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Molecular Identification of Cellulose Degrading *Trichoderma viride*

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The objective of the present study was to identify the cellulose degrading *Trichoderma viride*. Dilution plating was used to quantify the propagule numbers of *Trichoderma*, denaturing gradient gel electrophoresis (DGGE) and DNA sequence analysis were used to identify *Trichoderma* species. *Trichoderma viride* was identified based on the microscopic observations by Lactophenol cotton blue and scanning electron microscopy. It was ultimately confirmed based on DGGE and DNA sequence analysis and finally concluded by BLAST analysis by constructing a phylogenetic tree.

Key words: *Trichoderma viride*; Dilution plating; 18s RNA; BLAST.

The transition from conventional to organic management practices could influence soil microbial diversity, microbial composition and subsequently change nutrient cycling and soil quality[1]. Conventional farming systems characterized with extensive ploughing and application of synthetic fertilizers, fungicides, insecticides, and herbicides, which have been associated with loss of soil fertility, soil erosion, ground water pollution[2], as well as the inhibition of the activity of soil microorganisms including soil fungi[3]. Although many studies of microbial communities in soils from organic and conventional farming systems have been conducted[4-5], there is little information on the responses of *Trichoderma* community to alterations of soil management practices, and on the relationship between the propagule numbers of *Trichoderma* or *Trichoderma* species and disease incidence.

Trichoderma is a genus which includes species of free-living soil fungi, opportunistic, avirulent plant symbionts[6], asymptomatic endophytes[7], and parasites of other fungi[8]. It is

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often the major component of the mycoflora in soils of various ecosystems, such as agricultural farm soil, grassland, forest, marshes, deserts, and water[9-11]. *Trichoderma* species possess high reproductive capacity, ability to survive under very unfavourable conditions, efficiency in the utilization of nutrients, and capability to modify the rhizosphere[12]. Moreover, *Trichoderma* species have long been recognized as agents for the control of plant pathogenic fungi, and have the ability of promoting plant growth and development[13]. Due to the ecological importance of *Trichoderma* species and their application as the biocontrol agents in agricultural fields, it is crucial to understand the changes in community structure of *Trichoderma* subjected to the transitions from conventional to organic agricultural management systems, and the relationship between the *Trichoderma* communities.

Identification of *Trichoderma* species is extremely difficult, due mainly to the few morphological characters available for differentiation of closely related species[13-15]. The advancement of molecular biology especially the sequence analysis of 18S rRNA and internal transcribed spacer (ITS) sequences has accelerated *Trichoderma* species classification and identification[16-17]. One of the molecular techniques widely used for community analysis is denaturing gradient gel electrophoresis (DGGE)[18]. DGGE has been used to separate PCR product of the amplified 18S rDNA or ITS regions. The sequence analysis of purified PCR product amplified from excised DGGE bands is used to determine the phylogenetic placement of each individual band in the community. DGGE and DNA sequence analysis may give a more rapid and realistic view of soil *Trichoderma* diversity and their distribution in the agro ecosystem. Such approaches may lead to a better understanding of soil *Trichoderma* community responsible for suppression of soilborne plant pathogens, and may eventually lead to improved and more reliable disease control.

The objectives of this research were to quantify the abundance of *Trichoderma viride* using dilution plating, and identify *T. viride* species using microbial staining, DGGE as well as DNA sequence analysis.

MATERIAL AND METHODS

Soil sampling

The soil sample was collected from a depth of 15 inches depth in to the soil where there was high moisture content in the Oil palm plantations of Directorate of Oil Palm Research (ICAR), Pedavegi. Soil samples were collected in the location and were stored in coolers on ice until returning to lab. For soil dilution plating, the soils were transferred to a storage room and stored at 4°C until the time of analysis.

Screening for *Trichoderma viride*

Trichoderma viride was quantified using dilution plating[19]. Soil dilutions were done in diluted water agar (0.225 g agar plus 90 ml water) by subjecting the soil for enrichment with cellulose. Soil samples were analyzed for *Trichoderma* species using 10-fold serial dilutions of soil on selective media. *Trichoderma* medium (contained V-8 juice, 200 ml; water, 800 ml; agar, 20 g; and glucose, 1 g; neomycin sulfate, bacitracin, penicillin G, and chloroneb, 100 µg/ml; chlortetracycline hydrochloride, 25 µg/ml; nystatin, 20mu g/ml; and sodium propionate, 500mu g/ml; Alkylaryl polyether alcohol, 2.0 ml/l)[20] was used for quantification of *Trichoderma* spp. Data were expressed as number of colony-forming units (CFUs)/g of dry soil. Three replicates were used for each soil sample for population estimation and incubated[19].

Microscopic identification of *Trichoderma viride*

The isolated fungi were identified using Lactophenol cotton blue staining technique, scanning electron microscopy.

Lactophenol cotton blue Staining

The diameter of the mycelium, size of the conidia and conidiogenous cell foe each isolate was measured with the help of ocular micrometer. The fresh culture of different age starting from 5 to 25 days was used for the preparation of semi-permanent slides. By keeping a small bit of mycelium on the slide and mixing with a drop of Lacto phenol-cotton blue and slightly heated on the steam for fixing the stain then covered with cover slip and observed under binocular compound microscope. The observations regarding diameter of the mycelium, shape, length and bredth of conidia and conidiogenous cell were

recorded under microscope. The size of 100 counts of mycelial diameter, conidia and conidiogenous cell was measured in each isolate separately and their averages were worked out.

Scanning electron microscopy of *Trichoderma viride*

Scanning Electron microscopic studies were carried out by taking 24 h old cultures of test fungi and fixed in 6% buffered glutaraldehyde followed by post fixation in osmium tetroxide and then dehydrated in increasing concentration of ethyl alcohol. The samples were mounted on copper stubs with double sided adhesive tape, coated with gold polaron, AU/PD sputter-coater and scanned in SEM (Jeol JSM 5600, Japan) and photographed. The SEM studies were conducted at Ruska laboratories, Sri Venkateswara Veterinary University, Rajendranagar, Hyderabad.

Molecular identification

Primer design

Total 150 ITS sequences representing 92 *Trichoderma* species were retrieved from GenBank (National Center for Biotechnology information; http://www.ncbi.nlm.nih.gov) aligned using Claustal X [21], and the *Trichoderma* genus-specific primers (forward: 5[1]TACCAAACTGTTGCCTCGGCGG3[1] and reverse: 5[1]GATGAAGAAGGCAGCGAAATGC GATA3[1]) were designed based on the homologous regions specific to *Trichoderma* genus. The design of the primers was based on the comparison with the ITS sequence of *Saccharomyces cerevisiae* (AY130313).

DNA isolation and amplification of 18s RNA gene of *Trichoderma viride* by Polymerase Chain Reaction (PCR)

The template genomic DNA from *Trichoderma viride* was isolated following the protocol described[22]. In Polymerase Chain Reaction, the specific primers *Forward* and *Reverse* (Helini Biomolecules, Chennai) were used to amplify the genomic sequence of the open reading frame (ORF) of the gene. PCR conditions were 94°C for 2 min, and then 94°C for 1 min, 60°C for 1 min, 72°C for 3 min for a total of 30 cycles, with the extension at 72°C for 10 min.

Agarose gel electrophoresis

Required amount of agarose (w/v) was weighed and melted in 1X TBE buffer (0.9M Tris-borate, 0.002 M EDTA, pH 8.2). Then, 1-2 µl ethidium bromide was added from the stock (10 mg/ml H2)). After cooling, the mixture was poured into a casting tray with an appropriate comb. The comb was removed after solidification and the gel was placed in an electrophoresis chamber containing 1X TBE buffer. The products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 ratios and loaded into the well. Electrophoresis was carried out at 60V[23].

Eluting DNA from agarose gel fragments

Ethidium bromide stained agarose gel was visualized under a transilluminator. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column. The tube was centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA. The eluent was checked by running on an agarose gel and observed on a transilluminator for the presence of ethidium bromide stained DNA. The eluted DNA was used directly in manipulation reactions. This DNA fraction was subjected for sequencing (Helini Biomolecules, Chennai).

Sequencing and chimera checking

The eluted PCR product was directly sequenced using *Trichoderma* genus-specific primers without GC-clamp at Ohmlina Centre for Molecular Research, Chennai. Sequencing reactions were carried out with ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., USA). All sequences exhibiting less than 95% sequence similarity to existing sequences in GenBank were checked using CHIMERA-CHECK program at the Ribosomal Database Project (RDP) using default settings[24]. All representative sequences corresponding to bands were *Trichoderma* species.

Phylogenetic placement

The environmental sequences were compared to the sequences in GenBank using the BLAST algorithm[25] and RDP database[24] to search for close evolutionary relatives.

GenBank accession numbers

The representative sequence of the soil *Trichoderma* species was deposited in GenBank of National Centre for Biotechnology Information (NCBI). The GenBank accession number is GU131272.

RESULTS

Collection of soil sample

Different soil samples were collected from Oil palm plantations of Directorate of Oil Palm Research (ICAR), Pedavegi. The soil collected was sterilized with UV- rays and X- rays to avoid external contamination.

Isolation of the cellulose degrading Fungi

All the soil samples collected were subjected to enrichment, 100 grams of the soil sample was enriched by adding one percent of Cellulose in a sterilized 250 ml beaker. The enriched soil sample was incubated for 4 to 5 at 37°C [26] and kept at a temperature regulated shaking incubator for 4 to 5 weeks at 200 rpm/ min at 37°C and at pH 7.0.

Microscopic Identification

Morphological characteristics of screened species basing on Colony Colour and Substrate Pigmentation

Trichoderma species are filamentous fungi. Colonies of *Trichoderma* grow rapidly and mature in 5 days. At 25°C and on Potato Dextrose Agar (PDA) the colonies are wooly and become compact in time. From the front, the colour is white. As the conidia are formed, scattered blue-green or yellow green patches become visible. The T-celled spores (conidia) are produced successively from the tips of the phailidae and collect in small masses. *Trichoderma viride,* an aggressive toxin-producing micoparasite grow rapidly in field under favorable moisture, soil and temperature. The production of conidia from

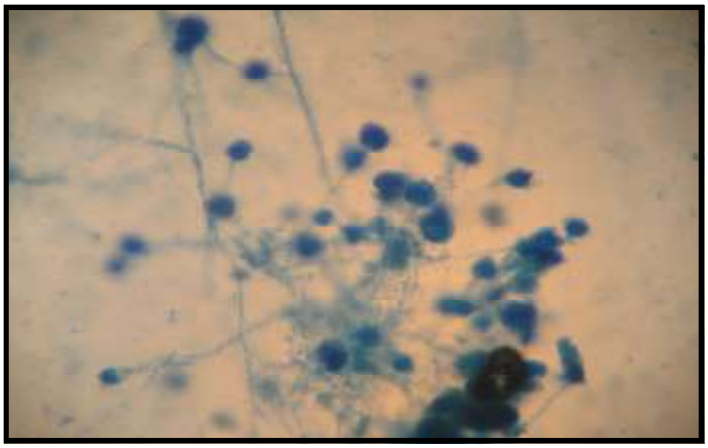

Fig. 1. Micrograph of *Trichoderma viride*

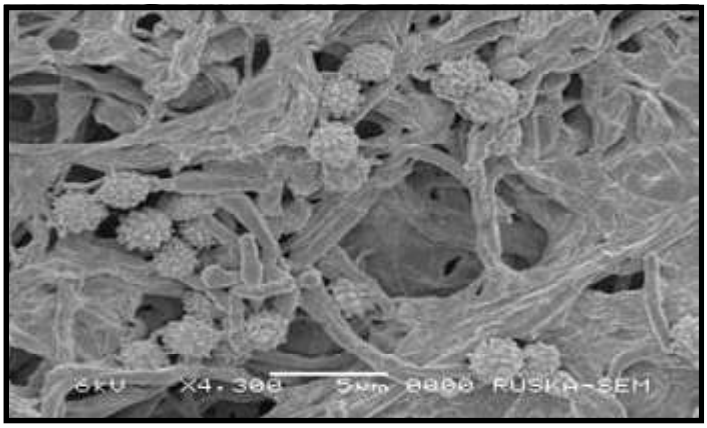

Fig. 1. Scanning electron micrograph of *Trichoderma viride*

effused conidiophores, or from conidiophores aggregated into fascicles or pustules. The observations noticed were in accordance with the findings[14;27;28]. Basing on the microscopic studies and cultural characterization, it may be concluded that the screened species may be *Trichoderma viride* (Fig.1).

Scanning Electron Microscopy of *T. viride*

Scanning electron microscopy studies revealed the mycelial characters of *Trichoderma viride* (Fig.2). The hyphae are well developed, profusely ranched, septate and hyaline. The mycelial strands have the shape of cylindrical, hyaline, septate. The conidiophores were mostly branched and cylindrical and rarely unbranched and oblong shaped and developed from the mycelium at right angles near the septa and appeared at first as tumor like projections. These gradually emerged to form hyphae like elements on the main hypha and are groups/clusters of conidiogenous cells. The conidiogenous cells/ phialides were single celled with globose to ellipsoidal base attenuating in to long sympodial rachis, which are conidia. Basing on the scanning electron microscopic studies, it may be concluded that the screened species may be *T. viride.*

Fig. 3. Agarose gel showing amplified 18s DNA.
Well 1: Amplified DNA of *T. viride;*
Well 2: DNA Ladder

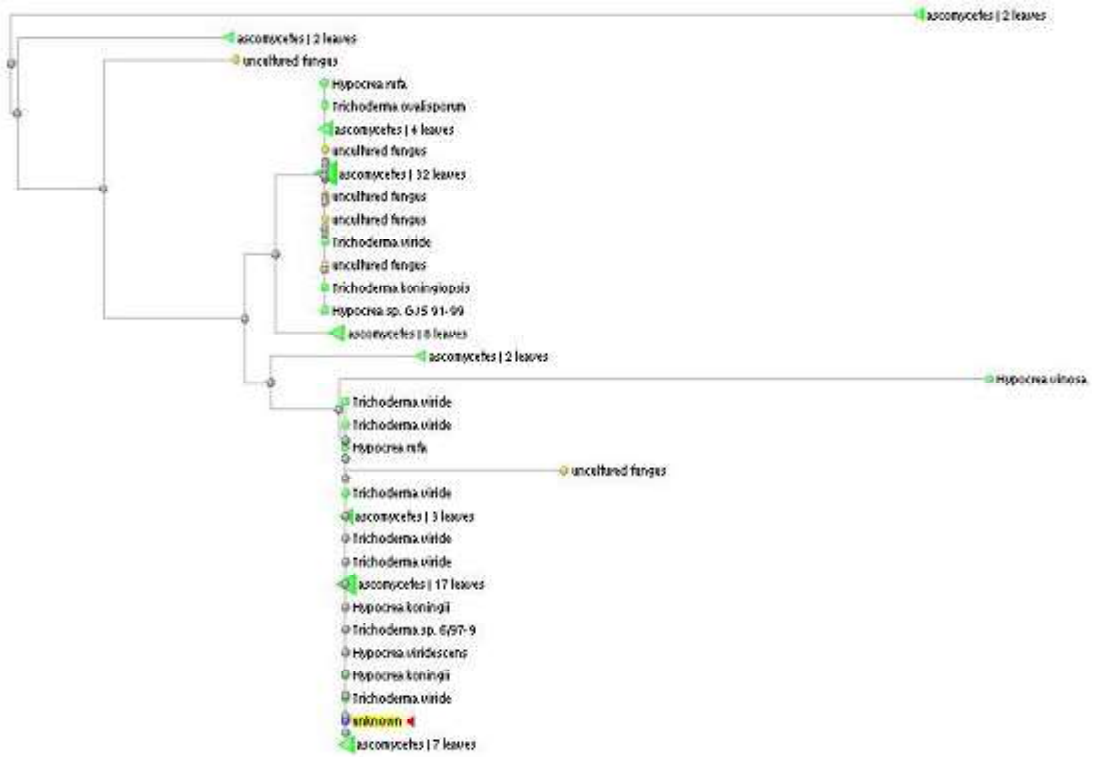

Fig. 4. Phylogenetic affiliation of *Trichoderma viride*

Molecular identification

Amplification of DNA coding for 18s r RNA

The genomic DNA of the three organisms was subjected for the isolation of the DNA coding for 18s rRNA by using Polymerase chain reaction (Fig.3). The bands were cut and eluted and the DNA so obtained was subjected for sequencing.

Sequence analysis

The sequence analysis demonstrated that all the corresponding bands on agarose gel belonged to *Trichoderma viride.* Upon sequencing of the amplified DNA, the data obtained corresponds to 600 base pairs for *T. viride.* The sequence so obtained was as follows:

TAGAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTT
ACAACTCCCAAACCCAATGTGAACCATACCAAACTGTTGCCTCGGCGGGGTCACGCC
CCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGGACCAACCAAACTCT
TTCTGTAGTCCCCTCGCGGACGTTATTTTTACAGCTCTGAGCAAAAATTCAAAATGAA
TCAAAACTTTCAACAATCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAG
TATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGT
CCGGCGTTGGGGATCGGGAACCCCTAAGACGGGATCCCCCGAAATACAGTGGCGGT
CTCGCCGCAGCCTCTCATGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGC
GTCCACGTCCGTAAAACACCCAACTTCTGAAATGTTGACCTCGGATCAGGTAGGAAT
ACCCGCTGAACTTAAGCATATCAATAAGCG

Sequence analysis

Sequences of the dominant DGGE bands revealed that *Trichoderma species* in tested soil was *T. viride* (100% similarity to the ITS sequences of *T. viride*) with the accession no. GU131272. This sequence can be found in NCBI Genome Databank. The phylogenetic tree was shown in Fig.4.

DISCUSSION

***Trichoderma* population in soils from organic, sustainable and conventional farming systems**

Indigenous *Trichoderma* species are known to have greater tolerance for broad spectrum pesticides than many other soil microorganisms and to colonize the treated soil more rapidly than other soil competitors[29]. Others showed that the application of sodium azide to soil resulted in the development of a large population of native *Trichoderma*[30]. Therefore, *Trichoderma* species may be affected to a lesser extent than other soil fungi following a soil disturbance, and are able to quickly colonized niches left by other organisms in conventional farms. In contrast, Bullock (2002)[19] found that propagule numbers of *Trichoderma* species were higher in soils amended with organic than synthetic amendments. However, the elevated *Trichoderma* intensity in this research was mainly related to colonization of the composts that was incorporated into fields by the fungus.

Comparison of species identification

We found that the ITS sequences of the saved colonies from soil dilution plating generally matched with those of the excised bands. The explanation might be that only sporulation spores can be isolated by dilution plating, whereas molecular method can detect the presence of the unsporulated species. This finding was confirmed by the research in disturbed and undisturbed soil[7], the sterile colonies of *Trichoderma* were recovered as root endophytes using molecular techniques.

Our results demonstrated that the *Trichoderma* ITS primers T230F and T397R enabled us to amplify *Trichoderma* species from soils and confirm their identity by BLAST search. The concentration of the gel and running time for electrophoresis should always be adjusted based on the GC content of the target DNA; these procedures will eventually maximize the band separation on agarose gel, and ensure that all related soil *Trichoderma* species have been included[31].

CONCLUSIONS

In general, soils are rich in microbial diversity. Therefore cellulose, which is a product of plant biomass and other waste materials, has to be degraded in the soil. *Trichoderma viride*, a biocontrol agent being used as biofungicide is also having the capability to degrade cellulose. In addition, our work suggest that the combination of soil dilution plating, DGGE and DNA sequence analysis are effective approaches to facilitate extensive examinations of the propagule numbers of *Trichoderma viride*, to reliably identify *Trichoderma viride* in soils with different management practices.

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