	CGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCMAAAGCCCCGGA ACCAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACGT ATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCT CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG GCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGG KATCGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAG CCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCG TAAAACACCCAACTT

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbette, 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al.*, 1991; Yao *et al.*, 1992) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the5.8S, and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [Bryan *et al.*, 1995]. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies.

In the present study, we focused on the ITS regions of ribosomal genes forthe construction of primers that can be used to identify *Trichoderma* spp. ITS region of rDNA was amplified using genus-specific ITS-1 and ITS4 primers (Table 3). The *Trichoderma* isolates were identified based on the amplification of the specific gene sequence with universal primer set ITS-1 (forward) and ITS-4 (reverse) sequenced and identified using NCBI BLAST. As per the BLAST search of the gene sequence given below, the isolates were identified as *Trichoderma asperellum* (ITC-1), *Trichoderma harzianum* (ITC-2), *Trichoderma harzianum* (ITC-3), *Trichoderma asperellum* (ITC-4) and *Trichoderma asperellum* (ITC-5). From the phylogenic tree, it was observed that *Trichoderma asperellum* strains (ITC-1, ITC-4 and ITC-5) were clustered together in the same group while *Trichoderma harzianum* (ITC -2 and ITC-3) were clustered separately in the separate group (Figure 1).

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