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Molecular variability of *Pythium aphanidermatum* causing damping-off of Bidi tobacco using RAPD technique

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Abstract
Damping-off, caused by *Pythium aphanidermatum* is one of the most serious disease in nursery of bidi tobacco. However, the incidence and severity of the disease differ from one location to other, one geographical area to other and even differs from country and region wise. The random amplified polymorphic DNA (RAPD) banding patterns of seventeen isolates of *Pythium aphanidermatum* generated by 5 random oligonucleotides showed a very high level of variation, allowing these isolates separated into two groups. This result revealed that two isolates genetically distinct from all other fifteen isolates.

Keywords: *Pythium aphanidermatum,* RAPD techmique

Introduction

Damping-off of tobacco caused by *Pythium aphanidermatum* is a very serious problem for successful nursery management. (Bhatt 1985, Manoranjitham *et al.,* 2001) [1, 7]. Understanding the disease epidemiology and host-pathogen interaction is greatly dependent on the knowledge of diversity of pathogen at field level as diverse population of a pathogen have different levels of interaction with the host under variable environmental conditions. Several DNA-based methods have been developed to detect and identify various *Pythium* species: DNA probes (Martin, 1991; Levesque *et al*., 1994; Matthew, Hawke & Pankhurst, 1995; Klassen, Balcerzak & de Cock, 1996) [8, 6, 10, 4], RFLP of PCR-amplified rDNA (Chen & Hoy, 1993; Rafin, Brygoo & Tirilly, 1995) [2], RFLP of mitochondrial DNA, RFLP of total DNA (Levesque *et al*., 1993; Descalzo *et al.,* 1996) [5,. 3], species-specific primers (Wang & White, 1996) [14] and karyotype analysis using field gel electrophoresis (Martin, 1995) [9]. One or a few isolates of *P. aphanidermatum* have sometimes been included in the studies, but no study has been addressed to the accurate and rapid identification of this fungus in association with other species of *Pythium* encountered in this crop environment. During recent years, numerous papers have described PCR using short arbitrary primers, RAPD (random amplified polymorphic DNA) (Williams *et al*., 1990) [15], as a useful method in genetic mapping, diagnosis in molecular taxonomy and evolutionary studies. In this paper we present results to show that RAPD can be used to study the variability among the isolates of *P. aphanidermatum*.

Materials and Methods

Collection of Samples: Infested soil samples showing typical symptom of damping-off of tobacco were collected from respective *bidi* tobacco growing areas of middle Gujarat. Five soil cores of 5 cm diameter and upto 15 cm deep were collected from five site of each tobacco nursery. A total 17 isolates of *Pythium* spp. were recovered from infested soil samples and placed in pots for the further studies.

Isolation of Pathogen: The pathogen was isolated using standard method suggested by Saha *et al.* (2002) [12] In this technique, bottle gourd fruits used as bait to stimulate pathogen growth. The fruits treated for 12 hours in a solution containing carbendazim (500 ppm) and streptocycline (100 ppm) to prevent bacterial and fungal contamination. The treated portion of the fruits transversely cut into 2-4 small pieces and then placed at a depth of 5-8 cm below the soil surface in contaminated soil (taken from a different area). The soil was irrigated and kept moist. After 24 to 48 hours, the fruits slices removed from the soil with minimal disruption to the pathogen-infected fruit.

Under aseptic conditions, each fruit slice was put into an air-filled plastic bag and maintained at room temperature for 24 hours., The entire bottleguard fruit slices were covered with white fluffy mycelial growth within 24 hours. The mycelial growth was observed under microscope and it was aseptically transferred to oat meal agar.

Table 1: Source of *Pythium* sp. isolates collected from *Bidi* tobacco growing areas of middle Gujarat

Isolates designation & place		Taluka	District
Pa1	Bhalej	Anand	Anand
Pa2	Napa		
Pa3	Visnoli	Petlad	
Pa4	Visnoli		
Pa5	Porda		
Pa6	Mahelav		
Pa7	Sundarana		
Pa8	Dharmaj		
Pa9	Dharmaj		
Pa10	Bhadran	Borsad	
Pa11	Vasna		
Pa12	Sisva		
Pa13	Kasor	Sojitra	
Pa14	Umba	Thasra	Kheda
Pa15	Arera	Nadiad	
Pa16	Vanoda	Mahisagar	Mahisagar
Pa17	Anand	Anand	Anand

Purification and maintenance of the pathogen
The fungus was purified by using hyphal tip method and pure culture was maintained by sub-culturing every ten days on plate of oat meal agar and preserved in refrigerator.

Identification of pathogen: The identification of pathogen which causing tobacco damping-off, grown on oat meal agar medium examined visually as well as microscopically for cultural and morphological characters.

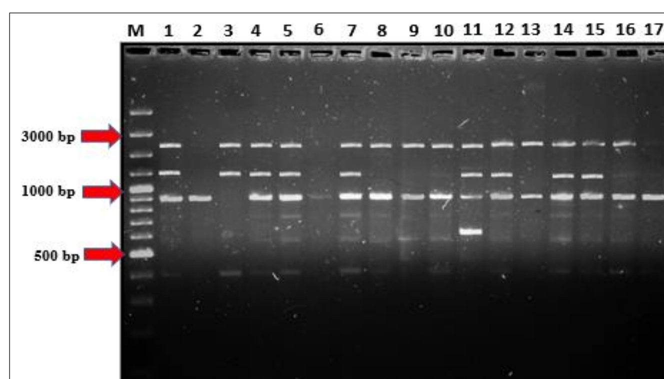
DNA extraction: The total genomic DNA was isolated from fungal mycelia using CTAB (Cetyl trimethyl ammonium bromide) DNA extraction protocol.

Amplification conditions: DNA was amplified by the RAPD technique (Williams *et al*., 1990) [15]. Approximately 1 ng of DNA was used in a 50 µl reaction volume containing 50 ml KCl, 3±0 ml $MgCl_2$, 10 ml Tris-HCl (pH 8±3), 200 µl each of dNTP, 0±2 µl primer and 0±75 units of Taq DNA polymerase. The reaction mixture was sealed with a drop of mineral oil. Amplifications were performed using 10-mer primers from kit A and kit B from Operon Technologies, Inc. Amplifications were performed in a Perkin Elmer Cetus thermal cycler programmed as follows: initial denaturation 7 min at 93° followed by 45 cycles of: denaturation 20 sec at 93°, annealing 1 min at 37°, extension 1 min at 72° with a final extension of 7 min at 72°. The amplification products were stored at 4° until analysis. Half of the amplification products were separated on a 1% TAE agarose gel at 120 V for up to 2 h (Sambrook, Fritsch & Maniatis, 1989) [13] and stained with ethidium bromide (1 µl ml−"). The fragments obtained were named following the convention of Paran, Kesseli & Michelmore (1991) [11]. The molecular mass of the fragments was estimated from a 123 bp molecular weight marker.

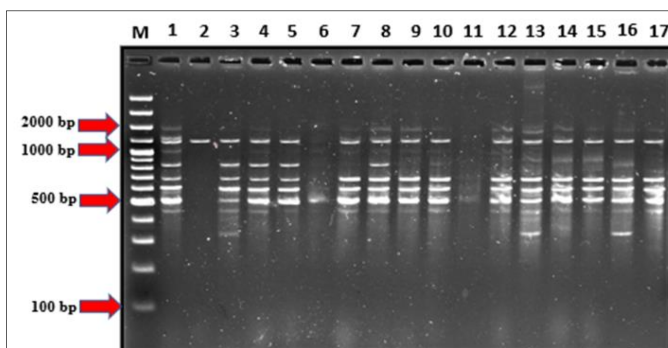
Random Amplification of Polymorphic DNA (RAPD) analysis: The RAPD primers used for the analysis of random amplification of polymorphic DNA to study the polymorphism present in the isolates of *P. aphanidermatum.* A total of 20 decamer primers belonging to OPA, OPB, OPC, OPE each series were screened for RAPD analysis Among 80 primers, 5 primers (OPA 1, OPA 3, OPB 8, OPB 12, OPE 3) were selected based on repeatability.

Results and Discussion
The present investigation was carried out with the seventeen isolates of *P. aphanidermatum* to study the molecular variability among these isolates. A total of 80 primers consisted of di-nucleotide repeat motifs were used for initial screening of Pa$_{14}$ isolate. Out of these, seventy-five primers gave no amplification at all, while five primers yielded clear banding patterns and were subsequently used to analyze the entire set of seventeen isolates. The fragment size was detected by comparing the amplicons with a 100 bp DNA ladder.
The present study (Table 1) showed a high level of genetic variability between seventeen isolates of *P. aphanidermatum*. All the primer OPA-1, OPA-3, OPB-12, OPB-8 and OPE-3 recorded highest polymorphism (100%). Five RAPD primers amplified a total of 21 scorable loci of which all 21 loci were polymorphic, with a range of 3 to 6 alleles per primer. Overall, *P. aphanidermatum* isolates exhibited a high level of genetic diversity. The maximum 6 alleles were generated by OPA-3 and the lowest numbers of alleles were generated by primer OPB-12 (3 alleles). This result showed the ability of RAPD to discriminate among isolates and suggested their application for species identification.

The amplification obtained with different RAPD primers as given below
Primer OPA 1 (CAGGCCCTTC)
A maximum of 4 DNA alleles were observed in OPA 1 primer. This primer showed 4 polymorphic loci out of 4 loci with 100% polymorphism and 0.65 PIC value.

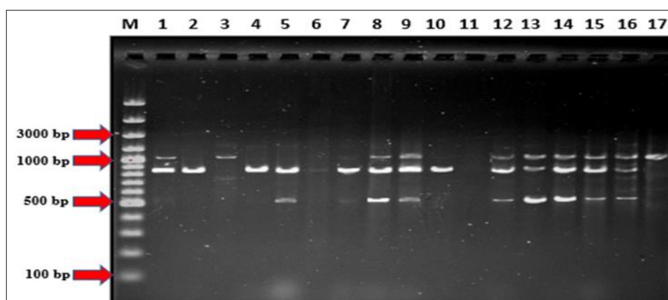


Primer OPA 3 (AGTCAGCCAC)
A maximum of 6 DNA alleles were found in OPA 3 primer. This primer showed 6 polymorphic loci out of 6 loci with 100% polymorphism and 0.78 PIC value.



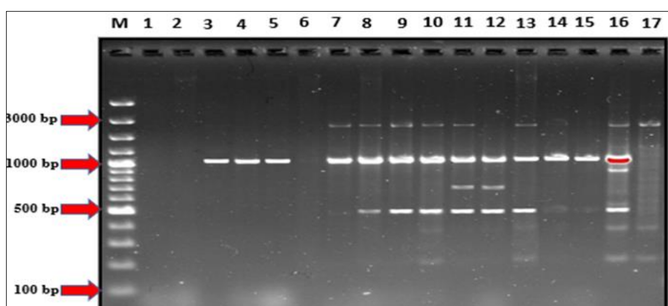
Primer OPB 12 (CCTTGACGCA)

A maximum of 3 DNA alleles were found in OPB 12 primer. This primer showed 3 polymorphic loci out of 3 loci with 100% polymorphism and 0.65 PIC value.



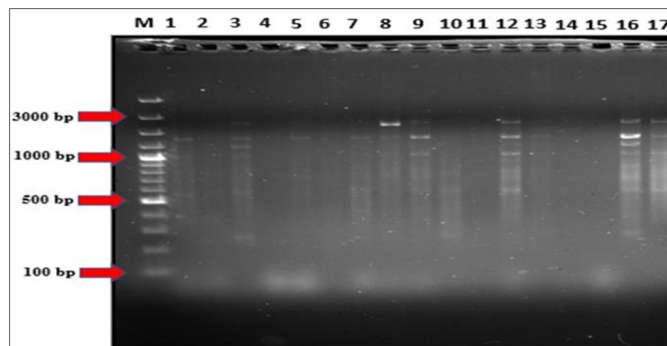
Primer OPB 8 (GTCCACACGG)

A maximum of 4 DNA alleles were observed in OPB 8 primer. This primer showed 4 polymorphic loci out of 4 loci with 100% polymorphism and 0.67 PIC value.



Primer OPE 3 (GTGACATGCC)

A maximum of 4 DNA alleles were observed in OPE 3 primer. This primer showed 4 polymorphic loci out of 4 loci with 100% polymorphism and 0.61 PIC value.



Pooled RAPD

A dendrogram was constructed according to Jaccard's coefficient (Jaccard, 1908) among the isolates of *P. aphanidermatum* using the Unweighted Pair Group Method of Arithmetical Averages (UPGMA) algorithm.

The dendrogram presented in Fig. 1 revealed that all the isolates were divided into two clusters A and B. Cluster A contains two sub-clusters A1 and A2. Cluster A1 contains further two sub-clusters a1 and a2, cluster a1 divided into more two sub- clusters *i.e.* I and II. Cluster I divided into two sub cluster i and ii. Cluster i contain seven isolates *i.e.* Bhalej (Pa_1), Visnoli (Pa_4), Porda (Pa_5), Umba (Pa_{14}), Arera (Pa_{15}) Sundarana (Pa_7) and Bhadran (Pa_{10}). while cluster ii contains five isolates *i.e.* Dharmaj (Pa_8), Dharmaj (Pa_9), Vanoda (Pa_{16}), Kasor (Pa_{13}) and Sisva (Pa_{12}). Cluster II contains single isolate Visnoli (Pa_3). Sub-cluster a2 also contain single isolates Anand (Pa_{17}). Cluster A2 contain one isolate Vasna (Pa_{11}). Cluster B contains only two isolates *i.e.* Napa (Pa_2), Mahelav (Pa_6)

Cluster A containing fifteen isolates which showed genetically similar, whereas cluster B containing only two isolates that was different from all the fifteen isolates. Among all the isolates, Napa (Pa_2) and Visnoli (Pa_3) were found high genetic variation with lowest similarity coefficient (0.071), while Umba (Pa_{14}) and Arera (Pa_{15}) isolates were showed genetically similar with highest similarity coefficient (1.00)`.

Table 2: Details of amplification obtained with different RAPD primers

Sr. No.	Primers	Primer sequence (5'-3')	Molecular weight range (Bp)	Total number of bands	Total number of loci	Number of polymorphic loci	Number of monomorphic loci	Polymorphism (%)	PIC Value
1	OPA 1	CAG GCC CTT C	665.96-2122.34	40	4	4	0	100	0.65
2	OPA 3	AGT CAG CCA C	283.27-1430.37	65	6	6	0	100	0.78
3	OPB 12	CCT TGA CGC A	554.54-1220.94	32	3	3	0	100	0.65
4	OPB 8	GTC CAC ACG G	492.33-2544.33	31	4	4	0	100	0.67
5	OPE 3	CCA GAT GCA C	1334.8-2513.27	19	4	4	0	100	0.61
Total				187	21	21	0	-	3.36
Average				37.4	4.2	4.2	0.0	100	0.67

PIC- Polymorphism Information Content

Table 3: Jaccard's similarity coefficient between seventeen isolates of *P. aphanidermatum* based on RAPD data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	1.000																
2	0.273	1.000															
3	0.643	0.071	1.000														
4	0.750	0.300	0.571	1.000													
5	0.769	0.250	0.600	0.833	1.000												
6	0.250	0.400	0.143	0.400	0.333	1.000											
7	0.692	0.273	0.533	0.750	0.769	0.364	1.000										
8	0.625	0.200	0.588	0.563	0.688	0.267	0.625	1.000									
9	0.529	0.200	0.500	0.471	0.588	0.267	0.625	0.875	1.000								
10	0.500	0.300	0.375	0.667	0.571	0.400	0.750	0.667	0.667	1.000							
11	0.200	0.111	0.188	0.308	0.267	0.222	0.385	0.294	0.294	0.417	1.000						
12	0.625	0.200	0.588	0.563	0.688	0.267	0.625	0.765	0.765	0.563	0.375	1.000					
13	0.600	0.231	0.471	0.533	0.667	0.308	0.714	0.867	0.867	0.769	0.333	0.750	1.000				
14	0.692	0.273	0.533	0.750	0.769	0.364	0.692	0.625	0.625	0.615	0.286	0.733	0.714	1.000			
15	0.692	0.273	0.533	0.750	0.769	0.364	0.692	0.625	0.625	0.615	0.286	0.733	0.714	1.000	1.000		
16	0.529	0.200	0.588	0.471	0.588	0.267	0.625	0.875	0.875	0.667	0.294	0.765	0.867	0.625	0.625	1.000	
17	0.538	0.200	0.500	0.357	0.400	0.182	0.538	0.600	0.600	0.462	0.143	0.500	0.571	0.429	0.429	0.600	1.000

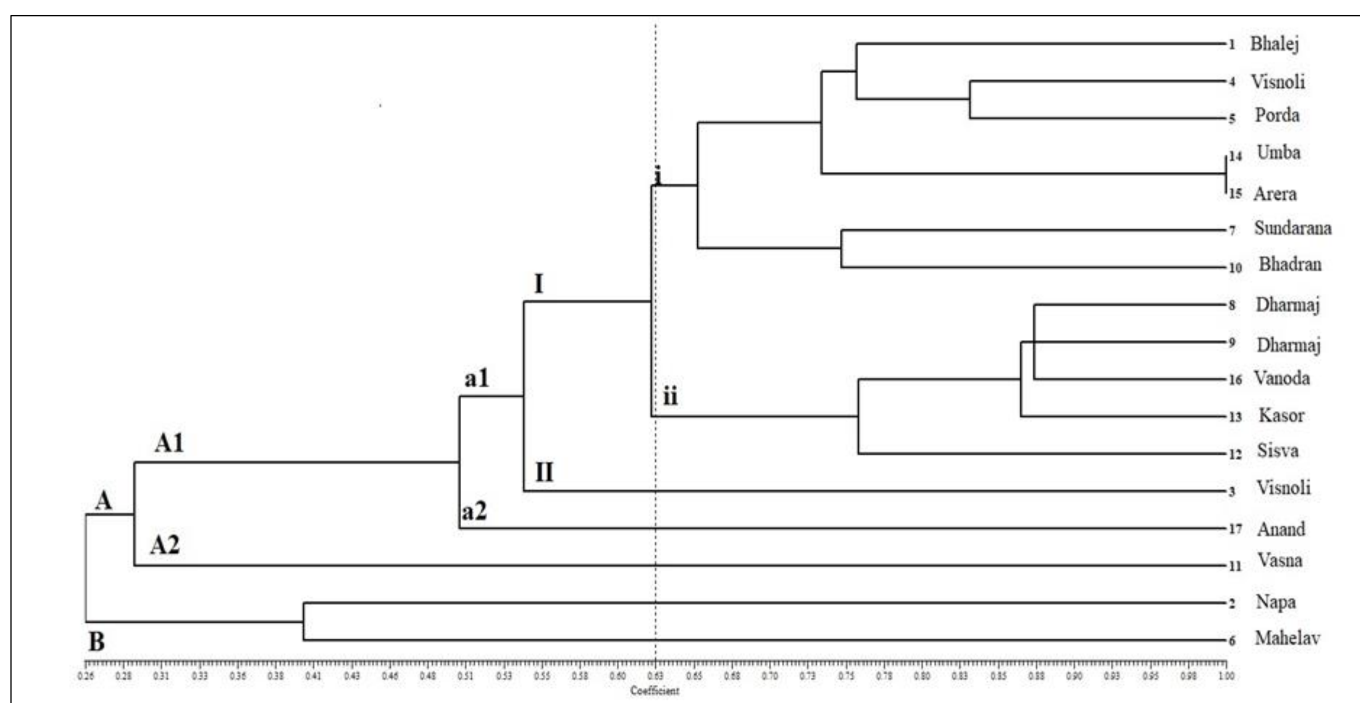


Fig 1: RAPD UPGMA dendogram of seventeen isolates of *P. aphanidermatum* based on Jaccard's similarity coefficient.

Conclusion

The molecular study revealed that the cent per cent polymorphism was recorded by the primer OPA-1, OPA-3, OPB-12, OPB-8 and OPE-3 out of 80 primers. Fifteen isolates come under cluster A, showed that these isolates are genetically similar, whereas cluster B containing only two isolates that were distinct from all the fifteen isolates. Among all the isolates, Napa (Pa2) and Visnoli (Pa3) were found high genetic variation with lowest similarity coefficient (0.071), while Umba (Pa14) and Arera (Pa15) isolates were showed genetically similar with highest similarity coefficient (1.00).

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