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Studies of genetic polymorphism in the isolates of *Fusarium solani*

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Abstract

Fusarium solani is the important pathogen causing wilt disease of guava in India. In the present investigation six representative isolate of *Fusarium solani,* collected from different places of India were subjected to analysis of genetic variability in terms of Carboxylesterases isozyme pattern and DNA polymorphism using RAPD-PCR. Pattern of Carboxylesterase revealed a similar isozyme cluster in the isolate namely, Allahabad (isolate-3), Faizabad, (isolate-4), Unnao (isolate-5) and Lucknow (isolate-6). Similar results were obtained when 10 randomly amplified polymorphic DNA markers (OPA1-OPA10) tested in the genome of F*usarium solani* and grouped on basis of obtained allelic data. This pattern of genetic variability in the isolate was also supported by the analysis of the similarity indices and UPGMA dendrogram.

Keywords: *Fusarium solani; Carboxylesterase; RAPD-PCR*

Abbreviations: DNA_deoxyribonucleic acid; RAPD-PCR_random amplified polymorphic deoxyribonucleic acid polymerase chain reaction; UPGMA_unweighted pair group method with arithmetic mean.

Introduction

Guava (*Psidium guajava* L.) is an important fruit crop and widely grown under subtropical and tropical climate. One of the major threats to guava cultivation is wilt disease. Wilt is reported to be caused by several pathogens but the most important fungus reported is *Fusarium solani* (Prasad *et al*., 1952; Chattopadhyaya and Bhattachariya, 1968; Misra and Pandey, 1996; Misra, 2006). Varied control measures including the chemicals and other non-chemical approaches applied against the control of *F. solani* have modified and resulted in heterogeneity among the isolate (Misra, 2006; Misra and Gupta, 2007). Hence, to precisely trace the genetic variability in the expanding geographical distribution of the *F. solani* isolates of guava an accurate and rapid identification of pathogens is necessary for appropriate management of plant diseases. In particular, genetic characterization of pathogenic variants of the plant pathogens prevalent in an area is required for efficient management and increase crop productivity. Molecular phylogenetic analyses have helped to clarify ambiguities in traditional classification systems of *Fusarium*. Genome organization and molecular mechanisms of pathogenicity are still not well understood in many *Fusarium* species (Michniewicz *et al*., 1999). Genetic distances among

Table1. Isolates and their cultural details collected from different guava growing areas

Culture	Location	Pathogenocity	Identification (Booth, 1971)	Spore Size (µm)				Septation of (Macroconidia)	Metabolite (colour) in culture
				Macroconidia		Microconidia			
				L	W	L	W		
F1	Rewa	100%	*Fusarium solani*	35.54	10.09	15.16	8.98	3-4	Reddish
F2	Ranchi	80%	*Fusarium solani*	33.19	8.55	15.76	9.56	3-5	Pinkish
F3	Allahabad	100%	*Fusarium solani*	35.62	8.91	16.94	5.91	3-5	Brownish
F4	Faizabad	100%	*Fusarium solani*	36.78	11.32	12.39	6.05	3-5	Yellowish
F5	Unnao	100%	*Fusarium solani*	33.27	8.13	14.86	5.66	3-5	No colour
F6	Lucknow	100%	*Fusarium solani*	36.07	10.13	15.44	7.42	3-4	Reddish

strains have been evaluated through analyses of pathogenicity, isozymes and molecular markers (Bateman *et al*., 1996; O'Donnell *et al*., 1996; Szecsi et. al., 1996). As conventional methods of identifying *Fusarium* spp. usually require time-consuming and laborious pathogenicity and vegetative compatibility analysis, therefore random amplification of polymorphic DNA (RAPD) analysis (Williams *et al*., 1990) has been applied widely in the detection and genetic characterization of phytopathogenic fungi including *Fusarium* spp. (Kim *et al*., 1993; Miller, 1996; Brown, 1998; Gulino *et al*., 2003).

Genetic characterization of *F solani* isolates causing wilt disease to the guava plants is important for the efficient management of *Fusarium* wilt and towards wilt resistant breeding line programme of guava cultivars. In this paper, we describe Carboxylesterase and RAPD-PCR patterns to illustrate their use as molecular markers in six representative isolate of *F.solani,* collected from different guava wilt affected places of India. This is the first report for the diversity analysis of *F. solani* isolates of guava from India. Since these technique has not been used in case of *F. solani* isolates of guava and may be reliable and efficient for the study of genetic variation in Indian *F. solani* isolates, hence it has been attempted and results obtained are represented in this communication.

Materials and methods

Collection of fungal isolates, pathogenicity assay

Isolate of *Fusarium solani* were collected from six different localities (isolate-1: Rewa, isolate-2: Ranchi, isolate-3:Allahabad, isolate-4: Faizabad, isolate-5: Unnao, isolate-6:Lucknow) of Nortern parts of India (Table 1). All isolates were grown as single spore culture on potato-dextrose agar (PDA, Hi Media) and isolated cultures were identified to *F. solani* as per method described by Booth (1971). The reference cultures were also sent to Indain Type Cultue Collection (ITCC).

Pathogenicity tests with six *Fusarium solani* isolates were performed on the guava seedlings of Allhabad safeda (since Allhabad safeda has no known resistance to *Fusarium* wilt) under greenhouse condition. Stem hole inoculation technique was employed in order to produce the typical symptoms of wilt and for a isolates of *Fusarium solani* three replicate were maintained. The temperature ranged from 22 to 32 °C during the test. Pathogenicity index was scored as described previously by Misra and Pandey (2000).

DNA extraction

Pure cultures of the isolates were maintained on PDA slants and incubated at $28^0 \pm 2^0$C for 6 days under controlled temperature and mycelia were aseptically transferred to flasks of potato-dextrose broth (PDB, HiMedia) and incubated for 5 days at $28^0 \pm 2^0$C without shaking. The mycelia were filtered from liquid medium through four cheesecloth layers. Total DNA was extracted according to the protocol of Abd-Elsalam *et al*. (2003).

Genetic analysis

Isoenzyme assay

The fresh mycelia were used Carboxylesterase analysis. The mycelia were dissected out and used for the enzyme extraction and gel electrophoresis by non-denatured polyacrylamide gel as described by Ho *et al*. (1985). Each lane of enzyme profiles was Subjected to gel documentation system (UVIPro, Germany).

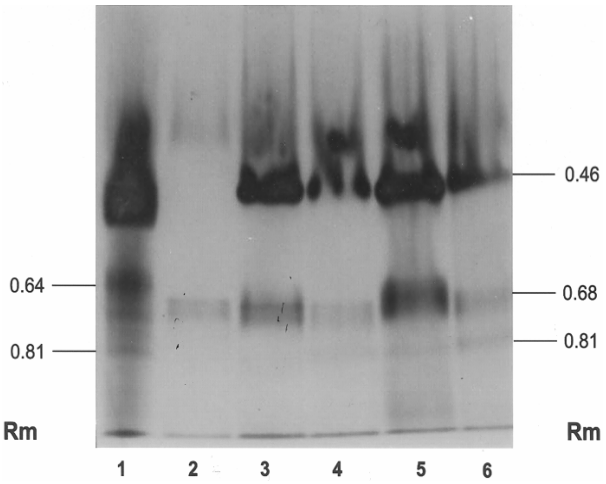

Fig 1 a. Native PAGE analysis of esterase isozymes of mycelial samples of *F. solani*

The dendrogram analysis was carried out using Bio profil 1D software.

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' (custom synthesized from Life Technologies, India) were used for RAPD marker studies.

Reactions and Conditions of RAPD-PCR

RAPD primer sets were used in Eppendrowf Master Cycler. RAPD-PCR was performed in 25 µl reaction volume containing 25 ng genomic DNA, 0.4 µl (5 pmole) primer, 1.5 µl dNTPs (25mM), 3µl of 10 X assay buffer with $MgCl_2$ (15mM), 0.5µl (3U/µl) of Taq DNA polymerase (Bangalore Genei Pvt.Ltd.). DNA was amplified by Eppendrowf Master Cycler programme to provide first denaturation for 5 min at 94^0C followed by 35 cycles of 1 min each at 94^0C and 35^0C followed by 2 min at 72^0C and final extension for 5 min at same temperature. 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 Carboxylesterase analysis and RAPD, by using Jaccard's coefficient (Jaccard, 1908). The data was subsequently used to construct a dendrogram using the unweighted pair group method of arithmetical averages (UPGMA) algorithm, as described by Sneath and Sokal (1973). All the computations were carried out using the NTSYS-software (Rohlf, 1998).

Results

Pathogenicity assay

All six isolates of *Fusarium solani* showed variability as they evaluated for their cultural and morphological assay and found to be pathogenic to guava seedlings of Allahabad safeda when tested under greenhouse conditions. Control plants did not develop any symptoms.

Isoenzyme analysis

The pattern of Carboxylesterase isozyme profile of *F. solani* in this study showed characteristics of genetic variability of each isolate. Fig 1a shows native PAGE analysis of Carboxylesterase isozymes of mycelial samples of *F. solani*. The isozyme pattern showed one slow moving esterase fraction at Rm 0.46 and three fast moving esterase fractions at Rm 0.64, 0.68 and 0.81. The slow moving esterase fraction was observed in all the isolate except the isolate-2 (Ranchi). The fast moving esterase fractions, Rm 0.68 and Rm 0.81, were found in all the isolate with differential staining activity. The fraction electrophoresis data Rm 0.64 was recorded to be unique to isolate-1 (Rewa). The similarity index analysis of all the Carboxylesterase fractions among various isolate resulted in the generation of 100% similarity among the isolate-4 to isolate–6 (isolate of Faizabad, Unnao and Lucknow; Table 2).UPGMA dendrogram also revealed these three isolate in one cluster, and Ranchi (isolate-2) and Rewa (isolate-1) were separated from the cluster.

RAPD analysis

Ten different (OPA-1 to OPA-10) random primers were tested with DNA samples isolated from various isolate. Of the primers used OPA-03 revealed a polymorphic pattern that enabled to distinguish various isolate of *F. solani* with product size of 350

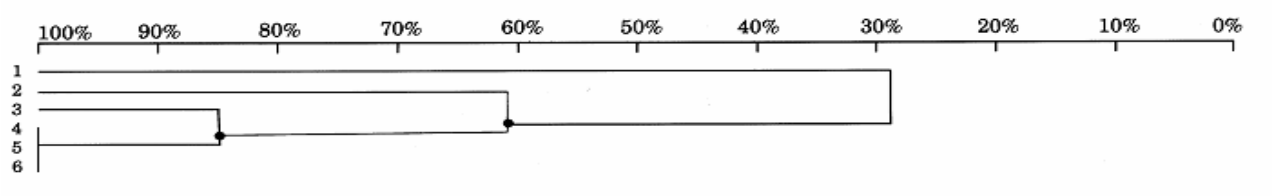

Fig 1b. Dendrogram with Homology Coefficient %: 0.0 (UPGMA) (Lane1-Rewa isolate; Lane 2-Ranchi isolate; Lane 3-Allahabad isolate; Lane 4-Faizabad isolate; Lane 5-Unnao isolate; and Lane6-Lucknow isolate)

Table 2. Similarity index for the Electreophoretic profile of esterases

	1	2	3	4	5	6
1	1.000					
2	0.250	1.000				
3	0.250	0.670	1.000			
4	0.440	0.570	0.860	1.000		
5	0.440	0.570	0.860	1.000	1.000	
6	0.440	0.570	0.860	1.000	1.000	1.000

Table 3. Similarity index of *F. solani* based on RAPD profiles

	1	2	3	4	5	6
1	1.000					
2	0.200	1.000				
3	0.400	0.000	1.000			
4	0.400	0.000	1.000	1.000		
5	0.200	0.000	0.750	0.750	1.000	
6	0.400	0.000	1.000	1.000	0.750	1.000

bps and 500 bps. Similarity indices and dendrogram analysis were also computed and presented (Fig 2a, b; Table 3). All the scorable fragments ranged from 100 to 1500 bps. Like isozyme analysis, RAPD results were also revealed the close relationship of isolate-3 to isolate-6 (isolate of Allahabad, Faizabad, Unnao and Lucknow).

Discussion

Pathogenicity tests are the only means of determining the pathological effect of fungal strains present in diseased plants or in soil samples. The pathogenicity study showed that the behavior of *Fusarium solani* isolates was homogeneous, with no variations in virulence. Studies of intra- and extracellular isozyme banding patterns (esterase, superoxide dehydro-genase, malate dehydrogenase, dihydrolipo- amide dehydrogenase and succinate dehydrogenase) of *Fusarium oxysporum* was done by Skovgaard and Rosendahl (1988) which corroborates our findings that isoenzyme can be successfully employed for genetic variability analysis of *F. solani* isolates of guava wilt. Yli-Mattila *et al*. (1996) work on differences in patterns of isozyme and RAPD-PCR polymorphisms in isolates of *Fusarium avenaceum* showed that amongst eight enzymes analysed clear isozyme polymorphism was detected in five enzymes, which could be grouped into 20 different electrophoretic phenotypes, and three main groups at the similarity level of 70% in unweighted pair group method with arithmetic average (UPGMA) analysis. Results indicate that the extent of isozyme and RAPD-PCR polymorphisms found in *Fusarium* strains potentially provides a method for identifying the fungi both at strain and species level. Different workers (Hyun *et al*., 1998; Migheli *et al*., 1998; Ibrahim and Nirenberg, 2000; Jana *et al*., 2003) have grouped *Fusarium* spp population from different plant host by using RAPD analysis and suggested that RAPD markers can be a quick and reliable alternative for differentiating isolates of *Fusarium* spp. into their respective pathogenicity group which corroborate the results of our study. RAPD applied to fungal studies would be useful in providing markers for identification purpose, furthermore revealed polymorphisms within reference isolates of *Fusarium solani* and established DNA fingerprints useful for genetic characterization and specific identification of *F. solani* isolates of guava. In this study, we assessed the suitability of Carboxylesterases isozyme pattern and RAPD techniques for rapid molecular characterization of *Fusarium solani* isolates.

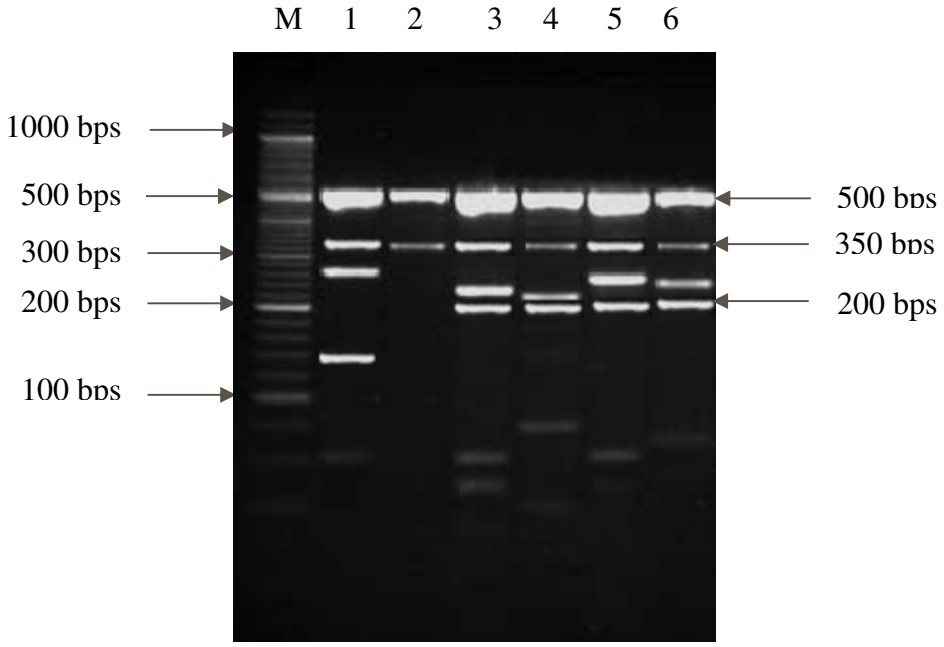

Fig 2a. Random amplified polymorphic DNAs generated by the Primer OPA-03 using genomic DNA of *F. solani* of different isolate (Arrow head indicates the isolate specific DNA fragments).

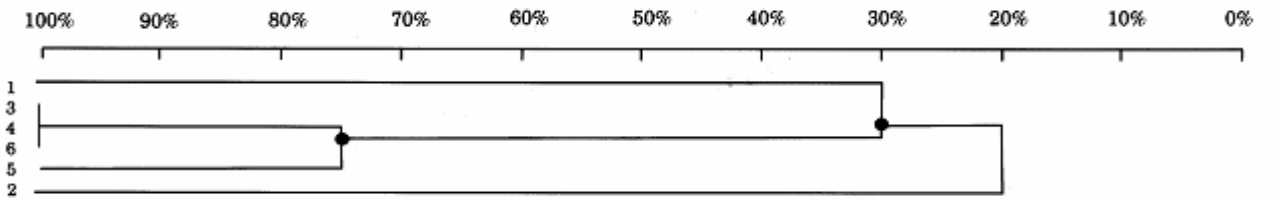

Fig 2b. *Dendrogram with Homology Coefficient %: 0.0 (UPGMA)[Lane M-20 bp Molecular weight standard marker; Lane 1-Rewa isolate; Lane 2-Ranchi isolate; Lane 3-Allahabad isolate; Lane4-Faizabad isolate; Lane 5-Unnao isolate; and Lane 6-Lucknow isolate].*

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