RESEARCH ARTICLE

Morphological and molecular characterization for identification of isolates of *Trichoderma* spp. from rhizospheric soils of crops in middle Gujarat

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ABSTRACT: *Trichoderma* spp. have gained immense importance as a component of biological control in integrated disease management. Three known isolates and five isolates of *Trichoderma* spp. collected from rhizospheric soils of tomato, brinjal, sorghum, tobacco and groundnut were characterized morphologically and by molecular tools. The isolates showed significant differences in terms of colony characters, sporulation, branching of conidiophores, phialospores colour and shape. RAPD and ITS analysis showed a comparable output grouping distinctly comprising of known isolate *T. viride* and isolates of *T. viride* from brinjal and groundnut in one cluster; known isolate *T. harzianum* and isolates of *T. harzianum* from tomato and tobacco in another cluster and known isolate *T. virens* and *T. asperellum* from sorghum in single cluster. RAPD analysis of eight isolates showed amplification of 391 bands. Analysis of phylogeny of isolates was done by sequencing the ITS region of ribosomal DNA using specific universal primers ITS 1 and ITS 4. Multiple nucleotide alignment of ITS 1, ITS 2 and 5.8s region depicted intra-specific and inter-specific variations in the ITS sequences among the three isolates of *T. harzianum* (known isolate), *T. harzianum* from tomato and tobacco which showed 97 and 83 per cent similarity. However, among the *T. viride* (known isolate) and *T. viride* isolates from brinjal and groundnut, per cent similarity was 84 and 95; while, known isolate of *T. virens* and *T. asperellum* from sorghum, it was 97 per cent. Thus the study was found useful for identification of *Trichoderma* spp. from rhizospheric soil of different crops.

Keywords: ITS, nucleotide alignment, phylogenetic relationships, RAPD, rhizospheric soil, *Trichoderma* spp.

Trichoderma spp. have gained importance globally as bio-control agents for eco-friendly management of plant diseases (Sharma *et al.*, 2014; Vyas and Mathur, 2002), because of their rhizospheric competence and survival ability (Gangwar *et al.*, 2013). Molecular technique *i.e.*, Random Amplified Polymorphic DNA (RAPD) suggested by Williams *et al.* (1990) has been exploited widely for genetic and taxonomic studies of *Trichoderma* spp. (Dodd *et al.*, 2004; Muthumeenakshi *et al.*, 1998). The ITS region of the ribosomal DNA is the most widely exploited sequenced DNA region for fungal identification based on nucleotide sequencing. It has been widely adopted for molecular systematic study at species and even within species level (Kulling *et al.*, 2002; Lee and Hseu, 2002).

Trichoderma spp. include strains which resemble and show rare distinctness in their colony characters and becomes difficult to identify accurately at species level based on their cultural and morphological characteristics. Molecular tools lay significant platform in identification of *Trichoderma* spp. at species level and also help in selecting the efficient antagonistic strains for biocontrol activity. Therefore, the efforts were made to characterize the available isolates morphologically and on the basis of molecular tools.

MATERIALS AND METHODS

Morphological characterization of *Trichoderma* spp.

Soil samples from rhizosphere of tomato, brinjal, sorghum, tobacco, groundnut grown in middle Gujarat were collected. The serial dilution plate technique as described by Lister (1878) was used for isolation of *Trichoderma* spp. Besides, three known specieses of *Trichoderma* viz., *T. viride*, *T. harzianum* and *T. virens* of Department of Plant Pathology, Anand were also used in the study to compare the authenticity of isolated *Trichoderma* spp. *Trichoderma* spp. were isolated on TS medium (Chet and Inbar, 1994) with certain modifications where TSM contained (gm/lit); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.2g; K_2HPO_4 -0.9g; KCl-0.15g; NH_4NO_3 -1.0g; Glucose-3.0g, Chloramphenicol-0.25g, Metalaxyl-0.3g, Penta-chloronitrobenzene-0.2g, Rose Bengal-0.15g, Captan-0.02g (post autoclaving), Agar-20g. The isolated *Trichoderma* spp. were cultured on the PDA medium. The morphological characterization and grouping of isolates was done based on the key given by Bissett (1991c) as well as literature *i.e.* “*Trichoderma*: A Review of biology and systematics of the genus” by Samuels (1996), *Trichoderma*: a guide to identification and biology by Samuels (2004) and “Monographic contribution on *Trichoderma* pers. ex Fr. by Nagamani *et al.* (2002).

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Molecular profiling and Identification of *Trichoderma* spp.

Growing of fungal mycelial mat and extraction of genomic DNA:

Fungal mycelial mat of 4 days old culture growing on potato dextrose broth (PDB) was harvested by filtering with filter paper Whatman No. 1. Genomic DNA was extracted from fungal mycelia with the protocol given by Raeder and Broda (1985) with certain modifications as described herein. The mycelial mat was crushed using pestle and mortar and homogenized with adding liquid nitrogen and incubated with lysis buffer containing 250mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS, for 1 hour at 65°C; followed by centrifugation at 12,000 rpm for 15 minutes. The supernatant was extracted by adding equal volume of water saturated phenol followed by centrifugation at 12,000 rpm for 15 minutes. Further, the supernatant was extracted using equal volume of phenol: chloroform: isoamyl alcohol in ratio of 25: 24: 1 by centrifugation at 12,000 rpm for 15 minutes. The aqueous phase was transferred in a fresh tube and chloroform was added in the ratio of 1: 4 v/v. The isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. The DNA was precipitated from the aqueous phase by adding chilled ethanol (100%) and pelleted by centrifugation at 12,000 rpm for 15 minutes followed by washing in 70% ethanol through centrifugation at 12,000 rpm for 15 minutes. The pellets thus obtained were air dried and suspended to dissolve in TE buffer having pH 8.0. Further, 2 µl of DNase free RNase A (MBI, Fermentas AG) was added to the dissolved DNA and incubated in a water bath at 37°C for 1 hour, followed by 60°C for 10 minutes for enzyme inactivation. The samples were stored at -20°C for further use. DNA was quantified using UV Spectrophotometer at 260 nm (Shimadzu UV-260, Columbia, MD, USA). DNA was also quantified by means of 0.8% Agarose gel electrophoresis followed by ethidium bromide visualization using 1 kbp DNA ladder (MBI, Fermentas AG, Hanover, MD, USA) as genomic markers.

Internal Transcribed Spacer (ITS) region analysis:

The ITS regions were amplified using the universal primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCTCCGCTTATTGATATGC-3') synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White *et al.*, 1990). Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µl, which contains 18.3 µl deionized water, 0.2 µl 10 X Taq pol buffer, 0.2 µl of 3 U Taq polymerase enzyme, 0.4 µl 2.5 mM dNTPs, 0.8 µl of 100 mM reverse and forward primers, 0.1 µl 2 mM MgCl₂ and 2.5 µl of 20 ng template DNA. PCR was programmed with an initial denaturing at 95°C for 5 min., followed by 35 cycles of denaturation at 94°C for 90 sec, annealing at 55°C for 2 min. and extension at 72°C for 3 min. and the final extension at 72°C for 5 min. in a Biometra Thermal Cycler. PCR product of 10 µl was mixed with loading buffer (2 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 1.2%

Agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis using 1X TBE buffer prepared from 5X TBE buffer at pH 8.0 along with 1 Kb DNA molecular weight marker. Electrophoresis was done at 80V for 2 hours using electrophoresis unit of Cleaver Scientific Co., U.K. The gel was observed in ultra violet light Trans-illuminator and image was documented using Alpha EaseFC4.0.0 Gel documentation system (Syngene, USA).

Nucleotide sequencing of PCR product and *in silico* analysis

The sequencing of the PCR product was done using automated sequencer ABI 3730 genetic analyser at Xcelris Genomics Co., Ahmedabad, India. Related sequences were obtained using BLAST software from the NCBI GENbank (<http://www.ncbi.nlm.nih.gov/blast/>) (Altschul *et al.*, 1997). BioEdit version 5.09 was used for multiple sequence alignment and pair-wise alignment (Hall, 1999). CLUSTAL W 1.6 matrix by the CLUSTAL X programme ver. 1.81 was used to create neighbourhood-joining boot-strap tree as given by Thompson *et al.* (1997).

Random Amplified Polymorphic DNA (RAPD) analysis

RAPD-PCR protocol was standardized for molecular characterization of *Trichoderma* isolates. Forty random primers of 10 bp long each (decamer) of OPA and OPE (Operon Technologies, USA) series were used to amplify genomic DNA of the isolates. Out of these, fourteen primers viz., OPA 3, OPA 12, OPA 13, OPA 15, OPA 16, OPA 17, OPA 18, OPA 20, OPE 3, OPE 4, OPE 5, OPE 6, OPE 8, OPE 10 which presented strong band resolution were chosen for the study. For optimization of component concentrations, PCR amplification was carried out with 20 ng/µl of genomic DNA, 2 mM MgCl₂, 1U Taq DNA polymerase, 1X PCR buffer with 15 mM MgCl₂, 0.6 M decamer primers and 0.2 mM of dNTP mix with the final volume of 25 µl. PCR reactions were carried out using Biometra Thermocycler using protocol involving denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min.; primer annealing at 38°C for 1 min.; primer extension at 72°C for 1.5 min. and final extension step at 72°C for 7 min. PCR product of 10 µl was mixed with loading buffer of 2 µl containing 0.25% bromophenol blue, 40% w/v sucrose in water and then loaded in 1.8% Agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis using 1X TBE buffer prepared from 5X TBE buffer at pH 8.0 along with 1 Kb as DNA molecular weight marker. Electrophoresis was done at 80V for 2 hours in electrophoresis unit of Cleaver Scientific Co., U.K. The gel was observed in a Trans-illuminator over ultra violet light and image was documented using Alpha EaseFC4.0.0 Gel documentation system (Syngene, USA).

Data analysis

The amplified products of RAPD were scored for the presence as "1" and for absence as "0" of the band obtained in the gel. The associations between isolates

at molecular level were evaluated by calculating the 'Jaccard's similarity coefficient' for pair wise comparisons based on the proportion of shared bands produced by the primers. Dendrogram was generated through similarity matrix which was subjected to cluster analysis by UPGMA. The computations were performed using the program NTSYS-PC. version 2.02h (Rohlf, 1997). The Jaccard's similarity matrix was subjected to principal component analysis (PCA). This coordination method were used of multi-dimensional solution of the observed relationships. PCA resolves complex relationships into a function of fewer and simpler factors based on distances or similarities between the operational taxonomic units. The principal axes through the hyper-ellipsoid were calculated to simplify the description of these clouds of points. The polymorphism percentage of DNA of eight isolates were calculated as per the formula suggested by Blair *et al.* (1999) where Polymorphism (%) = (Total number of bands - Number of monomorphic bands)/Total number of bands x 100.

Statistical analysis of data

Data of different investigations were analyzed by using variance technique (Steel and Torrie, 1980). The standard methods of analysis of variance for Completely Randomized Design were used in the experiments. The test of significance among the treatments was worked out by 'F' test. The appropriate standard error (S. Em. $\pm$) was computed in each case. For the treatments effects, which were found to be significant, the critical difference (CD) at 5% level of probability was worked out to compare two treatment means.

RESULTS AND DISCUSSION

Morphological characterization of *Trichoderma* spp.

The growth patterns of eight *Trichoderma* isolates cultured on PDA was determined which revealed

significant variations in nature of cultural growth, sporulation patterns, shape of phialides, branching of conidiophores, shape of conidia and ornamentation (Fig. 1). The Petri plates were completely occupied with mycelial growth of *Trichoderma* spp. within three to four days. All the eight isolates produced green coloured loosely floccose to compactly tufted colonies and sporulated profusely by producing masses of green spores in patches or in defined rings. These colonies were white in colour initially and turned to yellowish to greenish with prominent concentric rings in *T. viride* (known isolate), *Trichoderma* sp. from brinjal and groundnut; whereas, in *T. virens* (known isolate), *T. harzianum* (known isolate) and *Trichoderma* sp. from tobacco and tomato were with effuse concentric rings. The mycelial growth was floccose in *T. viride* (known isolate), *Trichoderma* sp. from brinjal and groundnut as well as in *T. virens* (known isolate) to arachnoid in *T. harzianum* (known isolate) and *Trichoderma* sp. from tobacco and tomato. Conidiophores were regular to irregularly branched with conical shape in *T. viride* (known isolate), *Trichoderma* sp. from brinjal and groundnut to pyramidal shape and compact to loosely arranged in *T. harzianum* (known isolate) and *Trichoderma* sp. from tobacco and tomato. Phialides were hyaline and globose in *T. viride* (known isolate), *Trichoderma* sp. from brinjal and groundnut to flask shaped forming large globoid masses in *T. harzianum* (known isolate) and *Trichoderma* sp. from tobacco and tomato. Phialospores were globose, ellipsoidal to sub-globose with light green to dark green colour (Table 1). Distinct characters viz., pale whitish mycelial colour, compact cottony mycelial form, loose tufts conidiation in concentric rings, repeated paired branched conidiophores and ampulliform phialide were observed in *Trichoderma* sp. isolated from sorghum. The cultural characteristics of the *Trichoderma* spp. were as per the description given in "*Trichoderma: A Review of biology & systematics of the genus*" by Samuels (1996) and "*Trichoderma: a guide to identification and biology*"

Fig. 1. Cultural growth pattern of (a) *T. viride* from brinjal; (b) *T. virens* (known Anand isolate); (c) *T. harzianum* from tobacco; (d) *T. viride* (known Anand isolate); (e) *T. viride* from groundnut; (f) *T. asperellum* from sorghum; (g) *T. harzianum* from tomato and (h) *T. harzianum* (known Anand isolate)

Table 1. Morphological characteristics of eight isolates of *Trichoderma* spp. cultured on potato dextrose agar (PDA) and water agar medium

Isolate	Colony growth rate (cm)	Colony colour	Mycelium form	Mycelium colour	Conidiation	Conidiophore	Phialide shape	Phialospore	Conidial colour
<i>T. viride</i> (Known isolate)	9	Dark green	Floccose to Arachnoid	Hyaline, White	Loose tufts in Concentric zones	Highly branched, irregular and compact	Sigmoid & hooked, narrow at base	Globose to obovoid	Dark green
<i>T. virens</i> (Known isolate)	9	Green to bluish green	Floccose	Hyaline, white	Effuse in concentric rings	Few Lateral branches at apex, irregular	Nine pin shaped, curved	Ellipsoidal	Green
<i>T. harzianum</i> (Known isolate)	9	Whitish green to bright green	Floccose to Arachnoid	Watery white	Loose tufts in ring like zones	Highly branched at lower portion, regular	Globose	Singly, Sub-globose	Green
<i>Trichoderma</i> from sorghum	9	Pale to Light yellow	Compact and cottony	Pale Whitish	Loose tufts in concentric ring	Repeated, paired branched	Ampulliform	Globose to sub-globose	Light green
<i>Trichoderma</i> from brinjal	9	Yellow to green	Arachnoid	White	Ring like zones	Moderately branched, irregular	Nine pin shape	Globose to Sub-globose	Green
<i>Trichoderma</i> from tomato	9	Pale to light green	Floccose to arachnoid	Watery white	Ring like zones	Branched, regular	Globose	Sub-globose	Dull Green
<i>Trichoderma</i> from tobacco	9	Light Green to green	Arachnoid	White	Ring like zones	Branched, regular	Globose	Sub-globose	Green
<i>Trichoderma</i> from groundnut	9	Yellow to green	Floccose	White	Concentric zones	Moderately branched, irregular	Nine pin shape	Globose to sub-globose	Dark green

by Samuels (2004) as well as 'Monographic contribution on *Trichoderma pers. ex Fr* by Nagamani *et al.* (2002).

Molecular characterization of *Trichoderma* spp.

Internal transcribed spacer region: All eight isolates i.e. identified *T. viride*, *T. harzianum*, and *T. virens* and *Trichoderma* spp. isolated from rhizospheric soils of brinjal, tomato, tobacco, sorghum and groundnut were identified further by nucleotide sequencing of ITS region of ribosomal DNA. ITS region of all eight isolates was amplified which gave amplicons ranging from 500-600 bp in size (Fig. 2). The sequences of PCR product of ITS

1, ITS 2 and 5.8s of rDNA region of eight isolates were identified by BLAST analysis and subsequently submitted to NCBI GENbank and TrichOkey version 2.0 (Table 2). The ITS sequences subjected for the multiple alignments analysis of ITS sequences by Tom Hall's offline tool BioEdit revealed both intra-specific and inter-specific variations. The variations were observed as 97 and 83 per cent between *T. harzianum* (known isolate) and *Trichoderma* sp. from tomato and tobacco; 84 and 95 per cent between *T. viride* (known isolate) and *Trichoderma* sp. from brinjal and groundnut and 97 and 97 per cent between *T. virens* (known isolate) and *Trichoderma* sp. from sorghum. Further when these

Table 2. NCBI GENBank accession numbers of isolates of *Trichoderma* spp.

Isolates	Identified as	Gene Accession No. of <i>Trichoderma</i> spp. deposited	E value	Percent identity (%)
<i>T. viride</i> (known isolate)	<i>T. viride</i>	KM875462	0.0	99
<i>T. virens</i> (known isolate)	<i>T. virens</i>	KM875461	0.0	100
<i>T. harzianum</i> (known isolate)	<i>T. harzianum</i>	KM875463	0.0	100
<i>Trichoderma</i> sp. from sorghum	<i>T. asperellum</i>	KM875460	0.0	100
<i>Trichoderma</i> sp. from brinjal	<i>T. viride</i>	KM875464	0.0	99
<i>Trichoderma</i> sp. from tomato	<i>T. harzianum</i>	KM875465	0.0	100
<i>Trichoderma</i> sp. from tobacco	<i>T. harzianum</i>	KM875467	0.0	99
<i>Trichoderma</i> sp. from groundnut	<i>T. viride</i>	KM875466	0.0	100

Fig. 2. ITS profile of eight isolates by distinct primer pair (ITS 1+ITS 4) M= Molecular ladder, 1= *T. viride* (known Anand isolate), 2= *T. virens* (known Anand isolate), 3= *T. harzianum* (known Anand isolate), 4= *T. viride* from brinjal, 5= *T. asperellum* from sorghum, 6= *T. harzianum* from tobacco, 7= *T. harzianum* from tomato, 8= *T. viride* from groundnut

sequences were aligned with NCBI database sequences, they showed 99 to 100 per cent similarity among themselves.

Phylogenetic tree constructed by using MEGA 5.10 software with nucleotide sequences of the sequenced ITS region of eight categorized isolates gave eight distinct phylogenetic sub-clades. Group-1: it consists of known isolate of *T. viride* only; Group-2: *T. viride* from brinjal and groundnut; Group-3: *T. harzianum* (known isolate); Group-4: *T. harzianum* from tomato and tobacco, and Group-5 consist of *T. virens* (known isolate) and *T. asperellum* from sorghum. Phylogenetic analysis in combined form of all the nucleotide sequences of the ITS region exhibited significant phylogenetic distance of about 100% (Fig. 4). The similarity *i.e.* 48% was found among isolates of group-1 and group-2 as well as group-3 and group-4; 72% among the isolates of the group-2; 70% between the groups-2 and group-3; 83% among the isolates of group-4 and 100% among the isolates of group-5. Multiple nucleotide sequence alignment revealed a number of gaps which were introduced in the alignment within the ITS region of rDNA. Also, similarity among the sequences of eight isolates was observed based on their conserved regions in rDNA.

Random Amplified Polymorphic DNA (RAPD) analysis: Analysis of DNA of eight isolates with 14 random decamer primers amplified 391 bands, with an average of 27 bands per primer (Table 3). Overall, *Trichoderma* isolates exhibited a high level of genetic diversity. The maximum of 56 bands were generated by primer OPA 18; whereas, OPE 3 generated 43 bands. Similarly, OPA-12 generated 39 bands, OPA-3 and OPA-13 generated 34 bands each; OPA-20 generated 38 bands; OPE-4 and OPE-10 generated 24 bands each;

OPA-16 and OPA-17 generated 20 bands each. Whereas, lowest of 7 bands were generated by OPA-15 (Table 3 and Fig. 3). This result established the potentiality of RAPD to differentiate among isolates and suggested their application for species level characterization and identification.

Dendrogram (Fig. 5) obtained using “Nei’s (1978) unbiased measures of genetic distance by UPGMA method” revealed two clusters *i.e.* Cluster A and Cluster B. Cluster A includes six isolates, where, sub-cluster A_1 consists of sub-cluster A_{11} , which consists of sub-cluster A_{121} contained single 1 (known Isolate *T. viride*) and A_{122} which includes sub-cluster consisting of A_{1221} includes two isolates *i.e.* Isolates 3 (known isolate *T. harzianum*) and Isolates 4 (*T. harzianum* from tomato); while A_{1222} consists of Isolate 8 (*T. viride* from groundnut) and Cluster

Fig. 3. RAPD profile of OPA-3 M= Molecular ladder, 1= *T. viride*, 2= *T. virens*, 3= *T. harzianum*, 4= *T. viride* from brinjal, 5= *T. asperellum* from sorghum, 6= *T. harzianum* from tobacco, 7= *T. harzianum* from tomato, 8= *T. viride* from groundnut

Table 3. RAPD analysis of eight isolates of *Trichoderma* spp.

Primer	Molecular weight range (bp)	No. of bands	No. of Loci	No. of polymorphic loci	Polymorphism (%)	PIC value
OPA-3	300-7742	34	9	9	100.00	0.85
OPA-12	210-3348	39	8	8	100.00	0.89
OPA-13	194-3304	34	8	8	100.00	0.82
OPA-15	2549-6801	7	4	4	100.00	0.61
OPA-16	212-2955	20	7	7	100.00	0.84
OPA-17	450-3407	20	8	8	100.00	0.80
OPA-18	554-3621	56	10	9	90.00	0.88
OPA-20	191-3516	38	12	12	100.00	0.90
OPE-3	449-7177	43	11	10	90.90	0.86
OPE-4	647-10,080	24	6	6	100.00	0.81
OPE-5	3024-10,700	15	5	5	100.00	0.73
OPE-6	2023-9442	23	5	4	80.00	0.72
OPE-8	360-3006	14	4	4	100.00	0.70
OPE-10	238-3461	24	8	8	100.00	0.80
Total		391	102	99	-	-
Average		27.92	7.28	7.07	97.14	0.80

Fig. 4. Phylogenetic tree of eight isolates through analysis of ITS sequences

A₂ consists of Isolate 7 (*T. harzianum* from tobacco). Sub-cluster A₂ of Cluster A consists of single isolate 6 i.e. *T. viride* from brinjal. Cluster B includes two sub-clusters B₁ and B₂ which consists of single isolate i.e. Isolate 2 (known isolate *T. virens*) and Isolate 5 (*T. asperellum* from sorghum). Similarity coefficient of all the eight isolates ranged from 0.28 to 0.85 with all 14 primers. Minimum genetic similarity was obtained between Isolate 5 (*T. asperellum* from sorghum) and Isolate 7 (*T. harzianum* from tobacco) included under cluster A and B as the similarity matrices value was 0.22. Whereas, the maximum genetic similarity of 0.85 was found between the Isolate 3 (known isolate *T. harzianum*) and Isolate 4 (*T. harzianum* from tomato) followed by Isolate 4 (*T. harzianum* from tomato) and Isolate 8 (*T. viride* from groundnut) with 0.70. The characterization at molecular level offers a distinct platform for identification and selection of efficient bioagents for efficient management of seed- and soil-borne plant pathogens.

The genetic distinctness of eight isolates belonging to *T. viride*, *T. harzianum*, *T. virens* and *T. asperellum* as

well as high degree of intra- and inter-specific variation existing among isolates authenticated their higher degree of polymorphism. The dendrogram created based on RAPD analysis was very much related to the phylogenetic tree obtained from ITS 1 and ITS 2 sequences. Singh *et al.* (2006) reported both inter- and intra-specific variation in the isolates based on analysis through RAPD markers and were able to differentiate the pathogenic isolates *T. harzianum* and *T. virens* inciting green mould disease in *Agaricus bisporus*. Similarly Muthumeenakshi *et al.* (1998) characterized different strains of *Trichoderma* spp. by RAPD markers into various groups based on their virulent characters infecting edible mushrooms. The results of the present investigation are in agreement with the results reported by Zimand *et al.* (1994) who reported that nine random primers could distinguished between strains of *Trichoderma* spp. The result of present investigation based on rDNA- ITS region corroborate with the result reported by Kulling *et al.* (2002) and Druzhinina *et al.* (2005). They characterized strains of *Trichoderma* based on sequencing of ITS 1 and ITS 2 region of rDNA

Fig. 5. Phylogenetic tree of eight isolates of *Trichoderma* spp. through analysis of RAPD using UPGMA based on Jaccard's coefficient 1= *T. viride* (known Anand isolate), 2= *T. virens* (known Anand isolate), 3= *T. harzianum* (known Anand isolate), 4= *Trichoderma* isolate from Tomato, 5= *Trichoderma* isolate from Sorghum, 6= *Trichoderma* isolate from Brinjal, 7= *Trichoderma* isolate from Tobacco, 8= *Trichoderma* isolate from Groundnut

and applied multigene approaches especially of the ITS region of the ribosomal operon for the identification of *Trichoderma* species as well as developing a DNA oligonucleotide barcode as a rapid method for the identification of species of *Hypocrea* and *Trichoderma*. It is a TrichOKey v 1.0-a software based program with user friendly web interface supported by a library of short, essentially invariable and species-specific oligonucleotide sequences from the ITS 1 and ITS 2 loci. Similarly Muthukumar and Sharma (2011) assessed twelve isolates of *T. harzianum* and *T. viride* against *Pythium aphanidermatum* and *Sclerotinia sclerotiorum*, wherein, the genetic distinctness was established through RAPD and ISSR which showed a comparable output grouping of isolates. Prameela Devi *et al.* (2012) reported the positive relatedness of molecular and morphological characters with antagonistic ability of *Trichoderma* species. Using these characteristics, the *Trichoderma* isolates were identified as *T. virens* (11 isolates), *T. asperellum* (15), *T. harzianum* (14) and *T. longibrachiatum* (32). Similar studies were carried out by Kumar *et al.* (2012), who analysed bootstrap values with 100 bootstrap replications which produced three major branches. On the basis of the bootstrap values, 34 *Trichoderma* spp. could be identified as *T. harzianum*, *T. brevicompactum*, *T. viride*, *T. ovalisporum* and *T. erinaceum* H. *orientalis* and *Pachybasium* A (*T. asperellum*). Kamala *et al.* (2015) assessed the genetic diversity of 193 *Trichoderma* strains from cultivated soils of nine districts of Manipur based on ITS sequences. Among 65 isolates of *Trichoderma*, 22 different types of *Trichoderma* spp. were found representing 4 well-separated main clades in which *T. harzianum* was found to be the most prevalent sp. The molecular and phenotypic data leads to the development of taxonomy

of all the 22 *Trichoderma* spp. Savitha and Sriram (2015) characterized and identified the sequences of ITS gene and translation elongation factor 1- α encoding gene (*tef1*) using the BLAST interface in TrichOKEY and TrichoBLAST. The phylogenetic analysis of the *Trichoderma* isolates were carried out based on the ITS and *tef1* sequences. Among 10 isolates of *Trichoderma* Th9, Th10, Th19, Tv10 and Tv115 were identified as *T. harzianum* isolates and Th16, Tv30 and Tvs7 were identified as *T. asperellum* and isolates Tvs5 and Tvs8 were identified as *T. virens*.

It is obvious from the present study that genetic diversity analysis through RAPD and ITS based markers as well as analysis of ORF's of isolates depicted a good number of conserved transcribing regions present in rDNA which encodes for large number of amino acids with protein mass of higher molecular weight. All results obtained lays a concrete platform of positive correlation between morphological and molecular based characterization to identify a potent strain of *Trichoderma* in terms of its genetic profile and for further use as a efficient biocontrol agent in plant disease management.

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