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EXPERIMENTAL ARTICLES

Comparative Molecular Analysis of *Fusarium solani* Isolates by RFLP and RAPD[1, 2]

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Abstract—*Fusarium solani* is an important pathogen causing wilt disease of guava in India. In this work, we analyzed seven representative isolates of *F. solani*, collected from different places of India, by restriction fragment length polymorphism (RPLP) using *Hind*III or *Dra*I restriction endonucleases and random amplified polymorphic DNA (RAPD). Pattern of restriction enzyme revealed a similar restriction cut type cluster in the isolate namely, Allahabad (isolate-3), Faizabad (isolate-4), Unnao (isolate-5) and Lucknow (isolate-6) region, while other cluster was consist of isolate from Ranchi (isolate-2) and Ludhiana (isolate-7). Slightly variable results were obtained when 10 randomly amplified polymorphic DNA markers (OPA01–OPA10) tested in the genome of *Fusarium solani* and grouped on basis of obtained allelic data. RAPD fingerprinting showed a higher variability than RFLP, and each isolate had a unique electrophoretic pattern with five of the ten primers used. Our results show that RAPD much efficient to distinguish between all *F. solani* isolate tested.

Keywords: *F. solani*, RAPD, RFLP.

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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* [1–4]. 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 [5]. 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 [6]. Genetic distances among strains have been evaluated through analyses of pathogenicity, restriction enzyme pattern and molecular markers [7, 8]. In recent years, much progress has been made in the development of molecular tools to identify fungi, such as restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). PCR-RFLP technique that enables easy and rapid identification of the fungi residing in the three phylogenetic clades of *F. oxysporum* defined by O'Donnell et al. [9]. 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 [10] has been applied widely in the detection and genetic characterization of phytopathogenic fungi including *Fusarium* spp. [11–14]. 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 work, we analyzed seven representing isolates of *F. solani*, collected from different guava growing major wilt affected areas of India by a combination of methods for genetic differentiation, including RFLP and RAPD, hence it has been attempted and results obtained are represented in this communication.

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Table 1. *Fusarium solani* isolates and their cultural details collected from different guava growing areas

Culture no.	Location	Pathogenocity	Identification (Booth, 1971)	Spore Size, µm				Septation of (Macroconidia)	Metabolite (colour) in culture
				Macroconidia		Microconidia			
				L	W	L	W		
1	Rewa, India	100%	*Fusarium solani*	35.54	10.09	15.16	8.98	3–4	Reddish
2	Ranchi, India	80%	*Fusarium solani*	33.19	8.55	15.76	9.56	3–5	Pinkish
3	Allahabad, India	100%	*Fusarium solani*	35.62	8.91	16.94	5.91	3–5	Brownish
4	Faizabad, India	100%	*Fusarium solani*	36.78	11.32	12.39	6.05	3–5	Yellowish
5	Unnao, India	100%	*Fusarium solani*	33.27	8.13	14.86	5.66	3–5	No colour
6	Lucknow, India	100%	*Fusarium solani*	36.07	10.13	15.44	7.42	3–4	Reddish
7	Ludhiana, India	50%	*Fusarium solani*	32.50	9.25	12.30	6.50	2–3	Yellowish

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 and isolate-7: Ludhiana) of Northern parts of India (Table 1). All isolates were grown as single spore culture on potato-dextrose agar (PDA, HiMedia) and isolated cultures were identified to *F. solani* as per method described by Booth [15]. The reference pure culture of the fungus was also sent to Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology, I.A.R.I., New Delhi-110 012 for identification. These identified cultures were used as reference culture and thus, *Fusarium* sp. under study were identified and confirmed. Pure cultures of the isolates were maintained on PDA slants under controlled temperature (Table 1).

Pathogenicity tests with seven *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 was from 22 to 32°C during the test. Pathogenicity index was scored as described previously by Misra and Pandey [16].

DNA Extraction

Pure cultures of the isolates were maintained on PDA slants and incubated at 28 ± 2°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 ± 2°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. [17].

Genetic Analysis

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'-AGGGGTCTTG-3'; 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 (25 mM), 3 µl of 10 X assay buffer with $MgCl_2$ (15 mM), 0.5 µl (3 U/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°C followed by 35 cycles of 1 min each at 94°C and 35°C followed by 2 min at 72°C 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 RFLP and RAPD analysis, by using Jaccard's coefficient [18]. 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 [19]. All the computations were carried out using the NTSYS-software [20].

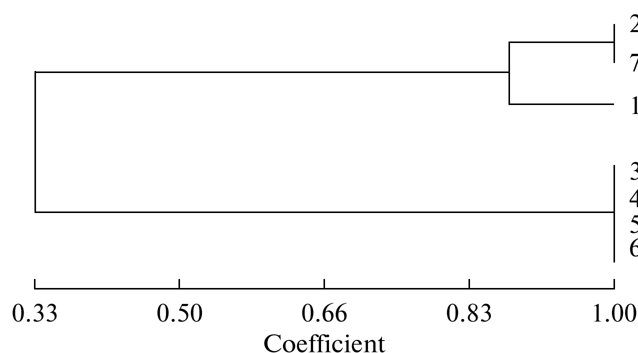
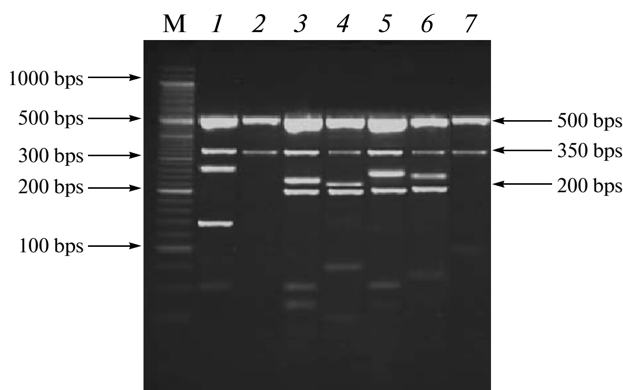


Fig. 1. Dendrogram inferred from the RFLP profiles obtained by digestion of total DNA of *F. solani* isolates with restriction endonucleases *Hind*III or *Dra*I.



1–7 = F. solani isolates,
M = Molecular wt. DNA Ladder (Life Technologies)

Fig. 2. Random amplified polymorphic DNAs generated by the Primer OPA03 using genomic DNA of *F. solani* of different isolate (Arrow head indicates the isolate specific DNA fragments).

RFLP Fingerprinting

Genomic DNA isolated as described above was digested with *Hind*III or *Dra*I (Invitrogen) and submitted to agarose gel (1.2%) electrophoresis. Analyses were performed as described for RAPD fingerprinting.

RESULTS AND DISCUSSION

All seven 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 (Table 1).

RFLP Fingerprinting Analysis

The total DNA restriction patterns of the *F. solani* isolates were used to construct a dendrogram, which clustered them into three groups (Fig. 1 and Table 2). The *Dra*I or *Hind*III restriction profiles of Ranchi (isolate-2), Ludhiana (isolate-7) were identical, while those of Faizabad, (isolate-4), Unnao (isolate-5), Lucknow (isolate-6), had identical patterns with *Dra*I, but *Hind*III produced two different bands (Table 2). However, analysis of their electrophoretic profiles positioned these strains together with Allahabad (isolate-3) and Faizabad, (isolate-4), forming a cluster with a similarity coefficient of 100%. *F. solani* isolate of Rewa region (isolate-1) had a distinct electrophoretic pattern and was allocated into a separate group, closer to the Ranchi/Ludhiana cluster. These results indicate that, as far as the separation of the *F. solani* isolate is concerned, restriction length polymorphism has a limited potential.

Table 2. Genotypic characterization of *F. solani* as revealed by RAPD and RFLP profiling analysis

F. solani isolate no.	RAPD pattern[a]	RFLP pattern[b]
2	aaaaaaaaaa	aa
7	bbbbbbbbbb	aa
1	cccccccccc	bb
3	ddddddddd	cc
4	deeeeeeeee	cd
5	efffffffff	ce
6	fggggggggg	ce

Note: [a] RAPD patterns obtained with different primers. Each letter defines a common pattern for primers 1 to 10, respectively.
[b] RFLP patterns obtained with restriction endonucleases *Dra*I and *Hind*III, respectively. Each letter defines a com mon pattern.

RAPD Analysis

Ten different (OPA01 to OPA10) random primers were tested with DNA samples isolated from various isolate. Genomic diversity of the *F. solani* isolates was also investigated by random amplified polymorphic DNA (RAPD) analysis. Each of the ten primers used generated electrophoretic DNA patterns for the isolates studied (Table 2). OPA03 revealed a unique banding pattern that enabled to distinguish the isolate of *F. solani* with product size of 350 and 500 bps, respectively. All the scorable fragments ranged from 100 to 1500 bps (Fig. 2). The analysis of these patterns produced highly congruent DNA fingerprint clustering, in overall agreement with the RFLP results (Figs. 1, 3). With the exception of primer 1, all primers produced unique patterns for Ranchi (isolate-2) and Ludhiana (isolate-7), allowing unequivocal differentiation of these isolates. As with RFLP, analysis of the RAPD fingerprinting patterns revealed three main clusters of isolates (Figs. 1, 3) with a similarity level of approximately 39%. Cluster I was formed by Ranchi (isolate-2), Ludhiana (isolate-7), with a similarity

level of 65%. Isolate Rewa (isolate-1) was located in a separate branch, closer to Ranchi (isolate-2), Ludhiana (isolate-7), but with a similarity level of 40%. Isolates Allahabad (isolate-3), Faizabad, (isolate-4), Unnao (isolate-5) and Lucknow (isolate-6) were located in a separate cluster, occupying distinct positions in the dendrogram and forming two subgroups, one cluster comprising isolates Allahabad (isolate-3) and Faizabad, (isolate-4) with 72% similarity and second cluster comprising isolates Unnao (isolate-5) and Lucknow (isolate-6) with 65% similarity, respectively. Cluster analysis of RAPD profiles supported the differences noted by visual observation of the electrophoretic profiles (data not shown). Furthermore, the profiles obtained by RAPD showed a higher level of variability than those obtained by RFLP, since, using primers 1 to 10, all isolates could be distinguished by at least one band (Table 2). These results show that RAPD is the most sensitive and convenient method tested to unequivocally identify *F. solani* isolates using whole genomic DNA.

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. Thirty-two isolates of *Fusarium* species, obtained from wilted Welsh onion were subjected to molecular characterization using five restriction enzymes (*Rsa*I, *Hin*fI, *Hae*III, *Scr*FI, and *Msp*I) and the phylogenetic analysis permitted the discernment of the three *Fusarium* species [21]. Yli-Mattila et al. [22] 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 [23–26] 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 restriction enzyme cut pattern and RAPD techniques for rapid molecular characterization of *Fusarium solani* isolates. In summary,

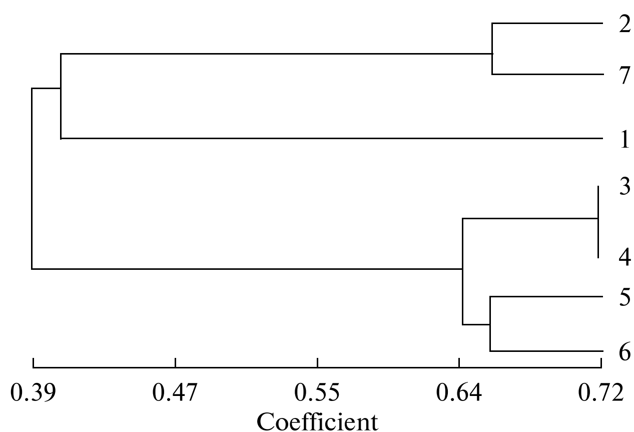


Fig. 3. Dendrogram inferred from RAPD profiles of the *F. solani* isolates. Similarities were calculated using Dice's coefficient, and clustering was achieved by UPGMA.

the molecular tools evaluating using RAPD allowed distinction of the seven *F. solani* isolate studied more efficiently than RFLP and RAPD profiling tested found to be very reliable. This finding agrees with the observation that the DNA sequences are highly conserved among the species and potentially useful to distinguish between distantly related organisms [27]. Also, RAPD utilizes fragment amplification of the whole genome, therefore being well suited to detect differences between closely related organisms.

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