

Full Length Research Paper

Genetic relatedness among *Trichoderma* isolates inhibiting a pathogenic fungi *Rhizoctonia solani*

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The mechanism and mode of action of the *Trichoderma* against *Rhizoctonia solani* is through coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm. Characterization of 17 biocontrol strains identified as "*Trichoderma*" and isolated from *R. solani* was carried out using RAPDs. A certain degree of polymorphism was detected and the cluster analysis grouped the 17 isolates into three groups: *T. harzianum*, *T. viride*, and *T. aureoviride*. Some of the RAPD markers were also useful for identifying strains of *T. aureoviride* within the complex collections from Raipur and Bilaspur in India.

Key words: Antagonism, mycoparasitism, random amplified polymorphic DNA, *Rhizoctonia solani*, *Trichoderma*.

INTRODUCTION

Trichoderma, a genus under Deuteromycotina, Hyphomycetes, Phialasporace, Hyphales, Dematiaceae has gained immense importance since last few decades due to its biological control ability against several plant pathogens (Kubicek and Harman, 1998). Researchers are interested in this genus because of its novel biological properties and biotechnological applications. *T. harzianum* is one efficient biocontrol agent that is commercially produced to prevent development of several soil pathogenic fungi. Knowledge concerning the behaviour of these fungi as antagonists is essential for their effective use since they can act against target organisms in several ways. Strains of *Trichoderma* can produce extracellular enzymes and antifungal antibiotics, but they may also be competitors to fungal pathogens, promote plant growth, and induce resistance in plants. The commercial use of *Trichoderma* BCAs must be preceded by precise identification, adequate formulation, and studies about the synergistic effects of their mechanism of biocontrol (Grondona et al., 1997).

The close morphological resemblance that exists between the species of *T. harzianum* and *T. inhamatum*; *T. viride* and *T. asperellum*; and *T. Koningii* and *T. Konilangbra* has been resolved clearly without any controversy using molecular and biochemical analyses (Samuels, 1996; Viterbo and Haran, 2001, Goes et al., 2002; Latha et al., 2002). Internal transcribed sequences (ITs) of the ribosomal DNA (rDNA) analysis (rDNA-ITS1) and universally primed polymerase chain reaction (PPCR) have been used to characterise isolates of *Trichoderma* (Cumagun et al., 1999). In the present investigation two PCR-based techniques (RAPD and restriction analysis of the amplified ITS1-5.8 S-ITS2 region of the nuclear ribosomal DNA) were used to distinguish Indian isolates of six *Trichoderma* species, namely *T. virens*, *T. pseudo*, *Koningii*, *T.hamatum*, *T. harzianum*, *T. viride* and *T. koningii*.

MATERIALS AND METHODS Collection and isolation of the fungus

The experimental material consisted of the seventeen strains of the *Trichoderma* sp., out of which ten strains were isolated from Sarkanda farm, Bilaspur (India) and remaining seven strains were isolated from the rhizosphere soil sample at I.G.K.V.V, Raipur (India). For isolation of *Trichoderma*, serial dilution technique was used. One ml aliquot of appropriate *Trichoderma* dilutions was

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Table 1. Random Amplified Polymorphic DNA's (RAPD) primers and their sequences.

Code	PRIMER SEQUENCE (53)
A-01	CAG GCC CTT C
A-4	AAT CGG GCT G
A-5	AGG GGT CTT G
A-20	GTT GCG ATC C
AA-11	AGA CGG CTC C
AA-3	TTA GCG CCC C
AA-04	AGG ACT GCT C
AA-7	CTA CGC TCA C
AA-06	GTG GGT GCC A
AA-09	AGA TGG GCA G
AA-14	AAC GGG CCA A
AA-15	ACG GAA GCC C
AA-17	GAG CCC GAC T
AA-18	TGG TCC AGC C
AC-03	CAC TGG CCC A
F-3	CCT GAT CAC C
F-12	ACG GTA CCA G

plated in potato dextrose agar (PDA) and the plates incubated at 28±2°C. Colonies of *Trichoderma* were observed on the medium after three days of incubation and were transferred aseptically to PDA slants for future use.

Morphological studies

Morphological observations were made from cultures grown at 28°C for about one week on potato dextrose agar (PDA), corn meal dextrose agar (CMB), oat meal agar (OMA), special nutrient agar (SNA) and *Trichoderma* selective medium (TSM). The radial growth of the fungus was measured by measuring the diameter of the colony in mm or cm. The sporulation was estimated based on number of conidia observed per microscopic field. Growth pattern were recorded depending on the presence or absence of aerial mycelium or subdued growth. Pigmentation of varying shades was recorded visually. All the stages were examined under LEICA phase contrast binocular light microscope and the selected specimen were microphotographed on KODAK 100 ASA film.

Antagonistic activity

The dual culture technique described by Dennis and Webster (1971) was used to test the antagonistic ability of *Trichoderma* against *Rhizoctonia solani*. The host fungus and *Trichoderma* were grown on potato dextrose agar (PDA) for a week at room temperature (28±2°C). Small blocks of the target fungus (*R. solani*) cut from the periphery were transferred to the Petri dish. After two days of growth, *Trichoderma* was transferred aseptically in the same plate at about 3 cm distance and were incubated at room temperature with alternate light and darkness for seven days and observed periodically. The interaction between *R. solani* and *Trichoderma* sp. were observed by using a phase LEICA Microscope and the selected specimen were microphotographed on KODAK 100 ASA film.

Fungal growth conditions and DNA extraction

Cultures were maintained on potato dextrose agar (PDA) at 25°C were grown in potato dextrose broth (PDB) for 48 h. Hyphae were

collected on filter paper in a Buchner funnel, washed with distilled water, frozen, and were used for DNA extraction. Genomic DNA was extracted using the method of Rogers and Bendish (1988). The genomic DNA was stored in 50µl TE buffer at -20°C for future use.

Random amplified polymorphic DNAs (RAPD)

Amplification was carried out for 45 cycles on PTC 100 (Programmable Thermocycler) of MJ Research Pvt. Ltd., USA. A set of 17 RAPD primers was tested across 17 *Trichoderma* isolates. All RAPD primers for analysis were obtained from Promega, USA (Table 1). The sample contained 80-100 ng of *Trichoderma* DNA, 1 mM each deoxynucleoside triphosphate, 5 U/µl of *Taq* polymerase, 10X PCR buffer with MgCl₂, 50 ng/µl of each primer in a total reaction volume of 20µl. The samples were heated to 94°C for 4 min and then subjected to 45 cycles of annealing at 36°C for 1 min., extension at 72°C for 2 min and denaturation at 94°C for 1 min followed by a final extension for 7 min. The combination of concentration which gives consistent profile with sharp bands was used for RAPD analysis.

The amplified RAPD products were separated on 1.5% agarose gel in 0.5X TAE buffer for 3.5 h at 100 V. The banding pattern was visualized on UV transilluminator and documented by using BIO-RAD gel documentation system.

RESULTS AND DISCUSSION

Morphological observations

Based on the observation of the conidiophore morphology the isolates were designated as *Trichoderma viride*, *Trichoderma harzianum* and *Trichoderma aureoviride*. In *T. viride*, the hyphae are septate, branched, smooth walled and colorless (Figure 1). The conidiophores were observed as less complicated and formed a aerial hyphae. They produced smaller branches, and ultimately a conifer-like branching system is formed (Figure 1C). All the branches stand at wide angles to the bearer and tips terminated by phialides. Phialides are formed in false whorls beneath each phialide. Generally not more than two or three phialides, which arise at right angle, were observed (Figure 1G). Occasionally they arise singly or in opposite pairs along the branches (Figure 1G). Phialides were ninepin shaped attenuated into long neck. Phialospores were globose or short obovoid, rarely broadly ellipsoidal. The phialospores are green coloured. In *T. harzianum*, conidiophores were seen as much branched, form loose tuft, which arise in distinct and continues ring like zones. The main branches mostly in groups of 2-3 and stand at right angle to the bearer and their length increased with the distance from the tip of the main branch which gave a conical or pyramidal appearance. In *T. aureoviride*, the hyphae are branched, septate and colourless (Figure 1A). Conidiophores formed compact distinct zones or irregularly dispersed on the surface of the colony. Dirty yellow to brownish yellow pigmentation with the development of needle shaped, golden yellow crystals is characteristic of *T. aureoviride*.

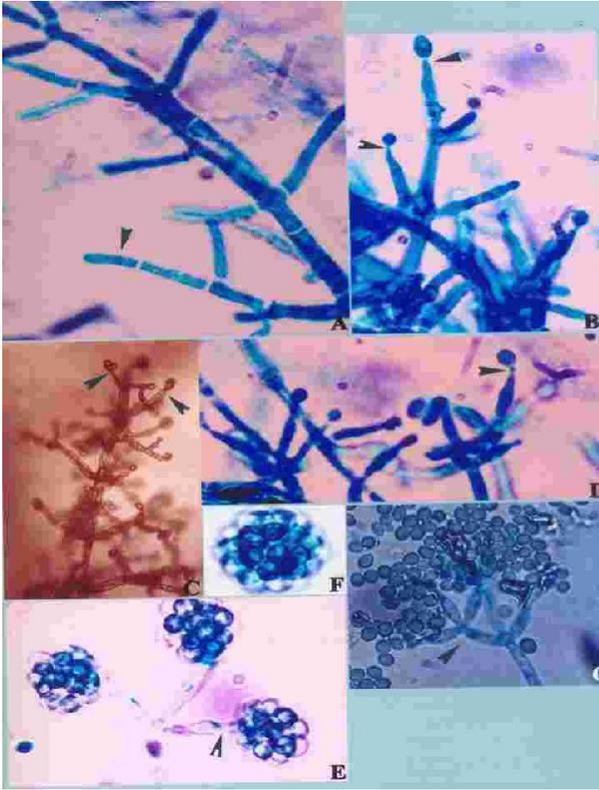


Figure 1. Morphological characters of the anamorph of *Trichoderma* sp. **a.** Conidiophores of *Trichoderma* sp. (100X); **b.** Phialide with a single phialospore borne at the tip (100X); **c.** Conifer shaped arrangement of the conidiophore (40X); **d.** Phialide with a single phialospore borne at the tip (100X); **e., f., g.** Spores produced successively at the tip of the phialides and form into globose gloccoid head (100X).

Gams and Bisset (1998) reported that the reverse side of colonies is often uncolored, buff, yellow, amber, or yellow-green, and many species produce prodigious quantities of thick walled spores (chlamydo spores) in submerged mycelium. Different species of Hypococ producing *Trichoderma* anamorph are identified (Bisset, 1991a,b,c, 1992; Samuals et al., 1999; Rifai and Webster, 1996) under different species name. The colour of the colony, growth formation and characters of chlamydo spores; disposition, branching, size and shape of the conidiophores; disposition, size and shape of phialides; size, shape, color and ornamentation of conidia are the main characters considered to identify the species (Bisset 1991a,b,c, 1992; Samuals et al., 1996).

Mechanism employed by *Trichoderma* for its mycoparasitic activity

One of the mechanisms observed to be adopted by *Trichoderma* to parasitize *R. solani* is by competition. *Trichoderma* suppressed the growth of *R. solani* through the overgrowth. Secondly, *Trichoderma* was observed to

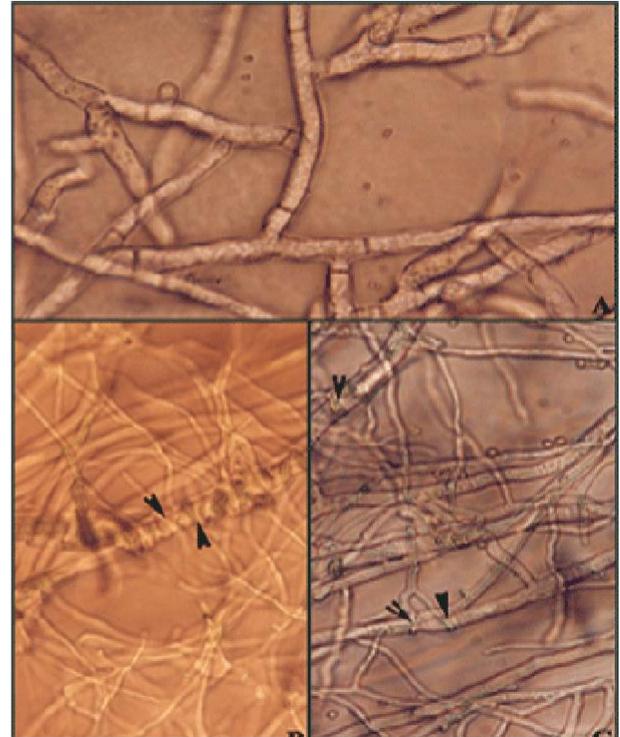


Figure 2. Mode of the action of the biocontrol agent. **a.** Mycelium of *R. solani* (40X); **b.** Coiling of the large hyphae of *R. solani* by *Trichoderma* sp. (40X); **c.** Initial interaction between the *Trichoderma* and *R. solani* (attachment of the *Trichoderma* hyphae by tips on the large hyphae of *R. solani*) (40X).

cluster around the *R. solani* by the formation of small tufts thus limiting the growth of the pathogen sheath blight. In both the cases formation of sclerotial bodies of *R. solani* were suppressed.

Interaction between the two fungi when documented through light microscopy indicated that the mode of action adopted by *Trichoderma* to parasitize *R. solani* (Figure 2A) was through coiling (Figure 2B). A sort of searching and recognition mechanism between the two is speculated. The mycelial tip of *Trichoderma* runs parallel with that of the mycelium of *R. solani*, and with its mycelial tips it sticks on to the large hyphae of *R. solani* (Figure 2C (arrow heads)). This process is further followed by a very rapid and excessive coiling on the target fungus. It is speculated that the process of sticking with the mycelial tips is further advanced by the production of enzymes such as chitinases and/or glucanases by the bio-control agent.

Weindling (1932) ascribed bio-control by *T. lignorum* of citrus seedling disease, incited by *R. solani*, to mycoparasitism and described in detail the mycoparasitism of *R. solani* hyphae by the bio-control agent, including coiling around pathogen hyphae, penetration, and subsequent dissolution of the host cytoplasm a phenomenon which occurred regardless of the supply of external nutrients to the host or mycopara-

Table 2. Summary of PCR based-RAPD analysis of 17 *Trichoderma* isolates using 17 Random primers.

Total No. of primers	17
Total No. of Bands amplified from polymorphic primers	102
Size range of amplification products	400 to 6000 bp
Total number as polymorphic bands (RAPDs) identified	85

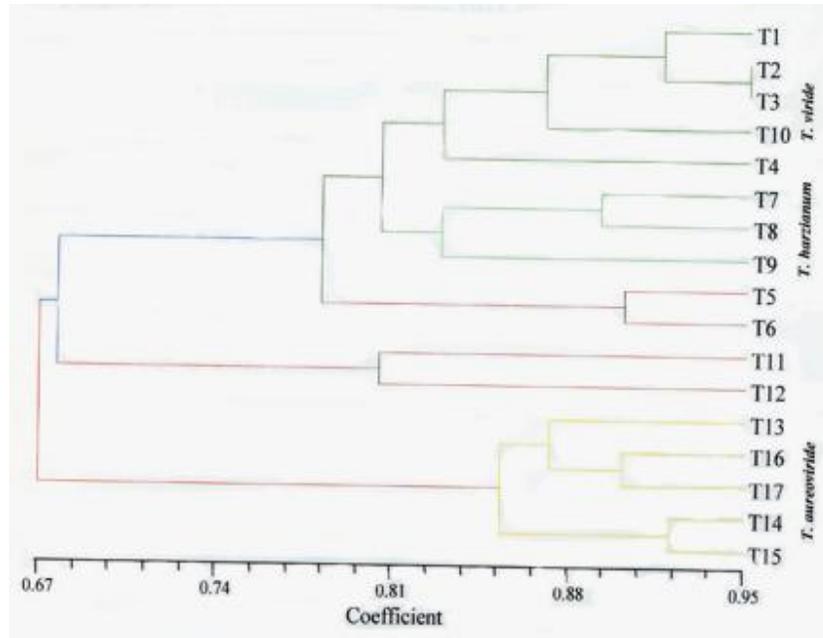


Figure 3. Dendrogram showing similarity coefficient of 17 *Trichoderma* isolates based on RAPD analysis.

site. Weindling (1934) also reported that a strain of *T. lignorum* produced a “lethal principle” that was excreted into the surrounding medium, allowing parasitic activity by the bio-control agent. In 1941 the “lethal principle”, was characterized and found to be toxic to both *R. solani* and *Sclerotinia americana*, and named as gliotoxin (Weindling, 1941).

DNA fingerprinting of subset of *Trichoderma* isolates

Amplified products were observed when the genomic DNA was subjected to RAPD analysis using 17 Random primers. Eighty five of the 102 bands were polymorphic. The RAPD data was computed into similarity matrix using NTSYS (Numerical Taxonomy System Biostatistics) computer programme. Similarity coefficient ranged from 0.67 to 0.95 (Table 2). A perusal of the dendrogram obtained (Figure 3) indicates that there was a major cluster consisting of 12 of 17 isolates, whereas five

isolates were found to be different from rest of the genotypes. The major cluster A and cluster B consisted of 12 and 5 isolates and shared 67% similarity. Major cluster A was further divided into subcluster A₁ and A₂ and consisted of 10, and 2 isolates, respectively, sharing 68.4% similarity. A distinct cluster B is further sub-divided into B₁ and B₂ and consisted of 3 and 2 isolates, respectively, and shared 85.2% similarity.

The light microscopic observation indicates that the isolates were divided into three groups *T. aureoviride*, *T. viride* and *T. harzianum*. All the isolates which produced yellow coloured crystals were grouped as *T. aureoviride*. The RAPD primers Ac3, AA17, Aa01, AA07, AA14, AA05 and A01 were able to discriminate *T. aureoviride* from the rest of the isolates. The isolates which did not produce pigmentation in the medium were grouped into two groups based on the morphology of spores and phialides. If we compare the clustering with that of the observations on vegetative morphology, three distinct clusters are formed consisting of 10, 2 and 5 isolates. The last cluster

consisting five isolates (#T13, T16, T17, T14 and T15) represented *T. viride*. The cluster which consisted of ten isolates was further grouped into two consisting of five (#T1, 2, 3, 10 and 4) and three (#T-7, 8, 9) isolates and represented *T. viride* and *T. harzianum*. The cluster representing *T. aureoviridae* was morphologically different from the other two groups, which was distinctly classified.

The close morphological resemblance that exists between the species of *Trichoderma* can be resolved clearly without any controversy using molecular data and similar observations has been reported by several workers (Bissett 1991a; Rehner and Samuels 1995; Lieckfeldt et al., 1998). *T. harzianum* is a species aggregate, grouped on the basis of conidiophore branching patterns with short side branches, short inflated phialides, and smooth and small conidia. *T. harzianum* has been divided in three, four, or five sub specific groups, depending on the strains and on the attributes considered (Grondona et al., 1997). Four biotypes (Th1, Th2, Th3, and Th4) were originally proposed based on its pathogenicity on mushrooms (Seaby, 1987).

Among the 17 isolates, all *T. harzianum* were classified in group I and the others in group II. In group I, there were minor variations which could be related to the source of origin from different climatic zones. There were 3 subgroups in group II. Subgroup I is composed of *T. auroviride* (T18 Udaipur), which was different from *T. hamatum* and *T. viride*. sSubgroup III comprises of *T. Hamatum* (T5 and T6 NRCG), *Trichoderma* spp. (T10 NRCG), *T. viride* (T25 ICRISAT, T17 Udaipur and T27 NARDI) and *T. longibrachiatum* (T16 Udaipur). Subgroup III has only *T. viride* (T20 and T22 Akola).

T. viride includes an aggregate of species with globose or subglobose to ellipsoidal warted conidia; most of them produce antibiotics and a typical coconut odor. Recently, it has been found that *T. viride* is a paraphyletic group, and an integrated morphological and molecular approach has confirmed the redefinition of types I and II of *T. viride* in two species. Type I is the true *T. viride* species, which also includes the anamorph of *Hypocrea rufa*, and is grouped together with strains of *T. atroviride* and *T. koningii* (Lieckfeldt et al., 1999). Type II represents the new species *T. asperillum* (Seaby, 1987; Lieckfeldt et al., 1998), which has ovoidal rather than globose condition and also darker and faster condition.

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