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RESEARCH ARTICLE

RAPD ANALYSIS OF *TRICHODERMA SPP.* ISOLATED FROM CHICKPEA FIELDS OF UTTAR PRADESH

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Abstract

Molecular characterization of the promising bio-control agents adopting Random Amplified Polymorphic DNA (RAPD) analysis helps to determine the diversity and identification. DNA (RAPD) analysis were employed in aggregation with dilution plating on semi selective medium for distinguishing and identification of *Trichoderma,* a potential bio-control agent utilized in compost amended mixes. Distinct and reproducible fingerprints were attained upon amplification of purified genomic DNA of *Trichoderma spp.* with random primers of Operon (OPH) series. The amount of genetic variation was figured out with a set of 20 RAPD primers. In most cases, the amplified fragments showed more than 50% polymorphism. It is evidenced that there was good genetic variability among the isolates collected from chickpea fields of Uttar Pradesh.

Introduction

Soil microorganisms influence ecosystems by contributing to plant nutrition **(George, *et al.*, 1995)**, plant health **(Smith & Goodman, 1999),** soil structure **(Wright & Upadhyaya, 1998)** and soil fertility **(Yao *et al.*, 2004).** It has been widely recognized, particularly in the last two decades that, majority of harsh environments are inhabited by surprisingly diverse microbial communities. Bacteria, actinomycetes and fungi are three major groups of soil inhabiting microorganisms. An estimated 1,500,000 species of fungi exist in the world **(Giller *et al.*, 1997).**

Trichoderma species are cosmopolitan soil fungus, known for their rapid growth, capability of utilizing diverse substrates and resistance to noxious chemical **(Mercedes *et al.*, 2001).** They are highly interactive in root, soil and foliar environmental. *Trichoderma* species are wide spread saprophytic soil-borne or wood decaying fungi, which appear well adapted to diverse abiotic stresses such as salinity and drought. Some species also cause substantial economic losses in commercial mushroom production while others act as bio-control agents **(Venkateswarlu *et al.*, 2008),** induce host defense responses in plants and cellulosic and hydrolytic enzymes and degrade organochlorine pesticides. Some of these fungi have substantial potential for a variety of commercial applications. The taxonomy of these fungi has been revised significantly and many new species are being recognized. Taxonomic identification of *Tricoderma* species is largely based on morphological traits in the division of the genus into five sections: *Trichoderma, Pachybasium, Longibrachiatum, Saturnisporum* and *Hypocrasium* **(Bissett, 1991a & Bissett, 1991b)**. *Trichoderma*, commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability microorganisms, such as *Trichoderma,* reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. In addition, the release of biocontrol agents into the environment has created a demand for the development of methods to monitor their presence or absence in soil. Therefore, monitoring population dynamics in soil is of much importance. Previous methods employed to identify strains of *Trichoderma spp*. in soil samples have included the use of dilution plates on selective media. However, this method does not distinguish between indigenous strains and artificially introduced ones **(Knudsen *et al.*, 1996).** The *Trichoderma* isolates were differentiated by mycelia growth rate and colony appearance, as well as microscopic morphological features, including phialides and phialospores **(Seaby, 1996).** These can also be

distinguished by randomly amplified polymorphic DNA (RAPD) - PCR, restriction fragment length polymorphisms in mitochondrial DNA and ribosomal DNA and sequence analysis of ribosomal DNA **(Castle *et al.*, 1998 & Ospina-Giraldo *et al.*, 1999).**

Molecular analysis of several strains, including some ex-type strains revealed that classification based on morphological data has been to a great extent erroneous resulting in re-classification of several isolates and species **(Samuels *et al.*, 1999).** The morphological differentiation between some species requires a high level of expertise and is highly time consuming in addition; these characters are not always present in culture, making morphological identification difficult **(Samuels *et al.*, 1999).** Attempts to improve the process have been made by combining morphological and physiological data but these do not necessarily reflect phylogeny **(Hermosa *et al.* 2000, Kuhls *et al.*, 1997 & Samuels *et al.*, 1999).** PCR based fingerprinting is widely applied for the characterization of *Trichoderma* strains and species for various purpose **(Venkateswarlu *et al.*, 2008).** It is recommended as a basic tool for proving the identity of stains especially with regards to comprehensive culture collections **(Sharma *et al.*, 2010)**. In the present study, genetic variability in nineteen isolates of *Trichoderma spp.* from different ecosystems were evaluated with twenty different RAPD markers.

Materials & Methods:

A total of 5 monoconidial (genetically pure single spore cultures) isolates of *Tricoderma spp.* **(Table 1)** were employed which were identified by Indian Type Culture Collection, IARI, New Delhi. These isolates isolated from rhizospere soil and gathered from different geographical locations of Uttar Pradesh.

Cultures were grown on potato dextrose agar (PDA) at 24 ± 1^0C. Colony descriptions are based on investigations on PDA under ambient day light conditions. The following colony characters were investigated to determine the sections and species of *Trichoderma* - colour of the fungus, colour of the metabolism produced in the media, growth of fungus, growth patterns (aerial, subdued, submerged or combination) and appearance (ringed, sectored, uniform, rough, smooth) **(Bissett, 1991a, Bissett, 1991b, Kumar *et al.*, 2011, Venkateswarlu *et al.*, 2008 & Kullnig-Gradinger *et al.*, 2002).** Microscopic studies were made from lactic acid mounts. Conidiophore structure and morphology were described from macronematous conidiophores taken from the edge of conidiogenous pustules or fascicles when conidia were maturing, usually after 4 to 7 days of incubation. Conidial morphological were documented after 14 days.

Trichoderma spp. were grown on 100 ml conical flask from actively growing colony of *Trichoderma* were transferred to each flasks aseptically in a laminar air flow. The flasks were incubated at 24^oC for 21 days. The mycelia mat was collected by passing the fluid through 3 layers cheese cloth. DNA extraction was done by the method of **(Sharma *et al.*, 2010)** with minor modifications. The fungal cell wall was disrupted by grounded with pestle and mortar in liquid nitrogen. The powdered mycelium then transferred to an extraction buffer which contained detergent Cetyl-Tri-methyl Ammonium Bromide (CTAB) and 2, β-mercaptoethanol, EDTA and polyvinyl pyrrolidone (PVP) and incubated for 1 hr at 60^oC followed by centrifugation at 12000 rpm for 15 minutes. The supernatant was then extricated with equal volume of phenol, chloroform, isoamyl alcohol (25:24:1) and centrifuged at 12000 rpm for 10 minutes. The aqueous phase was then transferred in a fresh tube and DNA was precipitation with chilled (100%) ethanol. DNA was decontaminated by centrifuging at 12000 rpm for 15 minutes and washed in 70 % ethanol. The pellets were air dried and suspended in TE buffer (pH 8.0).

The extraction of total genomic DNA from different isolates of *Trichoderma spp.* as per the above methodology was followed by RNAse treatment. Genomic DNA was re-suspended in 100 ml. 1X TE buffer and incubated at 37^0C for 30 min with RNAse. After incubation, the sample was re-extracted with PCI (phenol:chloroform:isoamyl alcohol 25:24:1 solution and RNA free DNA was precipitated with chilled ethanol as narrated earlier. Quantification of DNA was in ratio also done. Working concentration of DNA was adjusted to 20 mg/ml and stored at 4^oC. The DNA from all isolates produced clear shaped bands indicating good quality of DNA.

The methodology reported in reference was adopted with minor modification for carrying out PCR reaction to produce RAPD profiles. Amplification of DNA fragments was carried out by the PCR using 10 mer arbitrary primer. The reaction mixture consisted of 200 ml. of dNTP mix (fermentas) 15p mol of primer (operon), 5µl/ml of Tag polymerase (fermentas) and 25m M $MgCl_2$. DNA polymerase (fermentas) and 25m M $MgCl_2$. DNA amplifications were performed in thermocycler with one cycle of initial denaturation at 94^0C for 15 min. followed by 40 cycles of denaturation at 94^0C for 1 min, annealing at 35^0C for 2 min., extension at 72^0C for 1 min and final extension at 72^0C for 10 minutes. Amplified products together with marker (Lambda DNA/Eco+Hind III double digest; Bangalore, Genei) were resolved by 1.5 % agarose gel electrophosis (60 $Vcm^{-1)}$; Gels were photographed by gel documentation system (Uvitec). The extraction of total genomic DNA from Trichoderma isolates was followed by RNAse treatment **(Chakraborty *et al.*, 2010).** The quality and quantity of DNA was analysed with both

spectrophotometrically and in 0.85% agarose gel. The DNA from all isolates produced clear sharp bands, evidence of good quality of DNA**.**

Results & Discussions:

Soil samples were collected from different infected chickpea fields of Uttar Pradesh. These bio-agents were then isolated and purified and were preserved in PDA. These cultures were then identified as *Trichoderma spp. by* ITCC, Division of Plant Pathology, IARI for proper identification and accessioned as ITCC 7442, 7443, 7444, 7450 and 7451. Similar studies were also done by **Sharma *et al*., 2010** who characterized thirty isolates of *Trichoderma* obtained from rhizospheric soils of various crops from different locations of Uttarakhand, India and identified morphologically as *Trichoderma harzianum* and *T. virens*. They were compared using RAPD Markers to estimate the genetic variations**.**

In present investigation, it was found that the total number of reproducible band amplified were 67 out of which 50 bands were found to be polymorphic and the rest i.e. 17 was found monomorphic **(Fig 1).** Hence, the percentage of polymorphic was 74.63% and monomorphic was 25.37%.The number of bands per primer ranged from maximum of 10 (given by OPH – 11) to minimum of 4 (given by OPH – 1, OPH – 3 and OPH – 19) with an average of 7 bands per primer. Three RAPD primers (OPH – 7, OPH - 11 and OPH - 16) produced average or above average amplified products. Three RAPD primers (OPH – 7, OPH - 9 and OPH - 14) showed unique polymorphic bands. None RAPD primers showed unique monomorphic bands. Eight RAPD primers (OPH - 02, OPH – 06, OPH - 08, OPH - 10, OPH - 12, OPH - 15, OPH - 17 and OPH - 20) did not give any amplification **(Table-2).** The size of the amplified products varied from minimum of 100 bp *i.e*., 0.1 (OPH - 07, OPH - 09, OPH - 16, and OPH - 18) primers to maximum of 1175 bp *i.e*. 1.175 kb (OPH - 04). Thus, it was concluded that out of total 20 decamers of operon H primer series 12 primers namely OPH - 1, OPH - 3, OPH - 4, OPH - 5, OPH - 7, OPH - 9, OPH - 11, OPH - 13, OPH - 14, OPH - 16, OPH - 18 and OPH - 19 showed the amplification which was a good sign to predict the genetic diversity and molecular variability in the *Trichoderma spp*. isolated from chickpea fields of Uttar Pradesh.

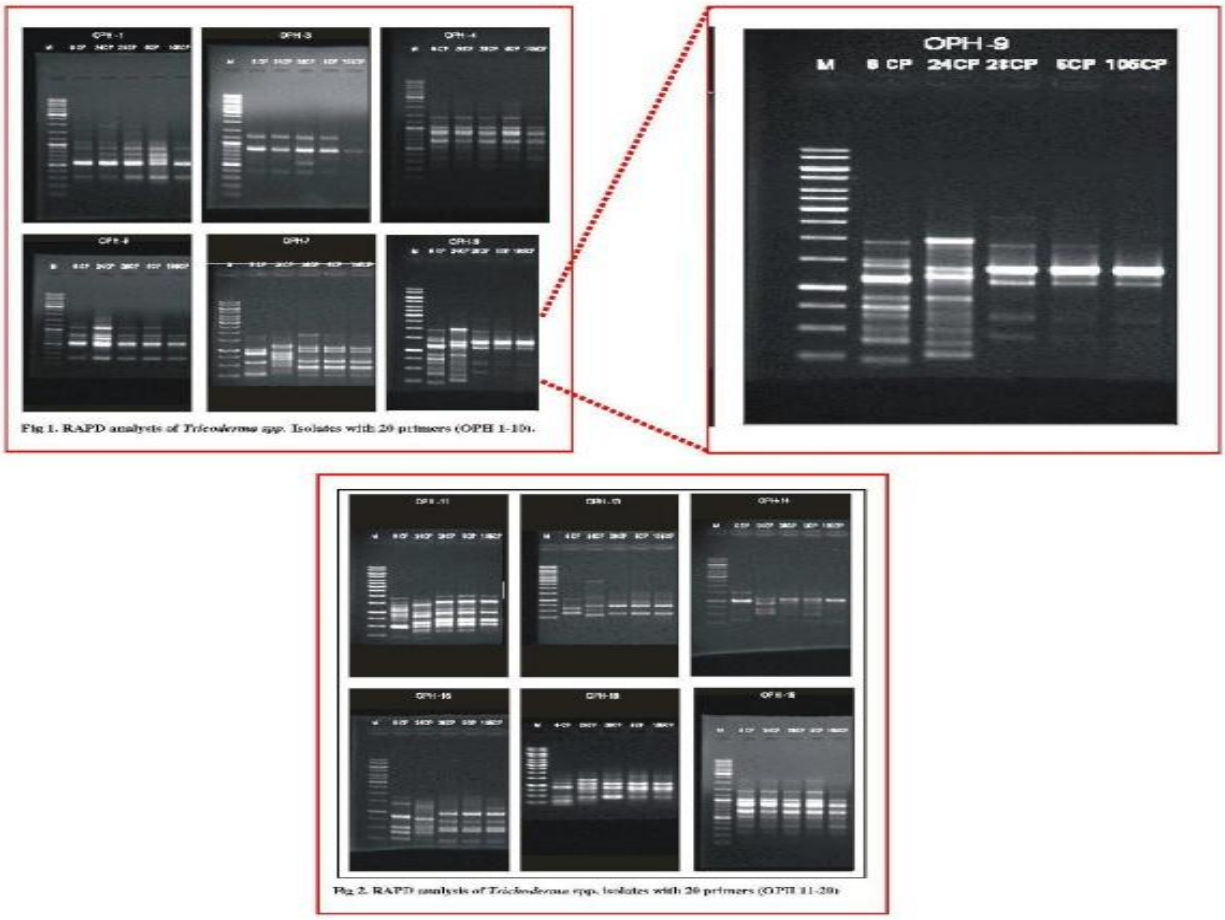
Table 1: List of used isolates

I.D. No	Culture type	Ref. No.	Source	Fungus identified
ITCC-7442/09	6 CP	01	Sultanpur	*Trichoderma atroviride*
ITCC-7443/09	24 CP	02	Sitapur	*Trichoderma atroviride*
ITCC-7444/09	28 CP	03	Barabanki	*Trichoderma longibrachiatum*
ITCC-7450/09	5 CP	04	Kanpur Nagar	*Trichoderma longibrachiatum*
ITCC-7451/09	105 CP	05	Etawah	*Trichoderma atroviride*

Table 2: Amplified fragments obtained with RAPD primers in *Trichoderma spp.*

Primer name	Sequence (5-3)	Total no. of bands	Poly-morphic bands	Mono-morphic bands	Percentage of polymorphism	Percentage of monomorphism
OPH-01	GGTCGGAGAA	4	3	1	75	25
OPH-02	TCGGACGTGA	-	-	-	-	-
OPH-03	AGACGTCCAC	4	3	1	75	25
OPH-04	GAAGTCGCC	5	2	3	40	60
OPH-05	AGTCCTCCCC	5	3	2	60	40
OPH-06	ACGCATCGCA	-	-	-	-	-
OPH-07	CTGCATCGTG	7	7	0	100	Nil
OPH-08	GAAACACCCC	-	-	-	-	
OPH-09	TGTAGCTGGG	5	5	0	100	nil
OPH-10	CCTACGTCAG	-	-	-	-	-
OPH-11	CTTCCGCAGT	10	9	1	90	10
OPH-12	ACGCGCATGT	-	-	-	-	-
OPH-13	GACGCCACAC	5	3	2	60	40
OPH-14	ACCAGGTTGG	5	5	0	100	Nil
OPH-15	AATGGCGCAG	-	-	-	-	-
OPH-16	TCTCAGCTGG	7	6	1	86	14
OPH-17	CACTCTCCTC	-	-	-	-	-
OPH-18	GAATCGGCCA	6	3	3	50	50
OPH-19	CTGACCAGCC	4	1	3	25	75
OPA-20	GGGAGACATC	-	-	-	-	-
Total		**67**	**50**	**17**	**74.63**	**25.37**

Fig 1. RAPD analysis of *Tricoderma spp.* Isolates with 20 primers (OPH 1-20).



Conclusion:

Results indicates that the *Trichoderma* isolates in the present study had very good diversity and there are strong possibility to get the isolates specific primers that will be utilized for identifying the particular *Trichoderma* isolates with good biological potential from the field isolates without going the cumbersome bioassay again. Moreover, this method is rapid, as the duration for complete extraction of DNA is much less compared to the other published protocol. This shows that there is a complete variability within the strains of *Trichoderma spp.* and it would enable us to develop a potential strain of *Trichoderma spp.* having competitive ability and also having growth promoting characters and in inducing resistance in plants. On the basis of results this can be said that amplification through PCR can be used in differentiation of strains with each other and act as function of genotypic finger printing. The most polymorphic RAPD primer obtained in this study can be effectively used in DNA fingerprinting of *Trichoderma spp*. and these primers may also be helpful in genome mapping and used as probe to identify homologous sequences in other isolates.

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