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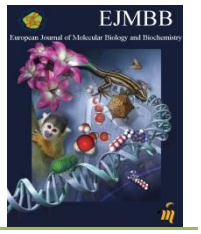

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *TRICHODERMA* ISOLATES OF TAMILNADU

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ABSTRACT

Fifteen isolates of *Trichoderma viride, Trichoderma atroviride* and *Trichoderma harzianum* obtained from rhizosphere soil of plantation crops, forest soil and agricultural field of Tamilnadu region were studied using morphological characters, molecular characterization using Random Amplified Polymorphic DNA and Inter Simple Sequence Repeats-Polymerase Chain Reaction. The morphological characters were based on the following characters included here viz., colony growth rate (after 7days in cm) at 28±1ºC, colony colour, reverse colony colour, colony edge, culture smell, conidiation, mycelia form, mycelia colour, conidiophores branching, phialide disposition, phialide shape, conidial shape, conidial wall etc. The isolates were grouped into three distinct clusters of *T.harzianum, T.viride* and *T.atroviride*. The similarity ranged from 50 to 91%. The maximum genetic variation in the *T. viride* group was observed between the isolates T4 and T7 (90%). In *T. harzianum* cluster variation ranged between T8 and T12 (88%). Followed by T3 and T5 (79%) in the *T. atroviride* group. On the other hand maximum genetic variation of 51 % was observed between the cluster I and followed by 55% between cluster II and cluster III.

INTRODUCTION

A huge part of interest in biocontrol is the diminution of plant diseases caused by soil-borne and foliar plant pathogenic fungi. Approximately 70% of all the most important crop diseases are caused by fungi or the fungus-like Oomycota [1]. Disreputable examples are species belonging to the genera *Sclerotinia, Rhizoctonia, Sclerotium, Botrytis, Phytophthora, Pythium* and *Fusarium*. The largest part of the formulations of commercially existing biocontrol products against plant pathogenic fungi contain the bacteria *Pseudomonas* and *Bacillus* or fungi belonging to the genus *Trichoderma* [2]. The advantages of use of biocontrol agents in disease management are the safety of handling, the self-perpetuation and therefore a less frequent need of application and a high degree of host specificity.

Identification based on morphological characters consent a relatively simple method for classification of *Trichoderma* as genus, but the species perceptions are complex to construe and there is considerable confusion over the application of specific names. Pioneers in *Trichoderma* like [3] and [4] observed certain cultural characters that could be used for identification and description of these species. Rifai [3] Classified the *Trichoderma* into nine species aggregates, further it was elaborated by Bissett [4,5,6] covering thirty five species, their classification reflected the importance of microscopic characters for delimiting the *Trichoderma* species. To resolve this genetic variation among the isolates random amplified polymorphic DNA (RAPD), and inter simple sequences repeats (ISSR) molecular markers were used and based on the sequence homology analysis was done.

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Members of the fungal genus *Trichoderma* were found to be useful as effective biological control agents for many diseases caused by soil borne pathogens. Weindling [8] gave the first report on *Trichoderma* as a potential biocontrol agent, since then various workers have speculated the existence of biological control ability of *Trichoderma* for over seventy years [9]. *Trichoderma* species can act as biocontrol agents through different synergistic mechanisms. However, it is difficult to predict the degree of synergism and the behavior of a biocontrol agent in a natural pathosystem.

MATERIAL AND METHODS

Isolation of *Trichoderma* isolates

The soil samples were brought to the laboratory air dried at room temperature.The *Trichoderma* isolates were isolated on *Trichoderma* selective media [10]. About 20ml of TSM medium was poured in petridishes and allowed to solidify. Serial dilution method was employed to isolate *Trichoderma* from soil samples. One gram of soil was added to 9 ml of sterile distilled water and was serially diluted up to the dilution factor of 10^{-4}. Thereafter 200µl aliquot of soil suspension was spread on TSM. The plates were incubated at 25±2ºC. Seventy five *Trichoderma* colonies appearing on the TSM medium were transferred to the petriplates containing Potato Dextrose Agar medium. Cultures of seventy five selected *Trichoderma* isolates were also maintained on PDA slants at 4ºC for further study.

Cultural and Morphological observations

The cultural characterization of the isolates was studied by the observations made from the microscopic slides. Each isolate was grown on PDA containing the antibiotics streptomycin at 25^0C. The cultural characters like colony color, growth rate, colony edge and culture smell were studied for all the 75 isolates. Finally the 15 representative isolates were taken under study for both cultural and morphological characterization. For observing the cultural characters slides were prepared from 3-7 days old cultures by cutting a thin section of the fresh hyphal tips with the help of an inoculation needle on to the slide in a drop of water or lactophenol - cotton blue solution. The morphological characters observed were formation and characters of chlamydospores; disposition, branching, size and shape of the conidiophores; disposition, size and shape of phialides; size, shape, colour and ornamentation of conidia with verification of the key provided by Rifai [3] and Bissett [4, 5, 6].

Genomic DNA extraction from *Trichoderma* isolates

Mycelium from fifteen *T.harzianum* strains was grown in liquid shake cultures (120 rpm) at 25°C in potato dextrose broth (Difco) for 96 h. DNA was extracted from 1g of freeze-dried mycelia powder. The samples were frozen in liquid nitrogen. The extraction was based on the Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) mini extraction method of with modification. The finely grounded mycelium was transferred to sterile centrifuge tube. Pre- warmed at 65^0C DNA extraction buffer [100 mM Tris HCl (pH 8.0), 1.4M NaCl, 50mM EDTA (pH 8.0) and 2% CTAB] was added to this, mixed well and incubated in a water bath at 65^0C with gentle shaking for 1 hr. after incubation an equal volume of chloroform isoamyl alcohol (241 v/v) was added gently to denature proteins and centrifuged at 10000 rpm at 25^0C for 10 min. The aqueous phase was transferred to a new sterile tube and DNA was precipitated with 0.6 volume of cold isopropanol and 0.1 volume of 3M sodium acetate (pH 5.2) at 15000 rpm for 25 min at 25^0C. The supernatant was poured off and the pellet was washed twice with cold 70% ethanol and dried at room temperature. The pellets were air dried and suspended on TE buffer (pH8.0).

Purification of Genomic DNA

The extraction of total genomic DNA from the *Trichoderma* isolates as per the above procedure was followed by RNAse treatment. Genomic DNA was re-suspended in 100ul of TE buffer and add 2µl of RNAse and incubated at 37C for 1 hr. After incubation the sample was re-extracted with phenol chloroform isoamylalcohol (25241) solution and RNA free DNA was precipitated with 0.1 M sodium acetate solution along with 100% chilled ethanol and centrifuged at 12000rpm 15min at 4^0C, washed with 70 % ethanol and air dried and dissolved in TE buffer. The quality and quantity of DNA was analyzed both spectrophotometrically and in 1% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

Random Amplified Polymorphic DNA (RAPD)

RAPD_PCR conditions for *Trichoderma* isolates in the present investigation were standardized. Six primers which present strong band resolution were chosen for the study viz., OPA 7, OPA 10, OPA 14, OPA 16, OPC 2 and OPE 5. The PCR amplification was carried out with 25ng of genomic DNA, 2.5 mM Mgcl2, 1 µl Tag polymerase, 1x PCR buffer, 1µl ISSR primer and 0.2 mM dNtps mix. The PCR cycle was as follows 5 minute at 95ºC, 30 seconds at 32ºC, and 1minutes at 72ºC for 32 rounds. The extension period was 8 minutes at 72ºC.

Inter simple sequence repeats (ISSR) analysis

The polymerase chain reaction was used for (ISSR) analysis. Three primers were tested for different annealing temperate of genomic DNA of the isolates. Out of these, three primers viz., ISSR1 (GAG AGA GAG AGA GAG AAC), ISSR2 (AGA GAG AGA GAG AGA GAT) and ISSR3 (YCT GTG TGT GTG TGT GT) consisting of anchored ISSR gave satisfactory amplification and band resolution. The PCR amplification was carried out with 25ng of genomic DNA, 2.5 MM Mgcl2, 1u Tag polymerase, 1x PCR buffer, 1µl ISSR primer and 0.2 mm dNtps mix. The PCR cycle was as follows 5 minute at

95ºC, 30 seconds at 32ºC, and 1minutes at 72ºC for 40 rounds. The extension period was 5 minutes at 72ºC.

The PCR products of both random amplified polymorphic DNA(RAPD) and ISSR reaction were resolved on 1.3%agrose gel in 1x TAE buffer stained with ethidium bromide (8 µl/100ml) and electrophoresis was carried out at 75 volts for 1.5 hrs and visualized under UV – Transilluminater. The gel was photographed using a gel documentation system.

Scoring and Data analysis

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system. All reproducible polymorphic bands were scored and analyzed by UPGMA cluster analysis protocol and computed *In silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System genome arranged in tandem repeats with each Biostastiscs, version 2.11W). The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, transcribed spacer (ISSR) regions have been used assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering program, and selecting the un-weighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc.

RESULTS

Morphological characters of *Trichoderma atroviride*

The mycelium composed of hyaline, septate, much branched and smooth walled hyphae. The mycelium became hairy from the formation of loose, scanty, aerial mycelium, which made the colonies floccose; the colonies became white to green at maturity and reverse remained deeply yellowish. Crowded irregularly branched conidiophores arose in compact or loose tufts which open from concentric zones. The main conidiophores were 2-2.5 µm length and produced smaller side branches (fig.1). All the branched stood at wide angles to the bearer and tip terminated by phialides.

Morphological characters of *Trichoderma viride*

Colonies on PDA were fast growing with 9cm in 5 days. Surface become smooth hairy, and dark green, typical coconut odour was emitted in old culture. The mycelium composed of hyaline, septate, highly branched and smooth walled hyphae. The mycelium became hairy from the formation of loose, scanty, aerial mycelium, which made the colonies arachnoid, the colonies became white to green in maturity and reverse remained deep orange. Moderately branched conidiophores arose in compact or loose tufts which open from broken ring like zones. Distinctly scattered conidia were formed on less complicated conidiophores rarely formed on the aerial or creeping hyphae. The main conidiophores were 2-2.5 µm length and produced smaller side branches (fig. 2). All the branched stood at wide angles to the bearer and tip terminated by phialides.

Morphological characters of *Trichoderma harzianum*

Growth of the colonies on PDA was rapidly was upto 9cm in 3 days at 28±1ºC. The mycelium was watery white with smooth walled hyphae, septate become hairy from the formation of loose scanty aerial mycelium which made the colonies arachnoid and somewhat whitish. The colonies became green to dark green with maturity and reverse remained uncolour. The older colonies emitted malt odour.

Highly branched conidiophores arose loosely in tufts which often broken or ring like zones. The main conidiophores were 3µm in length and produced smaller side branches (fig. 3). All the branches stood at wide angles to the bearer and tip terminated by phialides.

Three morphologically perceptible phenotypes were identified for *Trichoderma* isolates T1, 2, 3, 5 and 6 as *T. atroviride*, T4, 7, 9, 10 and 15 as *T. viride*, and T8, 11, 12, 13 and 14 as *T. harzianum* using keys provided by Rifai [3] and Bissett [5,6]. All the isolates were characterized on the basis of specific cultural and morphological characteristics. The isolates associated with *T. viride* showed white to green colony with comparatively faster growth but slower sporulation than that of the other isolates, concentric rings formation in the ellipsoidal conidia, floccose mycelium, crowded irregular conidiophore branching, ampulliform phialides (6-12x2.1-2.8 µm), smooth and ellipsoidal conidia (2.5-2.5 µm) with the 6.5 to 9 cm in 3 days of growth on TSM. While *T. harzianum* isolates were identified on the basis of floccose to arachnoid mycelium form, conidiophore branching patterns with multiple short side branches, short inflated phialides and smooth and subglobose conidia. The isolates belonging to *T. viride* were observed on the basis of similarity in typical coconut odour, wavy colony edge, moderately branched conidiophore, nine pin head phialide, globose to obovoid and smooth conidia.

RAPD analysis

The genetic relatedness among five isolates each of *Trichoderma harzianum*, *T.viride* and *T.atroviride* were analyzed (fig. 4). To generate reproducible polymorphisms all amplified products with the primers had shown polymorphic and distinguishable banding patterns (fig. 6) which indicate the genetic diversity of *Trichoderma* isolates. Total 134 reproducible and scorable polymorphic bands ranging from approximately 100bp to 1500bp were generated with 9 primers among the fifteen *Trichoderma* isolates (fig. 5). Relationships among the isolates were evaluated by cluster analysis of the data based on similarity matrix. The dendogram was generated by UPGMA using

NTSYS pc software (Fig. 8). Based on the results obtained all the fifteen isolates were grouped into three distinct clusters having isolates of *T. harzianum*, *T.viride* and *T.atroviride* in each cluster separately. The similarity ranged from 50 to 91%. The maximum genetic variation in the *T. viride* group was observed between the isolates T4 and T7 (90%). In *T. harzianum* cluster variation ranged between T8 and T12 (88%). Followed by T3 and T5 (79%) in the *T. atroviride* group. On the other hand maximum genetic variation of 51 % was observed between the cluster I and followed by 55% between cluster II and cluster III.The similarity matrix obtained through Jaccards similarity coefficients between the isolates indicated that maximum similarity of 100% between the isolates of *T. viride* T4 and T7 followed by 91% in between *T.harzianum* isolates T8 and T12 and 84% similarity was shown by T3 and T5 which give the close relatedness of *T.atroviride* isolates among themselves (fig. 7). Maximum genetic variation observed was between isolates T14 and T2 (64%). The minimum variation of 49% was observed between cluster I and followed by 58% between cluster II and III. ISSR marker was able to identify (fig. 8) variation between *T.harzianum*, *T.atroviride* and *T.viride* isolates.

Figure 1. Conidiophore structures of *Trichoderma atroviride*

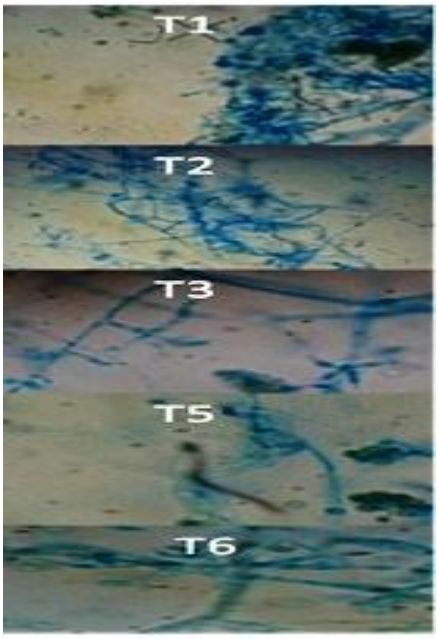


Figure 2. Conidiophore structures of *Trichoderma viride*

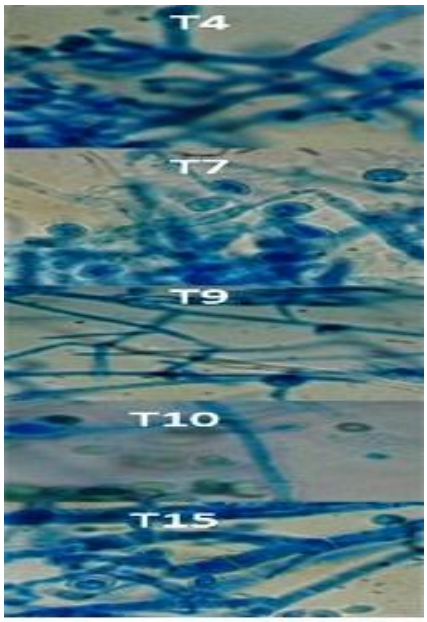


Figure 3. Conidiophore structures of *Trichoderma harzianum*

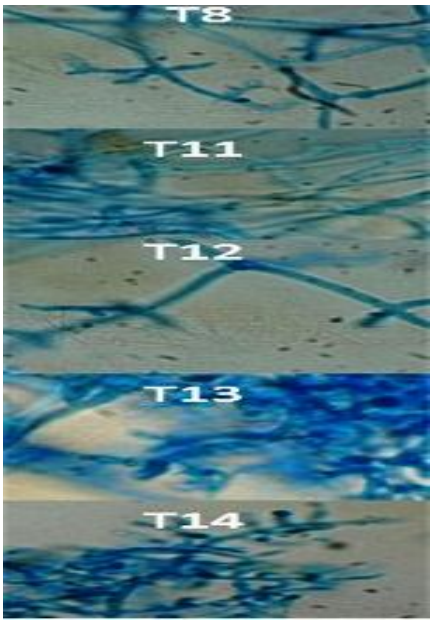


Figure 4. OPA-7 primer RAPD analysis of *Trichoderma* species

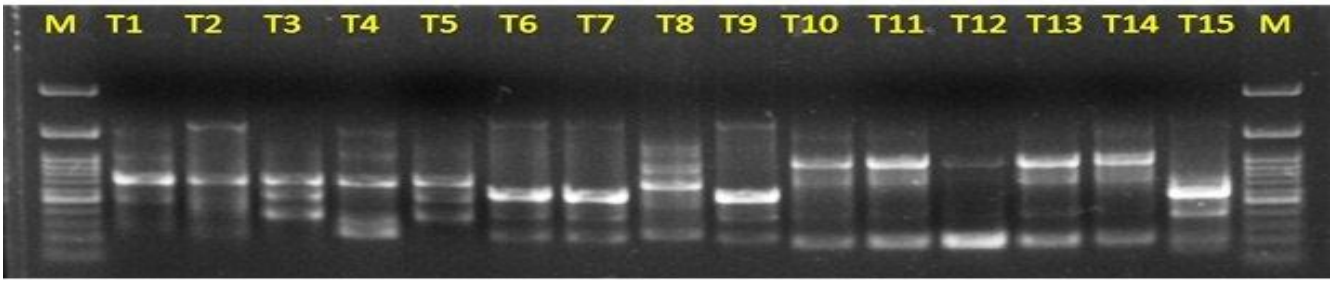


Figure 5. OPA-10 primer RAPD analysis of *Trichoderma* species

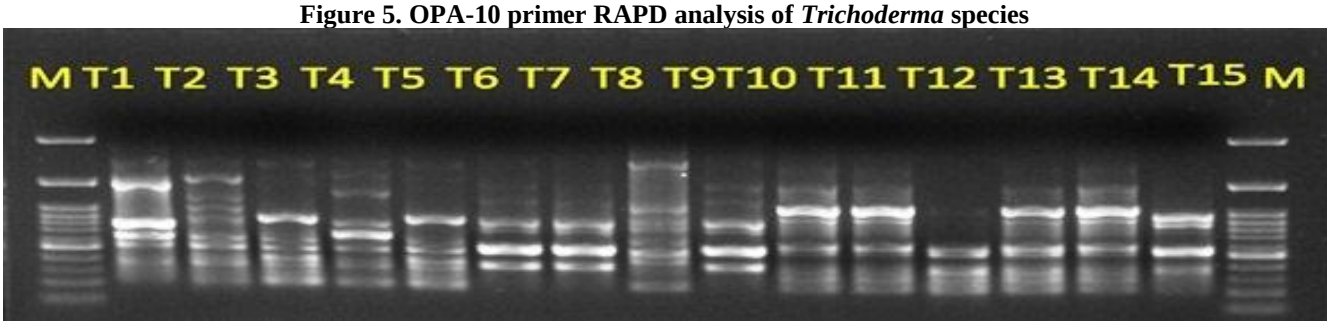


Figure 6. OPC-2 primer RAPD analysis of *Trichoderma* species

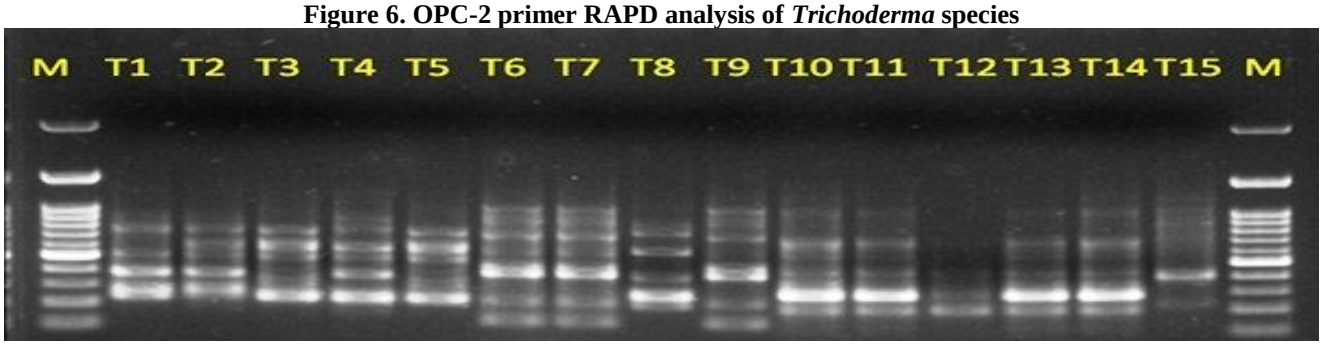
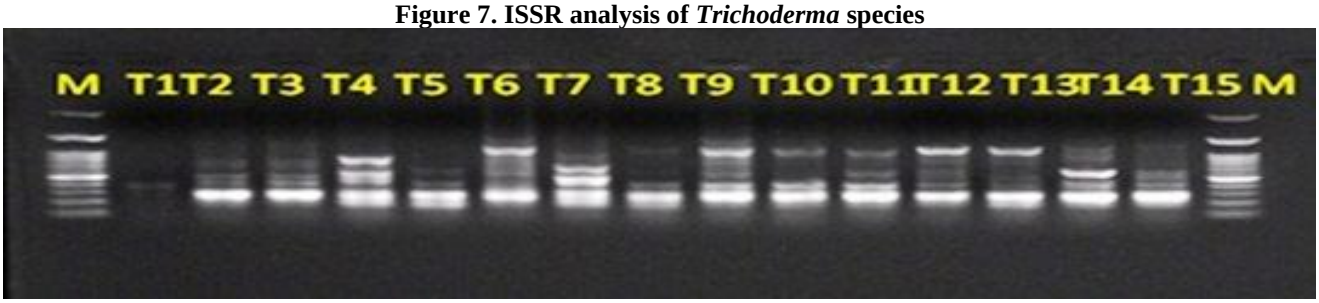
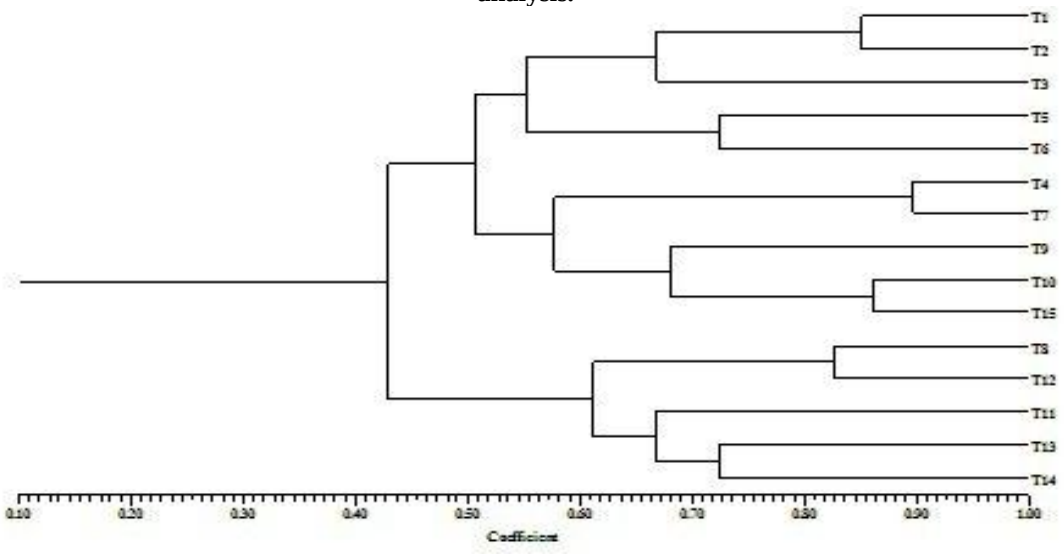


Figure 7. ISSR analysis of *Trichoderma* species



A profile of *Trichoderma* isolates characterization using RAPD and ISSR primers

Figure 8. Dendogram of *T.harzianum*, *T.atroviride* and *T.viride* isolates developed from both RAPD and ISSR data analysis.



DISCUSSION

Several workers identified intra and interspecific relationship in *Trichoderma* using RAPD as a tool. The genetic relatedness among *Trichoderma* isolates inhibiting pathogenic fungi *Rhizoctonia solani* and identified polymorphism among *T. viride* and *T. harzianum* isolates using RAPD. Since RAPD is often considered to be less reliable due to its low reproducibility than other molecular based fingerprinting tools; therefore other molecular markers such as ISSR (inter simple sequence repeats) was also used in stain and species discrimination in several fungi. ISSR identifies polymorphism at microsatellite loci and have high reproducibility. Thus in this study, we have compared the fingerprinting data generated by RAPD with ISSR (inter simple sequence repeats). RAPD analysis was based on six random and ISSR analysis with three primers revealed a great deal of intra and inter-specific variability amongst *Trichoderma* isolates. Most of the isolates were clustered in a similar fashion in both RAPD and ISSR based dendograms and correlated with the morphological data. But *T.atroviride* (T2), *T.viride* (T10) and *T.harzianum* (T8) were discriminated by ISSR markers only which revealed a close relationship between these *Trichoderma* isolates. The close relation between the *T. viride* isolates T14-15 and T7-9 were also distinguished by ISSR markers. The morphological characters were found to

be more significantly related with genotypically defined characters observed from RAPD as concluded from the Mantel test. This indicates that ISSR markers are more diverse and discriminating for intra and interspecific level divergence in *Trichoderma* as compared to RAPD.

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