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Morphological and Molecular Characterization of Lasiodiplodia Theobromae Causing Crown Rot of Banana

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ABSTRACT

Crown rot of banana caused by *Lasiodiplodia theobromae* is a serious post harvest problem leads to severe yield reduction. In survey, Negamam in Coimbatore district showed highest incidence of crown rot (62.0%). Among the varieties surveyed, Rasthali showed higher incidence than Poovan. Among the different methods tested, pin prick method recorded the maximum per cent disease index of about 80.91% by the isolate Lt_8. *L. theobromae* produces grey, grayish black and medium grey mycelium. Lt_8 produces maximum mycelium growth whereas Lt_7 produces least mycelia growth. Excellent pycnidial production was observed in Lt_8 whereas poor pycnidial production was observed at Lt_7

Keywords: Lasiodiplodia, Crown rot, Banana, Mycelium, Pycnidia.

Introduction:

Banana, the herbaceous flowering plant belonging to the genus *Musa* is one of the oldest fruit crop known to human race. It is cultivated in more than 120 nations of the world and ranks first in the global fruit production (Kamsu et al. 2019). India has highest area under banana cultivation in the world with 0.80 m ha, which accounts of about 15 per cent of area of world under banana cultivation. With a production of 29.2 m. tonnes, India shares 25 per cent of the global banana production while the productivity of banana in India is around 37.04 t/ha (Sathappan et al. 2019). Postharvest diseases account for a major loss in fruit market and developed countries suffer a loss of about 25 to 40 per cent while in developing countries the loss is around 50 percent (Bhale 2011; Hailu et al. 2013). One of the most common and serious postharvest disease that occurs in

dehanded banana during storage and marketing is crown rot. It is caused by a range of different fungi such as *Colletotrichum musae, Lasiodiplodia theobromae*, *Verticillium* spp., *Fusarium* spp. and *Cephalosporium* spp. (Abd-Alla et al. 2014). In India the fungi *Colletotrichum musae* and *Lasiodiplodia theobromae* has been the major pathogens in causing the crown rot disease of banana (Thangavelu et al. 2007). The visual symptoms don't appear until the fruit ripening stage (Ulrike Krauss and Andrea Johanson 2000). Crowns are black, soft and water soaked. Dark fungal growth may be developed in mass in the stem ends and the skin turns black in colour. Fungal growth may cover the skin with white to greenish black. The pulp may rot and fingers drop (Scot et al. 2006). This study aims to study the morphological and molecular characters of *Lasiodiplodia theobromae* causing crown rot of banana.

Materials and methods:

Market survey for the assessment of loss due to crown rot of banana:

A survey was conducted in Coimbatore, Erode, Cuddalore, Tirupur, Namakkal districts in Tamilnadu during 2018 – 2019 for the assessment of loss due to crown rot disease of banana. The assessment was conducted in markets and retail shops and the disease incidence in the fruits are examined. Disease incidence was estimated in different varieties by using the standard grade chart given below

Disease rating	Injury / decay
0	No infection
1	25% fruit surface infected
2	26-50% fruit surface infected
3	51-75% fruit surface infected
4	More than 75% fruit surface infected

Disease assessment was estimated using the above scale and per cent disease index was calculated using the following formula (Rose 1974),

$$\text{PDI} = \frac{sum\ of\ indivudual\ ratings \times 100}{Total\ number\ of\ leaves\ observed \times Maximum\ rating}$$

Isolation of *L. theobromae*:

The tissue bits from fruits showing the typical symptom of crown rot was taken and it was surface sterilized with 0.1% sodium hypochlorite solution. Then it was washed with three changes of sterile distilled water to remove the traces of surface sterilizing agent used. The washed sterile tissue bits were then kept in the Petri plates containing potato dextrose agar medium and incubated

at (28±2°C) for 5 -7 days. The colonies that develop on the plates were then sub cultured again on another Petri plates containing potato dextrose agar medium. Single spore isolation of fungus and single hyphal tip method was followed for obtaining pure cultures and it was maintained in PDA slants at 4°C (Aneja 2003). A total of about 10 isolates of *Lasiodiplodia theobromae* (LT1 to LT10) were collected from the districts of Tamil nadu.

Morphological and cultural characters of *L.theobromae* isolates

Fifteen ml of the PDA medium was poured into the sterile Petri plates and allowed to solidify. A 8mm disc of the *L. theobromae* culture was placed at the centre of the Petri plate and it was incubated at 28±2°C for 7 days. The mycelial growth of the isolates were measured at the end of the incubation period. Colony characters, linear growth, colony colour, sporulation and pycnidial production were also examined.

Sporulation:

To assess the sporulation, three mycelia discs of 8 mm diameter was taken from the periphery, middle and centre of the colony using a sterile cork borer and they were transferred to a test tube containing 5 ml of distilled water and shaken thoroughly for 5 min. From this 0.2 ml suspension was transferred to the slide and three such slides were prepared for each replication. The average counts of conidia from 15 microscopic fields were taken and the intensity of sporulation was grouped into five classes based on the grades given (Latha et al. 2013).

Sporulation	**Average number of conidia/microscopic field**	**Intensity**
Nil	0	-
Poor	<15	+
Moderate	15-30	++
Good	30-60	+++
Excellent	>60	++++

Molecular characterization of *L. theobromae*

DNA extraction

All the fungal isolates were grown in 100 ml of PDA broth for 7 days at 28±2°C. The genomic DNA was extracted and purified using the CTAB buffer method. Fungal mycelium was harvested and 2 g of dried mycelium were ground into fine powder in liquid nitrogen using mortar and pestle

until it forms a dry powder. 200 mg of powdered mycelium was transferred to 2.0 ml Eppendorf tubes and 1 ml of 20mM EDTA (pH: 8.0) and 2 % CTAB, mercaptoethanol (0.1) was added, vortexed and incubated at 65°C for 10 minutes. The mixture was transferred to clean tube and chloroform: isoamyl alcohol (24:1) was added in equal volume. The mixture was centrifuged at 10,000 rpm for 10 minutes. Equal volume of 5M Nacl and ice cold isopropanal was transferred to the supernatant taken in clean tube and mix well. It was incubated at 65°C for DNA precipitation or incubated overnight at -20°C. The content was centrifuged at 13000 rpm for 10 minutes and the pellet was collected by discarding the supernatant. The pellet was washed with 70% ethanol twice. 50µl of TE buffer or double sterile water was used for resuspending the pellet (10 mM Tris, 1mM EDTA, pH 8.0). PCR amplification & Sequencing of 18S rDNA of L. theobromae

Genomic DNA was isolated and quantity was measured using Nano Drop Spectrophotometer and the quality was determined using 2 % Agarose gel. A single band of high molecular weight DNA has been observed. 18S rDNA gene was amplified by 18SrRNAF and 18SrRNAR primers. A single discrete PCR amplicon band of 700 bp was observed when resolved on Agarose gel.

PCR amplification was carried out to amplify the internal transcribed spacer (ITS) region in the DNA of the Lasiodiplodia isolates.

Forward primer: ITS 1 5’ TCCGTAGGTGAACCTGCGG 3’

Reverse primer: ITS 4 3’ TCCTCCGCTTATTGATATGC 5’

The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 370x1 Genetic Analyzer in MEDAUXIN PVT. Ltd. Bangalore. Consensus sequence of 18S rRNA gene was generated from forward and reverse sequence. 18S rRNA gene sequence was used to carry out BLAST with the database of NCBI Gen bank data base. Based on maximum identity score first ten sequence were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed by using MEGA 7. Finally to acquire accession numbers, these sequences were submitted to NCBI (National Centre for Biotechnology Information) gene bank, USA.

Preparation of inoculum

Conidial suspension was prepared from 7 days old cultures of L. theobromae grown on PDA medium. Concentration of conidia in the suspensiom was adjusted to 1 x 105 conidia ml-1 using Haemocytometer (Martinez et al. 2008).

Pathogenicity test

Pathogenicity of the isolates of L. theobromae (LT1 to LT10) was tested using three methods such as pin prick method, mycelial inoculation method and spore suspension method in fruits. All the

three methods were tested with three replications and the virulent isolate among all the ten isolates was used for the subsequent studies.

Pin prick method

The fruits were completely cleaned with the sterile distilled water followed by surface sterilization with 2.0% sodium hypochlorite solution for one min and then with two changes sterile distilled water. The fruits were then dried at room temperature. The fruits were then wounded with a sterile pins and the conidial suspensions (1× 105 spores per ml) of each isolates were smeared and inoculated. Other set of fruits were inoculated without wounding. The fruits thus inoculated were kept in the moist chamber to maintain high humidity. Control was maintained by treating the fruit with sterile distilled water instead of pathogen. Observations were made regularly until the disease symptoms appears. After the development of symptoms, re-isolation from the symptom was done and it was compared with the original culture for confirmation. Similarly, the procedure was followed for other isolates (Divya Jagana et al. 2017b).

Mycelial inoculation method

The fruits were completely cleaned with the sterile distilled water followed by surface sterilization with 2.0% sodium hypochlorite solution for one min and then with two changes sterile distilled water. The fruits were then dried at room temperature. A mycelial disc of size 8 mm obtained from seven days old culture of L. theobromae was placed on the surface of the fruits. The fruits thus inoculated were kept in the moist chamber to maintain high humidity.

Control was maintained by placing 8mm of PDA medium. Observations were made regularly until the disease symptoms appears. After the development of symptoms, re-isolation from the symptom was done and it was compared with the original culture for confirmation. Similarly, the procedure was followed for other isolates (Abd-Elsalam et al. 2010).

Spore injection method

The fruits were completely cleaned with the sterile distilled water followed by surface sterilization with 2.0% sodium hypochlorite solution for one min and then with three changes in sterile distilled water. The fruits were then dried at room temperature. A hypodermic syringe was taken and was pre sterilized with 90 % ethanol before injecting the inoculum. The conidial suspension (1× 105 spores per ml) from the seven days old culture of L. theobromae was taken in the hypodermic syringe and inoculated into the fruit. The fruits thus inoculated were kept in the moist chamber to maintain high humidity. Control was maintained by treating with sterile distilled water instead of pathogen. Observations were made regularly until the disease symptoms appears. After the development of symptoms, re-isolation from the symptom was done and it was compared with the

original culture for confirmation. Similarly, the procedure was followed for other isolates (Fitzell 1979).

Disease assessment

For comparing the pathogenicity and virulence of the different isolates, the intensity of the disease incidence was assessed based on the development of the symptom in the crown region of the fruit. The following grades were used for the disease assessment.

Standard for assessment of banana crown rot disease (Akhtar and Alam 2002)

Disease rating	Injury / decay
1	None
3	Traces(after careful observation)
5	Slight
7	Moderate
9	Severe

Results and Discussion:

Market survey on the crown rot incidence of banana in districts of Tamil Nadu (2018-2019)

The systemic survey conducted during the year 2018 – 2019 in the retail markets of Coimbatore, Erode, Cuddalore, Tirupur, Namakkal and Dharmapuri districts and the results are presented in table1.

The percent disease index ranged from 40.71 to 62.0 in banana. Among the different isolates collected, the isolate collected from Negamam in Coimbatore district recorded highest crown rot incidence of about 62.0 per cent disease index followed by Pollachi (60.36%), Gobi (59.19%), Paapirattipatti (54.86%), Palladam (52.81%), Tiruchengode (48.40%), Annamalai nagar (43.98%), Rasipuram (40.71%), Sivapuri (41.12%) in the *C. Musae* decreasing order of merit. The variety Poovan collected from Perundurai recorded the least disease index of 40.71 per cent (Table 1). The results observed from the present study revealed that the variation in the disease incidence might be due to the occurrence of the isolates of pathogen differing in their virulence. The survey in the year 2009-2010 in Tamilnadu for crown rot of banana revealed an average post harvest disease loss of 0.30 to 10.20 per cent in different varieties caused by *L. theobromae* and (Jenisha 2018). Lokeshwari (2019) had surveyed for the post harvest disease in mango and recorded maximum of about 23.98 per cent disease index and minimum of about 06.94 percent.

Pathogenicity of L. theobromae isolates on banana:

Among the ten isolates of L. theobromae collected from different districts of Tamilnadu, the isolate (Lt8) collected from Negamam was found to be more virulent and recorded the maximum incidence of 79.49 per cent followed by Lt6 (78.16%) collected from Sivapuri. The isolates Lt1 and Lt10 showed 67.56 and 69.11 per cent of disease incidence and were on par. The isolate Lt7 collected from Rasipuram of Namakkal district was the least virulent which recorded the minimum (52.66 %) crown rot disease incidence (Table 2). The difference in the crown rot incidence of banana could be understood by the difference in the virulence of the isolates collected from different locations. The difference in the virulence of the isolates from the present study was similar with earlier reports of many workers (Syed et al. 2014; Nogueria et al. 2017; Munirah et al. 2017).

Cultural characteristics of L. theobromae isolates

The morphological variability of L. theobromae isolates collected from different locations were studied for their cultural characters such as mycelial growth, days taken for pycnidial production and amount of pycnidial production. The results of in vitro studies revealed the existence of wide variation in their morphological characters (Table 3).

Colour of mycelium:

The isolates of L. theobromae showed variations in colour of the mycelium. It varied from grey, medium grey and grayish black in the starting stage and later turns to black in colour due to enormous spore production (Table 3). The results obtained are in agreement with the earlier report by Muthukumar and Udhayakumar (2017).

Mycelial growth:

Among the isolates Lt8 showed the maximum growth in potato dextrose medium of about 90 mm followed by Lt1 (83.5 mm), Lt5 (82.15 mm), Lt4 (79.4 mm), Lt6 (78.8 mm) in the decreasing order of merit after three days of incubation. The isolate Lt7 recorded the minimum growth of about 64.4 mm. (Table 3). The results showed that the isolate with maximum mycelial growth produced higher disease incidence and were more pathogenic than the isolate with minimum mycelial growth. The per cent disease index varied because of the variability in the virulence of the pathogen. Similar changes in the mycelial growth was observed by Sangeetha et al. (2011) and Muthukumar and Udhayakumar (2017).

Sporulation and pycnidial production:

All the isolates of L. theobromae varied in their ability to produce pycnidia on potato dextrose medium. The pycnidial patterns also varied significantly. The pycnidia were concentrated in centre

or periphery or scattered all over the plates. The days taken to produce pycnidia were also varied from 14 to 18 days of inoculation. Excellent pycnidial production was observed in Lt8, good pycnidial production were found in Lt1, Lt4, Lt6, Lt10, moderate pycnidial production was observed in Lt2, Lt3, Lt5, Lt9 and poor pycnidial production was observed in Lt7 (Table 3). The mycelium, pycnidial pattern and pycnidial type of isolates also varied among the different isolates. Some produced pycnidia in stroma, some like mustard, some pycnidia devoid of stroma while some are pin head like (Table 4). Simiarly, Lokeshwari (2019) reported that the position of the pycnidia varies and were concentrated in centre, periphery and scattered. All the isolates took 15 to 17 days to form pycnidia. Similar reports were also observed by Muthukumar and Udhayakumar (2017).

In the present study, conidia were initially hyaline and aseptate and it becomes dark brown with single septations, ellipsoidal to oval in the later stage. Similar results were obtained by Sathya et al. (2017) where the conidia are ovoid to elongate. Similarly Borges et al. (2015) reported that the conidia were initially hyaline, unicellular and sub-ovoid to ellipsoid and the matured conidia were cinnamon to dark brown colour and thick walled. The results obtained was also in agreement with Muthukumar and Sangeetha (2011), where the conidia wereinitially unicellular, hyaline, sub-ovoid to cinnamon dark brown, thick walled, ellipsoidal and longitudinal striations.

Molecular analysis:

In the present study the results obtained from PCR amplification of ITS region with the primer pair of ITS1 and ITS4 produced a gene product of 700 bp confirming the isolates to be *Lasiodiplodia* sp.

Among the 10 isolates, 2 isolates were sequenced. The sequences of the isolates Lt1and Lt8 were identified as *Lasiodiplodia theobromae* through BLAST search in NCBI website (ww.blast.ncbi.nlm.nih.gov/Blast). The sequences were deposited in the Genbank with the accession numbers MT580363 and MT580364.

Table 1. Market survey for assessment of crown rot of banana

District	Market locality	Variety	Percent Disease Index (%)
Coimbatore	Pollachi	Rasthali	60.36b (50.9)
Erode	Perundurai	Poovan	40.74g (39.6)
Cuddalore	Annamalai nagar	Poovan	43.98f (41.5)
Tirupur	Palladam	Rasthali	52.81d (46.6)

Erode	Gobi	Rasthali	59.19b (50.2)
Cuddalore	Sivapuri	Poovan	41.12g (39.8)
Namakkal	Rasipuram	Poovan	40.71g (39.6)
Coimbatore	Negamam	Rasthali	62.0a (51.9)
Dharmapuri	Paapirattipatti	Rasthali	54.86c (47.7)
Namakkal	Tiruchengode	Poovan	48.40e (44.0)

Mean of three replications

Values in each column followed by the same letter are not significantly different according to the DMRT method (p=0.05)

Values in the parenthesis are aresine –transformations .

Table 2. Pathogenicity test of *L. theobromae* isolates by artificial inoculum

Isolate number	**Percent Disease Index (PDI %)**		
	Pin prick method	**Spore injection method**	**Mycelial inoculation method**
Lt1	67.56c (54.9)	58.32d (49.6)	42.98g (40.3)
Lt2	77.76a (61.3)	66.06b (54.3)	58.90cd (49.6)
Lt3	70.34b (56.7)	62.59c (51.9)	54.92e (47.2)
Lt4	63.30d (52.5)	58.29d (49.6)	47.74f (43.2)
Lt5	66.63c (54.3)	54.77e (47.2)	44.78g (41.5)
Lt6	78.16a (62.0)	70.29a (56.7)	60.08bc (50.7)
Lt7	52.66e (46.1)	50.93f (45)	39.71h (38.6)
Lt8	79.49a (62.7)	71.70a 57.4)	62.36a (51.9)
Lt9	70.41b (56.7)	64.60bc (53.1)	57.88d (49.0)
Lt10	69.11bc (56.1)	65.43b (53.7)	61.30ab (51.3)
Control	17.11f (24.3)	16.14g (23.5)	14.25i (21.9)

Mean of three replications

Values in each column followed by the same letter are not significantly different according to the DMRT method (p=0.05)

Values in the parenthesis are aresine –transformations .

. Morphological and cultural characteristics of *L. theobromae* isolates

Variety	Isolate number	Colour of mycelium	Mycelial growth (mm)	Pycnidial pattern	Pycnidial production	Days taken for pycnidial production
Rasthali	Lt1	Grey	83.5[b]	Centre	+++	16
Poovan	Lt2	Grayish black	74.7[e]	Scattered	++	14
Poovan	Lt3	Grayish black	75.7[e]	Periphery	++	15
Rasthali	Lt4	Grayish black	79.4cd	Centre	+++	15
Rasthali	Lt5	Grayish black	82.15bc	Scattered	++	17
Poovan	Lt6	Grayish black	78.8[d]	Scattered	+++	18
Poovan	Lt7	Grayish black	64.4[g]	Centre	+	16
Rasthali	Lt8	Grey	90.0[a]	Scattered	++++	15
Rasthali	Lt9	Grayish black	70.2[f]	Centre	++	15
Poovan	Lt10	Grey	77.0de	Periphery	+++	17

Mean of three replications

Values in each column followed by the same letter are not significantly different according to the DMRT method (p=0.05)

- : No pycnidia production

\+ : Poor pycnidia production

++ : Moderate pycnidia production

+++ : Good pycnidia production

++++ : Excellent pycnidia production

Table 4. Comparison of mycelia and pycnidial pattern of *L. theobromae* isolates

Isolate number	Mycelia type	Pycnidial pattern	Remarks
Lt1	Aggregate and fluffy	Centre	Pycnidia in stroma
Lt2	Medium fluffy	Scattered	Pycnidia devoid of stroma and mustard like
Lt3	Aggregate and fluffy	Periphery	Pycnidia mustard like
Lt4	Medium fluffy	Centre	Stroma big in size
Lt5	Medium fluffy	Scattered	Stroma is prominent
Lt6	Medium and not fluffy	Scattered	Pycnidia pin head like
Lt7	Medium and not fluffy	Centre	Stroma medium sized
Lt8	Aggregate and fluffy	Scattered	Pycnidia devoid of stroma and prominent
Lt9	Aggregate	Centre	Stroma big and prominent
Lt10	Aggregate and not fluffy	Periphery	stroma big in size and pycnidia mustard like.

Mean of three replications

Values in each column followed by the same letter are not significantly different according to the DMRT method (p=0.05)

- : No pycnidia production

\+ : Poor pycnidia production

++ : Moderate pycnidia production

+++ : Good pycnidia production

++++ : Excellent pycnidia production

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