

Original Research Article

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## Coconut Nut Rot Disease in India: Prevalence, Characterization of Pathogen and Standardization of Inoculation Techniques

R. Dheepa<sup>1\*</sup>, C. Goplakrishnan<sup>1</sup>, A. Kamalakannan<sup>1</sup>, S. Nakkeeran<sup>1</sup>,  
C.A. Mahalingam<sup>2</sup> and J. Suresh<sup>3</sup>

<sup>1</sup>Department of Plant Pathology, <sup>2</sup>Department of Agricultural Entomology, Tamil Nadu  
Agricultural University, Coimbatore -641 003, Tamil Nadu, India

<sup>3</sup>Tamil Nadu Agricultural University, Coimbatore - 641 003,  
Tamil Nadu, India

\*Corresponding author

### ABSTRACT

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Coconut nut rot disease is one of the major economic constraints which hampers coconut production in India. An extensive disease survey was conducted during July 2015 - September 2016 to assess the nut rot incidence in major coconut growing tracts of Tamil Nadu. The young palms of 5-10 years were found to be more susceptible to nut rot than matured palms. A fungal pathogen was found to be consistently associated with nut rot disease and identified as *Lasiodiplodia theobromae* based on morphological characters. A type culture of pathogen was submitted to Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi with the accession number of 10,517.17. The different methods of inoculation *L. theobromae* through pin prick along with spore suspension spray was found to be the best which record higher severity inoculated with seven month old coconuts.

### Introduction

*Cocos nucifera* L. is a member of the family Arecaceae and one of the most important perennial tropical crops. India is the third largest producer of coconut, after Indonesia and Philippines (Asian and Pacific Coconut Community (APCC) Statistical Year Book 2014). The coconut palm is predominantly grown widely southern states of India

including Tamil Nadu. India produced 20439.60 million nuts in an area of approximately 1975.81 million hectares with a productivity 10345 nuts per ha during 2015 (Horticulture Division, Dept. of Agriculture and Cooperation, Ministry of Agriculture, Govt. of India). Coconut production is limited by several factors, among them diseases play a major role. Nut rot is the major post-harvest disease of coconut in India, which causes

significant yield losses up to 60 per cent (Viana *et al.*, 2007). A fungus *L. theobromae* (Pat.) Griffon & Maubl. is the only species that has been reported to be associated with this disease (Taylor and Hyde, 2003). *L. theobromae* is a cosmopolitan soil-borne fungus that causes both field and storage diseases in plant species leading to economic losses. Coconut nut rot was first observed to cause severe damage to coconuts in the southern state of Kerala in the late 1990's (Nair, 2000).

*L. theobromae* (Botryosphaeriaceae: Botryosphaeriales: Dothideomycetes: Ascomycota) is cosmopolitan in distribution; however, this species is more commonly found in tropical and subtropical regions (Punithalingam, 1980; Marques *et al.*, 2013). This fungus is unique and destructive to crops because it can live endophytically in asymptomatic plant material and avoid detection by quarantine. Moreover, *Lasiodiplodia* species can ramify profusely when the host is under stress, causing significant yield losses (Slippers and Wingfield, 2007).

The accurate identification of pathogen is essential for devising proper control measures, quarantine programs, and to develop nut rot resistant coconut varieties. The present work was undertaken to assess the prevalence of nut rot in popular varieties of coconut in Tamil Nadu, Pathogens involved and its characterization, standardization of inoculation methods, mode of entry and susceptibility of different stages of nuts to *L. theobromae* infection.

## **Materials and Methods**

### **Survey and assessment of coconut nut rot**

A survey was conducted during 2015-2016 on the incidence of nut rot disease in different

coconut growing areas of Tamil Nadu, viz., Coimbatore (Thondamuthur, Thadagam, Chinniyampalayam, Devarayapuram, Negamam and Aliyar), Tanjavur (Vepankulam and Peravoorani), Tiruppur (Chinnampalayam and Thali), Dharmapuri (Kaveripattinam, Arasampatti and Bommidi), Kanyakumari (Marthandam), Erode (Thalavadi, Pandiyampalayam and Chennampatti), Vellore (Tirupattur), Salem (Edappaadi, Morepalayam, Karuppur and Tharamangalam), Ariyalur (Suthamalli) and Trichy (Kattuputtur). In each village, four fields were selected and four plots in each field having average nuts of 100 numbers were marked at random. Lesions on the surface of the nut was measured, using 0-3 scale; (where 0= no lesion; 1=25 per cent area affected; 2= 26-50 per cent area affected and 3=>50 per cent area affected) as per scale proposed by Lakshmanan and Jagadeesan, (2004).

### **Isolation and purification of fungal pathogen**

Seven-month-old nuts (cv. Local) showing malformation and cracking symptoms were obtained from Coconut farm, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. Diseased tissues from the edge of the lesion were collected and surface-disinfected with 0.5 per cent NaOCl for 3 min., then placed on potato-dextrose-agar (PDA) in 9 cm Petri plates, and incubated at 25±3°C for 7 days (Phipps and Porter, 1998). Stock cultures of *L. theobromae* were obtained using the hyphal tip transfer procedure (Rangaswami *et al.*, 1975) and maintained in tube slants of PDA at 10°C.

### **Morphological characterization of *L. theobromae***

Fifteen isolates of *L. theobromae* were sub cultured on PDA medium on Petri dishes (90

mm in diameter) for correlation of phenotypic and growth characters. Eight-millimetre disc from margins of actively growing seven-day-old fungal culture were inoculated on the centre of Petri dishes containing PDA medium. Colony colour and texture observations were recorded after seven days of incubation. Species differentiation was possibly made between them by studying the colony character, pycnidia and conidia morphology (Waller and Lenne, 2002). For further confirmation the culture has been sent to Indian Agricultural Research Institute.

### **Microscopic observations of *L. theobromae***

The conidia were scraped from infected nuts and mounted in a lactophenol solution on a microscopic slide. Measurements of the conidia were made using a light microscope (Labomed-IVU 5100) and photographed using a Labomed camera model LX400 with an image analyser – pixelpro programme.

### **Pathogenicity test**

In order to prove Koch's postulates, pathogenicity test was carried out. Seven month old coconuts (cv. Local) were collected from the university coconut farm, washed thoroughly under running tap water. The coconuts were blot dried and surface sterilized with 70 per cent ethanol. Five month old coconuts were injured with sterilized needle and 9 mm mycelial disk from virulent isolate of *L. theobromae* (LDt3TM1) was placed on coconuts. A thin layer of sterilized moist cotton was placed over the inoculated coconuts. Inoculated coconuts were kept in a humid chamber using plastic bags for fifteen days under laboratory conditions. Untreated controls were inoculated with plain PDA discs only. The fungus was reisolated from the artificially inoculated coconuts showing typical nut rot symptoms and the culture obtained was confirmed for its morphology and colony characters.

### **Methods of inoculation**

The virulent isolate of *L. theobromae* (LDt3TM1) was used for artificial inoculation of the seven month old coconuts using (cv. Local) collected from the university coconut farm. The spore suspension ( $5 \times 10^5$  spores/ml) of the pathogen was prepared using a seven day old PDA culture by grinding it with sterile distilled water in a pestle and mortar. The coconuts were washed with running tap water; surface sterilized with 70 per cent alcohol and washed with sterile distilled water. The following methods were used for inoculation of the coconuts.

- i. Coconuts inoculated with spore suspension after pin prick using sterile entomological pins.
- ii. Coconuts inoculated with spore suspension without pin prick.
- iii. Coconuts inoculated with mycelium after pin prick.

Coconuts inoculated with sterile distilled water after pin prick served as control. The inoculated coconuts surfaces were covered with moist cotton and coconuts were kept inside the moist chamber. Five coconuts were used for each method. Four replications were maintained. The infection was recorded after seven days and the Per cent Disease index was calculated.

### **Results and Discussion**

#### **Survey for the assessment of nut rot in coconut**

A survey was conducted in various districts of Tamil Nadu to assess the occurrence of *L. theobromae* infected coconuts. Malformed and cracked nuts in all stages were collected. Further observation revealed the presence of *L. theobromae* @ 50-100 conidia/microscopic field. The survey also revealed that the incidence of *L. theobromae* varied from 5.1 to 22.0 per cent (PDI) in different districts of Tamil Nadu. The lowest incidence of the

disease (PDI - 5.1, 6.19, 6.69, 6.83, 7.29, 8.48 and 9.56) was recorded in Thadagam (CBE), Panjapatti (Karur), Tirupattur (Vellore), Devarayapuram (Coimbatore), Chennampatti (Erode), Thalavadi (Erode), Suthamalli (Ariyalur) and Morepalayam (Salem) respectively. Maximum occurrence of the disease was recorded in Thondamuthur (Coimbatore), Peravoorani (Tanjavur), Pandiyampalayam (Erode) and Chinnampalayam (Thiruppur) (PDI - 22.00, 21.30, 19.40 and 18.40), followed by Vepankulam (Tanjavur) and Tharamangalam (Salem) (PDI- 18.00 and 17.50) respectively (Table 1). The disease was prevalent in all major Agro Climatic Zones of Tamil Nadu. The young palms of 5-10 years were found to be more susceptible to nut rot than matured palms. The pathogen responsible for this disease was isolated from the infected samples collected during survey and it was confirmed as *L. theobromae*, which causes brown lesions covering entire nut surface, later turning into whitish grey colour having shrivelled appearance (Fig. 1a; 1b). Similarly, the world wide post-harvest losses have been estimated as 50 per cent and much of this is due to microbial decay (EI- Ghaouth and Wilson, 1995). Post-harvest losses occur at various stages, viz., storage loss, transportation loss and loss at the level of wholesaler or retailer or consumer (Dasgupta and Mandal, 1989).

### **Morphology and Identification of the pathogen**

The mycelium of the isolated fungus grew uniformly in all directions and fully covered the surface of the media within 3 to 4 days. The colour of the colony changed gradually from light grey at four to seven days of incubation to black after two weeks of incubation. The bottom most part of the fungus only became darker after 3 weeks of incubation. Subsequently, the fungus

produced stromata and pycnidia. The pycnidia produced were initially soft but hardened when the culture matured at 4 weeks. The culture has sporulated only after 4 weeks of incubation. Fruiting bodies developed after 4 weeks of incubation (Fig. 2a). Liquid exudates were produced and became dry after five days. After they dried, the spores were released from the ostioles in the form of a black dust on the culture media (Fig. 2b). The main characteristics of *L. theobromae* are presence of paraphyses within the pycnidia, and conidia that are initially hyaline and aseptate but become brown and one septate with age, forming longitudinal striations due to the deposition of melanin granules on the inner surface of the wall. Type culture of pathogen was submitted to Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi with the accession number of 10,517.17. The results are more or less similar to those obtained from a morphological and cultural basis reported by Burgess *et al.*, (2003). In view of its widespread occurrence, wide host range and its known morphological variability (Punithalingam, 1980), it is possible that *L. theobromae* is composed of a number of cryptic species.

### **Morphological variability among *L. theobromae* isolates**

Among the fifteen isolates studied for their morphological characters, six isolates, namely PKLDt1, CMPLDt2, LDt3TM1, POLLDt4, KVPMLDt9 and TPRLDt13, showed a colony growth rate of 24.50 to 25.60 mm/day indicating their fast growing nature. These isolates filled the entire 90-mm diameter Petri dish surface in 96 h. Six isolates viz., LDt6VPN, KGMLDt7, UDULDt8, TVDLDt12, SMLDt15 and KSGLDt10 exhibited a moderate growth rate that ranged between 23.40 mm and 24.30 mm. The lowest

growth rate was observed in the isolates of ALILDt5, NGLLDt11 and EDPLDt14. The majority of the isolates showed vegetative growth, four isolates showed fluffy colony growth and three isolates were oppressed to the culture medium. Almost all the isolates showed a uniform growth pattern, except LDt6VPN, which exhibited an irregular growth pattern.

The colony colour of isolates was dull white (ALILDt5 and SMLDt15), cottony white changing to black (LDt6VPN and KGMLDt7), cottony greyish black (PKLDt1, CMPLDt2, LDt3TM1, UDULDt8, KVPMLDt9, TVDLDt12 and TPRLDt13), greyish white (POLLDt4 and KSGLDt10) and whitish (EDPLDt14, SMLDt15) (Table 2). Despite the fact that morphological and pathological variability existed among the isolates concerned, the slightest comparable relationship existed between morphologically similar isolates and their pathological behaviour (Mali *et al.*, 2005). Similarly, Slippers *et al.*, (2014) reported that, most frequently described morphological characteristics include conidial and paraphyses morphology and, to a lesser extent, cultural characteristics of growth and pigment production.

### **Microscopic observations of *L. theobromae***

Observation of pycnidia on the infected coconut mesocarp was often found to be dark brown to black. Immature conidia were sub ovoid to ellipsoid, apex rounded; truncate at the base, thick walled, hyaline and single celled, matured conidia dark brown, two celled with irregular longitudinal striations measuring 20.2- 25.3 x 10.3-3.9 mm observed under 40X magnification (Fig. 3a and 3b). Similarly, *Lasiodiplodia theobromae* is pleomorphic and member of the sphaerosporales with immersed to erumpent

or superficial, pilose or glabrous, simple or often aggregated pycnidia reaching upto 5 mm width, with or without stroma. The pycnidia are usually papillate with prominent ostiole. Conidia are initially hyaline, unicellular, ellipsoid to oblong, thick walled with granular contents. On maturity the conidia became 2-celled, dark brown, usually 20-30 X 10-15 mm, with differentially pigmented longitudinal bands resembling striation (Punithalingam, 1980).

### **Pathogenicity test**

In order to prove the Koch's postulates, the pathogenicity of *L. theobromae* isolate (LD3TM1) on coconut (cv. Local) was carried out. Pathogenicity test was conducted *in vitro* on fully matured and immature coconuts by following 'pin prick plus mycelial disc method'. The results revealed the development of symptom on all coconuts inoculated with the mycelial disc of *L. theobromae* within ten days, initially the inoculated nuts showed typical triangular yellow patches radiating from the perianth. Later yellow patches turned into brown lesions which covered more than 50 per cent of the nut surface (Fig. 4a).

Numerous black pycnidia were formed on the diseased lesions. Later, brown lesions turned into whitish grey, shrivelled and developed hardness (Fig. 4b). All the isolates were pathogenic to coconut. In the field also similar types of symptoms were recorded within 15 days on nuts inoculated with the fungus after pin pricks, underneath of the perianth.

Typical cracking symptoms were recorded when disease intensity reached grade 3. In 37 per cent of the diseased nuts, the fungus entered into the kernel through mesocarp, resulting in decay of the endosperm (Fig. 4c).

**Table.1** Survey on the incidence of coconut nut rot disease in coconut plantations of Tamil Nadu

Sl. No.	Place of collection	District	Variety	Age (Years)	Sole crop / Inter crop	Nut Rot Incidence (PDI)
1	Thadagam	Coimbatore	Pollachi tall	44	Sole	5.1
2	Chinniyampalayam	Coimbatore	Chawghat Orange	35	Sole	18.4
3	Devarayapuram	Coimbatore	Pollachi tall	13	Sole	6.7
4	Negamam	Coimbatore	Pollachi tall	28	Sole	12.5
5	Aaliyar	Coimbatore	Pollachi tall	20	Cocoa	11.4
6	Vepankulam	Tanjavur	Chawghat Orange	14	Sole	18.0
7	Chinnampalayam	Tiruppur	Chawghat Orange	08	Fodder	18.4
8	Thali	Tiruppur	West Coast tall	30	Sole	12.1
9	Kaveripattinam	Dharmapuri	Local variety	45	Sole	13.5
10	Kasargode	Kerala	Chawghat Orange	12	Sole	16.7
11	Marthandam	Kanyakumari	Local variety Orange	32	Sole	10.0
12	Thalavadi	Erode	W.C. tall	27	Pulses	7.3
13	Tirupattur	Vellore	Arasampatti tall	12	Sole	6.2
14	Edapaadi	Salem	Arasampatti tall	16	Sole	12.0
15	Suthamalli	Ariyalur	Arasampatti tall	30	Sole	8.5
16	Pandiyampalayam	Erode	Hybrid (DxT)	07	Turmeric	19.4
17	Thondamuthur	Erode	Hybrid (DxT)	23	Turmeric	22.0
18	Morepalayam	Salem	Hybrid (DxT)	13	Sole	9.6
19	Karuppur	Salem	Hybrid (DxT)	18	Sole	17.0
20	Arasampatti	Dharmapuri	Hybrid (DxT)	40	Sole	11.8
21	Tharamangalam	Salem	Hybrid (DxT)	32	Sole	17.5
22	Panjapatti	Karur	Local	60	Sole	6.2
23	Kattuputtur	Trichy	Hybrid (DxT)	12	Sole	14.0
24	Peravoorani	Tanjavur	Hybrid (DxT)	10	Sole	21.3
25	Bommidi	Dharmapuri	Hybrid (DxT)	23	Sole	15.7
26	Chennampatti	Erode	Hybrid (DxT)	35	Sole	7.0
<b>Mean</b>						<b>13.00</b>

**Table.2** Colony characters of *L. theobromae* isolates collected from different coconut growing regions of Tamil Nadu

Isolates	Colony growth rate (mm/day**)	Sporulation <sup>#</sup>	Colony texture and colour
PKLDt1	25.30 <sup>ab</sup>	+++	Raised, uniform, cottony to greyish black
CMPLDt2	25.20 <sup>ab</sup>	+++	Fluffy, uniform, cottony to greyish black
LDt3TM1	25.60 <sup>a</sup>	+++	Raised, uniform, cottony to greyish black
POLLDt4	25.60 <sup>a</sup>	+++	Fluffy, uniform, greyish white in colour
ALILDt5	22.00 <sup>e</sup>	++	Appressed, uniform, dull white
LDt6VPN	24.10 <sup>bc</sup>	+++	Raised, irregular, cottony white changing to black
KGMLDt7	24.00 <sup>bc</sup>	++	Raised, uniform, cottony white changing to black
UDULDt8	23.40 <sup>cd</sup>	++	Raised, uniform, cottony to greyish black
KVPMLDt9	25.00 <sup>ab</sup>	++	Raised, uniform, cottony to greyish black
KSGLDt10	24.30 <sup>abc</sup>	+++	Fluffy, uniform, greyish white in colour
NGLLDt11	22.50 <sup>de</sup>	+	Raised, uniform, dull white in colour
TVDLDt12	23.60 <sup>cd</sup>	++	Raised, uniform, cottony to greyish black
TPRLDt13	24.50 <sup>ab</sup>	+++	Fluffy, uniform, cottony to greyish black
EDPLDt14	22.50 <sup>de</sup>	+	Appressed, irregular, whitish in colour
SMLDt15	23.40 <sup>cd</sup>	+	Appressed, uniform, dull white

\*Mean of three replications

\*\*24 h (based on growth at 96 h);

<sup>#</sup> Sporulation after 30 days of incubation

+ = Poor sporulation (1-10/ microscopic field 40X magnification)

++ = Medium sporulation (11-50/ microscopic field 40X magnification)

+++ = Good sporulation (more than 100 spores/ microscopic field)

\* In a column, means followed by a common letter are not significantly different at the 5 % levels by DMRT.

**Table.3** Effect of different methods of inoculation on severity of coconut nut rot (cv. Local) under lab condition

Sl. No	Technique	PDI*
1	Pin prick + Spore suspension	68.50 <sup>a</sup> (55.86)
2	Spore suspension spray alone	41.80 <sup>b</sup> (40.27)
3	Pin prick + Mycelial inoculation	27.35 <sup>c</sup> (35.24)
4	Mycelial inoculation alone	31.53 <sup>d</sup> (24.52)
5	Control	0.00 <sup>e</sup> (1.28)

\*Mean of four replications.

In a column, means followed by a common letter is not significantly different at the 5 % level by DMRT.

Values in parentheses are arcsine transformed values.



Fig. 1a

Fig. 1a. Brown lesions along with pycnidia covering entire nut surface



Fig. 1b

Fig. 1b. later turning into whitish grey colour having shrivelled appearance

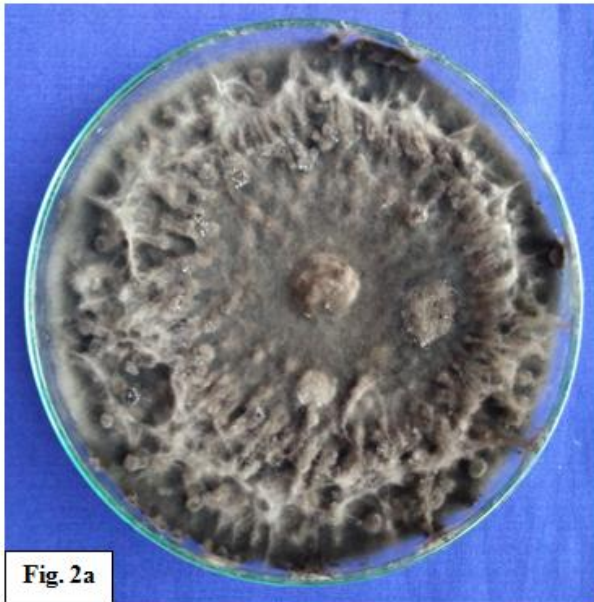


Fig. 2a

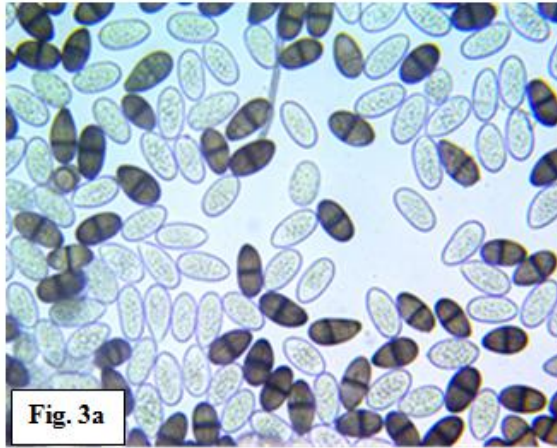
Fig.2a. Pure culture of *L. theobromae* produced fruiting bodies developed after 4 weeks of incubation



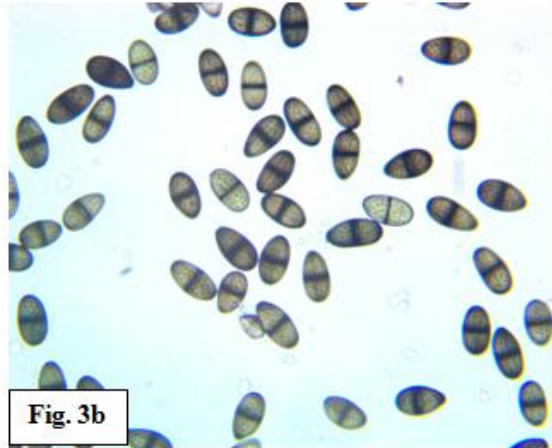
Fig. 2b

Fig.2b. Liquid exudates produced developing fruiting bodies





**Fig. 3a**



**Fig. 3b**

**Fig. 3a.** Immature conidia single celled and hyaline; **Fig. 3b.** Mature conidia were observed to be septate, oval shaped and brown in colour with the presence of irregular longitudinal striations @ 40X magnification



**Fig. 4a**



**Fig. 4b**

**Fig. 4a.** Yellow patches turned into brown lesions which covered more than 50 per cent of the nut surface

**Fig. 4b.** Dehusked coconut with rotting tissues



Fig. 4c

Fig.4c. Longitudinal splitting of nuts showing the fungus progressing into the endosperm



Fig. 4d

Fig. 4d. Control

In advanced stages, all nuts were malformed and developed hardness, and such nuts dropped prematurely. The coconuts maintained as control did not show any symptoms (Fig. 4d). Koch's postulates were fulfilled by consistently re-isolating *L. theobromae* from inoculated coconuts. Inoculations were repeated twice and obtained the same results. Similar results were found by Rosado *et al.*, (2016). In addition to devaluation of the crop due to esthetic damage, *L. theobromae* can also progress into the endosperm, rendering the coconut water unfit for consumption and resulting in significant monetary losses. Thus, *L. theobromae* was the most common and most aggressive species that is associated with the postharvest nut rot of coconut. Viana *et al.*, (2007) reported that the main symptoms are the blacking and cracking of basal parts of the fruits. Water oozing may occur as the infection progresses. The fungus *Lasiodiplodia theobromae* has been frequently isolated from the lesions.

### Methods of inoculation

Effect of different methods of inoculation of *L. theobromae* isolate (LDt3TM1) on seven month old coconut (cv. Local) was studied *in*

*vitro*. The different methods of artificial inoculation *viz.*, mycelial inoculation with pin prick, spore suspension spray with pin prick, spore suspension spray without pin prick, were tried on coconuts. The results indicated that the intensity of disease symptoms varied significantly with different methods of inoculation. Among the different methods of inoculation, pin prick with spore suspension spray was found to be the best which record a severity of 68.50 PDI followed by spore suspension spray without pin prick (41.80 PDI). A lowest PDI of 27.35 was recorded in mycelial inoculation with pin prick method (Table 3). The results of Rivera *et al.*, (2006) revealed that wounding or puncturing of agave and Chinese rose leaves before application of spores resulted in the highest per cent infection of *Colletotrichum gloeosporioides* when compared to spore suspension spray alone. Manjunath (2009) reported the same in noni plants with *C. gloeosporioides*. Similar findings were also reported in *Colletotrichum capsici* (Anand, 2002). These findings suggested that wounding or injuries are the major factors for the disease initiation and further development.

This work represents the detailed report of *L. theobromae* as a causal agent of post-harvest

disease coconut nut rot. The results of this study may be helpful for further studies on management measures, quarantine programs, or the development of coconut nut rot resistance coconut varieties.

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