ITS-RFLP BASED IDENTIFICATION OF *PHYTOPHTHORA MEADII* INFECTING RUBBER TREES

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Abnormal leaf fall caused by *Phytophthora* spp. is a serious disease of rubber *(Hevea brasiliensis)* in the traditional rubber growing regions of India. Twenty-five isolates from different rubber growing regions were subjected to molecular characterization and observed the variation if any. Internal transcribed spacers (ITS) of ribosomal DNA of the isolates were amplified with ITS 4 and ITS 6 primers. The rDNA- RFLP pattern was generated for all the isolates with different restriction enzymes. Among the different restriction enzymes tested, digestion was obtained only with two enzymes *viz. Msp 1* and *Alu 1*. Uniform RFLP pattern generated for all the isolates tested from *H.brasiliensis* belonged to *P.meadii*. The RFLP patterns of generated rDNA of isolates from *H.brasiliensis* were compared with other *Phytophthora* species *viz. P.capsici* from pepper and *P.palmivora* from coconut showed variation.

Key words: Abnormal leaf fall disease, *Hevea brasiliensis*, Natural rubber, Oomycetes, *Phytophthora meadii*

Abnormal leaf fall disease caused by *Phytophthora* is considered to be the most destructive disease of rubber *(Hevea brasiliensis)* in India causing an estimated crop loss of 5-49 per cent by different clones (Jacob, 2003). Four species of *Phytophthora viz. P.palmivora* (Butler), *P.meadii* (Mc Rae 1918), *P.nicotinae var parasitica* and *P.botryosa* have been reported to cause this disease in India (Thankamma *et al.*, 1968; Edathil and George, 1976; 1980; Edathil *et al.*, 2000). Other species like *P.faberi, P.hevea and P.citrophthora* have also been reported to cause this disease in other countries (Ramakrishnan and Pillai, 1961; Chee, 1969).

Proper identification of causal organism within a short time span is of importance in disease control. The earlier identification of the causal organism of abnormal leaf fall disease have been on the basis of the morphological characters. However, morphological identification may not be accurate for closely related fungi (Waterhouse 1970). Now PCR based molecular tools have been widely used to identify *Phytophthora* species accurately (Duncan *et al.*, 1987; Cook *et al.*, 2002). As the DNA sequence are unique for an individual and the variation can help in screening the varieties with in a population. Ribosomal DNA (rDNA) restriction digest

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analysis and sequence data have been reported as useful in identification of *Phytophthora* species (Cooke and Duncan, 1997; Cooke *et al.*, 2000; Chowdappa *et al.*, 2003 a, b). The internal transcribed spacers, ITS 1 and ITS 2 in the ribosomal DNA of different *Phytophthora* species were reported as distinct (Duncan *et al.*, 1987). Chowdappa *et al.* (2003 a,b) reported difference in the *Phytophthora* species infecting different plantation crops by rDNA and the restriction fragment length polymorphism (RFLP). In the present study the RFLP of amplified ITS region of ribosomal DNA was used for the identification of *Phytophthora* isolates from *Hevea* to detect any genetic variability among them.

Samples of leaf and infected petioles were collected during the year 2001 and 2002 from different rubber growing regions (Table 1). *P.capsici* and *P.palmivora* isolates were obtained from the phytonet culture collection maintained at the Indian Institute of Spice Research, Kozhikode. The pathogen was isolated and pure culture was developed by hyphal tip method (Brown 1924). One of the *Phytophthora* isolates from *Hevea* (isolate No.7) was identified at the International Mycological Institute, UK.

Total DNA was extracted from 1-2 g of the mycelium harvested from pure culture of the isolate grown on potato dextrose broth for three to four days at 24°C. The mycelium was washed thrice with double distilled water and powdered in liquid nitrogen. The extraction of total genomic DNA was done by a modified CTAB (Hexadecyl trimethyl ammonium bromide, BDH laboratory supplies, Poole, England) method (Doyle and Doyle, 1990). The buffer containing 100mM Tris HCL (pH8), 1.4M NaCl, 20mM EDTA (pH 8), 2 per cent CTAB and 20mM mercaptoethanol was pre-heated to 90°C and the suspension incubated at 65°C for 90 minutes with occasional mixing. After cooling the mixture to room temperature, equal volume of chloroform isoamyl alcohol (24:1) was added and mixed well to get an emulsion by inverting the tube several times. The mixture was centrifuged at 12,000 g for 10 minutes. The aqueous phase was

Table 1. **Source of isolates**

Serial No.	Location	*Hevea* clone / Crop species
1	Thodupuzha	RRII 105
2	Peruvanthanam	RRII 105
3	Puthukkad	RRII 105
4	Vaniyampara	RRII 105
5	Ottappalam	RRII 105
6	Malankara	RRII 105
7	Malankkara	FX 516 (IMI No. 387458)
8	Mundakkayam	RRII 105
9	Mundakkayam	RRII 105
10	Kaliyar	Fx 516
11	Malankkara	Fx516
12	Kaliyar	PB 235
13	Boyce estate	RRIM 600
14	Manarcad	RRII 105
15	Kaliyar	PB 217
16	Lahai estate	PB 28/59
17	Chethakkal	GT 1
18	Malankkara	PB 235
19	Vaniyampara	RRII 105
20	Echippara	RRIM 600
21	CES, Chethackal	RRII105
22	Moozhoor	RRII105
23	Enthayar	PB260
24	RRII	RRII 105
25	Echippara	RRII 105
26	*P. capsici* (IISR Calicut)	*Piper nigrum* L.
27	*P. palmivora* (IISR Calicut)	*Cocos nucifera*

transferred to another tube and re-extraction of the mixture with chloroform isoamyl alcohol was repeated thrice to remove polysaccharides from the DNA. To the final aqueous phase, 1/100 volume of 10mg mL^{-1} Ribonuclease A solution was added and the mixture was incubated at 37°C for 30 minutes. Genomic DNA was precipitated by the addition of 0.7 volume of ice cold isopropanol and centrifuged, at 8,000 rpm for five minutes. The pellet was further washed with pre-chilled 70 per cent ethanol twice, air dried and finally dissolved in TE buffer. The purity and concentration of DNA was estimated spectrophotometrically. The diluted DNA was stored at 4°C for assays.

PCR amplification of ITS region of rDNA was performed using the primers ITS4 (5'TCCTCCGCTTATTGATATCG3') and ITS6 (5'GAAGGTGAAGTCGTAACAAGG 3') synthesized by sigma Genosis Ltd. [U.K.], PCR was performed in a PTC 100 Mini Thermal Cycler (M.J. Research, USA) using 50µl reaction mixture containing 100 ng of template DNA with 0.5µM of each dNTP, three unit of Taq DNA polymerase and five µl of 10X PCR buffer. The PCR was programmed for a single step at 94°C for three minutes 35 cycles of 94 for 30 seconds, 55°C for 30 seconds, 72°C for seconds and followed by a single step at 72°C for 10 minutes (Cooke and Duncan, 1997)

Amplified rDNA was precipitated with distilled alcohol, dissolved in double distilled sterilized water and their purity was tested on two per cent agarose gel. The rDNA was digested with restricted enzymes *Msp* I and *Alu* I, *EcoR* 1 and *Rsa 1* (Gene banglore) according to manufacturer's instruction. The digested fragments were separated on two per cent agarose gel stained with Ethidium bromide.

The amplified rDNA when separated gave a single band of size 900 bp (Fig. 1) for all isolates from rubber and the isolates of *P.capsici* and *P.palmivora* conforming to the

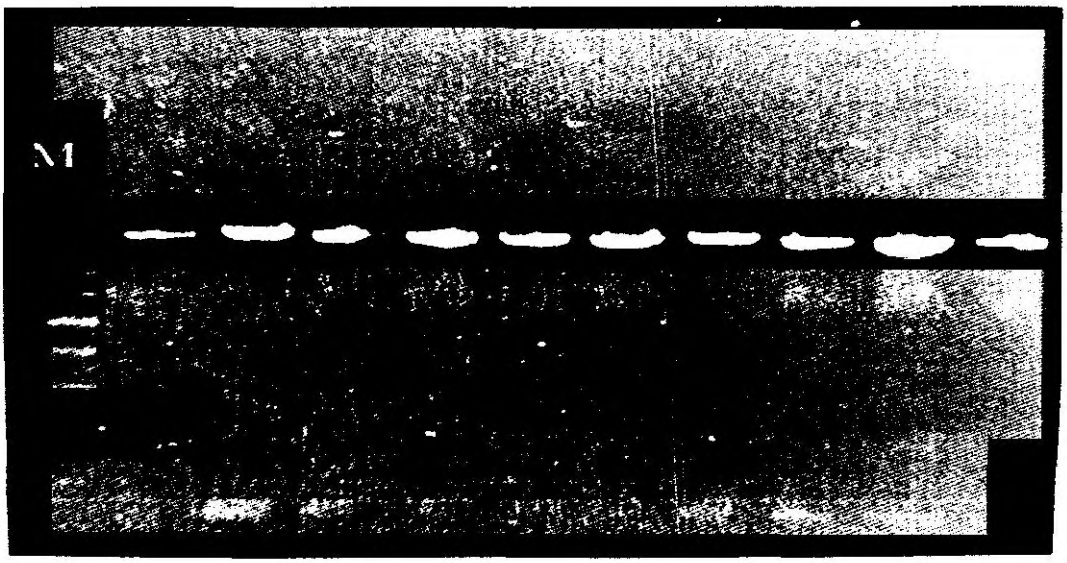

Fig. 1. Amplified rDNA for *Phytophthora* isolates (M- marker100bp ladder)

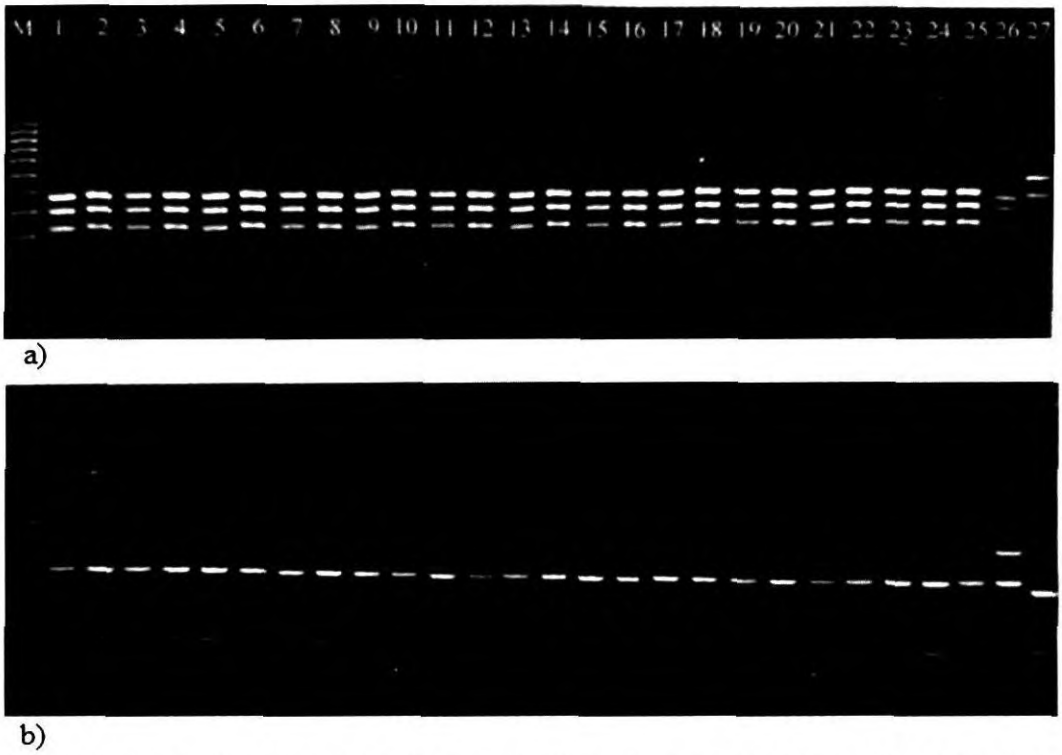

Fig. 2. ITS – RFLP patterns regenerated with the restriction enzymes (a) *Msp* 1 (b) Alu 1(M-Marker 100bp ladder, 1-25 *Phytophthora* isolates from *H. brasiliensis*, 26-*P.capsici*, 27-*P.palmivora*)

range reported for *Phytophthora* (Chowdappa *et al.*, 2003a).

When the amplified rDNA products were digested with the restriction endonucleases (*Msp* I, *Alu* I, *Eco* RI and *Rsa* I) digestion was obtained with only two enzymes *viz.* *Msp* I and *Alu* I (Fig. 2a and b) for all the isolates tested. The isolates from *H. brasiliensis* showed uniform restriction fragments (Table 2) and were similar to that of isolate NO. 7 identified by IMI as *P.meadii* on the basis of both morphology as well as ITS, RFLP.

The rDNA restriction length polymorphism detected among the fungal isolates were due to sequence variations in the ITS region of rDNA gene sequences. As these variations are highly specific for each species of fungi, it is the most effective method to compare the species within a genus. The uniform restriction fragment size of the ITS region digested with the two informative restriction enzymes clearly indicated that all the *Phytophthora* isolates from rubber belong to the species *P.meadii* and that they differ from *P.capsici* and *P.palmivora* although the identification and taxonomy of *Phytophthora* species are based on morphological characters the differences between the species are very little and the identification is often difficult (Erwin and Riberio, 1996; Appiah *et al.*, 2003). *P.meadii* is differentiated from *P.palmivora* on the basis of sporangial

Table 2. **Restriction fragment size (in bp) of *Phytophthora* rDNA, RFLP**

Isolates	Restriction enzymes	
	Msp I	Alu I
P. meadii	390,300,220	550,190
P. capsici	350,340,200	
P. palmivora	510,390	490,190

morphology and length of the pedicel. The sporangia of *P.meadii* are spherical to ovoid, caducious with medium pedicel length on sympodium, production of chlamydospores and formation of aplerotic oospores are rare (Oudemans *et al.*, 1991). *P.faberi* has been reported as the causal agent of abnormal leaf fall disease of rubber in Sri Lanka (Gadd, 1924) and latter *P.faberi* was merged with *P.meadii*. Recent studies could separate *P.meadii* isolates from *P.palmivora* on the basis of RFLP of ITS region of rDNA. *P.meadii* isolates from rubber and arecanut showed similarity on ITS, RFLP (Chowdappa *et al.*, 2003b).

The close similarities in morphological characteristics of *Phytophthora* species necessitate careful and detailed observation to avoid mis-identification. The present study has indicted that all *Phytophthora* isolates from rubber belong to *P.meadii* only and are clearly distinct from *P.capsici* and *P.palmivora* on the basis of rDNA RFLP. This is an easy method for accurate identification of *Phytophthora* species, a pre-requisite for adopting appropriate control measures based on epidemiology and pathogenicity of the species.

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