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Morphological and Molecular Variability of *Lasiodiplodia theobromae* Causing Stem End Rot of Mango in Tamil Nadu, India

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ABSTRACT

Sixteen isolates of Lasiodiplodia theobromae were collected from mango varieties grown in various regions of Tamil Nadu, India. The results revealed that most of the L. theobromae isolates exhibited significant differences in morphology, colour and spore size. However, all 16 isolates took 3–4 days invariably to cover the 90 mm Petri plates. Among the 16 isolates, the isolates LT 14 from Neelum produced abundant pycnidia and identified as a virulent isolate. These isolates were also analysed through Random amplified polymorphic DNA (RAPD) markers for genetic diversity. Polymerase chain reaction amplification of total genomic DNA of the 16 isolates of L. theobromae with 21 random primers generated numerous polymorphisms among the isolates, while many of the intense bands were shared among majority of the isolates. Cluster analysis also indicated a high degree of genetic variability within L. theobromae isolates from different banana cultivars. The 16 isolates were separated into two clusters namely A and B. The maximum similarity index of 85.40% was recorded between the isolates LT4 and LT5. The least similarity index of 41.60% was recorded with the isolates LT5 and LT7. Within the species of L. theobromae the genetic variability was high and it underlines and validates existence of significant intra-specific diversity in isolates of L. theobromae infecting different mango varieties

***Key words**: Mango; Stem end rot; Lasiodiplodia theobromae; RAPD analysis*

INTRODUCTION

The fungal pathogen *Lasiodiplodia theobromae* Patouillard [*Botryodiplodia theobromae* (Patouillard) Griffon and Maublanc] represents the sexual state of *Botryosphaeria rhodina* (Berk. and M.A. Curtis) Arx. It is an important opportunistic pathogen with worldwide distribution in tropical and subtropical regions causing different types of diseases in many plant species. It has a wide host range estimated to be more than 280 plant species[6,11,25], although with varied pathological effects on its hosts. *L. theobromae* causes shoot blight, die-back, twig blight, cankers, etc., mainly in woody plants[3,16]. *L. theobromae* is an economically important fungus known to cause major losses to mango, cocoa, banana and yam farmers[2,21]. Stem-end rot disease can render the mango fruits completely ineffective as it destroys the developed or developing fruits in field and storage condition[17].

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The molecular biology techniques are now used in conjunction with morphological and physiological markers for the analysis of populations. Few attempts have been made to organize *L. theobromae* isolates into groups on the basis of genetic characteristics. The invention and spread of polymerase chain reaction (PCR), different DNA fingerprinting methods have been introduced for the assessment of the genetic relationships among organisms. DNA polymorphism assay based on the amplification of random DNA fragments flanked by lamer of arbitrary nucleotide sequence by Random amplified polymorphic DNA (RAPD) has been used in the detection of variation among individuals[4]. RAPD fragments are useful in identifying variation and pathotypes in a wide variety of fungi. This technique has been used extensively as genetic markers in different fungal species aimed at establishing a correlation between genetic diversity and strain identification and differentiation[9]. It has permitted to study the population structure of many organisms that have fewer discernible morphological characters or one otherwise difficult to characterize using more traditional marker[14]. The relationships among pathogenicity in Botryosphaeriaceae strains from different geographical locations were demonstrated by RAPD technique[24]. It has been suggested that the comparison of a significant number of restriction sites data could be used to estimate genetic distance and generate characters for phylogenetic reconstructions[5]. In culture, the isolates from various mango cultivars are highly variable in morphology. The variability among the isolates of *L. theobromae* from various mango cultivars has been studied using RAPD technique. Among 16 isolates of *L. theobromae* infecting various cultivars of mango occurring at different commercial mango growing regions of Tamil Nadu as well as studying their cultural variability.

MATERIAL AND METHODS

Collection of pathogenic isolates

Pathogenic isolates of *L. theobromae* was obtained from various stem end rot infected mango fruits belonging to different cultivars namely, Neelum, Bangalora, PKM 1, Alphonso and Banganapalli from different mango growing regions and markets of Tamil Nadu *viz.*, Theni, Kanyakumari, Dharmapuri and Krishnagiri districts (Table 1). The infected fruits and leaves of mango showing typical anthracnose and stem end rot symptoms were collected from different districts. The pathogens were isolated by tissue segment method[19] on Potato Dextrose Agar (PDA) medium. Infected mango peels were cut into small pieces of 1.0 to 1.5 cm, surface sterilized with 0.1% mercuric chloride for one minute, washed in sterile distilled water thrice and blot dried with sterilized filter paper. Then the leaf bits were placed on Petri plates containing PDA medium. The plates were incubated at (28±2ºC) for the fungal growth. The cultures were purified by single spore isolation technique[20] and pure cultures were stored at 4ºC on PDA slants. The pathogenicity test was conducted for all the *L. theobromae* isolates and Koch's postulates were successfully proved in Neelum.

Morphological and cultural variability in *L. theobromae* isolates

Sterilized PDA medium was poured into sterile Petri plates, mycelial discs of 8 mm diameter cut from the periphery of a 10-day-old culture maintained on PDA medium was placed at the center of the Petri plate and incubated at room temperature for 10 days. Isolates of test fungi were examined for colony colour, topography, pigmentation, zonation, margin; days taken to cover the petri plate and sporulation were recorded by incubating the culture in dark at 28ºC. To assess the sporulation, three mycelia discs of 8 mm diameter from periphery, middle and centre of the colony were cut with a sterile cork borer, transferred to a test tube containing 5 ml sterile distilled water and shaken thoroughly for 5 min. From this, 0.2 ml suspension was transferred to a slide and three such slides were prepared for each replication. The average counts of conidia from 15 microscopic fields were taken, the intensity of sporulation was grouped into three classes namely, + Poor, ++ Medium and +++ Good.

Genetic variability of *L. theobromae* isolates

Genomic DNA was extracted from the suspension culture of *L. theobromae* by the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described[12]. The isolates of *L. theobromae* maintained in PDA slants were transferred into 250 ml conical flasks containing 200 ml potato dextrose broth and incubated at room temperature for seven days. After full growth, the individual isolates of *L. theobromae* mycelium were harvested by filtration through sterile filter paper and used for DNA extraction. To extract the DNA, 1g of mycelium from each isolate of *L. theobromae* was ground to fine powder in liquid nitrogen and incubated in 5 ml, 2% CTAB extraction buffer [10 mM trisbase (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2 %), mercaptoethanol (0.1 %) and PVP (0.2 %)] at 65ºC for 1 h. The suspension was added with equal volume of phenol-chloroform-isoamylalcohol (25:24:1) mixture. It was vortexed to mix two phases, followed by a centrifuge at 12,000 rpm for five min. The supernatant was transferred to a clean tube and mixed with equal volume of ice cold isopropanol. It was incubated at 25ºC for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70 per cent ethanol and again incubated for 15 min. The pellet was re-suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the DNA concentration was estimated spectrophotometrically. The DNA isolated was stored at -70ºC for further studies.

RAPD–PCR analysis

RAPD profiles were produced by the method [26]. Primers and all the reagents were obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. The primers used for analyses are OPA 02 (5’ TGC CGA GCT G 3’), OPC20 (5’ ACT TCG CCA C 3’), OPX (5’ GAG CGA GGC T 3’) OPX 9 (5’ GGA CAT GTA T 3’) P -1 (5’ GGT GCG GGA A 3’) P -2 (5’ GTT TCG GTC C 3’) P -3 (5’ GTA GAC CCG T 3’) P -6 (5’ CCC GTC AGC A 3’) S111 (5’ CTT TCC GCAGT 3’) S116 (5’ TCT CAG CTG G 3’) S1104 (5’ GAG GGA CCT C 3’) S1109 (5’ ACC ACG AGT G 3’) S1110 (5’ CAG ACC GAC C 3’) S1118 (5’ ACG GGA CTC T 3’) S1120 (5’ ACC AAC CAG G 3’) OPF 01 (5’ ACG GAT CCT G 3’) OPF 10 (5’ GGA AGC TTG G 3’) OPF 11 (5’ ACG GTA CCA G 3’) OPF 12 (5’ GGC TGC AGA A 3’) OPF 14 (5’ TGC TGC AGG T 3’) and C3(5’ CGG GCT TGG GT 3’). RAPD-PCR was carried out using an eppendorf master cycle gradient thermal cycler programmed for one cycle of denaturation at 94ºC for five min followed by 45 cycles of denaturation at 94ºC for one min, annealing at 37ºC for one min, extension at 72ºC for two min and a final extension at 72ºC for ten min. Each amplification mix (25µl) contained template DNA (50ng), ampli-taq polymerase (1unit), primer 20 µM, dNTPs (200 µM), and PCR buffer [1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.0), BSA (2 mg/ml)] and sterile distilled water. The RAPD-PCR products of *L. theobromae* were separated in 1.5% (w/v) agarose gel in 1x TAE buffer (0.4 M Tris, 0.2 M acetic acid, 10mM EDTA; pH 8.4) containing 0.5 µg/ml ethidium bromide. The PCR product along with gel loading buffer (6x containing 0.25% bromophenol blue, 0.25% xylene, cyndol FF and 3% glycerol) was loaded and electrophoresis was carried out at 50 V. Then, the gel was viewed in an UV illuminator.

Unweighted pair group method with arithmetic analysis

The banding patterns were scored for RAPD primers in each isolate starting from the small size fragment to large sized one. Presence and absence of each band in each isolate was coded as 1 and 0, respectively. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) described [22]. A dendrogram was constructed based on Jaccard’s similarity coefficient [10] using the marker data from different isolates with UPGMA.

RESULTS

Morphological and cultural characterisation of *L. theobromae* isolates from different mango cultivars

The results revealed that most of the *L. theobromae* isolates revealed significant

differences in fungal morphology in culture. Growth patterns of *L. theobromae* isolates at 28±2ºC showed significant differences in nature and colour of mycelia and sporulation pattern. However, all 16 isolates of *L. theobromae* took 3-4 days invariably to cover the 90mm Petri plates (data not shown). Colour of the isolates varied from grey, greyish white, white, greyish black, grayish brown, blackish grey and blackish white in early growth stages (Figure 1). Among the sixteen isolates of *L. theobromae*, the isolates LT1, LT2, LT6 and LT10 produced greyish white colour mycelium, LT3, LT8 and LT15 produced greyish black coloured mycelium, LT13 and LT14 produced blackish grey colour mycelium, LT5 and LT7 produced grey colour mycelium, LT4 and LT11 produced white colour mycelium, LT9 produced greyish brown colour mycelium, LT12 produced black colour mycelium. LT16 had blackish white colour mycelium. The topography of all the isolates exhibited aerial growth of mycelium. The isolate LT7 produced irregular margin, LT1, LT2, LT3, LT4, LT5, LT6, LT8, LT9, LT10, LT11, LT12, LT13, LT14, LT15 and LT16 produced smooth margin. The isolates of LT1 and LT14 produced concentric zonation and rest other showed no zonation. The isolates LT2, LT5, LT6, LT8, LT9, LT10, LT12, LT13, LT14, LT15 and LT16 produced black pigmentation. The isolates LT4 and LT11 produced dark black pigmentation. The LT1, LT3 and LT7 did not produce pigmentation. The isolate LT14 showed good sporulation followed by LT4, LT5, LT9, LT11 and LT16 when compared to other isolates. The variations in the conidial size were noticed in all the isolates. Among the isolates, growth of LT14 was fast and it covered the plate within 5 days but at later growth stages (2 week of incubation), all the isolates turned black due to enormous spore production (Table 2).

RAPD–PCR analysis

RAPD markers were better suited for differentiating isolates within a given species. Comparison of the 16 isolates of *L. theobromae* revealed numerous polymorphisms among the isolates, while many of the intense bands were shared among majority of the isolates. Among the 21 arbitrary primers, the primers namely, OPX 9, P-2, P-6, S111, S116, S1104, S1109, S1110, S1118, S1120, OPF 01, OPF 10, OPF 11, OPF 12, OPF 14 and C3 amplified fragments of similar molecular weight in all the isolates of *L. theobromae*. No variation in the genetic makeup was observed for these primers. The primers OPC-20, OPA-02, OPX, P-1 and P-3 synthesized the polymorphic bands under PCR reaction (Figure 2). The maximum similarity index (SI) of 85.40 % was recorded between the isolates LT4 and LT5. The least SI of 41.60% was recorded with the isolates LT5 and LT7. The dendrogram revealed that 16 isolates were classified into clusters A and B. Among the two clusters, cluster A was further divided into two sub- clusters C and D. C was further divided into three groups (Figure 3). In that the isolate LT13 and isolate LT14 have 83.30 % SI with other isolates (Table 3). The sub-cluster D consists of LT3 and LT 8 which were recorded 77.00 % SI with other isolates.

DISCUSSION

Sixteen isolates of *L. theobromae* were isolated from the infected fruits of different mango varieties collected from various localities of Tamil Nadu. The isolates widely varied in their morphological and growth characters. All the 16 isolates of *L. theobromae* produced either grey or greyish white, or greyish black or black coloured colonies on PDA. However, after 2 weeks all the isolates turned black due to enormous sporulation. Conidia of all the isolates were ovoid to elongate. All isolates of *L. theobromae* examined during this study agreed with the description[8,18]. In a similar study working with *L. theobromae* infecting citrus. The fungus produced greyish black colonies and black ostiolate pycnidia on V8 agar and conidia were ovoid to elongate[13]. In support of the present observations, while examining *L. theobromae* infecting eggplant had stated that the fungus produced greyish colonies with aerial hyphae and black ostiolate pycnidia

enmassed into stroma. Matured, two celled spores were found only in 2 to 3 weeks old cultures [27]. Classical microscopic features alone was difficult to characterize the isolate since cultural morphology varied widely on media and spore size varied significantly with environmental conditions (data not shown). The use of traditional methods (morphology, colony colour, conidia size, pycnidial production etc.) has not been satisfactory for differentiating between species, sub-species and between different isolates of pathogenic fungi, thus molecular approaches have gained popularity nowadays. Since the DNA markers detect variation directly at the DNA level and are not influenced by environment (test conditions), the PCR-based RAPD assay is fast, less cumbersome and very little template DNA is required for the analysis and these properties make RAPD very useful molecular technique to study genetic variability in pathogens. Presently characterization of isolates of plant pathogenic fungi based on DNA variability is considered useful. The information relating to variability in the isolates of *L. theobromae* from various banana cultivars is not available. The present study revealed the high degree of genetic variability with in *L. theobromae* isolates. The polymerisation products showed varied numbers of bands among the selected isolates of *L. theobromae*. The generation of a RAPD pattern relies on the binding of single short synthetic oligonucleotide primer of random sequence, to complementary DNA sequences and the amplification by PCR of the intervening DNA segments of variable length. The application of recently developed RAPD molecular markers in studies of genetic diversity has been shown to be useful for several fungal pathogens[15,26]. RAPD marker technique was successfully used in fungi like *Macrophomina phaseolina* in various crops by several researchers[1]. It has been used in fungi to study the genetic variation level of genus, species and or sub-species[7]. RAPD markers have been used for the intra-specific characterization of a number of pathogens. Analyzed RAPD pattern of 13 isolates of *B. theobromae*, which was isolated from different varieties of pear [23].

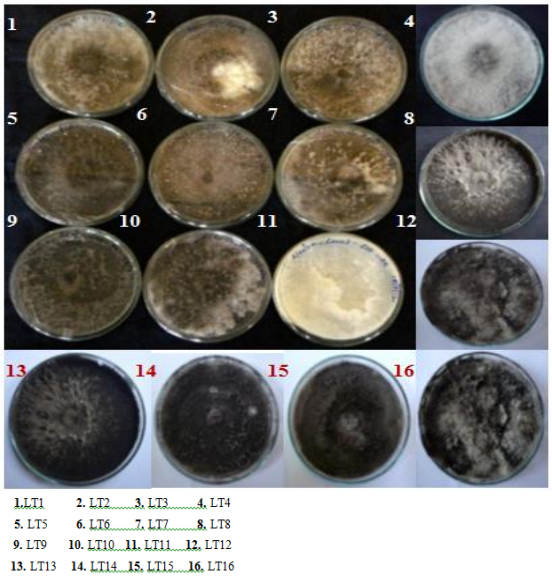

Fig. 1: Morphological variability among the isolates of *L. theobromae* from different mango varieties

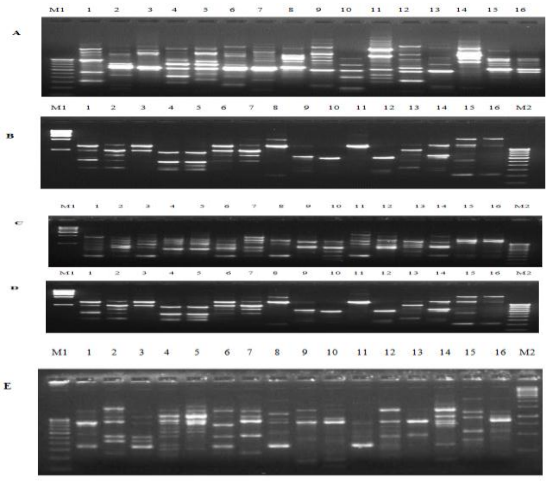

Fig. 2: Random amplified DNA polymorphisms of *L. theobromae* isolates amplified with primers (A) OPC-20, (B) OPA-02, (C) OPX, (D) P-1 and (E) P-3

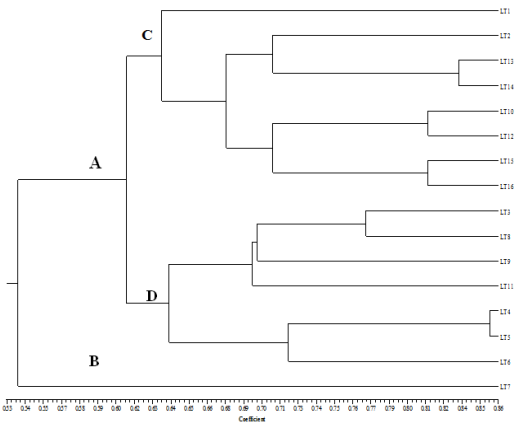

Fig. 3: Dendrogram for *L. theobromae* isolates using RAPD primer

Table 1: Isolates of *L. theobromae* used in the study

Isolate	Place of collection	Districts	Variety	Parts
LT1(CNF)	Cumbum	Theni	Neelum	Fruit
LT2(CNT)	Cumbum	Theni	Neelum	Twigs
LT3(PKMT)	Periyakulam	Theni	Neelum	Twigs
LT4(CBF)	Cumbum	Theni	Bangalora	Fruit
LT5(DPBLF)	Dharmapuri	Dharmapuri	Bangalora	Fruit
LT6(PKMF)	Periyakulam	Periyakulam	PKM 1	Fruit
LT7(KKBLF)	Kanyakumari	Kanyakumari	Bangalora	Fruit
LT8(KRBLF)	Krishnagiri	Krishnagiri	Bangalora	Fruit
LT9(CBEAL)	Coimbatore	Coimbatore	Alphonso	Fruit
LT10(KRBLF)	Krishnagiri	Krishnagiri	Bangalora	Fruit
LT11(KKNF)	Kanyakumari	Kanyakumari	Neelum	Fruit
LT12(KRBPF)	Krishnagiri	Krishnagiri	Banganapalli	Fruit
LT13(KKBPF)	Kanyakumari	Kanyakumari	Banganapalli	Fruit
LT14 (KKNT)	Kanyakumari	Kanyakumari	Neelum	Twigs
LT15(BONF)	Bodi	Theni	Neelum	Fruit
LT16(CBENF)	Coimbatore	Coimbatore	Neelum	Fruit

Table 2: Cultural characters of *L. theobromae* grown on PDA medium

Isolate	Colony colour	Topography	Margin	Days taken to cover petri plate	Pigmentation	Zonation	Sporulation
LT1	Greyish white	Aerial	Smooth	8	No	Concentric Zonation	+
LT2	Greyish white	Aerial	Smooth	7	Black	No	+
LT3	Greyish black	Aerial	Smooth	8	No	No	+
LT4	White	Aerial	Smooth	7	Dark black	No	++
LT5	Grey	Aerial	Smooth	7	Black	No	++
LT6	Greyish white	Aerial	Smooth	7	Black	No	+
LT7	Grey	Aerial	Irregular	7	No	No	+
LT8	Greyish black	Aerial	Smooth	7	Black	No	+
LT9	Greysih brown	Aerial	Smooth	7	Black	No	++
LT10	Grayish white	Aerial	Smooth	7	Black	No	+
LT11	White	Aerial	Smooth	7	Dark black	No	++
LT12	Black	Aerial	Smooth	7	Black	No	+
LT13	Blackish grey	Aerial	Smooth	8	Black	No	+
LT14	Blackish grey	Aerial	Smooth	5	Black	Concentric Zonation	+++
LT15	Greyish black	Aerial	Smooth	7	Black	No	+
LT16	Blackish white	Aerial	Smooth	7	Black	No	++

+Poor sporulation: 1-10 spores / microscopic field (100X);
++ Medium sporulation: 11-50 spores/ microscopic field (100X);
+++ Good sporulation: More than 100 spores/ microscopic field (100X)

Table 3: Similarity matrix of *Lasiodiplodia theobromae* isolates generated by RAPD analysis

	LT1	LT2	LT3	LT4	LT5	LT6	LT7	LT8	LT9	LT10	LT11	LT12	LT13	LT14	LT15	LT16
LT1	1.000															
LT2	0.520	1.000														
LT3	0.625	0.604	1.000													
LT4	0.583	0.479	0.583	1.000												
LT5	0.604	0.458	0.604	0.854	1.000											
LT6	0.708	0.604	0.708	0.666	0.770	1.000										
LT7	0.437	0.708	0.479	0.437	0.416	0.562	1.000									
LT8	0.687	0.541	0.770	0.687	0.708	0.729	0.500	1.000								
LT9	0.625	0.604	0.666	0.583	0.604	0.708	0.479	0.729	1.000							
LT10	0.645	0.583	0.604	0.604	0.625	0.645	0.500	0.666	0.729	1.000						
LT11	0.520	0.541	0.645	0.562	0.583	0.604	0.500	0.750	0.687	0625	1.000					
LT12	0.708	0.645	0.541	0.625	0.562	0.583	0.520	0.645	0.666	0.812	0.604	1.000				
LT13	0.666	0.687	0.583	0.625	0.604	0.750	0.645	0.604	0.666	0.645	0.604	0.666	1.000			
LT14	0.583	0.729	0.541	0.583	0.562	0.666	0.645	0.562	0.708	0.645	0.604	0.750	0.833	1.000		
LT15	0.645	0.708	0.520	0.604	0.541	0.604	0.666	0.625	0.645	0.708	0.583	0.687	0.729	0.687	1.000	
LT16	0.666	0.645	0.541	0.666	0.604	0.625	0.562	0.687	0.708	0.687	0.645	0.750	0.708	0.708	0.812	1.000

CONCLUSION

In the present study, 16 isolates of the fungus from various mango varities were also examined in relation to their variety of isolation or geographical locations. The clustering of RAPD dendrogram was not associated with geographical localities from which the isolates were obtained. The result of our studies underlines and validates existence of significant intra-specific diversity in isolates of *L. theobromae* infecting different mango varieties, and RAPD assay can be considered as an important tool to study the genetic variability among the *L. theobromae* isolates and it could be due to the fact that the isolates have been obtained from different mango varieties belonging to various genome.

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