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Morphological and Molecular Perspective of *Trichoderma reesei* (*Tr* (CSAU/7284) Isolated from Rhizospheric Soil

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Most isolates of the genus *Trichoderma* were found to act as mycoparasites of the many economically important aerial and soil-borne plant pathogens. *Trichoderma* has gained importance as an alternate for chemical pesticides and hence an attempt was intended to corroborate the positive connexion of molecular and morphological characters. A fungal strain of *Tr*(CSAU)/7284 (*Trichoderma reesei)* was isolated from a soil sample collected from CSA Farm, Kanpur, state, India. The universal primers were used for amplification of 25SrRNA sequence fragment and therefore the strain was characterized by using 25SrRNA gene sequence with the association of ITS marker. It's planned that the identified strain *Trichoderma reesei* be assigned because the kind strain of a species of the genus *Trichoderma* supported by phylogenetic tree analysis along with the 25SrRNA gene sequence search in Ribosomal Database Project, small subunit 232rRNA and large subunit rRNA databases. The sequence was deposited in GenBank with the accession number KM999966. Thus an integrated approach of morphological and molecular markers will be used to spot a superior strain of *Trichoderma* for its industrial exploitation.

Keywords: Biocontrol Agent, Internal Transcribed Spacer, *Trichoderma*, 25S ribosomal RNA gene.

Plant disease epidemics have created an ecologically imbalance system in trendy agriculture. Deterrence of such epidemics for the most part achieved through the employment of chemical fungicides has larger repercussion on environment and human health. Also, progressive confrontation in a midst of pathogen resistance to accessible chemical plant protectants has engrossed the necessity other ways of disease management. Fungi of the genus *Trichoderma* are necessary biocontrol agent against many soil borne phytopathogens. *Trichoderma*, commonly available in soil and root ecosystem, has gained immense importance since previous few decades as a result of its biocontrol ability against several plant pathogens. Some strains of *Trichoderma* like *T. harzianum*, *T. atroviride*, *T. viride*, *T. virens* and *T. koningii* are economical bio management agents that have the ability to inhibit infective agent growth within the soil, hence rising the health of the plant. Antagonistic microorganisms like *Trichoderma* reduce growth, survival of infective agent by totally different mechanism like competition association, mycoparasitism, hyphal interactions and enzyme secretion. Such small organisms are currently available commercially and are used in crop management and practices[1]. The employment of *Trichoderma* species as biological management agents has been investigated for over

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eighty years but it is only relatively recently that strains have become available commercially. Biocontrol agents are widely regarded by public as "natural" and non-threatening product, although risk assessments must clearly be carried out on their effects on non-target organisms. Moreover, information regarding the behavior of such antagonists is important for their effective use. Accurate and definitive fungal identification is important for proper sickness designation, treatment of related fungal infections. Characterization of fungal species using classical ways isn't as specific as the genotyping methods. rRNA is essential for the survival of all cells, and therefore the genes encoding the rRNA are extremely conserved within the fungal and alternative kingdoms. The sequences of the rRNA and proteins comprising the ribosome are highly preserved throughout evolution, as a result of they need advance inter- and intra molecular interactions to maintain the protein-synthesizing machinery reportable by[2,3,4]. *Trichoderma* species are common soil inhabitants and are effective in providing bio-control of soil borne pathogens due to antagonistic behaviors. The most important aspect of undefeated biological management ways includes the production, formulation and delivery system of bio-agents. The internal transcribed spacer (ITS) region of the rDNA is maybe the foremost widely sequenced Deoxyribonucleic acid region in fungi. It's usually been most halpful for molecular systematic study at species level, and even within species found by Kulling [5,6]. Ospina-Giraldo tried a primary phylogenetic analysis of the total genus of *Trichoderma* using sequence analysis of the ITS region of rDNA[7].

MATERIALS AND METHODS

Isolation and identification of *Trichoderma*

Soil samples were collected from rhizospheric soil of lentil crop of CSA Farm of Kanpur district, Indian. Isolate of *Trichoderma* species was isolated on potato dextrose agar (PDA) with low sugar medium at 25±2ºC at 7pH with serial dilution technique descrived by Jonson & curl[8]. Monoconidial (genetically pure single spore culture) isolate of *Tr*(CSAU)/7284 was used (Table 1), which was identified by slide mounting and microscopic observation based on phenotypic characters like colony colour and growth, size and shape of conidiophore, phialides and conidia, using the available literature[9,10,11] that was later confirmed by Indian Type Culture Collection (ITCC), Division of Plant Pathology IARI, New Delhi-12. The identification of *Trichoderma* isolates were confirmed both by morphological characters with Accession Number allotted by ITCC/7284 and reconfirmed by molecular characters (ITS) NCBI, GenBank Accession Number KM999966 (Table 1).

Molecular characterization

Genomic DNA isolation

Pure culture of the target fungal was full-grown nightlong in liquid PD Broth medium for the isolation of genomic DNA using a methodology delineated by Hiney[12]. The whole genomic DNA was extracted from isolate of *Trichoderma reesei* based on Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction methodology with minor modification[13]. Subsequently 10 microlitre of the reaction mixture was analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide at 8 V/cm and the reaction product was envisioned below Gel Doc/UV trans-illuminator.

PCR Amplification

PCR amplification of 5.8S-ITS region ITS1 and ITS2 regions together with the 5.8S gene in rRNA from species was amplified using the primer pair. The 25SrDNA amplified PCR product (100 ng concentration) was used for the sequencing with the single 25SrDNA 20F Forward, ITS1 primer: 5'-TCCGTAGGTGAACCTGCGG-3'and 22R Reverse ITS4 primer: 5'-TCCTCCGCTTATTGATATGC-3'synthesized by DNA Sequencer by (Merck laboratory, Bangalore). PCR amplification was carried out in a TPersonal thermocycler (Bio-rad) and 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 reportable by Zhang[14]. The PCR-amplification reactions were performed in a 50 ml mixture containing 50 mMKCl, 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, 40 ng/ml of template and 2.5U of Taq polymerase. Where cycle parameters included an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min,

primer annealing at 55°C for 2 min and primer extension at 72°C for 3 min and a final extension for 10 min at 72°C. Amplified product were separated on 1.2% agarose gel in TAE buffer, pre-stained with ethidium bromide (1mg/ml) and electrophoresis was carried out at 60 volts for 3 hours in TAE buffer. One Kb ladder (MBI, Fermentas) was used as a marker. The gel was observed in a trans-illuminator over ultra violet light. The desired bands were cut from the gel with minimum quantity of gel portion using QIAGEN gel extraction kit[15].

Sequence analysis

A comparison of the 25SrRNA gene sequence of the test strain was done using BLAST against non-redundant nucleotide (nr/nt) information determine by Thompson[16]. A number of *Trichoderma* sequences were selected on the basis of a similarity score of 90% with database sequences. Multiple sequence alignment of these selected homologous sequences and 25SrRNA gene sequence of test strain was performed using Clustal W reported by Saitou[17]. Subsequently, an evolutionary distance matrix was then generated from these nucleotide sequences in the dataset. A phylogenetic tree was then drawn using the Neighbour Joining method reported by Altschul[18]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics analysis) version 4.0 reported by Tamura[19]. We again compared the 25SrRNA gene sequence of test strain with different set of sequence database such as small subunit ribosomal RNA (SSU rRNA) and large subunitribosomal RNA (LSU rRNA) using Ribosomal RNA BLAST reported by Cole[20]. 25SrRNA gene sequence of test strain is also compared against those sequences, in Ribosomal Database Project found by Wang with using the RDP Classifier checks Program[21]. The sequencing data were compared against the GenBank database (http:// www.ncbi.nlm.nih.gov/BLAST/), where a nucleotide blast program was chosen to identify the homology between the PCR fragments and the sequences in the GenBank information. Additionally, the 5.8S-ITS sequences were compared to a TrichOKEY 2 program.

RESULTS AND DISCUSSION

Morphological characterization

The growth pattern of *Trichoderma* isolate after four days of incubation at 25±2ºC on PDA media showed significant similarity in nature of culture growth and sporulation patterns. The colony colour changes from watery white to Pale green yellow with the production of conidia. For microscopic characterization slides were prepared and stained with lactophenol cotton blue and observed under light microscope. The culture was found to be *Trichoderma reesei* by the following characteristics (Fig.1, Table 2).

Molecular characterization

A total of 974 bp of the 25SrRNA gene was sequenced and used for the identification of isolated fungal strain. Subsequently, 25SrRNA gene sequence based phylogenetic tree showing their relationships between the test strain *Tr*(CSAU)/7284 and selected representatives of the genus *Trichoderma* is given in Fig. 2. It is evident from phylogenetic analysis of 25SrRNA gene that the isolate *Tr*(CSAU)/7284 represents a genomic species in the genus *Trichoderma*. Comparison of test strain against known sequences of SSUrRNA and LSUrRNA databases showed that the gene sequence of isolate *Tr*(CSAU)/7284 has 90% sequence similarity (Score=546 bits, Expect=0.0) with 25SrRNA gene sequence of *Trichoderma* (Genbank Acc. No.: KM999966). Thus, data shows that the isolate *Tr*(CSAU)/7284 is a member of the genus *Trichoderma*. Similarity rank program classifier available at the Ribosomal Database Project classified the isolate *Tr*(CSAU)/7284 as a novel genomic species of the genus

Table 1. Identification of potential strain of *Trichoderma reesei*

S. No.	Name of Bioagent	ITCC Acc. No No.	GenBank Accession	Strain code	Source	GPS Location
T1	*Trichoderma reesei*	ITCC/7284	KM999966	*Tr* (CSAU)	CSA farm Kanpur Nagar	Latitude: 25° 82 34.8212" Longitude: 81° 592 2.9792"

Trichoderma with a confidence threshold of more than 90% (Fig. 2). The 25SrRNA gene sequence of isolate *Tr(*CSAU*)*/7284 was deposited in GenBank and allotted the accession number KM999966.

TrichOKEY

The nucleotide sequence (submitted and retrieved from NCBI) of *Tr*(CSAU) /7284 (*Trichoderma reesei*) is analyzed through *Trich*OKEY 2 program for their validation post molecular identification. This has confirmed the selected sequence as specific strain of *Trichoderma* reesei (Table 3). Oligonucleotide sequences which are present in known *Hypocrea/ Trichoderma* ITS1 - 5.8S RNA - ITS2 sequences, are used to identify the species at generic level[22].

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[23]. In this study, the method of isolation and identification of an unknown fungal isolate, isolated from CSA Farm of Kanpur district using 25SrRNA gene sequence as reported in bacterial rRNA gene to characterize the strain as a member of the *Trichoderma* sp. and the results obtained by two methods agree with each other[24]. The universal primers were used for amplification of the 25S rRNA gene fragment and strain characterized by using 25SrRNA gene sequence with the help of ITS marker. It is proposed that the identified strain *Tr*(CSAU)//7284 be assigned as a species of the genus *Trichoderma* based on phylogenetic tree analysis together with the 25S rRNA gene sequence search in Ribosomal Database Project, small subunit rRNA and large subunit rRNA databases. The sequence was deposited in GenBank with the accession number KM999966. Thus, an integrated approach of morphological and molecular markers can be employed to identify a strain of *Trichoderma* for its commercial exploitation. Previously similar results were also reported by Shahid[25],[26] and they also concluded that most of the *Trichoderma* species are morphologically very similar and were considered as a single species for many years. DNA methods brought additional valuable criteria to the taxonomy of *Trichoderma* which are being used today for studies that include identification and phylogenetic classification.

Table 2. Morphological and cultural characteristics of *Trichoderma reesei*

Morphological characteristic		Cultural characteristic	
Features	Species characters	Features	Speceis characters
Colony growth rate (cm)	5-6cm		
Colony colour	Pale green yellow	Conidiophore branching	Highly branched
Reverse colony colour	Light lemon yellow	Phialide shape	Legeniform
Colony edge	Smooth reguler surface	Phialide size	6.8-9.2x 1.9-2.1mm
Culture smell	No characteristic smell	Conidial shape	Enlarged in the middle or sub-globose
Mycelial form	Tufted, Crysty	Philospore size	2.6-3.0 x 2.8-3.4mm
Mycelial colour	Light yellow	Conidial wall	Smooth
		Conidial colour	Slight green
		Chlamydospore	No
		Spore Germination time	12 Hours

Table 3. ISTH *Trich*OKEY and *Tricho*MARK results for *Trichoderma* reesei

Input (Nucleotide Sequence)	*Trich*OKEY Results		*Tricho*MARK Results
Trichoderma reesei	Second anchor (GSH) was found in position 336	One genus specific hallmark (Anchor 2) is found.	Found 2 ITS anchors (ITS 1 and ITS4)

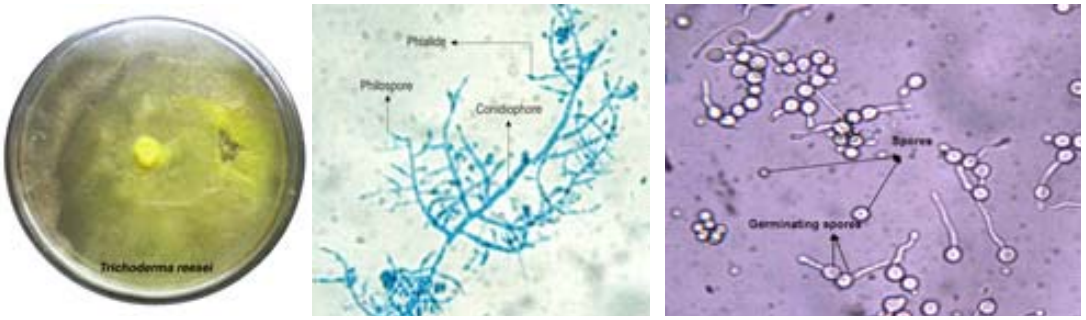

Fig. 1. *T. reesei* (a) Growth on PDA medium (b) microscppic observation (c) Spore Germination

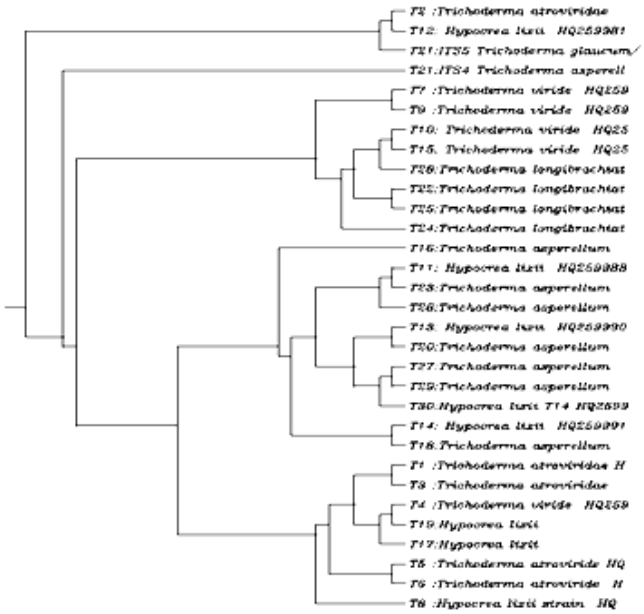

Fig. 2. Phylogenetic and molecular variability analysis of *Trichoderma* species

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