Genetic Variability and Relationships among Seventeen *Trichoderma* Isolates to Control Dry Root Rot Disease Using RAPD Markers

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Trichoderma spp. has been identified as potential antagonist of *Fusarium solani*, which is causing dry root rot of *Citrus*. A random amplified polymorphic DNA (RAPD) marker was used to estimate the genetic variation among 17 isolates of *Trichoderma*. These isolates were characterized using 20 random primers of the OPM series, out of which 16 primers gave a total of 145 DNA fragments, showing 91.8% polymorphism. The genetic distance between each isolate was calculated, and cluster analysis was used to generate a dendrogram showing the relationship among them. The isolates grouped into two major clusters, the first major cluster consisted of TCT_{14}, TCT_{17}, TCT_{13}, TCT_{12} and TCT_{16}. The remaining isolates in the second major cluster separated in two sub-clusters; the first cluster consisted of TCT_4, TCT_{10}, TCT_2, TCT_3, TCT_8, TCT_6, TCT_9, and the second sub-cluster consisted of TCT_1 TCT_{15} TCT_5, TCT_{11}, and TCT_7. The similarity matrix indicated that TCT_6 and TCT_{13} were genetically distinct as they showed only 22.6% similarity followed by TCT_5 and TCT_{16}; TCT_6 and TCT_{16} (25%), while the isolates TCT_4 and TCT_{10} were found to be genetically similar, as 66.7% similarity was observed between the isolates followed by 61.3% similarity between the TCT_2 and TCT_4 isolates.

Key words: Fusarium solani, Dry Root Rot Disease, *Citrus*

Introduction

Trichoderma spp. are antagonistic to other fungi and have shown promise as biological control agents for several soil-borne diseases (Papavizas, 1985; Jenson and Wolffhechel, 1995). Several potentially useful strains of *Trichoderma* for the biological control are difficult to distinguish from other strains found in the field. So there is a need to find ways to monitor these strains when applied to the natural pathosystem. The significance of genetic variation as one of several criteria for biodiversity evaluation is widely recognized (Humphries *et al*., 1995), and protection of genetic diversity is incorporated into many international conventions. Some of the genera such as *Trichoderma* contain species that are of great economic importance because of their production of enzymes, abiotics or use as biocontrol agents (Harman and Kubick, 1998). Since extracellular enzymes are highly substrate-dependent and looking into the nowadays growing interest in using biocontrol agents, a reliable and precise system for strain identification is important. More recently, the use of molecular markers has given a boost to the analysis of the accurate variation among various isolates of these bioagents. Latha *et al.* (2002) reported that the RAPD (random amplified polymorphic DNA) techniques can be used for distinguishing strains of bioagents. By using the RAPD procedure (Williams *et al*., 1990), which incorporates the PCR (polymerase chain reaction) technique without depending on a known DNA sequence, information can be generated on amplification patterns from only a small amount of DNA. The literature does not indicate any work on the molecular characterization of the genus *Trichoderma*, obtained from the rhizoplane of sweet orange, for the control of the dry root rot disease

caused by *Fusarium solani* (Kavitha *et al.*, 2004), which is a major disease (Gopal *et al.*, 1999, 2001) in the state of Andhra Pradesh, India. Therefore, the present investigation was carried out on the molecular characterization of native *Trichoderma* isolates obtained from the rhizoplane of sweet orange.

Materials and Methods

Trichoderma isolates

Seventeen isolates of *Trichoderma* spp. isolated from the rhizoplane of sweet orange for the management of the dry root rot disease caused by *Fusarium solani* were used in the present study (Kavitha *et al.*, 2004). The details of the isolates are listed in Table I.

Table I. *Trichoderma* isolates obtained from the rhizoplane of sweet orange for the control of dry root rot disease (*Fusarium solani)*.

Location	Accession no.
Saidapuram (Nellore Dist.)[a]	TCT_1
Saidapuram (Nellore Dist.)	TCT_2
Chillapuram (Nellore Dist.)	TCT_3
Malakapatnam (Nellore Dist.)	TCT_4
Chillapuram (Nellore Dist.)	TCT_5
Malakapatnam (Nellore Dist.)	TCT_6
Venkatagiri (Nellore Dist.)	TCT_7
Nallapareddipalli (Nellore Dist.)	TCT_8
Mopur Village (Nellore Dist.)	TCT_9
Nallapareddipalli (Nellore Dist.)	TCT_{10}
Gudur (Nellore Dist.)	TCT_{11}
Naidupalem (Nellore Dist.)	TCT_{12}
Petlur (Nellore Dist.)	TCT_{13}
B.N. Kandrigia (Chittoor Dist.)	TCT_{14}
B.N. Kandrigi (Chittoor Dist.)	TCT_{15}
Kodur (Kadapa Dist.)	TCT_{16}
Rajempeta (Kadapa Dist.)	TCT_{17}

[a] The names in parentheses are names of the districts in the state of Andhra Pradesh, India.

Fungal multiplication

Potato dextrose broth (PDB) was used to induce mycelial growth of the fungus for the extraction of DNA. 150 ml of medium were dispensed in 500 ml conical flasks and sterilized at 0.1034 MPa for 20 min. Each flask was inoculated with a 6 mm mycelial disc of the fungus, taken from the actively growing single spore cultures of different isolates growing on potato dextrose agar. The inoculated flasks were incubated for 10 d at (28 ± 1) °C in a BOD incubator. At the end of the incubation period, the mycelial mats were harvested by filtering through Whatman no. 1 filter paper, washed with sterilized water thrice, blot-dried and stored in Alfoils at −20 °C.

Nucleic acid extraction

DNA extraction was done according to procedures given by Murray and Thompson (1980) and Zolan and Pukilla (1986) with slight modifications. Mycelial mats were weighed (1 g), ground in liquid N_2 with a pre-chilled pestle and mortar. The powdered mycelium was transferred into centrifuge tubes carrying 25 ml of preheated (65 °C) 2% cetyltrimetyl-ammonium-bromide extraction buffer to make a slurry. The tubes were incubated at 65 °C for 1 h and stirred occasionally with the help of a sterile glass rod. The equal volume of chloroform/isoamyl alcohol (24:1) was added to each tube and mixed gently. Samples were centrifuged at $9168 \times g$ for 10 min at room temperature. The upper aqueous phase was precipitated with 0.6 volumes of ice-cold isopropanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) and spinned at $20627 \times g$ for 15 min at room temperature. The pellets obtained were washed with 70% ethanol and kept for drying at room temperature. Total nucleic acids obtained were dissolved in sterile distilled water and stored at −20 °C in small aliquots.

Purification of DNA

RNase treatment was applied to remove RNA from the total nucleic acids. 2 μl of RNase from a stock solution (1 μg/ml) were added to the nucleic acid extracts and incubated at 37 °C for 1 h. The DNA concentration of samples and their purity were determined by measuring ultraviolet absorbance at 260 nm and 280 nm in a spectrophotometer and rechecked by running samples along with an 1 kb molecular weight marker (MBI, Fermentas) on 1% agarose gel.

Optimization of polymerase chain reaction (PCR)

The PCR was optimized by varying the content of template DNA (25, 50, 75 and 100 ng), Taq DNA polymerase (0.5, 1.0 and 1.5 units) and $MgCl_2$ concentration (3, 5, and 7.5 mM). The standardized amplification assay was as follows: template DNA, 25 ng; Taq DNA polymerase (Genei, Bangalore, India), 0.5 units; $MgCl_2$, 5 mM; dNTP (Genei), 100 μM each of dATP, dGTP, dCTP, dTTP; primer (Operon Bio-technologies, Cologne, Germany), 1 μM; buffer (Genei), 1× in a reaction

volume of 25 μl. Different PCR protocols given by Pascual *et al.* (2000) and Lee and Taylor (1990) were tested for obtaining the best amplification of nucleic acids of the isolates under investigation. The PCR was performed using a palmcycler (Carbett Research, Mortalake, Australia) with the following temperature profile: initial denaturation at 94 °C for 2 min, followed by 45 cycles of denaturation at 92 °C for 1 min; annealing at 37 °C for 1 min; extension at 72 °C for 2 min with final elongation at 72 °C for 5 min.

Primer survey and selection

The preliminary primer screening was carried out using 20 primers from the OPM series (Operon Bio-technologies) for molecular variation analysis. The primers that gave reproducible and recordable amplification were used in the analysis of variability of the isolates.

Agarose gel electrophoresis

To 25 μl of amplification products obtained after the PCR, 2 μl of loading dye (bromophenol blue) were added and loaded into individual wells of 1.2% agarose in 1 × Tris/acetic acid/EDTA buffer. Electrophoresis was carried out at 60 V for 3 h, and thereafter the gel was stained with ethidium bromide (1 μg/ml). Detection of DNA was made on a transilluminator under UV light. The 1 kb ladder (MBI, Fermentas, Germany,) was also loaded in one lane as a marker.

Each amplification product was considered as a RAPD marker and recorded across 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 bands that could be reproducibly detected in the gel and 'O' for the absence of a band. The data matrix thus generated was used to calculate Jaccard's similarity co-efficient for each pairwise comparison. The co-efficients were calculated *in silico*, following Jaccard (1908), using the following formula: similarity co-efficient = a/n, where a is the number of matching bands for each pair of comparisons and n is the total number of bands in two samples observed.

The similarity co-efficients were subjected to the unweighted pair-group method on arithmetic averages (UPGMA) of cluster analysis to group the isolates based on their overall similarities. The SPSS 10.0 package was used for cluster analysis and subsequent dendogram preparation.

Results

Random amplification of polymorphic DNA

The PCR conditions were optimized in terms of content of template DNA, Taq DNA polymerase and $MgCl_2$ concentration. Varying contents of

Table II. Primer survey for the determination of polymorphism in *Trichoderma* isolates.

	RAPD primer set M (primer sequence)	Total no. of bands	Polymorphic bands
M-01	5′-GTTGGTGGCT-3′	8	7
M-02	5′-ACAACGCCTC-3′	10	8
M-03	5′-GGGGGATGAG-3′	12	10
M-04	5′-GGCGGTTGTC-3′	10	10
M-05	5′-GGGAACGTGT-3′	6	6
M-06	5′-CTGGGCAACT-3′	11	10
M-07	5′-CCGTGACTCA-3′	10	9
M-08	5′-TCTGTTCCCC-3′	4	7
M-09	5′-GTCTTGCGGA-3′	11	9
M-10	5′-TCTGGCGCAC-3′	9	8
M-11	5′-GTCCACTGTG-3′	7	7
M-12	5′-GGGACGTTGG-3′	12	10
M-13	5′-GGTGGTCAAG-3′	–	–
M-14	5′-AGGGTCGTTC-3′	8	8
M-15	5′-GACCTACCAC-3′	–	–
M-16	5′-GTAACCAGCC-3′	12	11
M-17	5′-TCAGTCCGGG-3′	–	–
M-18	5′-CACCATCCGT-3′	8	8
M-19	5′-CCTTCAGGCA-3′	–	–
M-20	5′-AGGTCTTGGG-3′	7	7
Total		145	135

template DNA from 25 to 100 ng in a reaction volume of 25 μl and 25 ng DNA gave the maximum number of reproducible bands, and thus was considered ideal and used subsequently in all analysis. Titration of different contents of Taq DNA polymerase and $MgCl_2$ showed that 0.5 units of Taq DNA polymerase and 5 mM $MgCl_2$ in the final reaction mixture gave optimum, reproducible and well resolved results. A higher or lower content resulted in either sub-optimal or lack of complete amplifications. The final amplification assay contained 25 ng genomic DNA, 0.5 units Taq DNA polymerase, 100 mM each of dDTPs, 5 mM $MgCl_2$, 0.6 μM primer and 1 × *Taq* buffer in a PCR reaction volume of 25 μl.

Primer selection and survey

The primer survey was carried out by using 20 primers from the OPM series of Operon Bio-technologies; four resulted in either sub-optimal or non-distinct amplified products. Therefore, the remaining 16 primers which gave reproducible and scorable bands with high percentage of polymorphism were used in this study (Table II). Hence, the final numerical analysis included 145 bands with 91.8% polymorphism resulting from 16-primer amplification. PCR amplification with these primers was done twice before scoring for the presence and absence of bands. The number of amplified products obtained was specific to each primer; it ranged from 6 to 12.

Sixteen selected primers gave a total of 145 amplified products, out of which 135 were polymorphic. Maximum polymorphism was showed in PCR with M-04, M-05, M-08, M-11, M-14, M-18 and M-20. These primers showed 100% polymorphism as all these bands obtained were polymorphic with a size ranging from 1000 bp to 1.5 kb. They were closely followed by M-06, M-07, M-10 and M-16 with 90.9, 90, 88.8 and 91.6% polymorphism, respectively.

The banding pattern of TCT_4 and TCT_{10} was found to be identical with many primers (M-02, M-07, M-10). In case of primers M-05 and M-04 the majority of bands was found to be common among all isolates. In case of primer M-16 the total number of bands was 12 out of which 11 were polymorphic. Hence, a common 1.5 kb band was present in all isolates except for TCT_5 and TCT_7. The size of amplified products varied from 500 bp to 3 kb. Jaccard's similarity co-efficients between

Table III. Jaccard's similarity co-efficients of 17 isolates of *Trichoderma* based on polymorphism obtained with 20 random primers of the OPM series.

Trichoderma isolate	TCT_1	TCT_2	TCT_3	TCT_4	TCT_5	TCT_6	TCT_7	TCT_8	TCT_9	TCT_{10}	TCT_{11}	TCT_{12}	TCT_{13}	TCT_{14}	TCT_{15}	TCT_{16}	TCT_{17}
TCT_1	1																
TCT_2	0.297	1															
TCT_3	0.419	0.443	1														
TCT_4	0.325	0.613	0.506	1													
TCT_5	0.467	0.337	0.373	0.406	1												
TCT_6	0.441	0.389	0.368	0.333	0.423	1											
TCT_7	0.411	0.372	0.288	0.333	0.379	0.419	1										
TCT_8	0.450	0.367	0.376	0.413	0.402	0.385	0.354	1									
TCT_9	0.374	0.359	0.368	0.294	0.366	0.376	0.361	0.432	1								
TCT_{10}	0.355	0.577	0.455	0.667	0.360	0.385	0.327	0.378	0.326	1							
TCT_{11}	0.402	0.352	0.413	0.367	0.384	0.394	0.355	0.324	0.355	0.388	1						
TCT_{12}	0.336	0.375	0.326	0.333	0.288	0.305	0.293	0.356	0.292	0.312	0.356	1					
TCT_{13}	0.380	0.380	0.333	0.367	0.308	0.226	0.276	0.362	0.275	0.347	0.361	0.432	1				
TCT_{14}	0.454	0.364	0.414	0.379	0.370	0.366	0.327	0.333	0.394	0.360	0.409	0.411	0.429	1			
TCT_{15}	0.514	0.352	0.373	0.391	0.395	0.342	0.391	0.387	0.342	0.324	0.431	0.318	0.360	0.432	1		
TCT_{16}	0.381	0.302	0.360	0.337	0.250	0.250	0.266	0.361	0.369	0.270	0.361	0.291	0.393	0.404	0.333	1	
TCT_{17}	0.372	0.304	0.327	0.333	0.377	0.308	0.308	0.327	0.360	0.327	0.391	0.347	0.394	0.537	0.402	0.382	1

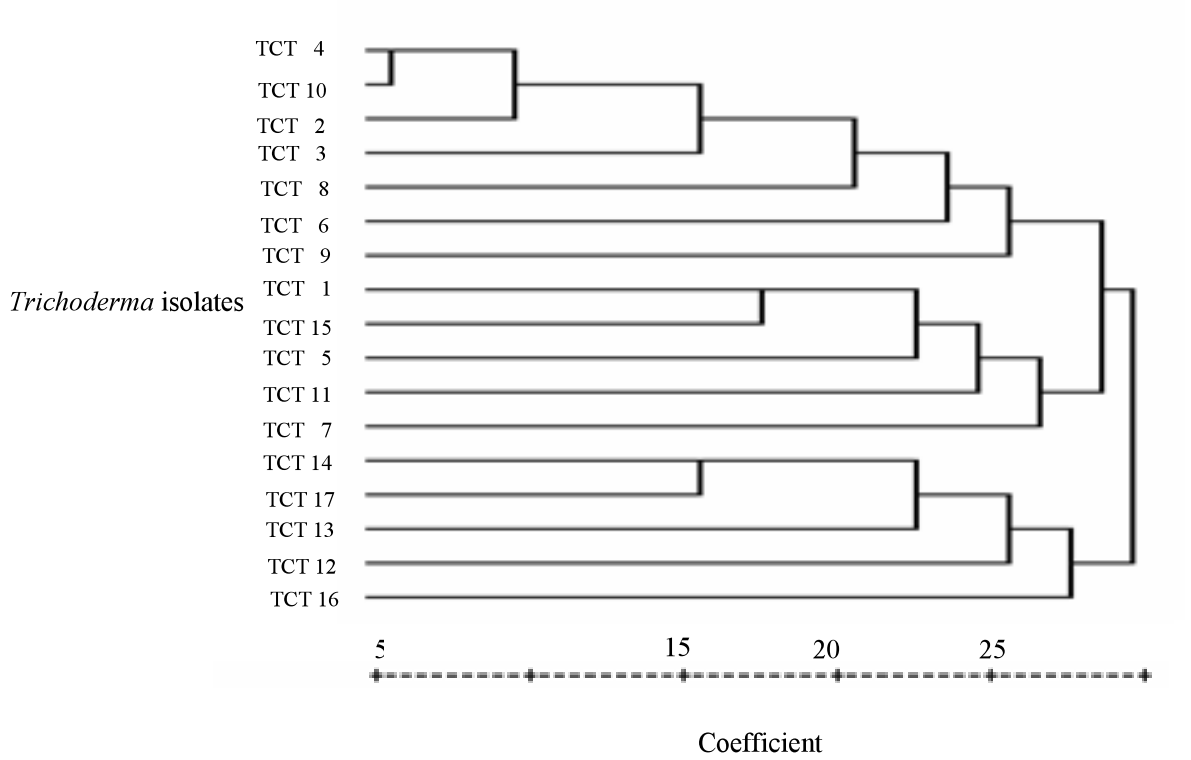

Fig. 1. Dendrogram depicting the variation among isolates of *Trichoderma* based on RAPD.

the isolates are presented in Table III. The similarity matrix thus produced indicated that TCT_6 and TCT_{13} were genetically distinct as they showed only 22.6% similarity followed by TCT_5, TCT_6 and TCT_{16} (25%). While the isolates TCT_4 and TCT_{10} were found to be genetically similar, as 66.7% similarity was observed between the isolates followed by 61.3% similarity between the TCT_2 and TCT_4 isolates.

The similarity co-efficients, subjected to the SPSS package to produce a dendrogram, show two major clusters (Fig. 1). TCT_{14}, TCT_{17}, TCT_{13}, TCT_{12} and TCT_{16} are in first major cluster and the remaining isolates are in the second major cluster which is further separated into two sub-clusters where TCT_4, TCT_{10}, TCT_2, TCT_3, TCT_8, TCT_6, TCT_9 are in the first sub-cluster and TCT_1 TCT_{15} TCT_5, TCT_{11}, TCT_7 are in the second sub-cluster. Among all the isolates, the isolates TCT_2, TCT_4, and TCT_{10} were found to be genetically similar when compared to other isolates.

Discussion

Accurate, reliable characterization of variation is not always possible using morphological characters; even when it is, identification of variations at the intraspecific level could be difficult. In recent years, a number of techniques has been developed based on comparisons of nucleic acids, which have been successfully applied to distinguish strains of a particular microorganism. These techniques include AFLP and RAPD. In the present investigations, RAPD, a PCR-based technique, has been deployed to assess the variability at the intraspecific level. In this technique, arbitrary short oligonucleotide primers targeting unknown sequences are used to generate amplified products that often show polymorphism within species (Welsh and McClelland, 1990; Williams *et al.*, 1990).

RAPD analysis offers the possibility to create polymorphism without any prior knowledge of the DNA sequences of the organism investigated. The patterns produced are highly polymorphic, allowing to discriminate between isolates of a species, if sufficient numbers of primers are screened. In the

present investigations, it was found that once the optimal RAPD conditions for a given species are established, the method works well for fungal samples, even on crude DNA extracts. Out of 20 primers screened for the amplification of DNA of isolates of *Trichoderma*, 16 were found to give reproducible and scorable bands with high percentage of polymorphism. Maximum polymorphism was observed in M-04, M-05, M-08, M-11, M-14, M-18 and M-20 showing 100% polymorphism as all the bands obtained were polymorphic for these primers.

Random PCR approaches are being increasingly used to generate molecular markers which are useful for taxonomy and for characterizing fungal populations. Since previous knowledge of DNA sequences is not required, any random primer can be tested to amplify any fungal DNA. The RAPD method has been successfully used to differentiate and to identify fungi at the intraspecific level (Gutheric *et al.*, 1992; Assigbetse *et al.*, 1994; Nicholson and Reozanoor, 1994) and at the interspecific level (Lehman *et al.*, 1992). Differences between isolates from different areas are therefore more easily detectable if the RAPD technique has been used to distinguish genetic variation among strains/isolates within a species (Cook *et al.*, 1996; Boyd and Carris, 1997).

In the present study, phylogenic analysis based on the similarity co-efficients amplified two major clusters having TCT_{14}, TCT_{17}, TCT_{13}, TCT_{12} and TCT_{16} in the first major cluster and the remaining isolates in the second major cluster, which in turn separated in two sub-clusters separating isolates TCT_4, TCT_{10}, TCT_2, TCT_3, TCT_8, TCT_6, TCT_9 (in the first sub-cluster) from TCT_1, TCT_{15}, TCT_5, TCT_{11}, TCT_7 (in the second sub-cluster). The selection of primers has great significance in pathotype strain identification and characterization and molecular mapping of the genome. These primers can also serve as a reference for integral comparison in future studies. In the present study, it was interesting to note that there was no instance of any two isolates sharing all the bands with any primer, which eliminated the possibility of the presence of duplicates among nine isolates. The usefulness of this technique in strainal identification and characterization has been established by many workers in the past. Schlock *et al.* (1994) analyzed parent strains and mutants of *Trichoderma harzianum* by PCR fingerprinting (RAPD). They clearly distinguished mutant and wild-type isolates by genomic fingerprinting and emphasized their importance for patent protection. In our study, the characterization of *Trichoderma* isolates by RAPD has proved useful in separating all the isolates from each other. It has also provided us with primer markers that can be used to separate and distinguish each isolate. TCT_4 and TCT_{10} isolates, which were shown to be the most effective strains for biological control of the dry root rot disease of citrus in earlier investigations (Kavitha *et al.,* 2004) gave a distinct banding pattern with most of the primers. This possibility of distinguishing different isolates by a rather simple technique of genomic fingerprinting based on PCR-RAPD could be of great importance for the use in patent protection of fungal strains of biotechnological use, where additional more easily detectable markers are not available.

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