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MOLECULAR CHARACTERISATION OF FUNGAL PATHOGENS CAUSING LEAF DISEASES IN RUBBER (*HEVEA BRASILIENSIS*)

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ABSTRACT

Identification of fungal races/pathotypes using morphological criteria alone is difficult. Hence, molecular markers were used in the present study to characterise the pathogen causing leaf diseases of rubber. Molecular characterisation of the *Colletotrichum* isolates showed two major RAPD profiles suggesting the possibility of involvement of two different fungi inciting different disease symptoms, raised spots, anthracnose and circular papery lesions on *Hevea*. RFLP analysis of ribosomal DNA spacers (ITS4/5 including 5.8S ribosomal DNA) also reflected two groupings of the *Colletotrichum* isolates confirming the existence of two different *Colletotrichum* species infecting *Hevea*. Nucleotide sequence analysis of the rDNAs revealed that the two groups of pathogens belonging to two different species of *Colletotrichum*, namely, *C. acutatum* and *C gloeosporioides*.

Genetic structuring of the *Corynespora* populations, another fungal pathogen of rubber, was carried out through RAPDs. Ten different genotypes/races of *Corynespora cassiicola* were identified from different rubber plantations in Kerala and Karnataka states. Wide genetic variability was noticed among the *Corynespora* isolates from Kerala. Knowledge about the existence of different *Corynespora* races would be very useful to trace the development of new virulent races among the populations for avoiding the outbreak of the disease.

INTRODUCTION

Rubber trees are prone to various leaf diseases caused by fungal pathogens. Among the pathogens, *Colletotrichum* and *Corynespora* cause mild to severe disease incidence in *Hevea* resulting in significant reduction in latex yield. *Colletotrichum* causes leaf disease in rubber with the development of three characteristic symptoms: raised spots, anthracnose and circular papery lesions. Earlier, another fungus, *Gloeosporium alborubrum* was considered to be the pathogen responsible for raised spot symptom in *Hevea*. However, the present day taxonomists suggested synonymy of the two genera *Colletotrichum* and *Gloeosporium* as they are morphologically indistinguishable. *Glomerella cingulata*, teleomorph of both the genera, has now been described as the causative agent of the disease. Therefore, taxonomical identity of the pathogen causing three different disease symptoms in *Hevea* is confusing and needs to be clarified.

Corynespora, another leaf pathogen has become a major threat to the present-day rubber plantations in almost all the rubber-growing countries. *Corynespora* leaf disease attained an epidemic scale affecting vast areas of rubber plantations in Indonesia, Sri Lanka, Malaysia and Thailand (Jacob, 1997). *C. cassiicola* was reported for the first time in India in 1958 as a pathogen on *Hevea brasiliensis* (Ramakrishnan and Pillay, 1961). The disease is of serious concern as the high yielding clone RRII 105 is susceptible to *Corynespora* infection. Severity of the disease in different rubber growing regions is highly variable, which may be attributed to several factors like climate, existence of virulent pathotypes and susceptibility of the host genotype under cultivation. Where a mild disease turns out to be an epidemic in a short time, development of new races of the pathogen could be suspected. Identification of races/pathotypes by cultural characteristics may not be accurate as it is based on morphological criteria that are often dependant on cultural conditions, leading to misidentification (Faris-Mokaiesh *et al.*, 1996). Hence, characterization of the *Corynespora* isolates at the molecular level appears to be very essential for identification of the races/pathotypes existing among the population.

Development of molecular techniques, such as PCR-based methods, has provided powerful tools for fungal diagnosis and taxonomy (Bridge and Arora, 1998). Random amplified polymorphic DNA assays (RAPD; Williams *et al.*, 1990) are being extensively used in fungal studies to distinguish within and between species (Raina *et al.*, 1997). Another molecular approach, restriction fragment

length polymorphism (RFLP) analysis of internal transcribed spacer (ITS) or inter generic spacer (IGS) of ribosomal DNA (Silva *et al.*, 1998; Hsiang and Mahuku, 1999) are useful in species delineation. Use of ribosomal DNA (rDNA), RFLP analysis and sequence data for species delineation in *Colletotrichum* have been reported by many authors (Buddie *et al.*, 1999; Freeman *et al.*, 1996; Sherriff *et al.*, 1994; 1995; Sreenivasaprasad *et al.*, 1993; 1994). However, there are only a few published reports on molecular characterisation of *C. cassiicola* isolates on rubber (Darmono *et al.*, 1997; Silva *et al.*, 1995; Silva *et al.*, 1998) as the pathogen is a relatively less known fungus than *Colletotrichum*.

Ribosomal DNA analysis along with RAPD markers were adopted to verify the pathogen identity in Colletotrichum leaf diseases of rubber (Saha *et al.*, 2002). Nucleotide sequence analysis of rDNA from the pathogen isolates was attempted in the present study to confirm the identity of two different fungi involved and to get an insight of the extent of the rDNA sequence variability between these two pathogens. In Corynespora leaf disease, RAPD profiling had been the choice of technique for molecular genetic structuring of the pathogen population from traditional rubber growing regions to trace the development of new aggressive races/ genotypes in a fungal population (Saha *et al.*, 2000). RAPD analysis of 16 more fungal isolates from different locations in Karnataka and Kerala, collected during the years 1999 and 2000 was carried out to identify existence of more groups if any.

MATERIALS AND METHODS

Colletotrichum isolates

The isolates from three typical symptoms, raised spots, anthracnose and papery lesions on leaflets of different clones of *H. brasiliensis* were collected from rubber plantations located at the Rubber Research Institute of India (RRII), Kottayam and other locations in Kerala state. Twenty-five isolates (14 from raised spots, 6 from anthracnose and 5 from papery lesions) were used in the present study (Table 1). Isolations were made by plating surface-sterilized pieces of diseased tissue on oat-meal agar (OMA). All isolates were purified by single-spore culture, maintained on OMA and stored at 5°C in the dark. Fungal isolates were identified based on culture characteristics and microscopic morphology.

Corynespora isolates

C. cassiicola showing typical symptoms on leaves of *H. brasiliensis* were collected from various rubber plantations and nurseries located at 19 different places in Kerala and Karnataka states of India (Table 2). Isolations were made by plating surface-sterilized pieces of diseased tissue on potato dextrose agar (PDA). All isolates were purified by single-spore culture, maintained on PDA and stored at 5 C in the dark. Thirty-six isolates of *C. cassiicola* were used in the present study that had been collected in different years.

Genomic DNA extraction

For extraction of genomic DNA, about 300-500 mg of mycelium was harvested by filtering through Whatman No.1 filter paper under vacuum, washed three times with sterile distilled water; immediately frozen in liquid nitrogen and powdered. Extraction and purification of the total genomic DNA were carried out following a modified CTAB (Hexadecyl trimethyl ammonium bromide) method (Saha *et al.*, 2000).

RAPD analysis

Pre-screened nine decamer Operon primers (OPA-18, OPB-10, OPB-12, OPB-17, OPB-18, OPD-11, OPE-18, OPI-06 and OPJ-20) for *Colletotrichum* and seven primers (OPA-01, OPA-02, OPA-03 OPA-04, OPA-10, OPA-18 and OPA20) fo *Corynespora* 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 unit of Taq DNA polymerase (Promega, USA), 2.5 ml of 10X DNA polymerase buffer (100 mM Tris-HCl (pH 9.0), 500 mM KCl, 20 mM $MgCl_2$). Amplifications were performed in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, USA) with an initial denaturation step 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 7 min. Amplified products were analysed along with a DNA marker, as molecular size reference, by electrophoresis on a 1.4% agarose gel in 1X TAE 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.

Ribosomal DNA (rDNA) RFLP analysis of Colletotrichum

Amplification of rDNA. Amplification of the internal transcribed spacer regions between the small (18S) and large (28S) nuclear rDNA (Fig. 3A) was achieved using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) supplied by Sigma Genosys, USA. The amplified fragment (ITS4/5) includes the 5.8S rDNA gene and the internal transcribed spacers (ITS1 and ITS2). PCR amplification was performed as mentioned earlier for RAPD assays, except for the annealing at 55°C.

Restriction enzyme digestion of amplified rDNA. PCR amplified rDNA products were purified and digested with the restriction enzymes *Acc* I, *Alu* I, *Sau* 3A I, *Eco*R I (Boehringer Mannheim), *Taq* I and *Xho* I (Promega) using the buffers and conditions recommended by the suppliers. The digested fragments were separated on 4% agarose gel, stained and visualized as described above.

Cloning and sequencing of rDNA

PCR amplified rDNA fragments were

Table 1. Source of *Colletotrichum* isolates[2]

Code No.	Host genotype	Location	Symptom
CG1	BD 10	RRII Farm, Kottayam	Raised spots
CG2	BD 10	RRII „	Raised spots
CG3	GT 1	RRII „	Raised spots
CG4	GT 1	RRII „	Raised spots
CG5	RRII 208	RRII „	Raised spots
CG6	RRII 105	RRII „	Raised spots
CG7	PB 280	RRII „	Raised spots
CG8	PB 310	RRII „	Raised spots
CG9	PB 217	RRII „	Raised spots
CG10	PB 217	RRII „	Raised spots
CG11	RRII 105	RRII „	Raised spots
CG12	RRII 105	Chervally Estate	Raised spots
CG13	RRII 105	Chervally „	Raised spots
CG14	RRII 105	Manickal Estate	Raised spots
CG15	Seedling	RRII Farm, Kottayam	Anthracnose
CG16	Seedling	RRII „	Anthracnose
CG17	Seedling	RRII „	Anthracnose
CG18	Seedling	RRII „	Anthracnose
CG19	PR 255	RRII „	Papery lesions
CG20	RRII 300	RRII „	Papery lesions
CG21	RRII 300	RRII „	Papery lesions
CG22	PB 235	RRII „	Papery lesions
CG23	Seedling	Kaliyar Estate	Anthracnose
CG24	PB 260	Manickal Estate	Anthracnose
CG25	RRII 105	Manickal Estate	Papery lesions

cloned in pGEM-T vector (Promega) after purification of excised gel band and transformed in to *E.coli* DH5á cells. Recombinant plasmids were isolated and purified using GFX Plasmid purification kit (Amersham Pharmacia biotech) and sequenced on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems) at the Mycrosynth GmbH. The BLASTN program was used to perform homology search of rDNA nucleotide sequences with the National Centre for Biotechnology Information (NCBI) sequence database. Two rDNA sequences of *Colletotrichum* were registered with the GenBank at NCBI.

Table 2. Source of the *Corynespora cassiicola* isolates

Code[3]	Location	State	Year	Host genotype
KL01/97	Mundakayam	Kerala	1997	RRII 105
KL02/98	Mallappally	"	1998	RRII 105
KL03/98	Rajagiri	"	1998	RRII 105
KL04/98	Teekoy	"	1998	RRII 105
KL05/97	Vakayar	"	1997	RRII 105
KL06/97	Karikattoor	"	1997	Nursery
KL07/99	Karikattoor	"	1999	Nursery
KL08/99	Chethackal	"	1999	Germplasm
KL09/99	Kolichal	"	1999	RRII 105
KL10/99	Kolichal	"	1999	RRII 105
KL11/00	Kolichal	"	2000	RRII 105
KL12/00	Kolichal	"	2000	RRII 105
KL13/00	Karikattoor	"	2000	Nursery
KR01/98	Kadaba	Karnataka	1998	RRII 105
KR02/98	Eswaramangala	"	1998	RRII 105
KR03/98	Sullia	"	1998	RRII 105
KR04/96	Nettana	"	1996	RRII 105
KR05/98	Jalsur	"	1998	RRII 105
KR06/98	Sampaje	"	1998	RRII 105
KR07/98	Nettana	"	1998	RRII 105
KR08/98	Devachalla	"	1998	RRII 105
KR09/98	Guthigar	"	1998	RRIM 600
KR10/98	Ivernadu	"	1998	RRII 105
KR11/99	Nettana	"	1999	RRII 105
KR12/99	Sampaje	"	1999	RRII 105
KR13/00	Nettana	"	2000	PB 311
KR14/00	Nettana	"	2000	Nursery
KR15/00	Guthigar	"	2000	RRII 105
KR16/00	Guthigar	"	2000	RRII 105
KR17/00	Shiradi	"	2000	RRII 105
KR18/00	Shiradi	"	2000	RRII 105
KR19/00	Sampaje	"	2000	RRII 105
KR20/00	KFDC, Barya	"	2000	RRII 105
KR21/00	Sullia	"	2000	RRII 105
KR22/00	Sampaje	"	2000	Nursery
KR23/00	KFDC, Barya	"	2000	RRII 105

RESULTS AND DISCUSSION

RAPD profiling of *Colletotrichum* isolates

Two major types of RAPD profiles were obtained among the three groups of isolates, viz. raised spots (R), anthracnose (A) and papery lesions (L) (Fig. 1A & 1B) which clearly indicated that the fungal isolates causing raised spot symptom in *Hevea* reacted differentially from the other two symptoms. With all the primers tested, 14 isolates from raised spot symptom could easily be distinguished from 11 isolates, which originate from anthracnose and papery lesion type of symptoms and these formed genetically two distinct groups. RAPD profiles also revealed genetic diversity within sub-groups and all the 25 isolates were different. Wide genetic variability was detected among the isolates from anthracnose and circular papery lesions, whereas, the isolates that originated from raised spots showed comparatively less variations among themselves. More than 90% genetic dissimilarity was recorded between these two groups (data not shown). Although, the present day fungal taxonomy suggests that both the genera, *'Gloeosporium'* and *Colletotrichum* are synonymous, there are sufficient reasons to believe the existence of two distinct groups after conducting the molecular characterization of fungal isolates through RAPD markers. Grouping of the *Colletotrichum* isolates causing anthracnose and circular papery lesions together on the basis of RAPD profiles suggested that in both these cases symptoms were developed due to infection caused by *Colletotrichum gloeosporioides* as already identified earlier from circular papery lesions in *Hevea* (Rajalakshmy & Joseph, 1988).

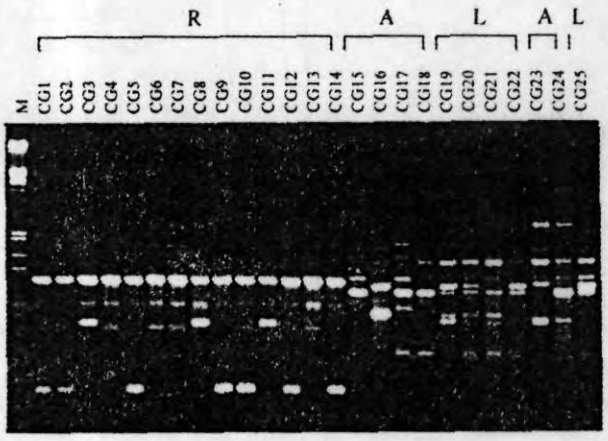

Fig. 1. Random amplified polymorphic DNA fingerprints of 25 *Colletotrichum* isolates generated with the primer OPB-10, clearly discriminated the fungal isolates associated with raised spot symptom (R) from anthracnose (A) and papery lesions (L). Lane M: molecular weight marker (lambda DNA/ *Eco*RI+*Hind* III).

rDNA RFLPs

Amplified rDNA product (internal transcribed spacers including 5.8S rDNA, ITS4/5) was ~0.6 kb in size and length and polymorphism could not be detected among the isolates as reported in *Colletotrichum* isolates from strawberry (Buddie *et al.*, 1999). Restriction digestion of the amplified rDNA product was obtained with the four enzymes, AluI, Sau3AI, EcoRI and TaqI, which yielded two specific restriction patterns (Fig. 2).

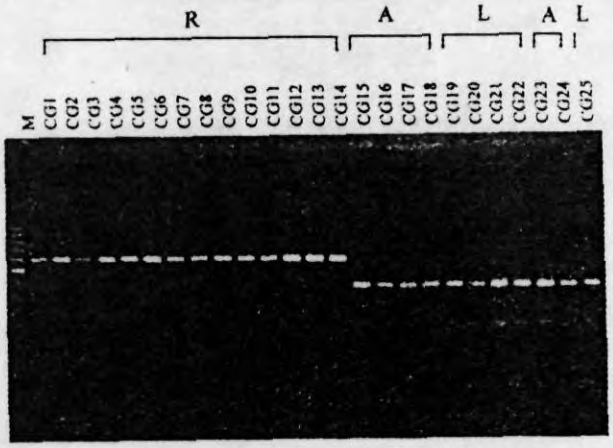

Fig. 2. Restriction fragment length polymorphisms of PCR amplified rDNA from all the isolates of *Colletotrichum* causing three different symptoms on *Hevea*. Restriction digest with *Alu*I that yielded distinct pattern for raised spot isolate (R), which differed from the other two isolates having identical restriction profiles causing anthracnose (A) and papery lesions (L). Lane M: 100 bp ladder molecular weight marker.

The isolates causing raised spots could clearly be differentiated from other *Colletotrichum* isolates originating from anthracnose and papery lesions through restriction profiles of the rDNA reflecting the same groupings as revealed from RAPD studies. The rDNA restriction length

polymorphisms detected among the fungal isolates were generated due to sequence variations in the ITS regions, which are rapidly evolving regions of ribosomal DNA/rRNA gene sequences and often best studied for comparing species and closely related genera.

Nucleotide sequence data of the rDNA revealed that the fungus causing raised spots is different from the other two and having 99% rDNA(ITS4/5) sequence homology with that of *Colletotrichum acutatum* infecting other crop species. Similarly, ribosomal DNA sequence from the fungus causing anthracnose showed high degree of homology with *Colletotrichum gloeosporioides* rDNA sequences. Thus, molecular evidences suggested that the fungus causing raised spot type of symptom in rubber is distinct from that causing anthracnose and papery lesion, the latter two being similar. Alignment of rDNA sequences of these two *Colletotrichum* species revealed more nucleotide sequence variability in the ITS1 region compared to ITS2. Nucleotide variations were also detected at three positions of the conserved 5.8S ribosomal DNA. The International Mycological Institute, UK also confirmed the identification of the isolate causing raised spots (IMI 383015) as *Colletotrichum acutatum* Simmonds (P.F. Cannon, pers. comm.). Other two isolates causing anthracnose (IMI 383016) and papery lesions (IMI 383017) were identified as *Colletotrichum gloeosporioides* as expected.

Species delineation in *Colletotrichum* through morphological studies (especially of conidium shape and size) may not be easy. Sutton (1992) suggested that relationships within the genus *Colletotrichum* were unlikely to be resolved using morphology alone. Morphological plasticity and overlapping of phenotype make the taxonomic criteria unreliable for the accurate delineation of *Colletotrichum* species. On the other hand, molecular characterisation of *Colletotrichum* isolates revealed considerable levels of variation in rDNA RFLPs and random amplified polymorphic DNA, which could successfully be used in the identification of *Colletotrichum* isolates from rubber, at the species level.

RAPD profiling of *Corynespora* isolates

RAPD analysis provided a rapid method to distinguish *Corynespora* isolates and to assess the variability existing among them. RAPD assays, carried out initially with 20 isolates, were repeated with 16 more isolates, which had been collected during 1999-2000. Based on amplification profiles of the initial 20 isolates, developed by the primer OPA4 (Fig. 3), Kerala isolates (KL01-KL08) could only be distinguished in to six categories. There was no polymorphism detected among the Karnataka isolates (KR01-KR12) with the same primer. Amplification profiles of the Karnataka isolates were very similar to that of three isolates from Kerala, KL02/98, KL03/98 and KL05/97. Three Kerala isolates, KL01/97, KL06/97 and KL04/98 showed extensive polymorphisms in RAPD profiles and easily distinguishable from others. Two Kerala isolates, KL07/99 and KL08/99, collected from seedling nursery at Karikattoor and wild *Hevea* germplasm at Chethackal respectively, could also be distinguished from all other isolates based on the amplification profile generated by OPA4. However, the isolates from Karnataka showed uniform amplification profile with all primers except with the primer OPA18 (Fig. 4), which could detect polymorphisms among the Karnataka isolates. Ultimately, seven genotypes could be identified among the 20 isolates through RAPD profiling. In the second set of 16 *Corynespora* isolates, three more new genotypes, KR13/00, KR21/00 and KL12/00 belonging to Karnataka and Kerala state respectively, were identified through RAPD profiling with three informative primers for *Corynespora*, OPA3, OPA4 and OPA18 (Fig. 4). Isolates, KR21/00 from Sullia and KL12/00 from Kolichal showed more variability than the KR13/00 from Nettana. Detection of a new genotype with multi-loci polymorphisms among the isolates from same host RRII 105, collected in recent years from Kolichal is of significance, as there was no variation detected in the earlier collections form the same location and therefore, the genotype might be considered as a newly evolved one. However, rest 13 *Corynespora* isolates, showing unique RAPD profiles with all the primers, were very similar to one of the genotypes detected earlier.

Existence of ten different races of the pathogen (seven from initial set of 20 isolates and three from second set of 16 isolates) could be evidenced by the genetic variability detected

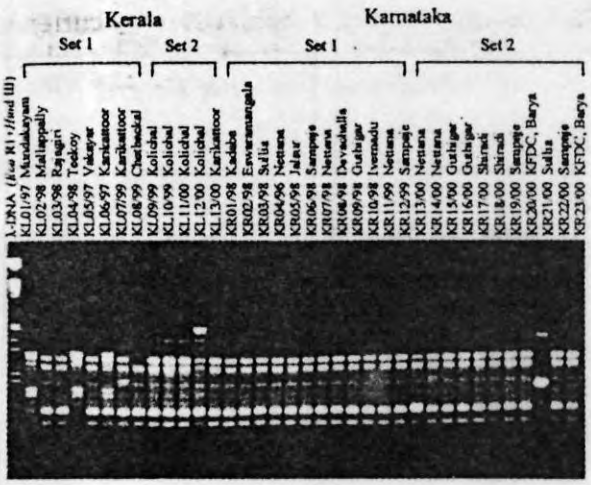

Fig. 3A

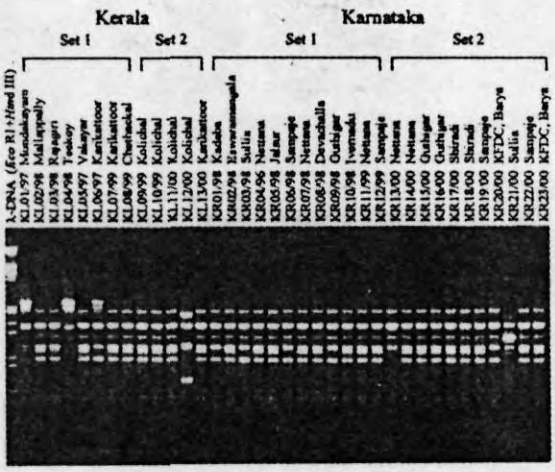

Fig. 3B

Fig. 3. RAPD profiles of *Corynespora* isolates generated by the primer OPA-04 (FIG. 3A) and OPA-18 (FIG. 3B). Eight types of DNA fingerprints detected among the isolates with the primer OPA-04. Seven isolates, KL01/97, KL 04/98, KL06/97, KL07/99, KL08/99, KL12/00 and KR20/00 showed distinct patterns, which could be identified individually. Polymorphisms noticed in four Kerala isolates: KL01/97, KL04/98, KL06/97 and KL12/00 with the primer OPA-18 could also be discriminated. Amplification profiles of 23 Karnataka isolates with the same primer (OPA-18) revealed the existence of four genotypes/races. Among these four types, KR21/00 showed extensive variations in RAPD profiles.

through RAPDs among the isolates from both the states. However, in Kerala more numbers of races have been identified compared to Karnataka. The reason may be attributed to the long history of rubber cultivation in Kerala compared to that in Karnataka and genetic modification of the pathogen could have taken place over the years in old rubber plantations as the different host populations act as a powerful selection force on pathogen population and vice-versa (Burden, 1987). Existence of different pathotypes of *C. cassiicola* on rubber was reported from Sri Lanka (Silva *et al.*, 1998) and Indonesia (Darmono *et al.*, 1996), where the disease became epidemic.

Selection intensities on virulent and avirulent genotypes on susceptible hosts are likely to vary spatially and temporarily within and between years because plant pathogen populations are very sensitive to host genotypes and environmental variations. Variability among the isolates, KL06/97 and KL07/99, collected in two different years, from the same location of the Kerala state, i.e., Rubber Board Central Nursery, Karikattoor could be attributed to the highly heterozygous nature of the seedling populations raised each year in the nursery. Genetic uniformity noticed among the three isolates collected in different years from Nettana in Karnataka, might be due to the same host genotype. Detection of a variable genotype, KR13/00, among the recent isolates from the same location might be attributed to the difference in host genotype (Table 2). Three isolates from Kerala viz., KL01/97, KL04/98 and KL06/97 having very similar morphological features with other isolates, revealed high degree of genetic variability in the present RAPD studies. This proves the notion that taxonomic consideration, based solely on phenotype, may be subjected to ambiguities as it is induced by the environmental conditions.

In conclusion, the present study confirmed the existence of *Colletotrichum acutatum* as the major pathogen of Colletotrichum leaf disease. Ten different genotypes of *C. cassiicola* including three newly detected genotypes are associated with rubber, in India. Specific RAPD profile/ fingerprints generated for each individual genotype could effectively be used in rapid screening of the pathogen in both the leaf diseases in *Hevea* for effective disease management.

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