Journal of Microbiology (2013) Vol. 51, No. 4, pp. 405–412
DOI 10.1007/s12275-013-2638-3

Molecular Detection and Genotyping of *Fusarium oxysporum* f. sp. *psidii* Isolates from Different Agro-Ecological Regions of India

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(Received November 19, 2012 / Accepted March 22, 2013)

Twenty one isolates of *Fusarium oxysporum* f. sp. *psidii* (Fop), causing a vascular wilt in guava (*Psidium guajava* L.), were collected from different agro-ecological regions of India. The pathogenicity test was performed in guava seedlings, where the Fop isolates were found to be highly pathogenic. All 21 isolates were confirmed as *F. oxysporum* f. sp. *psidii* by a newly developed, species-specific primer against the conserved regions of 28S rDNA and the intergenic spacer region. RAPD and PCR-RFLP were used for genotyping the isolates to determine their genetic relationships. Fifteen RAPD primers were tested, of which five primers produced prominent, polymorphic, and reproducible bands. RAPD yielded an average of 6.5 polymorphic bands per primer, with the amplified DNA fragments ranging from 200–2,000 bp in size. A dendrogram constructed from these data indicated a 22–74% level of homology. In RFLP analysis, two major bands (350 and 220 bp) were commonly present in all isolates of *F. oxysporum*. These findings provide new insight for rapid, specific, and sensitive disease diagnosis. However, genotyping could be useful in strain-level discrimination of isolates from different agro-ecological regions of India.

Keywords: *Psidium guajava* L., RAPD, RFLP, *Fusarium oxysporum* f. sp. *psidii*, diagnostic, genotype, ITS

Introduction

Guava (*Psidium guajava* L.), which is grown under tropical and subtropical conditions, is one of the major fruit crops of India. It is commercially cultivated in more than 60 countries (Rajan *et al*., 2007). Presently, guava is being grown all over the sub-tropical and tropical regions of the world because of its high dietary value and also its good flavour. Guava fruit contains high amounts of Vitamins A, B1 (Thiamin), B2 (Riboflavin), and vitamin C. In India, it is grown on an area of 2.05×10^5 hectares with a total annual production of 2.46×10^6 MT and an average productivity of 12.0 MT/ha during 2010-11 (NHB, 2012). During the last decade, fruit production is declined significantly. In addition to other factors, diseases contributed significantly toward lowering guava fruit production.

A number of pathogens are reported to cause guava wilt, but the most common fungus associated with wilt disease is *Fusarium oxysporum* f. sp. *psidii* (Prasad *et al*., 1952; Chattopadhyaya and Bhattachariya, 1968; Misra and Pandey, 1996; Misra, 2006; Mishra *et al*., 2013). *Fusarium* exists saprophytically, but some are well-known for inducing wilt (O' Donnell *et al*., 2000; Jurgenson *et al*., 2002; Schroers *et al*., 2005). There is an urgent need to develop assay methods for rapid molecular detection of *F. oxysporum*. Polymerase chain reaction (PCR) is a specific, sensitive, and rapid assay for molecular detection. It has resolved genetic variation among isolates within or between formae speciales of *F. oxysporum* (McDonald, 1997).

RAPD and PCR-RFLPs are widely accepted for assessing genetic diversity, genome mapping, and molecular diagnostics of number of fungal species (Manulis *et al*., 1994; Annamalai *et al*., 1995). The ribosomal DNA (rDNA) genes have been employed to analyze major evolutionary events because they are highly conserved. In contrast, the rDNA internal transcribed spacers (ITS1 and ITS4) are more variable; thus, they have been used for investigation of species-level relationships (Bruns *et al*., 1991; Samuels and Seifert, 1995). The ITS region has been successfully used to identify and discriminate closely related fungal species (Bryan *et al*., 1995). Molecular approaches have been developed for fungal systematic studies, including RAPD analysis (Voight *et al*., 1995), specific diagnostic PCR primers (Nicholson *et al*., 1998) and also DNA sequencing (Appel and Gordon, 1996; O'Donnell *et al*., 1998a). However, the methods more currently used are often based on the analysis of ribosomal RNA gene sequences that are universal and contain both conserved and variable regions, allowing discrimination at different taxonomic levels (Guadet *et al*., 1989; Edel *et al*., 1995). The aim of the present study was to detect and characterize genetic variation within the *F. oxysporum* f. sp. *psidii* isolates from different agro-ecological regions of India.

Materials and Methods

Isolation of fungal isolates and pathogenicity assay

Twenty one Fop isolates were identified from infected guava

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roots and soil samples from different agro-ecological regions of India. The purified and identified isolates were stored on potato dextrose agar (PDA) medium at 4°C in the Laboratory of Molecular Plant Pathology, Central Institute for Subtropical Horticulture, Rehmankhera, Lucknow, India. The reference culture of *F. oxysporum* from guava root, culture Fop-48 (NAIMCC-F-00813), was obtained from NAIMCC, National Bureau of Agriculturally Important Microorganism (NBAIM), Mau, U.P., India. Pathogenicity tests were carried out using the stem hole inoculation technique on Allhabad safeda guava seedlings under greenhouse conditions.

Genomic DNA extraction

Total genomic DNA was isolated from fresh mycelium of *F. oxysporum* as per the mini-prep protocol described by Cenis (1992). The total genomic DNA was resuspended in 50 µl of Tris-EDTA buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA) and kept at -20°C for further molecular study.

Primer design and PCR conditions

PRIMER3 software was used to design new primers based on existing sequence data (Accession no. HM102501) in the NCBI GenBank. A set of primers, BKP1 and BKP2, was designed and synthesized for specific detection of a 183 bp amplicon from the ITS region of *F. oxysporum* f. sp. *psidii*. Standard PCR was performed in 25 µl reaction volumes. Each reaction consisted of 1 µl genomic DNA (20 ng), 0.5 µM primers, 0.5 mM of each dNTP, 2.5 µl 1× PCR buffer, 2.5 mM $MgCl_2$, and 1.25 U Taq DNA polymerase (Fermentas, Germany). The thermal conditions were set up as: an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturaton at 94°C for 1 min, annealing at 53°C (optimum temperature of these primers) for 1 min, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. Upon completion, 10 µl of the PCR product were analysed by 2.0% agarose gel electrophoresis with ethidium bromide, at 8 v/cm. The gel was then visualised under a UViPro gel documentation transilluminator.

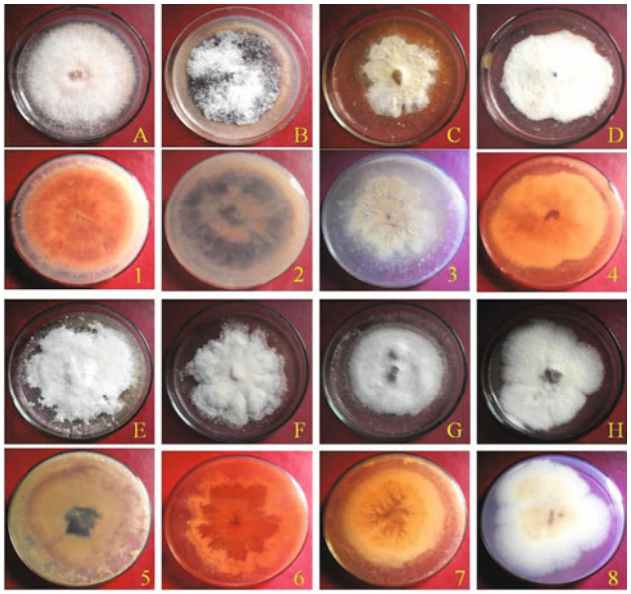

Fig. 1. *F. oxysporum* f. sp. *psidii* (Fop) isolates, isolated from different agro-climatic region. (A-H) shows colony colour and growth pattern of Fop isolates while, (1-8) shows metabolite colour.

RAPD analysis

Fifteen arbitrary 10-mer primers (Table 2) were synthesized (Eurofins Genomics India Pvt. Ltd, India) and used for RAPD marker studies. RAPD was performed in a 25 µl re-

Table 1. List of *F. oxysporum* f. sp. *psidii* isolates indicating geographic location and colony colour used in this study

S. No.	Culture No.	Geographic origin	Colony colour	Wilt symptom (%)	Source of isolation
1	Fop10	Chandigarh	Light brown	100	Soil
2	Fop14	Chandigarh	Dark yellow	70–80	Soil
3	Fop18	Ranchi	Light brown	100	Soil
4	Fop37	Rewa	Pink	100	Soil
5	NAIMCC-F- 00813(Fop 48)	Farukhabad	Brown	70	Root, Soil
6	Fop148	Rewa	No colour	50	Root
7	Fop 23	Kanpur	Pale yellow	50	Root
8	Fop 24	Kanpur	Pale yellow	100	Soil
9	Fop123	Allahabad	Cream colour	100	Soil
10	Fop44	Farukhabad	Brown	50	Soil
11	Fop125	Allahabad	Creamy red	50	Root
12	Fop127	Allahabad	Cream colour	50	Root
13	Fop128	Allahabad	Reddish brown	50	Root
14	Fop19	Ranchi	Pale yellow	50	Root
15	Fop137	Ranchi	Cream colour	100	Soil
16	Fop146	Rewa	Pink	100	Soil
17	Fop147	Rewa	Pink	50	Soil
18	Fop201	Lucknow	Reddish brown	100	Soil
19	Fop203	Lucknow	Pink	50	Soil
20	Fop134	Allahabad	Pink	50	Root
21	Fop135	Allahabad	Cream colour	50	Soil

Table 2. Sequences of reliable RAPD primers and the number of scorable polymorphic bands of each primer

S. No.	Primer	Sequence detail	No. of amplified bands	No. of polymorphic bands	Polymorphic ratio (%)
1	OPA 01	CAGGCCCTTC	Non significant	-	-
2	OPA 02	CTGGGGACTT	7	4	57.14
3	OPA 03	CCCAAGGTCC	8	3	37.5
4	OPA 04	TCACCACGGT	10	9	90
5	OPA 05	GGTCTAGAGG	8	4	50
6	OPA 06	AAGCGGCCTC	9	5	55.56
7	OPA 07	GAAACGGGTG	Non significant	-	-
8	OPA 08	GTGACGTAGG	7	5	71.42
9	OPA 09	GGGTAACGCC	8	8	100
10	OPA 10	GTGATCGCAG	9	8	88.89
11	OPA 13	CAGCACCCAC	13	13	100
12	OPB 08	GTCCACACGG	Non significant	-	-
13	OPD 18	GAGAGCCAAC	7	4	57.14
14	OPD 19	CTGGGGACTT	12	12	100
15	OPX 01	CTGGGCACGA	Non significant	-	-
Average		-	6.5	5	76.92
Total		-	98	75	-

action volume containing 25 ng genomic DNA, 10 pmole primer, 0.5 mM of each dNTP, 2.5 µl of 1× PCR buffer with $MgCl_2$ (15 mM), and 0.5 µl (5 U/µl) of Taq DNA polymerase (Fermentas). The reaction mixture was amplified using a Mastercycler® thermal cycler (Eppendorf, Germany) with PCR conditions as: first denaturation for 5 min at 94°C followed by 36 cycles of 1 min at 94°C, annealing at 36°C for 1 min followed by 2 min at 72°C; and final extension for 5 min at 72°C. PCR products were resolved by 2% agarose gel electrophoresis with ethidium bromide in the Tris-Boric acid-EDTA running buffer. A DNA ladder was used for band size estimation and the gel was visualized using a Gel Doc™ system (UVPro, Germany). The NTSYS 2.02e software package (Rohlf, 1998) was used for generation of a dendrogram of genetic relationships.

Amplification of ITS region and RFLP analysis

A standard PCR reaction was performed using an ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCC TCCGCTTATTGATATGC-3′) primer pair (White *et al.*, 1990). The PCR amplification was carried out in a 25 µl reaction mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.5 µM of each primer, 1.25 U Taq polymerase, and 10 ng genomic DNA. The following cycling conditions were used for PCR amplification: 95°C, 4 min (denaturation), 54°C, 1 min (annealing), 72°C, 1 min (extension) for 35 cycles on a Mastercycler® thermal cycler (Eppendorf). The PCR products were checked after separation on a 1.2% agarose gel with ethidium bromide (EtBr) and visualization under a UV transilluminator. About 10 µl of PCR products were digested separately using seven restriction enzymes that included *Alu*I, *Eco*RI, *Rsa*I, *Taq*I, *Hin*dIII, *Msp*I, and *Hae*III (Fermentas) as per the manufacturer's instructions. The restriction fragments were separated by electrophoresis in 2% agarose gels (Banglore Genei, India) using 1× TBE as running buffer. The band data from the restriction analyses were scored as the presence or absence of a restriction site, indicated as 1 or 0 respectively. These data were then used to generate a dendrogram (NTSYS-PC2.02e) to analyze the relationships among *F. oxysporum* isolates. Cluster analysis was performed on the similarity matrix with the SAHN program using UPGMA and a dendrogram of the outcome was generated.

Results

Phenotype and pathogenicity assay

As shown in Fig. 1, the *F. oxysporum* f. sp. *psidii* isolates were observed to have a number of colours. The mycelia were various colours (e.g. pink, brown, yellow, pale yellow, red, reddish brown, cream, and creamy red). Pigments of colours (e.g. red, yellow, grey, brown, purple, and violet) were released into the medium. Stem hole inoculation technique

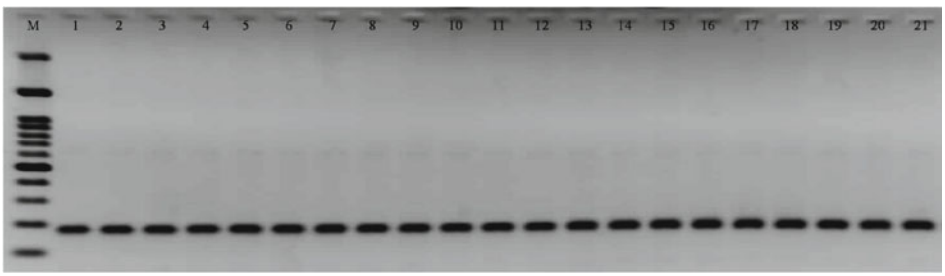

Fig. 2. PCR amplification of ITS region with specific primer BKP-1 and BKP-2. Lanes: 1-21, *F. oxysporum* f. sp. *psidii* isolates; M, 100 bp DNA Ruler.

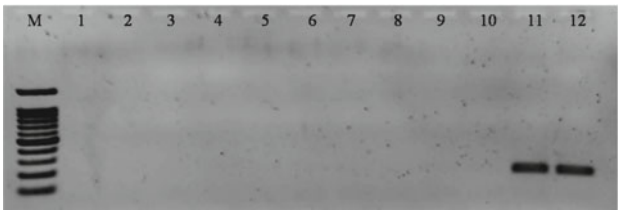

Fig. 3. PCR amplification of ITS region with specific primer BKP-1/BKP-2. Lanes: 1-2, *F. solani*; 3-4, *F. oxysporum* f. sp. *cubense*; 5-6, *F. oxysporum* f. sp. *ciceris*; 7-8, *F. moniliformae*; 9-10, *Colletotrichum* sp.; 11-12, *F. oxysporum* f. sp. *psidii* isolates; M, 100 bp DNA Ruler.

is used for the pathogenicity trial in guava plant as previously described by Misra and Pandey (2000). The pathogenicity trail found natural wilt symptoms. The wilting was recorded for artificially inoculated guava plants. All 21 isolates were observed to be highly pathogenic to the Allahabad safeda genotype guava seedlings (Table 1).

Specificity of the PCR amplification

This study was initiated to develop and evaluate the efficacy of PCR primers that were designed from the known sequences of the rDNA ITS region (GenBank accession no. HM102500.1). The specificity of the newly designed primers was validated in the isolates of *F. oxysporum*. Primer pair BKP1/BKP2 was designed for rapid, reliable and specific detection of guava wilt pathogen *F. oxysporum* f. sp. *psidii*. All the isolates were amplified and the size of the product was consistently 183 bp. The optimized PCR parameters for the specific amplification by this species-specific primer pair were: 53°C for annealing temperature, 2.5 mM $MgCl_2$ concentration, and 0.5 µM primers. There was no amplification observed for other fungal species tested, which indicates that these primers are highly specific for molecular detection of *F. oxysporum* f. sp. *psidii* isolated from these

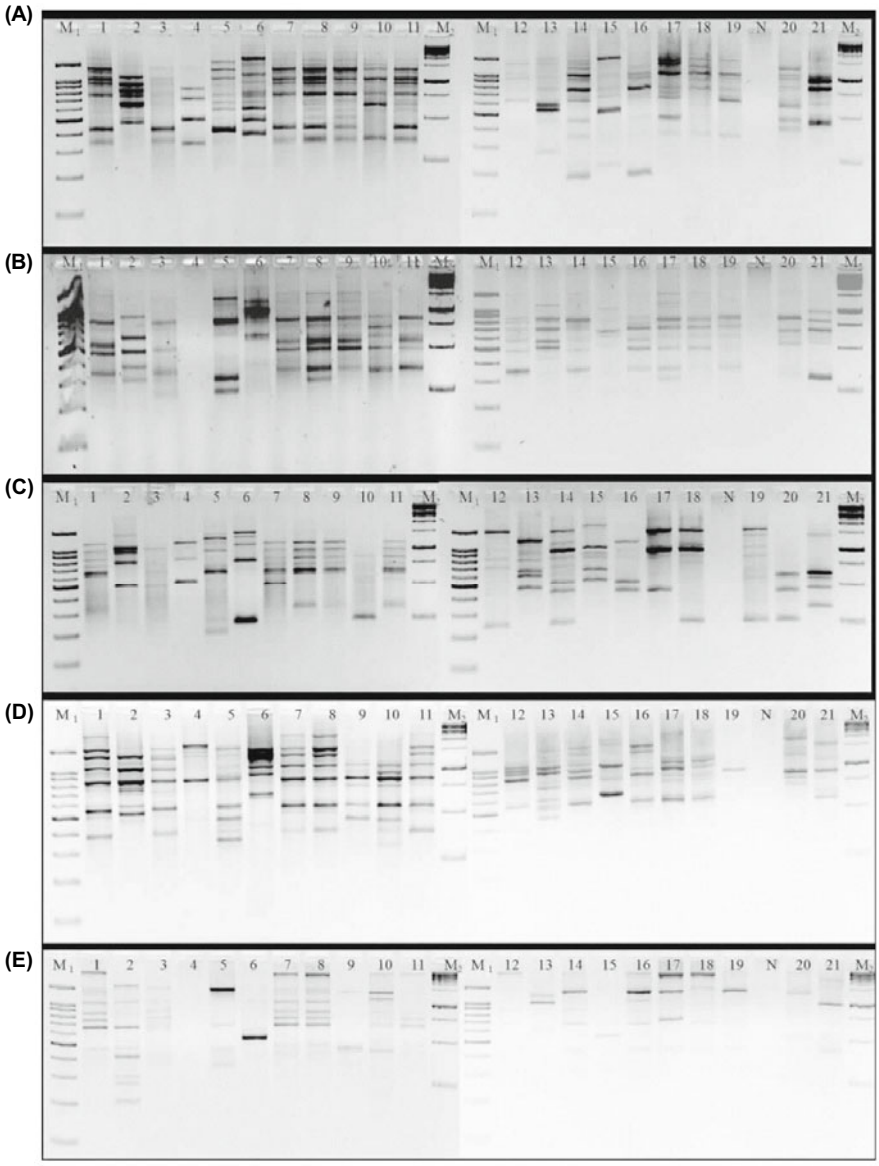

Fig. 4. RAPD profiles of *F. oxysporum* f. sp. *psidii* isolates. Amplification of the genomic DNA was performed using primers OPA-04, OPA-09, OPA-10, OPA-13, and OPD-19. Lanes: M1, 100 bp DNA ladder; M2, 1 kb DNA ladder (Biochem); 1–21, *F. oxysporum* f. sp. *psidii* isolates; N, Negative control.

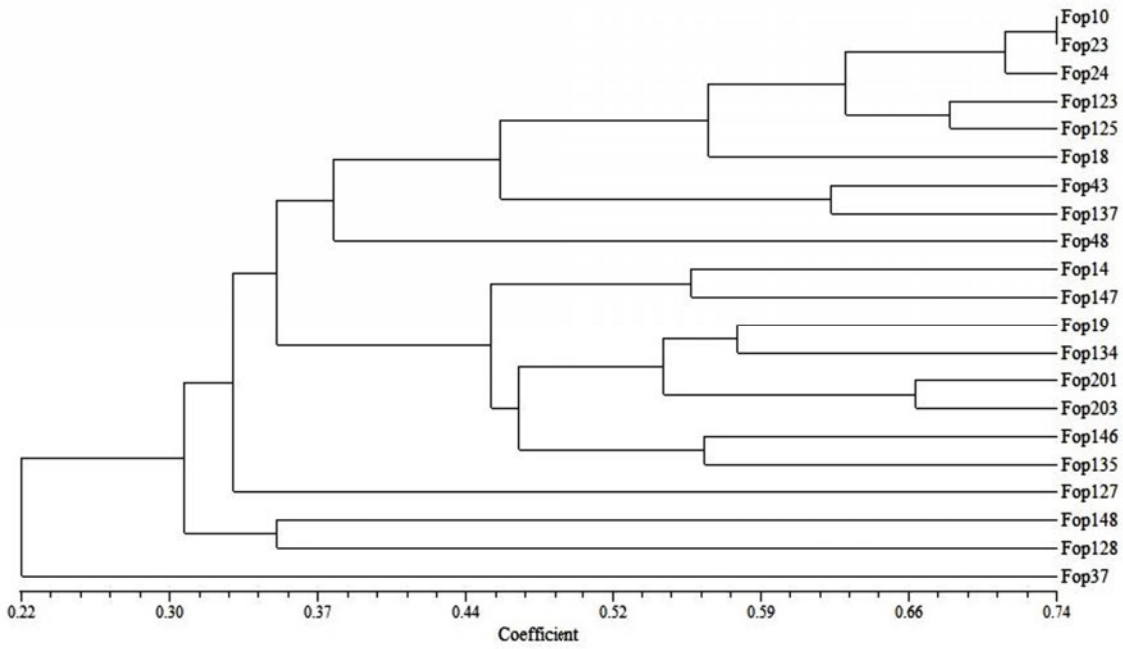

Fig. 5. Dendrogram of 21 *F. oxysporum* f. sp. *psidii* isolates derived from common data of RAPD fingerprints generated by UPGMA based on Genetic distance calculated from Presence (1) or absence (0) of polymorphic bands with OPA-4, OPA-9, OPA-10, OPA-13, and OPD-19 primers.

agro-ecological regions (Figs. 2 and 3). All the isolates were also tested using existing primers (ITS universal primers ITS1 and ITS4), which also gave positive amplification.

RAPD analysis

In the present study, genetic diversity of the 21 *F. oxysporum* f. sp. *psidii* (Fop) isolates collected from different states of India was examined. A total of fifteen RAPD primers were tested and five primers, OPA-4, OPA-9, OPA-10, OPA-13, and OPD-19, showed a high degree of polymorphism among the isolates (Fig. 4). Relatedness among the isolates was estimated by means of scorable polymorphic DNA bands, and the genetic similarity of isolates based on RAPD was assessed using Jaccard's coefficient. The constructed dendrogram (Fig. 5) and 3-D distribution of the isolates are shown (Fig. 6). The 98 reproducible DNA fragments that were scored for generation of the dendrogram showed a wide range of diversity within the pathogenic isolates, with 22–74% genetic similarities. For 75 polymorphic bands that were scored, the average polymorphic ratio was 76.53% (Table 2). In RAPD, bands of the phenotypically identified Fop showed variability in banding patterns with band sizes ranging from 200–2,200 bp. A cluster analysis was performed to estimate the variability and group the isolates. The dendrogram based on UPGMA cluster analysis of the RAPD data showed three main clusters (Fig. 5). Fop-10, 14, 18, 19, 23, 24, 43, 48, 123, 125, 127, 134, 135, 137, 146, 147, 201, and Fop-203 clustered in a major clade and also showed variability within a clade. In the second clade, Fop-128 and Fop-148 were isolated from Allahabad (U.P.) and Rewa (M.P.) respectively, fell into a single group with 35% similarity. The Fop-37 isolate, from Rewa (M.P.), was completely different from all the isolates having only 22% genetic similarity.

RFLP analysis of ITS region

Amplification with the universal primers for ITS1 and ITS4 resulted in a fragment of approximately 570 bp present in all the isolates. No variation was observed in the ITS + 5.8S rDNA region among *F. oxysporum* f. sp. *psidii*. There were seven restriction endonucleases such as *Alu*I, *Eco*RI, *Rsa*I, *Taq*I, *Hin*dIII, *Msp*I, and *Hae*III used for amplified

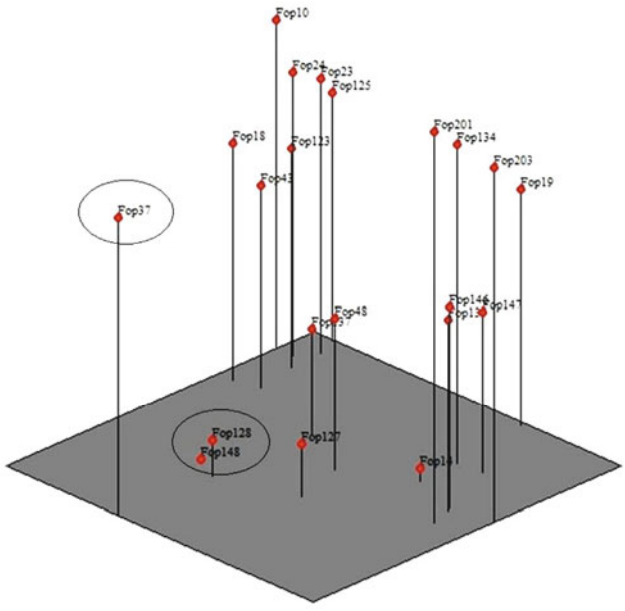

Fig. 6. Three-dimensional view of score plot resulted from principle coordinate analysis of RAPD data.

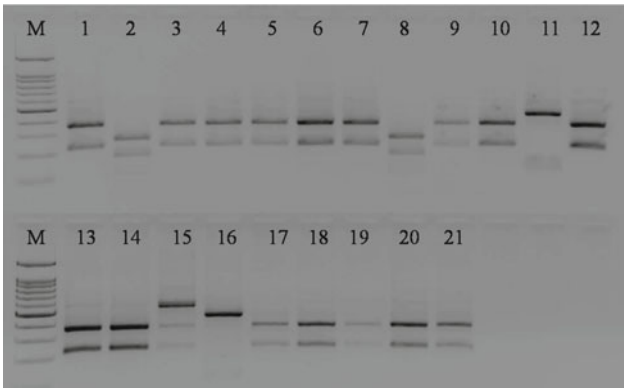

Fig. 7. Agarose gels showing restriction patterns of PCR-amplified rDNA digested with *Msp*I. Lanes: M, 100 bp DNA ladder; 1-21, Fop Isolates.

ITS region of *F. oxysporum* f. sp. *psidii*. We found only *Msp*I gave prominent and discriminating banding patterns in RFLP analysis (Fig. 7). Enzymatic digestions of the ITS region generated specific RFLP markers for each isolate and subsequently revealed some intra-specific variations. The results indicate that RFLPs generated by restriction enzyme digestion with *Msp*I of PCR-amplified rDNA can be used for discrimination of *F. oxysporum* f. sp. *psidii* isolates. The ITS region of the rDNA appeared to be highly divergent in length and nucleotide composition among the Fop isolates. As shown in Fig. 8, we obtained the 3 major clades in RFLP data. The most of isolates such as Fop-10, 18, 19, 28, 23, 37, 48, 123, 127, 128, 134, 135, 148, 201, and 203 present in main clade. Two isolates (Fop-14 and Fop-24) were present in same clade having the similar restriction pattern. Fop-125 and Fop-146 were present in a third clade have different restriction sites which were isolated from Allahabad (U.P.) and Rewa (M.P.), respectively.

Discussion

The rDNA genes have been employed to analyze major evolutionary events because it is highly conserved. Whereas the rDNA internal transcribed spacer is more variable thus, it has been used for investigation of the species-level. In present study, we have used a set of primers which help to diagnose *F. oxysporum* f. sp. *psidii*. The primer pair BKP1 and BKP2 were designed for *F. oxysporum* f. sp. *psidii* isolate that was targeted the 28S ribosomal RNA and 28S-18S ribosomal RNA intergenic spacer region. No cross reactivity was found with other species such as *Fusarium solani*, *F. oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *ciceris*, *F. moniliformae*, and *C. gloeosporioides*. The primer set BKP1-BKP2 appeared to be specific for *F. oxysporum* f. sp. *psidii,* giving positive amplification for the isolates from different agroecological regions. These primers could be useful for molecular detection of pathogenic isolates of *F. oxysporum* f. sp. *psidii* of guava.

In the present study, the genetic relatedness among the isolates of *F. oxysporum* f. sp. *psidii* (Fop) was analyzed by random primers to generate reproducible polymorphisms. All amplified products showed banding patterns that indicated the presence of genetic diversity. RAPD revealed polymorphisms within reference isolates of *F. oxysporum* that might be useful for genetic characterization and specific identification of Fop isolates of guava. Similarly, RAPD has been used previously to study inter- and intra-specific variation in twelve *Fusarium* species that were isolated from cotton growing areas in Egypt (Abd-Elsalam *et al.*, 2003). A correlation between genetic similarity and geographic origin has been reported (Assigbetse *et al.*, 1994). RAPD gives more comprehensive information regarding the genetic variability among the pathogen isolates as it is based on the entire genome of an organism (Achenback *et al.*, 1997). RAPD-PCR polymorphisms have been found in *Fusarium* and used for

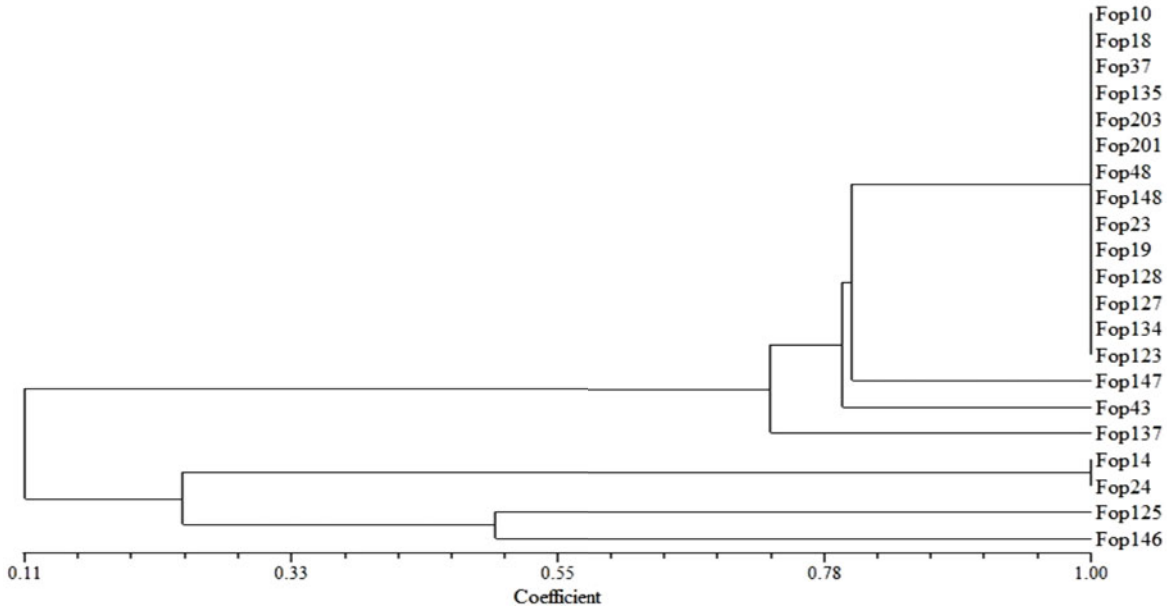

Fig. 8. UPGMA dendrogram showing relationships among the 21 isolates of *F. oxysporum* f. sp. *psidii* based on restriction site data.

identification of these fungi at the species level (Gupta *et al*., 2012). Similarly, RAPD was used for a quick and reliable discrimination of isolates of *Fusarium* species (Hyun and Clark, 1998; Migheli *et al*., 1998; Ibrahim and Nirenberg, 2000; Jana *et al*., 2003). For ease of interpretation, the genetic similarity values for RAPD data from the current study were subjected to Principal Coordinate Analysis (PCoA) to obtain graphical representations of the relationships among the 21 genotypes. The 3-D distribution of samples was generated on the basis of polymorphic and monomorphic bands that were produced by the 98 fragments (markers) obtained by RAPD-PCR (Fig. 6). The PCoA revealed a pattern in which the individuals were assigned separately, and the 3-D PCoA provided a better graphical illustration and also a clear separation of samples on the basis of genotypes.

For the restriction patterns generated by restriction enzyme *Msp*I, a single group was easily defined, having 350 and 220 bp size fragments, except that Fop-14, 24, 125, and 146 have a different product size. Fop-14 and Fop-24 are in another group with 250 bp and 200 bp fragments, while Fop-125 and Fop-146 have 420 and 150 bp fragments (Fig. 7). The reference isolate used in this study also had the same restriction pattern with 350 and 220 bp restriction products. Some isolates from the major clade identified using RAPD data (Fop-10, Fop-18, Fop-23, Fop-201, Fop-203, and Fop-223) showed a similar grouping in the RFLP dendrogram. The genetic variation of the ITS + 5.8S rRNA within the closely related isolates was low and sometimes no variation was observed. This region was not very informative for determining genetic variation within isolates of *F. oxysporum* f. sp. cubense (Foc) (Leong *et al*., 2009). Donaldson *et al.* (1995) examined the usefulness of primer sets designed to amplify introns within conserved genes in filamentous ascomycetes, and used them to differentiate 35 isolates representing 6 different species of *Fusarium* found in association with conifer seedlings.

Conclusion

The present investigation showed the high level of genetic variability in *F. oxysporum* f. sp. *psidii* (Fop), pointing out the dissimilarities within these isolates obtained from different agro-ecological regions of India. This study demonstrates the reliability of RAPD and RFLP analysis of rDNA, as tools for addressing questions on the geographical distribution of populations of this pathogen. In this study, the *F. oxysporum* isolates were all found to be closely related, regardless of the location and guava cultivars. Nevertheless, isolates of *F. oxysporum* f. sp. *psidii* are different from each other, and have a high level of genetic variation. These molecular techniques would be useful for species and strain differentiation for a wide variety of fungi, and could be applicable in studies of epidemiology, diagnosis, virulence and molecular taxonomy.

Acknowledgements

Authors are grateful to Head, Division of Crop Protection and also Director, Central Institute for Subtropical Horticulture, Rehmankhera, Lucknow for their encouragement and support. We gratefully acknowledged to Indian Council of Agricultural Research for Financial support to RKM, VS, and AJM.

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