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RAPD-PCR OF TRICHODERMA ISOLATES AND *IN VITRO* ANTAGONISM AGAINST *FUSARIUM* WILT PATHOGENS OF *PSIDIUM GUAJAVA* L.

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Abstract: The paper concerns randomly amplified polymorphic DNA RAPD-PCR analysis of seven *Trichoderma* species isolates and their *in vitro* antagonism against wilt pathogens of *Psidium guajava* L. viz. *Fusarium oxysporum* f. sp. *psidii* (*F. o.* f. sp. *psidii*) and *Fusarium solani*. Out of 10 RAPD oligonucleotides (OPA 1–OPA 10) tested, seven markers OPA 1, 3, 5, 7, 8, 9 and 10 efficiently differentiated the isolates of *Trichoderma* and showed reproducible banding patterns. A total of 248 bands were obtained from these markers along with a 61.84% per cent similarity among the seven isolates of *Trichoderma*. From the seven isolates of *Trichoderma*, isolates *T. harzianum*, *T. virens* and *T. viride* were evaluated for *in vitro* efficacy against *F. o.* f. sp. *psidii* and *F. solani*. Per cent inhibition was maximum by direct use of *Trichoderma* spp. in dual cultures against both pathogens, *F. o.* f. sp. *psidii* and *F. solani*. Two species, *T. virens* and *T. viride* were superior in inhibiting the growth of both *Fusarium* spp. *Fusarium* isolates showed intra species variability.

Key words: *Trichoderma* spp., RAPD-PCR, antagonism, *Psidium guajava* L., *Fusarium oxysporum* f. sp. *psidii*, *Fusarium solani*

INTRODUCTION

Guava (*Psidium guajava* L.) is an important fruit crop of subtropical countries. In India, it is grown in almost all of the states. Wilt is the most destructive disease of guava and causes a 5–60% loss in guava production in India. Various pathogens are reported to cause wilt in guava, but *Fusarium oxysporum* f. sp. *psidii* (*F. o.* f. sp. *psidii*) and *F. solani* are more widely reported as causal agents of the disease (Prasad *et al.* 1952; Misra 2006).

Advances in molecular biology techniques have provided the basis for uncovering a virtually unlimited number of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism. The polymerase chain reaction (PCR) technique has created new ways of revealing DNA polymorphisms among closely related genotypes with high sensitivity, via a fast and easy-to-perform protocol. Zimand *et al.* (1994) used RAPD markers obtained from 9 arbitrary primers to distinguish strains of *Trichoderma*. Ten of the strains identified as *T. harzianum* exhibited similarities, and it was possible to distinguish the isolate T-39, used commercially as a biocontrol agent for *Botrytis cinerea*. For efficient taxonomic identification of *Trichoderma* spp., Fujimori and Okuda (1993) examined 74 strains by RAPD profiles and the results were consistent with the morphological, physiological and ecological data, which suggested that the technique can aid to eliminate strains duplicated in a program for microbial selection. Using RAPD Schlick *et al.* (1994) analyzed strains of *T. harzianum* and mutants induced by gamma radiation originated from a wild isolate, The analysis verified that with RAPD it was possible to differentiate all the mutant strains for at least one primer, concluding that the method was valuable for identification and fast differentiation of strains.

The disease is soil-borne and difficult to control by chemicals. The effect of chemicals is also hazardous for the soil and environment. This can be seen when after application and after the effect of the chemicals diminishes, pathogens become more virulent and aggressive (Misra and Pandey 1999). Hence, control of soil borne diseases by the biocontrol method is the best solution. Bioagents multiply in the soil, if proper moisture and organic matter are maintained. Biocontrol is a dynamic process and the frequent application of bioagents is not required. *Trichoderma* sp. has shown its effectiveness towards the control of wilt pathogens of guava (Misra 2006). Culture filtrate and volatile compounds released by these bioagents have also been found effective against different pathogens (Dennis and Webster 1971; Maththeis and Roberts 1992; Tapwal *et al.* 2004; Eziashi *et al.* 2006).

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The aim of the present investigation was to characterize *Trichoderma* spp. isolates using PCR-fingerprinting with RAPD primers (RAPD-PCR method) and to evaluate the ability of *Trichoderma* spp. isolates to suppress the growth of *Fusarium* wilt pathogens of *Psidium guajava* by *in vitro*.

MATERIALS AND METHODS

Isolation of bioagents

Seven isolates of *Trichoderma* spp. were obtained from guava fields. The guava fields which were in different locations (Table 1). These isolates were identified based on taxonomic keys (Raper and Thom 1949; Rifai 1969), pure cultured, and maintained on potato dextrose agar (PDA, Hi-Media), then analyzed for genetic variability. Finally three species isolates of *Trichoderma* obtained from guava field soil, *T. harzianum* (Tri 1), *T. virens* (Tri 2) and *T. viride* (Tri 7) were subjected to *in vitro* bio-control assay.

Genetic analysis

DNA extraction

Pure cultures of the *Trichoderma* isolates were maintained on PDA slants and incubated at 28±2°C for 6 days under controlled temperature. Mycelia were aseptically transferred to flasks of potato-dextrose broth (PDB, Hi-Media) and incubated for 5 days at 28±2°C without shaking. The mycelia were filtered from the liquid medium and total DNA was extracted according to the protocol of Abd-Elsalam *et al*. (2003).

RAPD- primers

Ten oligodecamers OPA 01-5′ CAGGCCCTTC 3′; OPA 02-5′ TGCCGAGCTG 3′; OPA 03-5′ AGTCAGCCAC 3′; OPA 04-5′ AATCGGGCTG 3′; OPA 05-5′ AGGGGTCTTG3′; OPA 06-5′ GCTCCCTGAC 3′; OPA 07-5′ GAAACGGGTG 3′; OPA 08-5′ GTGACGTAGG 3′; OPA 09-5′ GGGTAACGCC 3′; OPA 10-5′ GTGATCGCAG 3′ (Life Technologies, India) were used for RAPD marker studies.

Reactions and conditions of RAPD-PCR

RAPD-PCR reaction was done in Eppendorf Master Cycler in 25 µl reaction volume containing 25 ng genomic DNA, 0.4 µl (5 pmole) primer, 1.5 µl dNTPs (25 mM), 3 µl of 10 X assay buffer with $MgCl_2$ (15 mM), 0.5µl (3 U/µl) of Taq DNA polymerase (Bangalore Genei Pvt. Ltd.). The reaction profile was as follows: denaturation for 5 min at 94°C followed by 35 cycles of 1 min each at 94°C and 35°C, followed by 2 min at 72°C, and a final extension for 5 min at 72°C. PCR products were resolved by horizontal electrophoresis using agarose gel (1.2%) with TAE buffer (1%) containing ethidium bromide.

Cluster analysis

The genetic similarity of isolates was assessed, based on RAPD data, using Jaccard's coefficient (Jaccard 1908). The data was subsequently used to construct a dendrogram with the unweighted pair group method of arithmetical averages (UPGMA) algorithm, as described by Sneath and Sokal (1973) using the NTSYS-software (Rohlf 1998).

***In vitro* biocontrol assay**

Isolation of pathogens

Five isolates each of *F. o.* f. sp. *psidii* (Fop) and *F. solani* (Fs) isolated from wilted guava roots from different guava growing areas of India were used in the present study (Table 2). These isolates were subcultured on potato dextrose agar (PDA, Hi-Media) and incubated at 28±1°C for 6 days. The morphological and cultural characterizations of the cultures grown on PDA were studied and compared with those mentioned by Booth (1971). The reference pure culture of the fungus was also sent to the Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology, I.A.R.I., New Delhi-110 012 for the identification of: *F. solani* [ITCC No. 5208 (F20) and 5212 (F15)] and *F. oxysporum* f. sp. *psidii* [MTCC No. 3326 (F24) and 3327 (F30)] was sent to the Microbial Type Culture Collection for identification. These identified cultures were used as reference species. Pure cultures of the isolates were maintained on PDA slants under controlled temperature at 28±1°C. Pathogenicity of these isolates was also confirmed.

Table 1. The origin of *Trichoderma* spp. isolates used in the present study

No.	Source	Location	Cultural No.	Bio-agent
1.	Rhizosphere of wilted guava plant	Pinjour garden, Punjab , India	Tri 1	*T. harzianum*
2.	Rhizosphere of wilted guava plant	Allahabd, Utter Pradesh, India	Tri 2	*T. virens*
3.	Rhizosphere of wilted guava plant	PAU, Punjab, India	Tri 3	*T. harzianum*
4.	Rhizosphere of wilted guava plant	Kausambi, Utter Pradesh, India	Tri 4	*T. virens*
5.	Rhizosphere of wilted guava plant	Dharwad, Karnataka, India	Tri 5	*T. harzianum*
6.	Rhizosphere of wilted guava plant	Dharwad, Karnataka, India	Tri 6	*T. viride*
7.	Rhizosphere of wilted guava plant	Rewa Agriculture College Rewa, Madhya Pradesh, India	Tri 7	*T. viride*

Table 2. The characteristics of *Fusarium* spp. isolates of guava used in the present study

Isolate No.	Location	Disease severity level	Identification	Spore Size [µm]				Sporulation Conidia		Spore septation of Macro-conidia	Colour of released metabolite in the media
				macro		micro					
				L	W	L	W	macro	micro		
F. oxysporum f. sp. *psidii*											
F10	Chandigarh	50%	Booth, 1971	31.72	4.76	12.49	4.85	+ + +	+	3-5	light brown
F18	Ranchi	100%	Booth, 1971	44.82	4.51	10.05	4.96	++	+ + +	5-7	brown
F24	Kanpur	50%	MTCC 3326	38.61	5.12	13.73	4.69	+ +	+ +	5-7	pale yellow
F30	Unnao	50%	MTCC 3327	39.50	6.87	9.61	8.32	+	+	5-7	pale yellow
F38	Rewa	50%	Booth, 1971	40.88	6.18	9.56	5.48	+	+ +	4-5	dark yellow
F. solani											
F2	West Bengal	100%	Booth, 1971	35.54	10.09	18.16	8.98	+	+	3-4	violet
F12	Chandigarh	50%	Booth, 1971	23.79	7.50	13.76	7.56	+ +	+	3-5	pinkish colour
F15	Ajmer	100%	ITCC 5212	35.62	8.91	16.94	5.91	+	+ +	3-5	pinkish
F20	Puskar	100%	ITCC 5208	36.08	10.35	14.39	6.70	+ +	+ +	3-5	pinkish
F29	Unnao	100%	Booth, 1971	33.27	8.13	14.86	5.66	+ + +	+	3-5	pink yellow

+ little sporulation; + + moderate sporulation; + + + profuse sporulation

Inhibition of pathogenic fungi by bioagents using the dual culture technique

Inhibition of 5 isolates each of *F. o.* f. sp. *psidii* and *F. solani* were evaluated by 3 isolates of *Trichoderma* [*T. harzianum* (Tri 1)], *T. virens* (Tri 2) and *T. viride* (Tri 7) using the dual culture technique (Watts *et al.* 1988). Mycelial discs of 5 mm each of *Fusarium* sp. and all bioagents obtained from actively growing colonies were placed on the two halves of the solidified PDA plates. Plates were incubated at 28±1°C for 6 days and growth of *Fusarium* sp. was recorded. Percentage of inhibition was calculated. Mean PI value of four replicates per isolate was calculated (Grondona *et al.* 1997).

Determination of antifungal properties of culture filtrate of bioagents

Three isolates of *Trichoderma* spp. [*T. harzianum* (Tri 1)], *T. virens* (Tri 2) and *T. viride* (Tri 7) were separately inoculated into 100 ml of potato dextrose broth per conical flask and incubated at 28±1°C for 6 days. After incubation for 6 days, the cultures were filtered through 0.22 mm Millipore filters. The aliquots (2 ml), of these filtrates were mixed with 25 ml of PDA at 45°C. Petriplates were rotated gently so that the culture filtrate properly mixes in the medium. Mycelial discs (5 mm diameter) of the pathogens obtained from actively growing colonies were placed gently in the center of the PDA petriplates. The petriplates were incubated at 28±1°C for 6 days. The growth rate of the pathogens were recorded in 4 replicates by measuring the diameter of colonies. Per cent inhibition was calculated for each replicate (Hutchinson and Cowan 1972).

Determination of the effect of the volatile compound

The bottom part of petriplates containing solidified PDA media were inoculated in the center with 5 mm discs of each of the *Fusarium* sp. isolates and all the 3 bioagents (*Trichoderma* isolates) obtained from actively growing colonies. These plates were placed on each other so that the bottom parts of the plates contained bio-agents and the upper half of the plates contained pathogens. These inverted plates were sealed with parafilm and incubated at 28±1°C for 6 days. The radial growth of the pathogen was measured on the 6th day. The volatile compounds released by bioagents were intended to affect the growth of pathogens. Per cent inhibition was calculated according to Tapwal *et al*. (2004).

RESULTS

RAPD analysis

Ten different (OPA 1 to OPA 10) random primers were tested with DNA samples of *Trichoderma* spp. isolates. Out of twenty RAPD markers tested OPA 01, 03, 05, 09, 11, 15 and 19 which were amplified in all isolates having a product size of 0.564 kbp and 0.125 kbp with OPA 01, 2.322 kbp and 0.564 kbp with OPA 03, 0.564 kbp and 0.125 kbp with OPA 05, 2.027 kbp and 0.564 kbp with OPA 09, 4.361 kbp, 2.322 kbp and 0.564 kbp with OPA 11, 4.361 kbp and 0.125 kbp with OPA 15, 2.027 kbp, 0.564 kbp and 0.125 kbp with OPA 19 respectively along with other polymorphic alleles (Fig. 1). The largest amplified RAPD product was 6.557 kbp and the smallest was 0.125 kbp. The product of 0.564 kbp was amplified with almost all RAPD markers tested. The number of scorable bands for corresponding primers ranged from 1 to 10 with an average of five bands. A total of 248 bands were scored against 7 isolates of the *Trichoderma* spp. A 4.66% polymorphism was also found in individual isolates but this polymorphism was not statistically significant ($p > 0.05$) among the samples. The average similarity per cent, based on amplified RAPD primers, was 61.84%. A dendrogram constructed using the UPGMA method showed two major groups consisting of A) *T. harzianum* and *T. virens* isolates, and B) *T. viride*. It proved that *T. harzianum* and *T. virens* are genetically closely related species, with morphological similarity (Fig. 2).

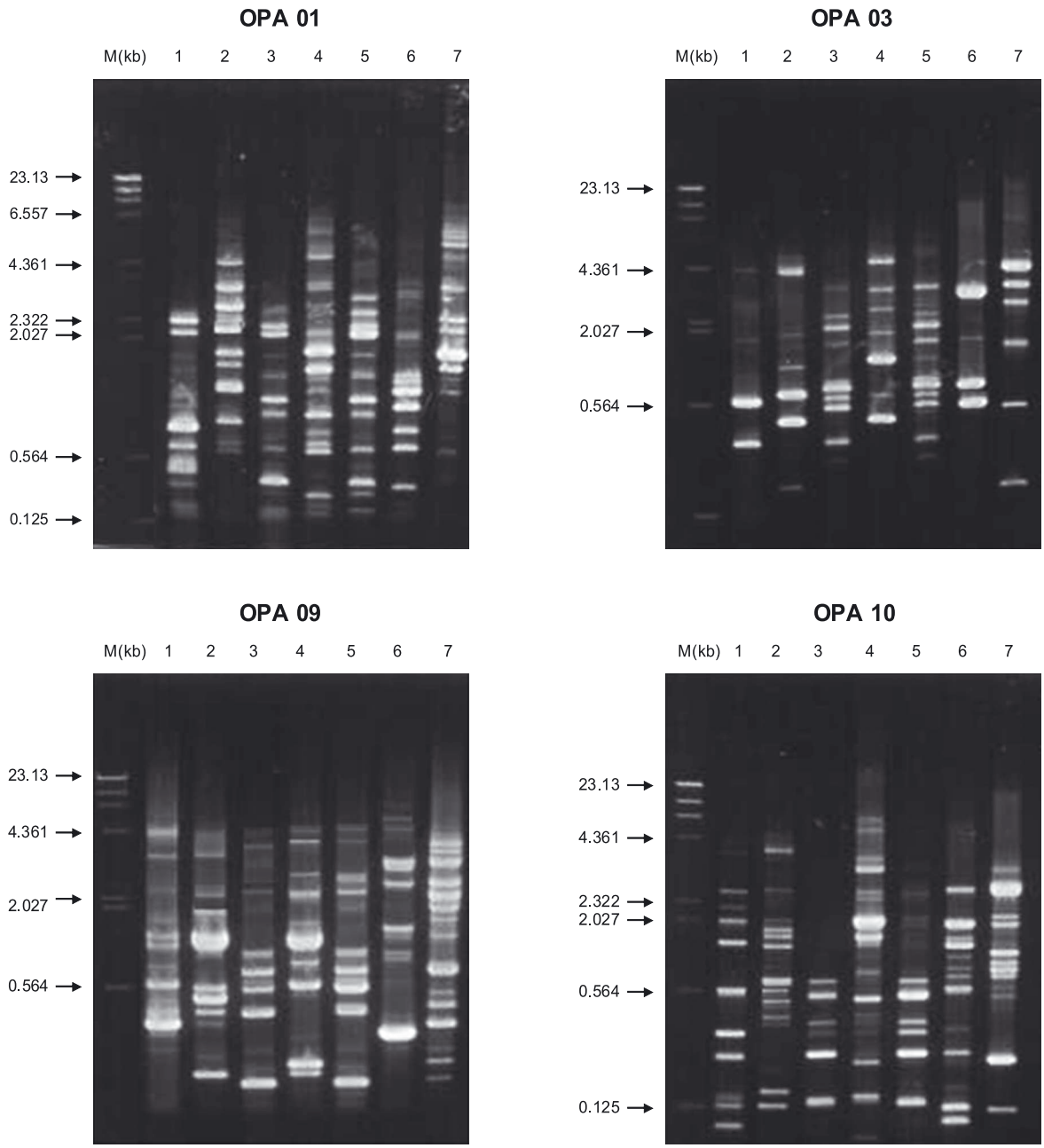

Fig. 1 Banding patterns of *Trichoderma* spp. isolates obtained using RAPD primers OPA 01, 03, 09 and OPA 10; lane 1–7 = *Trichoderma* spp. Isolates (1, 3 and 5 = *T. harzianum*; 2 and 4 = *T. virens*; 6 and 7 = *T. viride*) M – molecular weight size markers (λ DNA treated by Hind III)

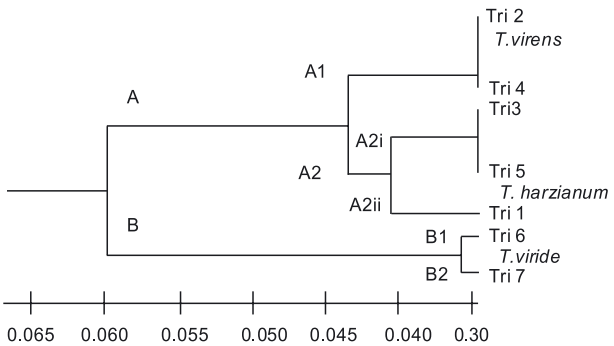

Fig. 2. Dendogram based on an allelic banding pattern obtained from RAPD markers

***In vitro* biocontrol assay**

When three species of *Trichoderma* i.e. *T. harzianum* (Tri 1), *T. virens* (Tri 2) and *T. viride* (Tri 7) were directly evaluated against *F. o.* f. sp. *psidii* and *F. solani* isolates, it was found that *T. harzianum* (Tri 1), *T. virens* (Tri 2) and *T. viride* (Tri 7) showed variable results against the different isolates of *Fusarium oxysporum* f. sp. *psidii*, while Tri 2 was slightly superior against *F. solani* isolates (Table 3).

Tri 7 isolate of *T. viride* was the best of all the isolates of both the *Fusarium* sp. (Table 3). In the case of the volatile compound Tri 2, it was superior for Fop isolates, and Tri 2 and Tri 7 were superior for Fs isolates (Table 3). In general, Tri 1 was less effective compared to Tri 2 and Tri 7; however, Tri 7 seemed to be better, compared to Tri 1. The direct inhibition by culture was more effective than toxic metabolites released by isolates of *Trichoderma* spp. in culuture filtrate or action of volatile compound.

Table 3. *In vitro* evaluation of *Trichoderma* sp. (Tri) isolates, culture filtrate and volatile compound against *F. oxysporum.* f. sp. *psidii* (Fop) and *F. solani* (Fs) isolates causing *Fuarium* wilt of guava

F. o. f. sp. *psidii* isolates	Per cent inhibition of *Fop* in presence of Tri isolates			*F. solani* (Fs) isolates	Per cent inhibition *Fs* in presence of Tri isolates		
	T. harzianum (Tri 1)	*T. virens* (Tri 2)	*T. viride* (Tri 7)		*T. harzianum* (Tri 1)	*T. virens* (Tri 2)	*T. viride* (Tri 7)
Dual culture technique							
Fop 10	63.44b	64.83b	68.96a	Fs 2	58.39c	67.15a	65.69b
Fop 18	61.81c	67.36a	65.97b	Fs 12	61.31c	65.69b	65.69b
Fop 24	60.99c	65.24b	65.24b	Fs 15	59.57d	68.08a	62.41c
Fop 30	67.13a	65.73b	65.03b	Fs 20	66.67a	62.41d	65.25b
Fop 38	66.19a	63.31c	63.30c	Fs 29	64.34b	64.33c	67.14a
CD at (p = 0.05)	1.207	1.031	1.092	CD at (p = 0.05)	0.940	1.136	0.928
Culture filtrate							
Fop 10	35.85a	35.85	41.38a	Fs 2	32.11b	32.11b	40.14a
Fop 18	36.11a	35.42	39.58b	Fs 12	32.11b	31.38b	37.95b
Fop 24	34.04b	34.75	35.46c	Fs 15	34.75a	34.75a	36.88c
Fop 30	34.96b	34.96	41.95a	Fs 20	35.46a	34.75a	36.88c
Fop 38	33.09c	33.81	40.28b	Fs 29	35.66a	35.66a	38.46b
CD at (p = 0.05)	1.414	N.S.	1.163	CD at (p = 0.05)	0.929	0.917	1.053
Volatile compounds							
Fop 10	28.27a	33.79a	28.96b	Fs 2	26.27b	27.73c	27.73c
Fop 18	28.47a	32.64b	33.33a	Fs 12	24.81c	29.19b	27.73c
Fop 24	26.24b	31.91b	29.78b	Fs 15	30.49a	29.78b	31.91a
Fop 30	23.77c	32.17b	32.86a	Fs 20	25.53c	29.79b	30.49b
Fop 38	20.86d	30.21c	27.34c	Fs 29	26.57b	32.86a	32.17a
CD at (p = 0.05)	1.098	1.022	1.150	CD at (p = 0.05)	0.833	1.204	0.957

a, b, c, d – statistically analyzed at par values of the inhibition per cent

DISCUSSION

Molecular markers offer a means of constructing quality control tests that are essential throughout the developmental processes of these biocontrol agents. In the case of *Trichoderma* spp., the quality control test is being evaluated through the production of polymerase chain reaction (PCR) fingerprints by use of semi-random primers designed to primarily target intergenic, more variable areas in the genome (Dubey and Suresh 2006).

Arisan-Atac *et al.* (1995) studied 11 strains of *T. viride*, 2 strains of *Hypocrea rufa* and 9 other species of *Trichoderma* with relation to the RAPD profile and their ability for controlling *Cryphonectria parasitica* through pairing *in vitro*. Gomez *et al.* (1997) analyzed the RAPD profiles of strains of *T. harzianum* and classified them in different groups according to their capacity for control of plant pathogenic fungi. Muthumeenakshi *et al.* (1998) genetically characterized 15 strains of *T. harzianum*, aggressive for edible mushrooms in the United States and England, using RAPD. The strains were designated "*T. harzianum* group 4", presenting a high homogeneity degree. Comparison of the molecular data of group 4 with group 2 (the causal agent of the epidemic green mould in industrial mushrooms in England) indicated that the isolates of *T. harzianum* group 4 were different from that of group 2. Most recently Gopal *et al.* (2008) investigated RAPD markers to estimate the genetic variation among 17 isolates of *Trichoderma.* and found them genetically similar showing a 91.8% polymorphism, which corroborates with the observations of our investigation. The RAPD technique

was found to be advantageous over other molecular techniques for the genetic characterization of *Trichoderma* spp. due to the possibility of detecting DNA polymorphisms for very closely related strains (Bardakci 2001; Misra and Gupta 2009).

Many studies in the past have proved that *Trichoderma* spp. was a potential biocontrol agent of several soil borne pathogens (Chet and Inbar 1994). Kumar *et al.* (2007) tested three *Trichoderma* spp. i.e. *T. virens*, *T. viride* and *T. harzianum* against *F. moniliforme* var. *subglutinans* and found them effective. Isolates of *Trichoderma* spp. grew considerably faster than pathogenic *Fusaria* under the same conditions. The rapid growth gives *Trichoderma* an added advantage in competition for the space and nutrient with plant pathogenic fungi, even before it develops its arsenal of mycotoxins (Simon and Sivasithaparam 1988). *Trichoderma* was also found to control many crop diseases (Singh and Singh 2004; Kidwai *et al.* 2006). Our studies clearly indicated that *Trichoderma* spp. is a suitable antagonistic agent against *F. o.* f. sp. *psidii* and *F. solani* isolates.

Gupta *et al.* (2003) reported responses of different isolates of *T. harzianum* and *T. viride* against *F. udum in vitro* and *in vivo*, which supports our findings. The consortium of bioagents shall be further investigated. It was concluded that RAPD markers can easily designate and characterize *Trichoderma* spp. both at the isolate as well as at the species level. In the present investigation OPA 01, 03, 09 and OPA 10 RAPD primers were found to be informative in genetic variability studies for isolates of *Trichoderma* spp.

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POLISH SUMMARY

RAPD-PCR IZOLATÓW GRZYBA *TRICHODERMA* ORAZ ANTAGONIZM *IN VITRO* W STOSUNKU DO PATOGENÓW WYWOŁUJĄCYCH FUZARYJNY UWIĄD W UPRAWACH *PSIDIUM GUAJAVA* L.

Praca dotyczy analizy losowej amplifikacji polimorfizmów DNA (RAPD)-PCR siedmiu gatunków izolatów grzyba z rodzaju *Trichoderma* oraz ich antagonizmu *in vitro* w stosunku do sprawców uwiądu roślin *Psidium guajava* L. takich jak grzyby z gatunku *Fusarium oxysporum* f.sp. *psidii* oraz *Fusarium solani*. Sośród 10 testowanych olinukleotydów (OPA1 – OPA10), siedem markerów OPA-1, 3, 5 ,7, 8 i 10 efektywnie różnicowało izolaty grzyba *Trichoderma* i dawało powtarzalny wzór rozdziału DNA. Całkowitą liczbę 248 pasm otrzymano w przypadku wymienionych markerów wraz z wielkością 61,84% podobieństwa pomiędzy siedmioma izolatami grzyba *Trichoderma.* Spośród siedmiu izolatów grzyba *Trichoderma* najbardziej efektywne przeciwko patogenom *Fusarium oxysporum* f. sp. *psidii* i *Fusarium solani* były gatunki: *T. harzianum*, *T. virens* oraz *T. viride*. Procent inhibicji był najwyższy po bezpośrednim zastosowaniu grzyba *Trichoderma* spp. zarówno w przypadku patogena *F. oxysporum* f. sp. *psidii* jak też *F. solani*. Gatunki *T. virens* i *T. viride* wykazywały silniejsze właściwości hamowania wzrostu obydwóch gatunków *Fusarium* spp. Izolaty *Fusarium* charakteryzowała wewnątrz gatunkowa zmienność.