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First report of leaf blight caused by *Alternaria alternata* on *Hevea brasiliensis* in India

C. Bindu Roy*, Cina Ann Zachariah, C. Kuruvilla Jacob and Thakurdas Saha
Rubber Research Institute of India, Kottayam 686 009

Abstract

Alternaria leaf blight caused by *Alternaria alternata* was observed on rubber (*Hevea brasiliensis*) tree, the source of natural rubber latex of commercial value. Initial symptoms appeared on young leaves as minute spots, which enlarged with the growth of the leaves. A characteristic browning and blackening of veins forming a 'fishbone' or 'railway track' symptom was noticed at later stage of the disease. This disease symptom appeared similar to that of the devastating Corynespora leaf disease, caused by *Corynespora cassiicola* on *Hevea brasiliensis*. Twenty-six isolates from two states of India, Kerala and Karnataka, were characterized using random amplified polymorphic DNA (RAPD) analysis and two major profiles were detected. Cluster analysis resolved the isolates into two major groups. Restriction analysis of the PCR amplified ribosomal DNA including the flanking internal transcribed spacers of representative isolates from both the groups also revealed two distinct RFLP patterns reflecting the same grouping as detected through RAPDs. The amplified rDNA from representative isolates of both the groups was cloned and sequenced. Sequence analysis revealed significant homology of one group of isolates with *A. alternata* and the other with *C. cassiicola*. This is the first report of this disease from India.

Key words : *Alternaria, corynespora*; cnternal transcribed spacers; Random Amplified Polymorphic DNA (RAPD); Restriction Fragment Length Polymorphism (RFLP).

Introduction

Hevea clones are susceptible to various foliar diseases. In rubber, severe form of Corynespora leaf disease caused by *C. cassiicola* resulted in a characteristic browning and blackening of veins of leaves forming a 'fishbone' or 'railway track' symptom. The disease symptoms initially appear as minute spots on leaves, which enlarged with the growth of the leaves. The spots are circular or irregular with a brown or papery centre surrounded by a dark brown ring and a yellow halo. However, association of *Alternaria* with similar symptom was noticed while working with the pathogen *C. cassiicola*. Earlier Martin (1947) reported *Alternaria* infection on *Hevea* in Mexico and the species was identified as *A. castilloae*. In this paper, an attempt was made to study if the association of *Alternaria* could develop a symptom, which resembles with the Corynespora leaf disease of *Hevea*.

Advances in molecular biology, particularly the polymerase chain reaction (PCR) assay, have provided exciting opportunities for the development of rapid and precise nucleic acid-based tests for the detection of various plant pathogens. The RAPD allows quick assessment of genetic variability among fungal isolates in various taxa, and has been used to study variability among fungi (Inglis *et al.*, 2001). The internal transcribed spacer (ITS) regions of rDNA in fungi are also useful for molecular systematic studies (Roy *et al.*, 2005; Saha *et al.*, 2002). Because of its higher degree of variation than other genic regions of rDNA (SSU and LSU), variation among individual rDNA repeats can be observed within the ITS regions. This has proven particularly useful for separation of fungal taxa at the genus to species level, because the rate of accumulation of mutations in these regions often approximates to the rate of speciation (Bruns *et al.*, 1991; Lee and Taylor, 1992). With the advancement of molecular techniques, several studies have examined genetic relationships using

* For correspondence

a variety of molecular methods among isolates of *Corynespora* (Saha *et al.*, 2000; Saha *et al.*, 2002) as well as *Alternaria* (Pryor and Gilbertson, 2000; Pryor and Michailides, 2002). The present study is aimed at grouping the isolates from similar symptom on *H. brasiliensis* using molecular tools.

Materials and Methods

Isolation of the pathogen

The pathogen showing symptoms of pinpoint to 7 mm lesion on leaves was isolated from *H. brasiliensis* collected from rubber plantations in Kerala and Karnataka during 2004 (Table 1). Isolations were made by plating surface-sterilized pieces of diseased tissue on potato dextrose agar (PDA) medium. All isolates were purified by single-spore culture (Ho and Ko, 1997), maintained on PDA and stored at room temperature. Twenty-six isolates were used in the present study.

Genomic DNA extraction

Six mycelial plugs (5 mm diameter) were taken from 6-day-old culture of each isolate, transferred to potato dextrose broth (PDB) and incubated at 25°C for 4 days. Mycelium was harvested by filtering through Whatman No.1 filter paper under vacuum, washed thrice with sterile distilled water, dried in liquid nitrogen and powdered. Extraction and purification of the total genomic DNA were carried out following a modified CTAB (Hexadecyl trimethyl ammonium bromide, Sigma Co., St. Louis, USA) method optimised for *Corynespora cassicola* (Saha *et al.*, 2000).

RAPD analysis

Twenty decamer primers (Table 2) selected at random from Operon primer kits (Operon technology Inc., USA) were used for PCR amplifications. Amplifications were performed in a total volume of 25 ml by mixing 50 ng of template DNA with 10 picomoles of single primer, 0.2 mM of each dNTP, 0.7 units of Taq DNA polymerase (Genei, Bangalore) and 2.5 ml of 10X DNA polymerase buffer. Amplifications were performed in a thermal cycler (Bio-Rad, USA) with an initial denaturation at 94°C for 3 min, followed by 40 cycles of 30 sec at 94°C, 1 min at 37°C and 2 min at 72°C with a final extension at 72°C for 10 min. Amplified products were analyzed along with a DNA marker, as molecular size reference, by electrophoresis on a 1% agarose gel in 1X TBE buffer. The gels were stained with ethidium bromide and viewed on UV transilluminator. To check the reliability of the method, the amplification reactions were performed twice. Amplification products were scored on the basis of their presence or absence and variations in the intensity of the same band across the isolates were not considered in the analysis. Pair-wise comparisons of the RAPD profiles of the isolates, based on both unique and shared amplification products, were employed to calculate genetic distance (GD). GD was calculated according to the following equation:

$$GD_{xy} = (N_x + N_y) / (N_x + N_y + N_{xy})$$

where, N_x is the number of bands in isolate x and not in isolate y, N_y is the number of bands in isolate y and not in isolate x and N_{xy} is the number of bands in isolates x and y (Link *et al.*, 1995). The data were subsequently used for cluster analysis to construct a dendrogram. All calculations were made using the TREECON programme (Van de Peer and De Wachter, 1994).

Ribosomal DNA RFLP analysis

Amplification of rDNA

Amplification of the internal transcribed spacer (ITS) regions between the small (18S) and large (28S) nuclear rDNA was achieved using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) supplied by Sigma Genosys, USA. The amplified fragment included the 5.8S rDNA gene and the internal transcribed spacers (ITS1 and ITS2) (Fig. 1). PCR amplification was performed in a total volume of 50 ml containing 100 ng of template DNA with 0.5 mM of each primer, 0.2 mM of each dNTP, 2 units of Taq DNA polymerase and 5 ml of 10X DNA polymerase buffer. Amplifications were performed in a thermal cycler with an initial denaturation step at 94°C for 3 min, followed by 45 cycles of 30 sec at 94°C, 1 min at 55°C and 2 min at 72°C with a final extension at 72°C for 7 min. Amplified products were separated on 1% agarose gel in 1X TBE buffer. The gels were stained with ethidium bromide and viewed on a UV transilluminator.

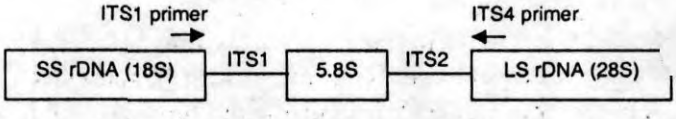

Fig. 1. Schematic representation of the repeat unit of nuclear ribosomal DNA. The open boxes represent the genes for ribosomal subunits. The arrows represent the positions of the PCR primers (ITS1 and ITS4) used for amplification of the 5.8S rDNA and the internal transcribed spacer regions ITS1 and ITS2.

Restriction enzyme digestion of amplified rDNA

PCR amplified rDNA products were purified using GFX-Gel band purification kit (Amersham Biosciences) and digested with the restriction enzymes *Alu* I, *Bam* HI, *Eco* R I, *Hae* III, *Hind* III, and *Sau* 3A I using the buffers and conditions recommended by the suppliers.

Table 1. Source of isolates used in the study

No	Code	Location*	Host	Symptom	% infection
1	Cc 105	Neeria, KN	GT 1	0.2 - 1 mm lesions on leaves	<10
2	Cc 106	Nettana, KN	GT 1	2 - 3 mm lesion on leaves, veins and raised spots	<10
3	Cc 107	Nettana, KN	GT 1	2 - 3 mm lesion on leaves, veins and raised spots	20-Oct
4	Cc 108	Nettana, KN	GT 1	3 - 5 mm lesion on leaves	20-Oct
5	Cc 109	Nettana, KN	RRII 105	0.2 - 5 mm lesion on leaves.	>80
6	Cc 110	Neeria, KN	PB 311	2 mm lesions on leaves	<10
7	Cc 111	Neeria, KN	RRII 105	0.2 - 3 mm lesion on leaves	20-Oct
8	Cc 112	Guthigar, KN	RRII 105	3 - 5 mm lesion on leaves extending to the vein	20-Oct
9	Cc 113**	Karmayi, KN	RRII 105	0.1 - 3 mm lesion and raised 0.2 spots	20-Oct
10	Cc 114**	Guthigar, KN	RRII 105	3 - 6 mm lesion on leaves	20-Oct
11	Cc 115	Adoor, KL	RRII 105	0.2 - 6 mm lesion on veins	20-Oct
12	Cc 116	Sampaje, KN	RRII 105	0.2 - 3 mm lesion on leaves	20-Oct
13	Cc 117	Nikkiladi, KN	RRII 105	1 - 5 mm lesion on leaves and veins	60-70
14	Cc 118	Kakkinje, KN	RRII 105	0.2 mm sized lesions	<10
15	Cc 119	Sheradi, KN	RRII 105	2 - 3 mm lesion on veins	20-Oct
16	Cc 120	Champamthotti, KL	RRII 105	1 - 4 mm lesion on leaves	>50
17	Cc 121	Thadikadavu, KL	RRII 105	3 mm lesion on leaves	>50
18	Cc 122	Malakkal, KN	RRII 105	6 - 7 mm lesion on leaves and veins	30 to 50
19	Cc 123	Irikkoor, KN	RRII 105	0.2 - 4 mm lesion on leaves and veins	10 to 20
20	Cc 124	Peringom, KL	RRII 105	1 mm lesion on leaves and veins	20
21	Cc 125	Pranavom, KL	RRII 105	0.2 - 5 mm lesion on leaves and veins	<10
22	Cc 126	Rayarom, KN	RRII 105	0.2 mm lesion on leaves	20-Oct
23	Cc 127	NSS, KN	RRII 105	0.2 mm lesion on leaves	<10
24	Cc 128	Peravoor, KL	RRII 105	0.2 - 5 mm lesion on leaves	20-Oct
25	Cc 129	Palar, KN	RRII 105	0.2 mm lesion on leaves	<10
26	Cc 130	Nettana, KN	GT1	Raised spots	<10

* KN – Karnataka; KL - Kerala, ** Later identified as *Alternaria* isolate

The digested fragments were separated on 2% agarose gel, stained and visualized as described above.

Cloning and sequencing of 5.8 S rDNA and ITS regions

Cloning of the PCR product was carried out using the pGEM-T vector (Promega, USA). Ligation was performed overnight at 16°C with T4 DNA ligase. Ligated products were transformed into JM 109 high efficiency competent cells. After transformation, the recombinants were selected by blue/white screening in X-gal medium. Two of the positive clones bearing the insert were sequenced at Avesthagen, Bangalore, using vector directed primers T7 and SP6 to get the complete ITS1 and ITS2 sequences along with the 5.8S rDNA. DNA sequences were aligned and sequence comparison was done through BLAST analysis at the NCBI (National Centre for Biotechnology Information, USA) site.

Results and Discussion

RAPD analysis

RAPD analysis is rapid and efficient in identifying variation among the pathogens and is therefore ideal for genetic structuring of plant pathogens. RAPD primers used in the study amplified reproducible RAPD profiles for all the 26 isolates. A total of 197 fragments were scored of which, 185 (93.91%) showed polymorphism (Table 2). The number of amplified DNA fragments generated by individual primers varied from four to 15. There was a high degree of similarity in RAPD profiles of all the isolates except two of them, Cc 113 and Cc 114, collected from Karmayi and Guthigar regions of Karnataka respectively, infecting RRII 105 clone of *H. brasiliensis*. Interestingly, these two isolates showed a distinctly different pattern with all the 20 primers tested (Figs. 2 a-c). Genetic relationships among the isolates were analysed using distance matrix data, based on pair-wise comparison of the RAPD profiles. Cluster analysis using UPGMA program produced a dendrogram in which the 26 isolates resolved into two major clusters. Cluster I contained 24 isolates with a genetic distance of less than 30% and cluster II had only two isolates, Cc 113

Table 2. Details of primers and the fragments amplified by RAPD profiling

Primer code	Nucleotide Sequence (5' to 3')	Number of Amplified fragments	Number of Polymorphic fragments	Polymorphism index
OPA-02	TGCCGAGCTG	6	3	0.5
OPA-03	AGTCAGCCAC	8	6	0.75
OPA-04	AATCGGGCTG	9	9	1
OPA-07	GAAACGGGTG	5	5	1
OPA-08	GTGACGTAGG	4	4	1
OPA-10	GTGATCGCAG	14	14	1
OPA-14	TCTGTGCTGG	4	4	1
OPA-17	GACCGCTTGT	10	10	1
OPA-18	AGGTGACCGT	11	11	1
OPA-19	CAAACGTCGG	5	5	1
OPA-20	GTTGCGATCC	11	9	0.82
OPB-06	TGCTCTGCCC	9	9	1
OPB-07	GGTGACGCAG	11	10	0.91
OPAB-11	GTGCGCAATG	15	15	1
OPAB-14	AAGTGCGACC	15	14	0.93
OPAC-05	GTTAGTGCGG	9	9	1
OPAC-12	GAGAGGCTCC	12	10	0.83
OPAE-14	GAGAGGCTCC	15	15	1
OPAF-17	TGAACCGAGG	11	11	1
OPAJ-11	GAACGCTGCC	13	12	0.92
	Total	197	185	

and Cc 114, having a genetic distance of over 75% with the isolates from cluster I. High degree of genetic differentiation (>75%) noticed between these two groups prompted us to assume the involvement of two different organisms in disease development although they were causing similar disease symptom. The isolates from cluster I sub-clustered into five distinct groups. Sub-cluster 1 contained 13 isolates of which 9 were from Karnataka and 4 from Kerala. The second sub-cluster had only two isolates, Cc 107 and Cc 112, both isolated from two estates of Karnataka, Nettana and Guthigar, infecting GT1 and RRII 105 respectively with a disease incidence of only 10-20% (Table 1). The sub-clusters 3 and 4 contained one isolate each from Kerala (Cc 110) and from Karnataka (Cc 124) isolated from RRII 105 and PB 311 respectively. In both the cases mild disease incidence was observed. The fifth sub-cluster contained 7 isolates of which three were from GT1 and the other four from RRII 105. The dendrogram revealed that all the isolates were genetically different with a genetic distance less than 20%. The pathogen isolated from the same host was found to group into different clusters, for example, five isolates of the pathogen infecting the clone GT 1 were grouped into three different clusters. However, out of the five isolates, three (Cc 105, Cc 106,

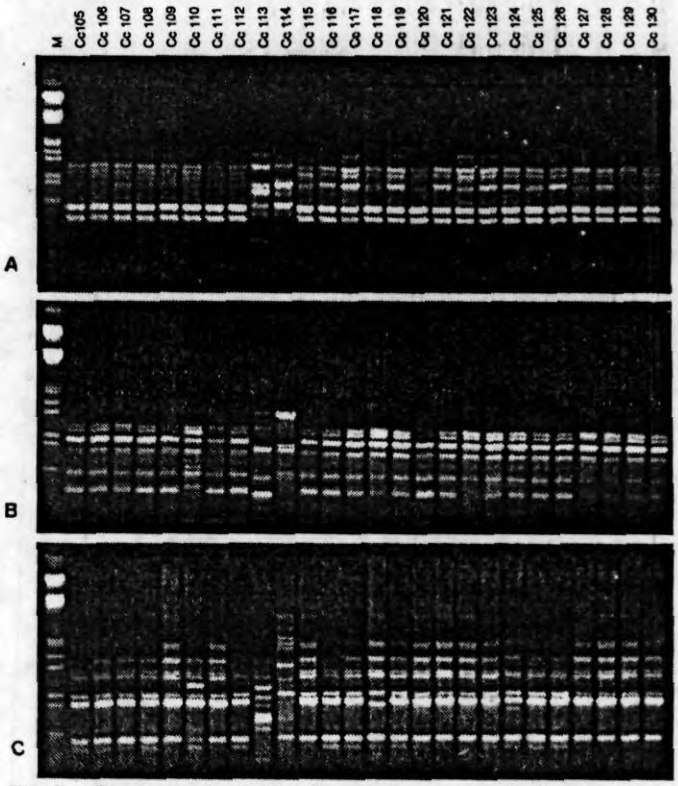

Fig. 2. Representative RAPD fingerprints generated with the primers OPA-04 (a), OPB-07 (b) and OPA-20 (c). Lane M: molecular weight marker (l-DNA/*Eco* RI + *Hind* III).

and Cc 108) were grouped together in one cluster showing close relation among them. The isolates exhibited little correlation between the geographical origin of the isolate and their RAPD group (Table 1) in different rubber growing regions of Kerala and Karnataka. This indicates that each pathotype might have originated from one point and eventually spread to different areas. This situation could have arisen in one of several ways, including the wind dissemination of spores between adjacent regions or through the transfer of diseased planting material from nurseries to rubber plantations. RAPD techniques have been used to determine the amount of interspecific genetic variability, which is required for the development of long-time disease management strategies (De Oliveira *et al.*, 2002; Tigano *et al.*, 2003).

rDNA-RFLP analysis

PCR-RFLP analysis of nuclear rDNA characterizes restriction sites within the highly variable ITS region. The internal transcribed spacer (ITS) regions of ibosomal DNA (rDNA) have been most useful for molecular systematic studies and delineation of species. This region evolve rapidly, suggesting that differences in the size and restriction endonuclease digestion pattern of the ITS region might be detected within fungi (Nazar *et al.*, 1991; Lee *et al.*, 1993) and hence are often studied for comparing within fungi (Anderson *et al.*, 2003; Chowdappa *et al.*, 2003a; 2003b; Watanabe, 2004). In our study, the amplified rDNA product (internal transcribed spacers including 5.8S rDNA) was identical in size (~0.6 kb) (Fig. 3). Restriction digestion of the amplified rDNA product with two representative isolates from both groups of the dendrogram (Cc 108 and Cc 129, the isolates showing wide divergence among cluster I and Cc 113, Cc 114 from cluster II), obtained with six restriction enzymes (*Alu* I, *Bam* HI, *Eco* R I, *Hae* III, *Hind* III, and *Sau* 3A) yielded two unique patterns (Fig. 4). The rest of the isolates of cluster I also showed similar restriction profiles with the two representative isolates (Cc 108 and Cc 129), indicating that only two restriction profiles existed among the 26 isolates, which reflect major grouping (cluster I and II) of the pathogen. The digestion products were reproducible and the results were similar to those of the RAPD analysis in that the isolates from both the groups digested with all the restriction endonucleases revealed two different digestion profiles, suggesting a significant degree of nucleotide sequence variation in the rDNA region of the isolates from both the groups (Fig. 4). The variation in the rDNA-ITS region of representative isolates was further checked by cloning the amplified rDNA onto pGEM-T vector and DNA sequence analysis. The sequences revealed homology of one group of isolates with *Corynespora cassiicola* and the other group with *Alternaria alternata*. The results of the RAPD and rDNA-RFLP analyses are corroborated with results of sequence analysis. Our observation based on molecular studies clearly showed that two pathogens belonging to two different genus incite similar disease symptom on rubber. Identity of the pathogen was confirmed by the International Mycological Institute, UK.

In conclusion, molecular analysis using RAPD and rDNA-RFLP techniques revealed the existence of A. alternata as one of the pathogens of rubber causing leaf blight disease, which is very similar to the disease symptom caused by another devastating pathogen C. cassiicola. The pathogenicity tests assessed using three methods: in vivo inoculation, detached leaf technique and leaf wilt bioassay, confirmed that both these pathogens are involved in the development of a similar disease symptom on rubber. In the present study, out of 26 isolates with similar disease symptom on rubber, only two were found to be Alternaria (7.7%) and the rest were Corynespora (92.3%). However, more number of samples is to be evaluated from similar disease symptoms to ascertain the contribution of Alternaria in the

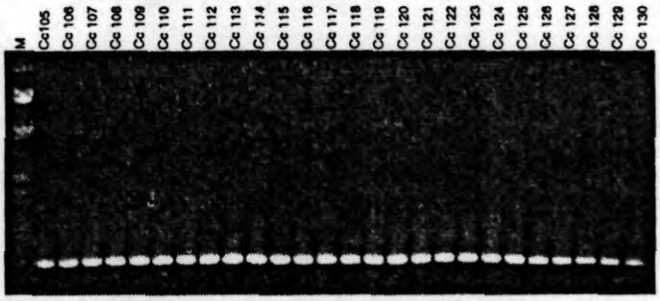

Fig. 3. Gel photograph of the PCR amplified ITS regions between the small (18S) and large (28S) nuclear rDNA from the 26 isolates used in the study. The amplified portion includes ITS 1 – 5.8 S rDNA – ITS 2 regions. Lane M: molecular weight marker (l-DNA/*Eco* RI + *Hind* III).

development of leaf blight disease in Hevea. For efficient disease management the knowledge of the pathogen is essential. As there are two pathogens associated with the same disease symptom, efficient control may depend on development of strategies for simultaneous suppression of both. At present there is only one report of Alternaria leaf blight caused by Alternaria castilloae in H. brasiliensis in Brazil (Martin, 1947). This study reports for the first time Alternaria leaf blight on H. brasiliensis caused by A. alternata in India.

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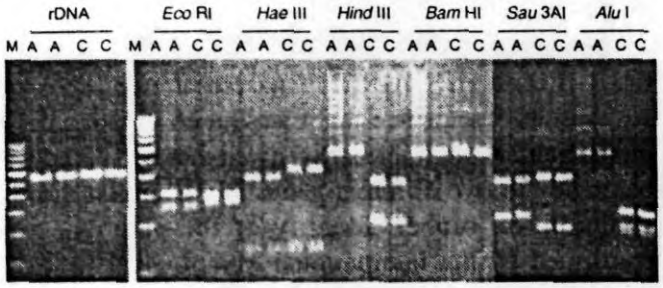

Fig. 4 Gel photograph of restriction fragment length polymorphisms of PCR amplified rDNA from representative isolates of both the groups (A: *Alternaria* and C: *Corynespora*) using six different restriction enzymes revealing sequence variation between the two groups of isolates. Lane M: 100 bp ladder molecular weight marker.

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