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Pathogenicity variation, morphological and cultural characteristic of *Lasiodiplodia theobromae* isolates

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Abstract
The stem end rot caused by *Lasiodiplodia theobromae* is one of the most common and serious diseases of mango. In the present study, the pathogenicity variation of *L. theobromae* was assessed by Pin-prick method, spore injection method and mycelial inoculation method. The data revealed that the level of pathogenicity varied between the isolates. Among the seven isolates of *L. theobromae*, the isolate LT_6 recorded the maximum disease incidence of 81.94 per cent in pin prick method, 73.82 per cent in spore injection method and 62.91 per cent in mycelial inoculation. In the morphology and culture character assessment, the isolate of *L. theobromae* showed variability with respect to colony characters. Most of the isolates produced greyish black on the PDA medium in Petri dishes. The pigmentations like greyish black, grey, blackish were predominant in isolates. Among the isolates of *L. theobromae*, the maximum mycelial growth (90.00 mm) was recorded by the isolates LT_6 followed by LT_5 recorded 88.37 mm each at three days after inoculation. All the isolates of *L. theobromae* varied in their ability to produce pycnidia on PDA. The pycnidial population was found to be excellent with LT_6 isolate.

Keywords: *Lasiodiplodia theobromae*, pathogenicity variation and culture characters

Introduction
Mango (*Mangifera indica* L.) is inextricably connected with the folklore and religious ceremonies of India. It belongs to the family *Anacardiaceae* and it is called as "King of fruits" (Hayes, 1953) [11]. It has been cultivated in India for 6000 years (Palanivel *et al.*, 2015) [25]. India has the richest collection of mango cultivars and is regarded as the "National fruit" of the country. Mango cultivation is an important agribusiness in India. It is the National fruit of India and Pakistan, and also the National tree of Bangladesh. Cultivation of mango is believed to have been originated from South East Asia (Litz *et al.*, 2009) [18]. Even though, it has the largest area, the productivity is very low due to a number of diseases. Mango trees are affected by several fungal and bacterial diseases, of which Stem-end rot is the most common disease which results in sporadic losses of upto 20 % (Johnson *et al.*, 1997) [14]. This disease is caused by *Lasiodiplodia theobromae*, a cosmopolitan fungus occurring predominantly throughout tropical and subtropical regions (Burgess *et al.*, 2006) [5]. The disease is a serious threat for the mango industry because it is difficult to control (de Oliveira Costa *et al.*, 2010). It is more prevalent in fruits harvested from older trees (Coates *et al.*, 2009) [7].

This pathogen 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 (Sutton, 1980; Khanzada *et al.*, 2006 and Domsch *et al.*, 2007) [29, 15, 9] although with varied pathological effects on its hosts. *L. theobromae* causes shoot blight, die-back, twig blight, cankers, etc., mainly in woody plants (Cedeno *et al.*, 1995; Mohali *et al.*, 2005) [6, 22]. Stem-end rot disease can render the mango fruits completely ineffective as it destroys the developed or developing fruits in field and storage condition.

Symptom produced by this pathogen is a rapid fruit decay which accelerates from the pedicel proceeds to the fruit pulp, affecting a large area by watery necrosis which results in the total decay of fruit. Usually it starts appearing as water soaked diffuse lesions emerging from the stem end and quickly become dark coloured. Infected fruit may split open as they collapse. A straw-coloured fluid drains from the stem-end or from splits in the side of the fruit. Steel-grey mycelium may cover the surface of fruit (Prakash, 2004) [26]. Flesh of infected fruit has an off flavour. The infection spreads all over the fruit surface within 7 days. The fungi exist endophytically in the mango tree, spread systematically through the vascular system and express symptoms in

pre- and post-harvest stages. But the post-harvest phase is the most damaging and economically significant phase of the disease worldwide. The present study is aimed for the better understanding about pathogen and to assess the pathogenicity variation and morphological and cultural characteristic of *L. theobromae* isolates.

Materials and Methods

Isolation of *L. theobromae*

The pathogen causing stem end rot disease in mango was isolated from diseased fruit samples collected from different tracts of Tamil Nadu. The infected tissue bits were separated with a sterile blade and surface sterilized with 1 per cent sodium hypochlorite solution for 1 min. and subsequently washed three times with sterile distilled water. Then they were transferred into a sterile Petri dish containing Potato Dextrose Agar (PDA) medium (Ainsworth, 1961) [1] amended with streptomycin. The plates were then incubated at room temperature (28±2 °C) for 3-4 days. The emerging colonies were sub cultured on to PDA slants. Single hyphal tip method was followed for making pure culture and maintained on PDA slants (Aneja, 2003) [3]. *L. theobromae* was isolated from seven locations and designated as LT_1 to LT_7 (Table 1). The identity of all isolates was confirmed by microscopic observations based on morphological characteristics as per the key suggested by (D'Souza and Moniz (1967).

Preparation of inoculum

Conidial suspension was prepared from 10 days old culture of *L. theobromae* grown on PDA medium. Concentration of conidia in the suspension was adjusted to 1×10^5conidia ml^{-1} using haemocytometer (Martinez *et al*., 2008) [20].

Pathogenicity test

Pathogenicity of all the seven isolates of *L. theobromae* was tested by pin-prick method, spore injection method and mycelial inoculation method were followed to test the pathogenicity in fruits. The experiment was conducted in a completely randomized block design with three replications. The highly virulent isolate of *L. theobromae* was used for subsequent studies.

Pin-prick method

The conidial suspension of *L. theobromae* isolate containing 1×10^5 conidia ml^{-1} were inoculated into fruits showing uniform size, free from bruise or blemish at full three quarter stage of maturity by pin prick method (Swinburne, 1976) [30]. Inoculation was done at two equidistant points and placed in perforated polythene bags to maintain high humidity and incubated at room temperature (28 ± 2°C). The symptom expression was recorded at 6 days after inoculation. The fungus was reisolated from the lesions of infected fruits and its identity was confirmed.

Mycelial inoculation method

The mycelial disc from seven days old cultures of *L. theobromae* was placed to the surface of the fruits showing uniform size, free from bruise or blemish at full three quarter stage of maturity. Inoculation was done at two equidistant points and the fruits thus inoculated were kept in moist chamber, to maintain high humidity and incubated at room temperature (28 ± 2°C). The symptom expression was recorded at 6 days after inoculation. The fungus was reisolated from the lesions of infected fruits and its identity was confirmed.

Spore injection method

For artificial inoculation of the pathogen, hypodermic syringe was used for injecting the conidial suspension (1×10^5 conidia ml^{-1}) of each isolate. The hypodermic syringe was pre sterilized with 90 per cent ethanol before injecting the inoculums. The fruits were injected with conidial spore suspension. The inoculated fruits were incubated in moist chamber, to maintain high humidity. The inoculated fruits were sprayed with sterile distilled water to ensure high humidity to favour conditions for conidial germination and infection. The fungus was reisolated from the lesions developed and its identity was confirmed.

Morphological and cultural characteristics of the isolates

Mycelial growth

Fifteen ml of the sterile PDA medium was poured in to sterile Petri plates and allowed to solidify. An 8 mm culture disc of *L. theobromae* was placed at the centre of the Petri plate and incubated at room temperature (28±2 °C) for 4 days. The radial growth of the isolates was measured at the end of the incubation period. In addition, the mycelial colour and the pycnidial production (D'Souza and Moniz, 1967) of the isolates were recorded. For each isolate three Petri plates were maintained.

Sporulation

Conidial suspension was prepared from 7 days old cultures of *L. theobromae* by flooding the plates with 10 ml of sterile distilled water, which was then shaken to dislodge the conidia. The number of conidia of each isolate was estimated in a haemocytometer (Williamson and Tandon, 1965) [32].

Assessment of loss due to stem end rot in mango

Stem end rot incidence was recorded using the following scale. Scale for assessing anthracnose in fruits (Akhtar and Alam, 2002) [2]

Table 1

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

Disease assessment

Disease incidence was estimated by using the different scale and Per cent Disease Index was calculated using the following formula.

$$PDI = \frac{\text{Sum of individual ratings}}{\text{Total number of fruits graded} \times \text{Maximum disease grade}} \times 100$$

Results and Discussion

Virulence of different isolates of *L. theobromae*

The data presented in Table 2 revealed that the level of pathogenicity varied between the isolates. Among the twenty isolates of *L. theobromae*, the isolate LT_6 collected from Villupuram market recorded the maximum disease incidence of 81.94 per cent in pin prick method, 73.82 per cent in spore injection method and 62.91 per cent in mycelial inoculation. This was followed by the isolate LT_3 and LT_2 in the decreasing order of merit. The Isolate LT_5 was the least virulent as it recorded the least incidence in pin prick method, spore injection method and mycelial inoculation (60.77, 52.34 and 40.19%, respectively). The Isolate LT_6 was found to be more virulent when compared to other isolates. The variation in stem end rot incidence could be well attributed to the difference in virulence of the *L. theobromae* isolates prevalent in the respective areas. The differences in virulence obtained in this study agree with the earlier reports of many workers (Meah *et al*., 1991; Hong *et al*., 2012; Kumari *et al.*, 2017) [21, 12, 16].

Cultural characters of *L. theobromae* isolates

Colour of mycelium

The isolates of *L. theobromae* showed variation with respect to colony colour. The colour of the isolates varied from grey to greyish black (Table 3). *Lasiodiplodia* species are highly variable, as manifested by colony morphology, colour of mycelium, pycnidial shape, presence and shape of paraphyses, conidiophore, conidial shape, appressoria, pigmentation, fungicide sensitivity, pathogenicity and other traits (Muthukumar and Sangeetha, 2011; Li *et al.,* 2013 and Syed *et al.* 2014) [23, 17, 31]. The results obtained from the colony characters of *L. theobromae* are in agreement with the reports given by Johnson *et al.*, 1989 [13]; Borges *et al.*, 2015 [4] and Satya *et al.*, 2017.

Mycelial growth

Among the isolates of *L. theobromae*, the maximum mycelial growth (90.00 mm) was recorded by the isolate LT_6 followed by LT_5 recorded 88.37 mm each at three days after inoculation. The minimum mycelial growth (61.26 mm) was recorded in LT_2 (table 3). Physiological studies not only form the basis for culturing pathogens but also prerequisite for effective planning and preparation for a successful crop protection. It also provides the vital information that has direct bearing on vigour disease incidence, pathogenesis and cultural characteristics of pathogen. Similar such variations in the mycelial growth among the isolates of *L. theobromae* were observed by earlier workers (Martin, 1958; Williamson and Tandon, 1965 and Satya *et al.*, 2017) [19, 32]. The results of the present experiments revealed that the isolates of *L. theobromae* with faster mycelial growth were more pathogenic and produced higher disease incidence. The present results corroborate with the findings of Muthukumar and Sangeetha (2011) [23], who reported that the isolates of *L. theobromae* with faster mycelial growth as more pathogenic.

Pycnidial production

All the isolates of *L. theobromae* varied in their ability to produce pycnidia on PDA. The days taken for pycnidial production was also varied from 15 to 17 days of inoculation. Excellent pycnidial production was observed in isolate LT_6, good pycnidial production was observed in LT_5 and LT_7, moderate pycnidial production was observed in LT_2, LT_3 and LT_4, whereas poor pycnidial production observed by the isolate LT_1. Similar such

variations with regard to the pycnidial production of *L. theobromae* were observed by earlier workers (Sangeetha *et al.*, 2011; Muthukumar and Udhayakumar, 2017) [23, 24].

Table 2: Isolation of *Lasiodiplodia theobromae* in major markets of Tamil Nadu

Variety	Isolate number	Place of collection	District
Shenduram	LT_1	Thiruvennainallur	Villupuram
Imam Pasand	LT_2	Chidambaram	Cuddalore
Mallika	LT_3	Oothangarai	Krishnagri
Bangalora	LT_4	Attur	Salem
Alphonso	LT_5	Palakodu	Dharmapuri
Neelam	LT_6	Villupuram	Villupuram
Local variety	LT_7	Sirkazhi	Nagapattinum

Table 3: Pathogenicity test of *L. theobromae* isolates by artificial inoculation on var. Neelam

Isolate number	Per cent disease index (PDI)		
	Pin-prick method	Spore injection method	Mycelial inoculation method
LT_1	69.34^{e}	58.77^{f}	45.95^{f}
LT_2	75.01^{c}	64.94^{c}	58.05^{c}
LT_3	78.63^{b}	69.78^{b}	60.12^{b}
LT_4	67.59^{f}	58.93^{e}	47.78^{e}
LT_5	60.77^{g}	52.34^{g}	40.19^{g}
LT_6	81.94^{a}	73.82^{a}	62.91^{a}
LT_7	71.28^{d}	61.73^{d}	51.83^{d}
Control	16.13^{h}	15.38^{h}	14.19^{h}

*Values are means of three replications. In a column, means followed by a common letter are not significantly different at 5% level by DMRTs C.D (P = 0.05%).

Table 4. Morphological and cultural characteristics of*L. theobromae* isolates

Cultivars from pathogen isolated	Isolates number	Days taken to cover the Petri plate	Colour of the mycelium	Mycelial growth (mm)	Pycnidial pattern	Pycnidia production	Days taken for pycnidial production
Shenduram	LT_1	3	Greyish black	66.28^{f}	Centre	+	17
Imam Pasand	LT_2	4	Grey	61.26^{g}	Periphery	++	14
Mallika	LT_3	4	Grey	84.67^{c}	Scattered	++	15
Bangalora	LT_4	3	Grey	82.87^{d}	Periphery	++	16
Alphonso	LT_5	3	Greyish black	88.37^{b}	Centre	+++	17
Neelam	LT_6	3	Grey	90.00^{a}	Scattered	++++	17
Local variety	LT_7	4	Greyish black	75.62^{e}	Periphery	+++	14

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