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Molecular characterization of *Phytophthora nicotianae* associated with diseases of horticultural crops by RFLP of PCR internal transcribed spacer region of ribosomal DNA and AFLP fingerprints.

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ABSTRACT

Molecular characterization of *Phytophthora* isolates from tobacco, tomato, carnation, gerbera, crossandra and periwinkle was carried out using internal transcribed spacer(ITS) regions of rDNA gene repeat and amplified fragment length polymorphism (AFLP). All the isolates of *Phytophthora* were identical in morphology and ITS-RFLP patterns, indicating *P. nicotianae* and *P. parasitica* are synonymous. However, based on AFLP, four sub groups were evident within population of *P. nicotianae* (*P. parasitica*): Group I, includes isolates from tomato, tobacco and periwinkle. Isolates from carnation represents Group II. Isolates from gerbera falls under group III. The crossandra isolates formed a Group IV. Thus, present study showed the existence of four molecular sub groups within population of *P. nicotianae* (*P. parasitica*) in India.

Key words: *Phytophthora nicotianae*, AFLP, ITS-RFLP

INTRODUCTION

Phytophthora parasitica (sensu Dastur,1913) and *P. nicotianae* (sensu Waterhouse, 1963), are pathogenic to a wide range of hosts ranging from tree crops to herbaceous plants(Oudemans and Coffey, 1991). *Phytophthora nicotianae* has been reported as causal agent of diseases of tobacco, crossandra, carnation, gerbera and periwinkle and *P. parasitica* on tomato in India (Mehrotra and Aggarwal, 2001). These species were mainly identified based on morphological features (Stamps *et al,* 1990; Sarma *et al*, 2002)Morphological criteria have limitations in understanding genetic diversity and genetic distance between isolates of *P. nicotianae* and *P. parasitica.* Protein patterns (Erselius and de Vallavielle, 1984), serological analysis (Morton and Dukes, 1967), DNA - RFLP patterns (Forster and Coffey, 1991) and isozyme patterns (Oudemans and Coffey, 1991) were found to be useful in understanding genetic divergence between isolates belonging to *P. nicotianae* and *P. parasitica*. Endonuclease restriction digestion analysis of the ITS region of ribosomal DNA (ITS-RFLP) (Cooke and Duncan,1997; Crowford *et al*, 1996; Chowdappa *et al*, 2003a, b, c; Chowdappa and Sarma, 2003) and AFLP finger prints were successful to separate species and also differences. The objective of the present study is to understand molecular diversity of *Phytophthora* isolates from tobacco, tomato carnation, gerbera, crossandra and periwinkle in India based on ITS-RFLP and AFLP fingerprinting.

MATERIAL AND METHODS

Fungal isolates

Phytophthora nicotianae isolates from tobacco, tomato, perwinkle, gerbera (05 isolates each), carnation and crossandra (04 isolates each) collected from Bangalore, Mysore, Tumkur and Hassan districts of Karnataka were used in this study. Reference cultures of *P. nicotianae* (IMI 3406160 and IMI 235604) were also included for comparison.

Morphological studies

Colony morphology of isolates, on carrot agar medium(CA) after three days of incubation in the dark at 24 ± 1ºC, was studied. Sporangia were produced on CA using mycelium-agar-disk-in-water method (Al-Hedaithy and Tsao,1979). Observations were made on length and breadth of sporangia, presence of papilla, caducity and shapes. Observations on chlamydospore production were made on CA following incubation at 18ºC in the dark. The mating types of isolates were determined by taking 5 mm dia. discs from advancing margins of 3-day- old cultures of opposite mating types and placed them 20 mm apart on CA. The inoculated plates were incubated

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at 20^{O}C in the dark for 15-20 days. The influence of temperature on vegetative growth on CA was determined in the dark at temperature ranging from 6 to 39ºC with an increment of 3^{O}C.

Molecular studies

The fungal mycelium was grown in 100 ml of V8 juice in a 250 ml flask for 3 days at 25°C on orbital shaker at 100 rpm. DNA was extracted from freeze dried vegetative mycelium following a method of Raeder and Broda (1985). PCR amplification of the ITS region was performed using with universal primer pair ITS1 and ITS 4 (White *et al* ,1990). Each 50 µl reaction mixture contained 100 ng of DNA, 50 pmol of each primer, 200 mm each dNTP, 5 µl of 10 x PCR buffer and 10 units of Taq DNA polymearse enzyme. PCR was carried out with following cycling parameters. A 4 minute denaturation at 94°C, followed by 34 cycles of 60s at 94°C, 60s at 55°C, and 1.5 min at 72°C and then a 5 min extension at 72°C. PCR products (5µl) were electrophoresed in a 2 % agarose gel with Tris-Borate-EDTA (TBE) buffer, stained with ethidium bromide and visualised under UV illumination. PCR product was digested with restriction enzymes namely *Hinf* I, *Msp* I, *Alu* I, *Hae* III, *Rsa* I, *Taq* I and *EcoR* V. Restriction digestions were performed in 10 µl reaction containing 5 µl PCR product, 1 µl 10 x restriction buffer, 3.6 µl PCR grade water, 0.1 µl Bovine serum albumn and restriction enzyme (3 U/reaction) at 37°C for overnight (16h). Digestion products were electrophoresed in 2.5% LE agarose. The size of restriction fragments was determined by comparison of fragments migration with that of known marker fragments (100bp molecular size ladder).

Amplified fragment length polymorphism (AFLP) analysis was carried out as per the method of Muller *et al* (1996). Fungal genomic DNA (500 ng) was digested by *Pst* (20 units), ligated to *Pst* adaptor at 37°C for 4h and pre- amplification was carried out at 94ºC for 4 min for initial denaturation followed by 34 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 60ºC, extension for 1.5 min at 72ºC and a final extension step of 5min at 72ºC. Pre - amplified PCR products were then diluted 1:100 in TE buffer and amplified with five AFLP primers, A(5'-GACTGCGTACATGCAGGT3',B(5'GACTGCGTACATGCAGGA3'), C,5'GACTGCGTACATGCAGGC3'), D (5'GACTGCGTACATGCAGAC3'), E(5'GACTGCGTACATGCAGCG-3') each with two selective bases at its 3' end underlined, adopting above thermal cycling parameters. The PCR products were electrophoresed in 2% agarose gels in Tris-Borate-EDTA (TBE) buffer and visualized under UV after staining with ethidium bromide

Table 1. Sporangial measurement in *P. nicotianae* isolates

Isolate No	Length*(µm)	Breadth (µm)	L/B ratio(µm)	Mating type
Tobacco				
OH 37	41.50 ± 1.21	29.50 ± 0.77	1.40 ± 0.77	A2
OH 38	43.07 ± 0.93	31.92 ± 0.61	1.33 ± 0.02	A2
OH 39	41.00 ± 1.08	30.20 ± 0.71	1.37 ± 0.02	A2
OH 40	36.70 ± 1.21	24.92 ± 0.54	1.47 ± 0.36	A2
OH 41	40.93 ± 1.91	27.80 ± 0.69	1.41 ± 0.04	A2
Tomato				
OH 42	38.50 ± 1.67	28.50 ± 1.14	1.30 ± 0.06	A2
OH 43	42.50 ± 0.86	32.25 ± 0.74	1.30 ± 0.02	A2
OH 44	40.00 ± 1.30	29.57 ± 0.83	1.37 ± 0.05	A2
OH 45	40.50 ± 0.77	29.00 ± 0.63	1.39 ± 0.03	A2
OH 46	39.00 ± 1.71	25.50 ± 1.00	1.38 ± 0.08	A2
Periwinkle				
OH 47	41.66 ± 1.45	28.54 ± 1.23	1.43 ± 0.03	A2
OH 48	42.75 ± 1.14	30.25 ± 0.96	1.41 ± 0.06	A2
OH 49	41.25 ± 1.53	28.75 ± 0.78	1.43 ± 0.02	A2
OH 50	41.66 ± 1.63	30.41 ± 1.09	1.36 ± 0.03	A2
OH 51	39.58 ± 1.81	27.91 ± 1.37	1.41 ± 0.06	A2
Carnation				
OH 52	38.61 ± 1.52	27.22 ± 1.32	1.41 ± 0.02	A2
OH 53	38.50 ± 1.67	28.50 ± 1.14	1.30 ± 0.06	A2
OH 54	44.44 ± 1.39	30.55 ± 1.51	1.46 ± 0.06	A2
OH 55	38.75 ± 1.39	27.25 ± 1.20	1.45 ± 0.48	A2
OH 56	46.50 ± 2.74	31.50 ± 1.53	1.46 ± 0.03	A2
Crossandra				
OH 57	43.25 ± 1.17	28.75 ± 1.28	1.53 ± 0.06	A2
OH 58	45.00 ± 1.32	31.11 ± 1.42	1.46 ± 0.05	A2
OH 59	40.00 ± 1.23	27.00 ± 0.93	1.48 ± 0.04	A2
OH 60	46.38 ± 2.28	30.31 ± 1.34	1.53 ± 0.05	A2
OH 61	40.55 ± 1.70	27.77 ± 1.32	1.46 ± 0.05	A2
Gerbera				
OH 62	45.50 ± 1.88	30.25 ± 1.24	1.49 ± 0.04	A2
OH 63	41.66 ± 1.63	30.41 ± 1.09	1.36 ± 0.03	A2
OH64	41.25 ± 1.53	28.75 ± 0.78	1.43 ± 0.02	A2
OH 65	44.07 ± 1.00	30.47 ± 0.90	1.44 ± 0.04	A2
OH 66	42.91 ± 1.49	28.33 ± 1.27	1.51 ± 0.05	A2

*Mean of 100 sporangia; ± standard deviation

RESULTS AND DISCUSSION

Phytophthora nicotianae isolates isolated from tobacco, tomato, periwinkle, carnation, crossandra and gerbera displayed no distinctive colony morphology (dense whitish aerial mycelium). Most isolates produced spherical, ovoid, ellipsoid and persistent sporangia on irregular / loose sympodial sporangiophore (Fig1). Sporangial sizes ranged from 36.70 to 46.50 x 24.92 to 32.25 µm (Table1). L:B ratios of sporangia ranged from 1.31 to 1.53. Chlamydospores were produced readily by all isolates on CA incubated in the dark at 18°C. Chlamydospores were single, spherical, terminal or intercalary and pale yellow in colour (Fig 1). The mean chlamydospores diameter ranged from 20 to 30 µm (Table 2). None of the isolates produced sexual structures. When paired with isolates of *P. capsici* of known mating types, all isolates produced sexual

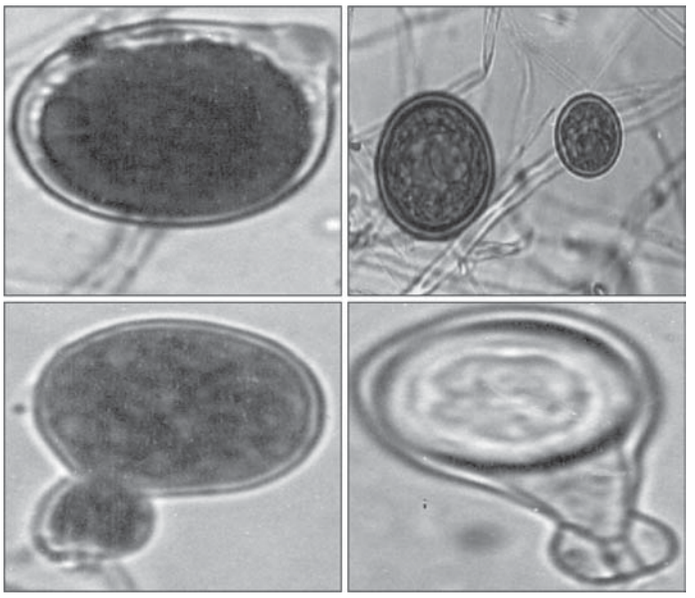

Fig 1. Morphology of *P. nicotianae* isolates, Top left, sporangia, Top right, chlamydospores, Bottom left, oogonia, Bottom right, oospores

structures (Fig 1). Size of oogonia ranged from 26 to 29 µm and oospores varied from 24 to 27 µm (Table 2). Minimum temperature for growth of all isolates was 9°C; optimum was 24 - 30°C; and maximum was 33°C.

Thus, the present study showed that sporangial, chlamydospore, oogonial and oospore characteristics of *P. nicotianae* isolates from periwinkle, carnation, gerbera, tobacco and crossandra are similar to those of *P. parasitica* isolates from tomato. Waterhouse (1963) created two morphological varieties; *P. nicotianae* var. *nicotianae* and *P. nicotianae* var. *parasitica*, which were based on Ashby (1928) microspore and macrospora, respectively. Further morphological studies of Ho and Jong (1989) in isolates from tobacco and other hosts clearly showed that var. *nicotianae* and var. *parasitica* could not be differentiated. Separation of the varieties *nicotianae* and *parasitica* based on morphological criteria was also questioned (Gallegly,1983). Oudemans and Coffey (1991) and Hall (1993) reported that oogonia , oospore and sporangial dimensions are similar among two groups.

When DNA was amplified with universal primer pair (ITS1 and ITS4), there was single band of 920 bp (Fig 2) for all isolates of *P. nicotianae* and reference cultures of *P. nicotianae* (IMI 3406160 and IMI 235604). All isolates had identical ITS - RFLP patterns when PCR product was digested with enzymes *Hinf 1, Msp I, Alu I, Hae III* and *Rsa I* (Fig 3). Restriction fragment sizes of the ITS region of rDNA digested with different enzymes are also identical.

Table 2. Dimensions of chlamydospores , oogonia and oospores of *P. nicotianae* isolates

Isolate	Chlamydospores (µm)	Oogonia (µm)	Oospores (µm)
Tobacco			
OH 37	24.38 ± 1.75*	28.34 ± 2.10	25.90 ± 2.00
OH 38	22.78 ± 2.30	27.26 ± 1.90	26.24 ± 1.75
OH 39	28.20 ± 2.60	28.75 ± 2.00	25.34 ± 2.30
OH 40	23.34 ± 3.24	25.20 ± 1.95	24.37 ± 2.30
OH 41	23.28 ± 1.95	27.32 ± 2.60	25.20 ± 2.60
Tomato			
OH 42	27.34 ± 2.02	29.75 ± 2.72	26.37 ± 2.27
OH 43	28 .28 ± 2.78	27.14 ± 2.02	25.40 ± 2.50
OH 44	23.38 ± 2.50	27.30 ± 2.50	26.32 ± 2.71
OH 45	26.72 ± 2.31	28.14 ± 2.60	26.37 ± 2.80
OH 46	24.32 ± 1.31	27.20 ± 1.90	25.27 ± 1.70
Periwinkle			
OH 47	20.24 ± 1.32	28.14 ± 2.60	25.32 ± 2.75
OH 48	26.32 ± 2.34	28.20 ± 2.80	26.97 ± 2.60
OH 49	26.78 ± 2.14	27.20 ± 1.97	24.32 ± 2.32
OH 50	28.24 ± 3.21	27.30 ± 2.30	25.84 ± 2.00
OH 51	24.32 ± 2.20	27.10 ± 2.02	26.80 ± 2.72
Carnation			
OH 52	28.45 ± 2.10	28.32 ± 2.00	27.40 ± 1.72
OH 53	30.25 ± 1.78	27.20 ± 1.80	26.68 ± 2.34
OH 54	28.75 ± 2.24	27.45 ± 2.20	27.35 ± 2.40
OH 55	25.62 ± 3.14	28.47 ± 2.15	26.40 ± 1.80
OH 56	25.34 ± 2.45	27.90 ± 2.70	25.21 ± 2.30
Crossandra			
OH 57	26.25 ± 2.41	29.30 ± 1.60	24.00 ± 2.26
OH 58	20.35 ± 2.35	26.40 ± 2.30	25.34 ± 1.60
OH 59	20.75 ± 2.48	25.30 ± 1.60	26.73 ± 1.70
OH 60	24.34 ± 1.82	28.17 ± 1.20	27.40 ± 1.20
OH 61	25.75 ± 2.35	26.30 ± 2.00	24.37 ± 1.78
Gerbera			
OH 62	27.32 ± 1.32	27.34 ± 2.06	26.75 ± 1.80
OH 63	26.31 ± 2.71	26.48 ± 2.68	25.30 ± 2.07
OH 64	28.25 ± 2.24	27.84 ± 2.50	25.24 ± 2.01
OH 65	26.32 ± 2.18	26.34 ± 1.85	25.70 ± 2.07
OH 66	22.35 ± 2.14	27.84 ± 2.00	26.87 ± 2.80

*Mean of 100 reproductive structures ± standard deviation

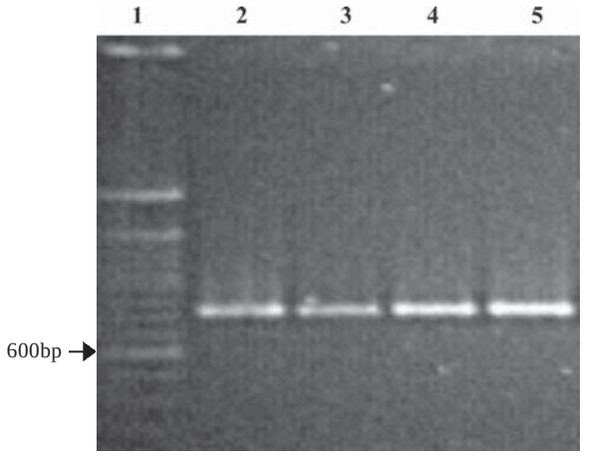

Fig 2. PCR amplification of ITS region of r DNA
Lane1, Marker, 100bp ladder, Lane2, Periwinkle, Lane 3, gerbera, Lane,4,carnation, Lane,5, crossandra

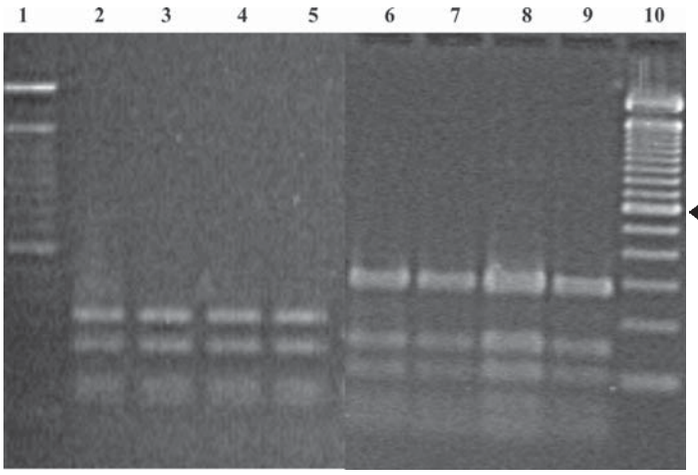

Fig 3. ITS-RFLP patterns generated with *Hinf I* (Lane 2-5)and *TaqI*(Lane 6-9). Lane 1and 10, Marker (100bp) Lane2 and 6,periwinkle, Lane 3and 7, gerbera, Lane,4 and 8,carnation, Lane,5 and 9, crossandra

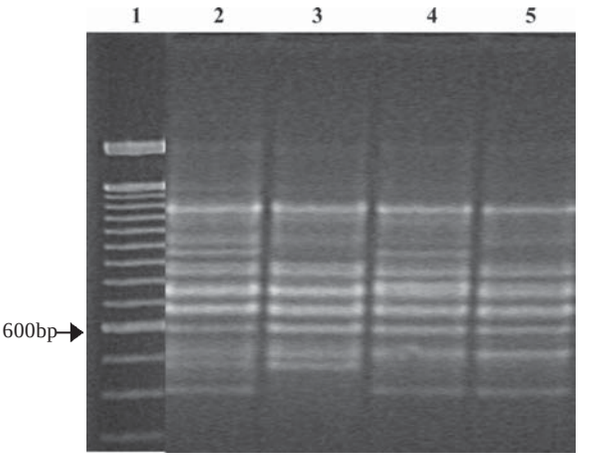

Fig 4. AFLP finger prints of *Phytophthora nicotianae* isolates Lane 1, bp ladder(size marker), Lane2,periwinkle, Lane 3, gerbera, Lane,4,carnation, Lane,5, crossandra

Protein profiles (Erselius and de Vallavielle,1984), serological reactions (Morton and Dukes, 1967), RFLP-DNA patterns (Forster and Coffey,1991) of *P. nicotianae* isolates from tobacco were almost identical to those of *P. nicotianae* isolates from other crops. ITS- RFLP data obtained in the present study also showed that *P. nicotianae* isolates from tobacco, tomato, periwinkle, carnation, gerbera and crossandra were identical. As varieties *nicotianae* and *parasitica* are identical based on morphological and molecular criteria, many researchers proposed (Ho and Jang,1989; Oudemans and Coffey,1991; Hall,1993) that the name *P. nicotianae* be eliminated and that all isolates from tobacco and other hosts should be merged under the name *P. parasitica*.

Among the isolates of *P. nicotianae*, four AFLP finger print groups were evident based on the combined results of five primers (A,B,C,D and E). The AFLP finger prints generated with primer E is shown in Fig 4. Group I includes isolates from tomato, tobacco and periwinkle. Isolates from carnation represents Group II. Isolates from gerbera falls under group III. The crossandra isolates formed a Group IV. Although there was no intra specific ITS variation among isolates of *P. nicotianae*, host specialized groups from tobacco, tomato, periwinkle, gerbera, carnation, and crossandra were found based on AFLP fingerprints.

There is considerable amount of evidence to show that some isolates of *P. nicotianae* are host-specific. For instance, isolates from okra and sesame were host specific, while isolates from *Peperomia obtusifolia* and *Saintpaulia ionatha* had different host ranges (Ho and Jong,1989). Isolates from carnation and tomato were more virulent on carnation and tomato respectively than other hosts. Thus, the present study clearly indicated that *P. nicotianae* isolates from tobacco, periwinkle, carnation, gerbera and crossandra and *P. parasitica* isolates from tomato were identical. The AFLP finger prints were useful in identifying host specialized groups within *P. parasitica* (*P. nicotianae*). While screening the accessions for disease resistance, host specialized AFLP molecular groups should be used rather than morphologically defined isolates.

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