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Morphological and molecular diversity study among the isolates of *Trichoderma* using RAPD markers

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Abstract
Species of *Trichoderma* are most commonly exploited fungus as a biological control agent and the present study involves the diversity study on molecular basis. Morphological methods have been diminished due to the use of molecular techniques which have made advances in systematic characterization of *Trichoderma* spp. Twelve different random primers viz., OPA-01, OPA02, OPA03, OPA-04, OPA-05, OPA06, OPA07, OPA08, OPA09, OPA-10, OPA12 and OPA-13 were screened for generating polymorphism among the randomly selected isolates under the study out of which three primers namely OPA04, OPA05 and OPA10 were selected to amplify with all the seven isolates. *Trichoderma* isolates differed in their morphological behavior while on molecular basis, maximum polymorphism was observed in the randomly selected isolates. All the bands observed were polymorphic in nature indicating the genetic diversity among *Trichoderma* spp.

Keywords: Diversity, morphology, primers, amplification, molecular techniques and polymorphism

Introduction
In biological control beneficial microorganisms such as specialized fungi and bacteria are involved in attacking and controlling plant pathogens which cause diseases. *Trichoderma* is one of the small group of beneficial fungi which is applied commercially as biological control agents against fungal pathogens [1]. *Trichoderma* thrives in the leaf litter or mulch in orchard situations and it requires a minimum organic carbon level of one per cent to ensure proliferation in cropping locations.

The genus *Trichoderma* belongs to class Ascomycota and order Hypocreales is a filamentous fungus widely distributed in the soil, plant material, decaying vegetations and wood. Species in this genus are of great economic importance as source of enzymes, antibiotics, plant growth promoters, xenobiotic degraders and as most commercial biofungicides [2]. Hjelijard and Tronsmo (1998) reported that genus *Trichoderma* are potent mycoparasites as they attack and parasitize plant pathogens and therefore they are commercially applied as biocontrol agents [3]. Use of *Trichoderma* fungi in biological control has many advantages such as reducing the effect of pesticides entering the environment and the changing conditions favourable to the plants. It helps in increasing nutrient uptake from soil [4], reduce the toxic metabolites produced by other rhizospheric microorganisms or pesticides [5], stimulate plants for producing chemical defenses compound and induces resistance in the plants [6], induce mycoparasitism or directly attack to other pathogenic fungi [7] and improve root system and plant growth [8].

The physiological and phenotypic characters, isozyme and molecular marker are used to identify *Trichoderma* [9]. The strategies to identify *Trichoderma* using DNA markers are sequence analysis of internal transcribed spacer (ITS) region, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD) and chromosome and karyotyping analysis [10].

Larralde *et al.* (2008) reported *in vitro* antagonism assays which is useful in identification of biocontrol potential of *Trichoderma* strains [11]. The protein fingerprinting and RAPD technique as well as relationship between their antagonistic capability and RAPD profiles were analysed [12]. The results of molecular and biochemical analysis revealed 96.8% polymorphism for proteins and 87% for RAPD. The similarity indices ranged from 77.8% to 29.6% and 69% to 32% for proteins and RAPD.

In addition to RAPD, a simple marker technique called sequence related amplified polymorphism (SRAP) based on two primer amplification has also been used for studying molecular diversity in *Trichoderma* species by Li and Quiros (2001) who found its superiority over RAPD [13]. Venkateswarulu *et al*. (2008) reported molecular characterization and identification of isolates of *Trichoderma* species [14]. Morphological methods have been diminished due to the use of molecular techniques which have made advances in systematic characterization of *Trichoderma* spp [15].

Sathiyavathi and Parvatham (2011) characterized *Trichoderma* spp. on molecular basis by partial 18S rRNA sequencing using ITS primer set ITS 1/ ITS 4 target to ITS region of r DNA complex and found presence of novel strain named *Trichoderma* sp. MS 2010, an efficient producer of laccase and xylanase for industrial use [16]. In *Trichoderma* sp. amplified rDNA fragment of 500-600 bp was observed using ITS PCR [17].

Shahid (2012) focused on the molecular identification and analysis of the genetic variability of a specific strain of *Trichoderma* based on antagonistic and RAPD analysis in some leguminous crops (pigeonpea, chickpea and lentil) produced in Uttar Pradesh [18]. The taxonomic determination of *Trichoderma harzianum* and *T. viride* was confirmed by the molecular analysis using ITS-RFLP and PCR with specific primer [19].

In recent years, the development of *Trich* OKEY and *Tricho*BLAST facilitates the identification of *Trichoderma* and *Hypocrea* based on oligonucleotide DNA barcode. *Trich*OKEY is a program used to identify *Trichoderma* and *Hypocrea* based on several genus-specific hallmarks located within the ITS1 and ITS2 sequences [20]. By 2006, International Submission on *Trichoderma* and *Hypocrea* Taxonomy listed 104 *Hypocrea/Trichoderma* species which have been characterized at molecular level [21].

Genetic variability among the isolates of *Trichoderma* species against *Colletotrichum gloeosporioides* was studied using molecular techniques like RAPD and ITS-PCR [22]. Shahid *et al*. (2013) characterized molecularly the strains of *Trichoderma longibrachiatum* by using universal primers (ITS-1& ITS- 4) for the amplification of 28S rRNA gene fragment that produced a sharp band of about 700 bp on the gel [23]. Molecular characterization helps in determining the diversity and identification of *Trichoderma* by using RAPD [24]. Sagar *et al*. (2011) examined the genetic variations by PCR-based Random Amplified Polymorphic DNA (RAPD) Marker employing decamer primers and produced scorable bands of which all were polymorphic [25].

Material and Method

Isolation and identification of *Trichoderma* spp

The rhizospheric samples were collected randomly from different locations of districts of Himachal Pradesh (India) and were well mixed to form a single composite sample for the isolation of residential antagonistic microorganisms particularly *Trichoderma* spp. The isolations were made by serial dilution method described by Johnson in 1957. From the composite sample one gram of soil was added aseptically to 100ml of sterilized distilled water in 250ml of flask. The soil was thoroughly mixed by constant shaking on stirrer homogenizer and subsequently serial dilutions were made from this solution up to 1 × 107. Simultaneously, potato dextrose agar (PDA) medium was prepared through the addition of rose bengal (0.03g/l), chloramphenicol (0.4g/l), and streptomycin sulfate (0.03g/l) after autoclaving the medium, pH 6 and poured into sterilized Petri plates aseptically. There after 1 ml of soil diluent was spreaded uniformly on Potato Dextrose Agar (PDA). These Petri plates were kept for incubation at 25 ± 1 °C for about 120 hours in case of fungi. The emerging colonies of *Trichoderma* spp. thus obtained were picked and transferred to PDA slants. Cultures were purified by single spore isolation in case of fungi and later maintained on PDA slants. Identification of fungi was done on the basis of morphological characters as described by Gilman (1957) in his book "A manual of soil fungi" and "A revision of the genus *Trichoderma*" by Rifai (1969).

Fungal Cultures

Mycelial discs of 4 mm diameter of *Trichoderma* isolates were cut from periphery of an actively growing 7 day old culture on PDA and transferred to 250 ml conical flask containing 100 ml of sterile potato dextrose broth for its multiplication. The resultant fresh growth of mycelial mat was harvested and excess of moisture was completely removed through sterile blotting paper and used for DNA extraction.

DNA extraction from *Trichoderma* isolates

The total genomic DNA from different isolates of *Trichoderma* was extracted from vegetative mycelium using the procedure adopted by Reader and Broda (1985) [26]. Mycelial mat of 200 mg was grounded in the pre-sterilized pestle and mortar with liquid nitrogen until fine powder was obtained. About 50 mg of freeze-dried ground mycelium in an eppendorf tube was resuspended in 500 µl of extraction buffer. To this 350 µl of phenol and 150 µl of chloroform was added and thoroughly mixed. The suspension was centrifuged at 13000 rpm for 1 hr. The upper aqueous phase was transferred to a new eppendorf tube. To this, 25 µl of RNAse (10%) was added and incubated to 37°C for 30 min. The solution was extracted with chloroform isoamylalchohol (24:1). The upper phase was transferred to an eppendorf tube and 0.54 volume (270 µl) of isopropanol was added and mixed thoroughly by inverting the tubes. DNA precipitated visibly into lump was observed. The tube was spun at 12,000 rpm for 10 min to pellet the DNA and the remaining liquid was removed. The pellet was rinsed with 70 percent ethanol two times, dried under vaccum and resuspended in 100 µl TE buffer (pH 8.0). The DNA samples were stored at -20 ℃ for further studies.

Qualitative and quantitative verification of DNA

The quality and quantity of DNA was analyzed by running 2 µl of each sample mixed with 2 µl of 10X loading dye in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands in one percent agarose gel indicating the good quality of DNA. The DNA has been quantified by comparing with the marker (Genei, Bangalore).

RAPD profiles through polymerase chain reaction (PCR)

Twelve different random primers viz., OPA-01, OPA02, OPA03, OPA-04, OPA-05, OPA06, OPA07, OPA08, OPA09, OPA-10, OPA12 and OPA-13 (Operon technologies Inc.,) were screened for generating polymorphism among the randomly selected isolates under the study. The experiment was repeated thrice and results were reproducible.

PCR amplifications were carried out in 0.2 ml eppendorf tubes with 25 µl reaction mixture which consists of 2.5 µl of 1 X Taq buffer, 2 µl of 25 mM $Mgcl_2$, 4 µl of primer (25

picomolar / µl), 0.5 µl of 100 mM dNTP mix, 0.6 µl of Taq polymerase enzyme (conc. 3U µl-1) and 13.4 µl of sterile PCR water (Genei, Bangalore) and 2 µl (20-25 ng) of DNA sample. Amplification was carried out by 5 minutes of initial denaturation of 94°C followed by 45 cycles of denaturation of 94°C for 1 minute; annealing at 37°C for 1 minute; extension at 72°C for 2 minutes with final elongation at 72°C for 5 minutes [27]. Amplified PCR products were subjected to 1.0 per cent agarose gel electrophoresis with 1.0 X TAE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide (1 mg mL-1) staining. The DNA banding profiles were documented in the gel documentation system (Alpha Innotech) and compared with 1 kb DNA ladder (Genei, Bangalore).

Scoring and data analysis

Each amplified band was considered as RAPD marker and recorded for all samples. Data were entered using a matrix in which all observed bands or characters were listed. The RAPD pattern of each isolate was evaluated, assigning character state '1' to all the bands that could be reproducible and detected in the gel and '0' for the absence of band. The data matrix thus generated was used to calculate Jaccard similarity coefficient for each pair wise comparison. The coefficients were calculated *In Silico* following Jaccard (1908). The similarity coefficients were subjected to Unweighted Pair-Group Method on Arithmetic Average (UPGMA) cluster analysis to group the isolate based on their overall similarities. Statistical Package for Social Science (SPSS) package was used for the cluster analysis and subsequent dendrogram preparation.

Results

Morphological characters of *Trichoderma* isolates

The isolated species of *Trichoderma* were morphologically characterized on the basis of colony color, reverse color, colony edge, mycelia, color, conidial size and growth rate. It was evident from the Table 1 that the colony color varied from snow white to white and light green, green, dark green to dirty green whereas reverse color of some isolates was orange while some represented no color and only one was yellowish. Colony edge was also observed which varied from smooth, effused to raised type. There was no variation seen in mycelial color of all the isolates, under microscope it was observed to be hyaline only. Presence of water droplets on the surface of mycelium was prevalent in almost all the isolates but more prominent in *T. hamatum*, *T. virens* and *T. viride*. The average growth diameter of the colony was 8-9 cm in 5 days with full green colored sporulation. However, the length and width varied from 5-10 × 5-7µm in all the isolates. On the basis of morphological description and their comparison with the keys given in "A revision of the genus *Trichoderma*" by Rifai (1969), the isolates were identified as *T. viride, T. hamatum, T. virens, T. polysporum, T. harzianum, T. piluliferum*, and *T. koningii*.

Table 1: Morphological descriptors used for the characterization of native isolates of *Trichoderma* spp.

Name of Strain	Colony color	Reverse color	Colony edge	Mycelial color	Growth-Diameter(cm)	Conidial size (µm) Length	Width	Species Identified
Trichoderma sp. (S1)	Green	Colorless	Smooth	White	7-8	5µm	5µm	*T. viride*
Trichoderma sp.(S2)	Dark Green	Colorless	Raised	White	7-8	5µm	5µm	*T. polysporum*
Trichoderma sp.(S3)	Light Green	Orange	Raised	White	7-8	7µm	5µm	*T. hamatum*
Trichoderma sp. (S4)	Green	Orange	Smooth	White	7-8	5µm	5µm	*T. virens*
Trichoderma sp.(S5)	Dark Green	Orange	Smooth	White	8-9	7µm	7µm	*T. polysporum*
Trichoderma sp.(SR1)	Light Green	Colorless	Smooth	White	8-9	5µm	5µm	*T. harzianum*
Trichoderma sp. (SR2)	Light Green	Orange	Smooth	White	8-9	7µm	5µm	*T. viride*
Trichoderma sp.(SR3)	Dirty Green	Yellowish	Raised	White	8-9	7µm	7µm	*T. koningii*
Trichoderma sp. (SR4)	Snow White	Colorless	Effuse	White	8-9	10µm	5µm	*T. piluliferum*
Trichoderma sp.(B1)	Dark Green	Orange	Smooth	White	7-8	5µm	5µm	*T. polysporum*

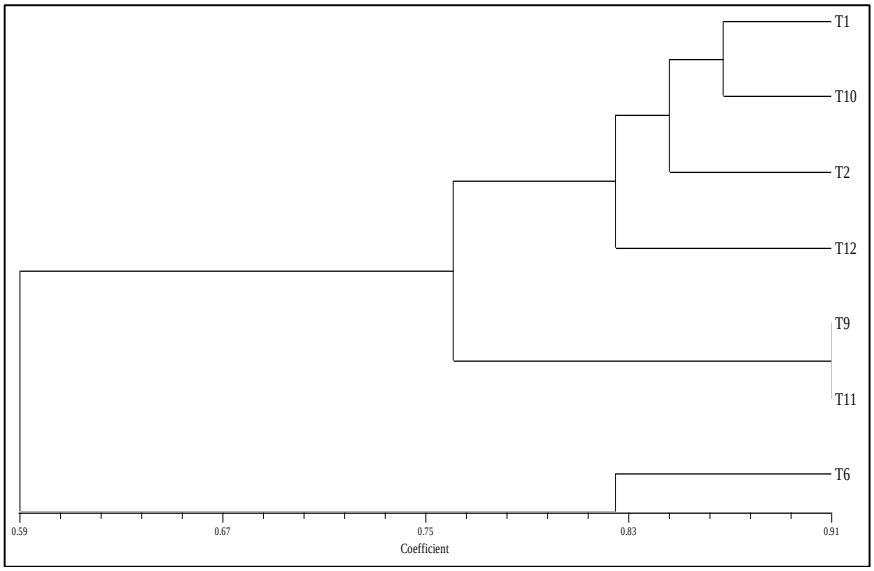

Plate 1: Dendogram generated using UPGMA analysis showing polymorphism between isolates of *Trichoderma* spp. using RAPD markers.

Molecular diversity study among *Trichoderma* spp.

To study the molecular diversity of *Trichoderma* spp., twelve different RAPD primers were screened out using 2 isolates of *Trichoderma* and out of which three primers namely OPA04, OPA05 and OPA10 were selected to amplify with all the seven isolates. OPA05 revealed high resolution bands and distinct polymorphic amplified products in comparison to OPA04 and OPA10. A dendrogram constructed (Plate 1) using the Unweighted Pair Group Method of Arithmetic Means (UPGMA) method resulted in segregation of three clusters. Cluster I was further sub divided into two sub clusters, in which *T*1 (*T. harzianum*), *T*10 (*T. polysporum*) and *T*2 (*T.viride*) were clustered in first sub cluster and *T*12 (*T. viride*) were clustered in second sub cluster. *T*9 (*T. virens*) and *T*11 (*T. harzianum*) were clustered in II cluster and *T*6 (*T. viride*) belonged to III cluster. Cluster I included isolates *T*1, *T*2 and *T*10 their color varied from light green and green to dark green while Cluster II contains isolates *T*9 and *T*11 which were green and light green in color and were isolated from Solan and Sirmour district (H.P, India) whereas, Cluster III consists of green colored isolate *T*6 collected from Solan.

Discussion

The macro and microscopic characters of *Trichoderma* spp., the major and remarkable macroscopic features in species identification were the colony features, including diameter after 7 days, color of conidia, mycelial color, colony reverse, colony texture and shape whereas microscopic characteristics were identified on the basis of conidial head; conidia shape, roughness and vesicle serration [28]. The growth patterns and sporulation patterns were varied among different *Trichoderma* isolates recorded [29]. He also noticed that conidial wall patterns and shape were rough and subglobose among *T. harzianum*, while they were smooth and globose to ovoid among *T. viride*.

In molecular diversity study it was found that three primers namely OPA04, OPA05 and OPA10 were amplified with all the eight isolates. A dendrogram constructed by using UPGAM method revealed three clusters in which I cluster is further sub divided into two more sub clusters. The first sub cluster consists of *T*1 (*T. harzianum*), *T*10 (*T. polysporum*), and *T*2 (*T. viride*) and *T*12 (*T. viride*) were clustered in second sub cluster. In the II cluster isolate *T*9 (*T. virens*) and *T*11 (*T. harzianum*) were clustered while *T*6 (*T. viride*) was clustered in III cluster. Similar findings were observed by Mathews *et al*. (2010) who reported that primers viz., OPA-3, OPA-5, PA-8, OPA-9 and OPA-10 generated reproducible polymorphism among the nine isolates of *Trichoderma* [22]. Amplified products with all the primers showed polymorphic and distinguishable banding pattern indicating the genetic diversity among all isolates of *Trichoderma*. Gupta *et al*. (2010) found that dendrogram constructed using the UPGMA method showed two major groups consisting of *T. harzianum* and *T. virens* isolates, and *T. viride* which proved that *T. harzianum* and *T. virens* are genetically closely related species, with morphological similarity [30]. Similar findings were illustrated by Chakraborty (2010) that the genetic relatedness among eleven isolates of *Trichoderma viride* and eight isolates of *Trichodema harzianum* were analyzed by using six random primers OPA-1, OPD-6, OPA-4, A-5, AA-04 and AA-11 to generate reproducible polymorphisms which had shown polymorphic and distinguishable banding patterns which indicate the genetic diversity of *Trichoderma* isolates [17].

Conclusion

The diversity study of newly isolated *Trichoderma* strains on the basis of morphological and molecular characters revealed that all the *Trichoderma* isolates differed in their morphological behavior while on molecular basis, maximum polymorphism was observed in the randomly selected isolates. All the bands observed were polymorphic in nature indicating the genetic diversity among *Trichoderma* spp. Their morphology was studied on the basis of colony color, reverse color, mycelial color, growth of mycelium and conidial size. The colony color varied from snow white to white, light green, green, dark green to dirty green and reverse color varied from yellowish to orange while the mycelial color was observed to be white in color and their conidial size varied from 5-10×5-7µm.

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