Research Article

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Characterization of *Lasiodiplodia theobromae* causing leaf blight disease of coconut

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Abstract

Coconut leaf blight pathogen *Lasiodiplodia theobromae* (Pat.) was characterized based on morphological, cultural characters and Internal Transcribed Spacer (ITS) sequences. Pathogen isolates collected from various coconut growing areas of Tamil Nadu, India showed significant differences in the colony morphology, colour, spore dimensions and fruiting bodies. Molecular characterization by partial sequencing of ITS region confirmed the identity of pathogen as *L. theobromae*. Among the several methods of inoculation employed to establish the pathogenicity, pinprick method with spraying of conidial suspension (10^5 spores mL^{-1}) and spot application of mycelial mat (5 mm disc) at the inoculation site produced typical necrotic spots and lesions on coconut leaves of West Coast Tall, Arasampatti Tall, Chowghat Orange Dwarf and Chowghat Green Dwarf. Koch's postulates were established to confirm pathogenicity. The result of the study helps to streamline the existing management strategies.

Keywords: Coconut, leaf blight, *Lasiodiplodia theobromae*, pathogenecity, PCR

Introduction

Coconut (*Cocos nucifera* L.) is an important oil seed and plantation crop in India and India ranks third in coconut production in the world (21665 million nuts). Among the states of India, the four southern states contribute to nearly 70 per cent of coconut production. Maximum nut production and productivity has been recorded in Kerala (7429 million nuts and 13732 nuts ha^{-1}) followed by Tamil Nadu which produced 6171 million nuts with a productivity of 13423 nuts ha^{-1} (Coconut Development Board, 2015-16). Though, coconut is affected by many diseases in India, the occurrence of these diseases is restricted to specific locations. For instance, in Tamil Nadu, the basal stem rot disease is more prevalent in eastern parts of the state *viz*., Thanjavur district, while, the leaf blight disease of coconut is restricted to certain pockets *viz.,* Coimbatore, Tirupur, Krishnagiri and Dindigul districts.

Leaf blight disease of coconut remains a problem especially in aged palms. The pathogen persists between seasons on infected leaflets and dead palm debris. The symptoms initially appear on the leaflets of matured outer/lower fronds and subsequently spread to other fronds leaving top most leaves including the spindle, unaffected. The disease progression starts from the distal end of the leaflets and spreads towards the midrib then end. As the disease become severe, most of the fronds would be affected and ultimately resulted in nut reduction.

Lasiodiplodia theobromae (Ascomycota: Dothideomycetes: Botryosphaeriales: Botryosphaeriaceae) affects many field and horticultural crops in tropical and subtropical regions including damage during storage and leads to heavy economic losses (Punithalingam, 1976; Pavlic *et al*., 2007). The pathogen could cause severe damage and lead to significant losses

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especially when the host plants are in stress (Slippers and Wingfield, 2007).

L. theobromae causes seedling and shoot blight, twig blight, cankers and die-back, collar and crown rots *etc*., mainly in fruit and tree crops such as avocado, apple, pear, peach, mango, avocado, Citrus spp., pine and banana (Sharma *et al.*, 1984; Cedeno *et al.*, 1995; Mohali *et al.*, 2005; Sulaiman *et al*., 2012; Bharani Deepan and Ebenezar, 2017).

Several pathogens including *Fusarium, Pestalotia, Alternaria, Colletotrichum* and *Helminthosporium* have been reported as foliar pathogens on coconut. The impact of any potential fungal pathogen affecting coconut has to be studied to develop a suitable disease management strategy. *L. theobromae* has caused severe damage to coconut in the southern states of Tamil Nadu and Kerala (Punithalingam, 1980). Though the pathogen was identified through morphological techniques, molecular confirmation, pathogenicity and variability studies are still lacking. Morphological identification and PCR-based detection of coconut leaf blight causing fungus, *L. theobromae*, has been reported in different crops (Norhayati *et al.*, 2016), but has not yet been attempted in coconut. Therefore, this study was designed to characterize *L. theobromae* under *in vitro* condition. Identification of pathogen is essential to study the epidemiology, to develop proper management strategies and also to quarantine the exotic pathogens to prevent the spread.

Materials and methods

Sample collection

Coconut leaflets showing typical symptoms of leaf blight disease were collected from farmers' field at three different locations *viz.*, Coimbatore, Tiruppur and Krishnagiri in Tamil Nadu State. The leaflets collected were kept in polythene packs and stored in refrigerated condition until further use.

Isolation of the fungi

Single spore isolation method using plain agar was used to obtain pure culture of *L. theobromae* and identified based on morphological descriptions (Wang-Ching Ho and Wen-Hsiung Ko, 1997).

Morphological characterization

Morphological characters *viz*., mycelia, conidial size, shape, colour and time of sporulation and development of fruiting body (size and arrangement) were recorded as per the methodology of Punithalingam (1976). Keys generated by Burgess *et al.* (2006) were used to compare the conidia of the isolated cultures.

Molecular identification of the pathogen

Isolation of genomic DNA

Mycelial mats harvested from 25 days old cultures were dried and ground into fine powder using liquid nitrogen in a pre-cooled pestle and mortar. Total genomic DNA was extracted by CTAB method (Murray and Thompson, 1980).

PCR amplification

The PCR amplification of the fungal ITS-rDNA region was performed by using forward (ITS 1; 5'-TCCGTAGGTGAACCTGCGG-3') and reverse primers (ITS 4; 5'-TCCTCCGCTTAATTGATATGC-3') by following standard protocol described by White *et al.* (1990). Amplified products were sequenced, aligned (Clustal X) and identified using BLAST.

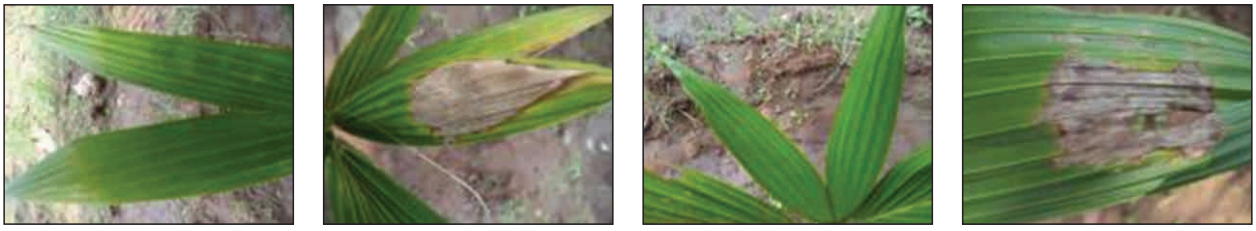
Reaction of tall and dwarf varieties of coconut to pathogen

Source of genotypes

Two tall varieties *viz.*, Arasampatti Tall and West Coast Tall (WCT) and two dwarf varieties *viz*., Chowghat Orange Dwarf (COD) and Chowghat Green Dwarf (CGD) were obtained from Coconut Research Station Aliyarnagar. The seedlings were raised in mud pots under glasshouse with potting mixture. Six month old seedlings were used for artificial inoculation of test pathogen. Ten seedlings under each were used for inoculation.

Preparation of conidial suspension

The conidia of 30 day old cultures of *L. theobromae* from PDA were washed with 10 mL of sterile distilled water, filtered through three layers of gada cloth, centrifuged at 10,000 rpm for 15 minutes and adjusted to 10^5 conidia per mL using sterile distilled water. The viability of conidia was examined by plating different dilutions on PDA media and the spore concentration was measured by using haemocytometer (Parker *et al.*, 1995).



Mycelial mat | **Pinprick + Mycelial mat** | **Spore suspension spray** | **Pinprick + Spore suspension spray**

Fig. 1. Pathogenecity of *L. theobromae* on different inoculation techniques

Artificial inoculation of pathogen

Four methods of inoculation (placing of mycelial mat with and without pinprick method and spraying of spore suspension with and without pinprick method) were used for artificial inoculation of pathogen (Fig. 1).

The conidial suspension (10^5 spores mL^{-1}) was prepared from 30 days old purified *L. theobromae* culture in sterile distilled water (10 mL) and sprayed separately on 6 months old seedlings of each varieties (10 seedlings per variety) during cool evening hours and covered with moist polythene bag. In same method of inoculation instead of spraying, the mycelial mat (5 cm diameter) was placed on leaf surface of the seedling. The seedlings sprayed with sterile water alone were maintained as control. All seedlings were observed periodically from the day after inoculation until the 25th day after inoculation. Samples were collected from the typical symptoms developed in inoculated seedlings and re-isolated to compare with original isolates.

The pathogenicity was carried out on coconut seedlings of tall (Arasampatti Tall and WCT) and dwarf (COD and CGD) varieties and this experiment was conducted on both winter (December, 2016) and summer (March, 2017) seasons. During this period, the weather parameters like temperature and relative humidity (RH) were recorded.

Results

Symptomatology

The pathogen caused severe damage in adult palms (above 30 years old) and mild damage in young palms. Heavily infected coconut palms exhibited delayed flowering when compared to healthy palms and the incidence was severe in older/matured fronds and the younger fronds were mostly free from disease. The affected leaflets showed minute yellow dots initially and start drying from the tip towards middle rachis. Drying spread to entire leaf let and shows a charred or burnt appearance from distance. In the fronds, irregular necrotic spots with dark brown margins appeared on leaf lets of older fronds and turned into dark brown in colour on maturation with black powdery mass. Under severe conditions, symptoms of dark grey to brown lesions with wavy or undulated margins appear on nuts from the apex. The affected nut was desiccated, shrunken, deformed and dropped prematurely. The pathogen penetrated into the kernel through mesocarp, resulted in decaying of endosperm (Fig. 2).

Morphological characteristics

The pathogen was isolated from the infected samples from three different locations of Tamil Nadu. The mycelium of the isolated fungus grew vigorously and in 3-4 days, it completely covered the surface of the media in Petri plates. Initially, the colonies were white and the colour gradually changed to light grey between the 4th and the 7th day. The colour turned dark grey/black two to three weeks after incubation (Fig. 3) and remained black. The fungus produced pycnidia at 22- 24 days after incubation which were initially soft in nature and became hard later. It produced liquid exudates initially, which dried up in 3-4 days. The size of pycnidia varied from 82 to 204 µm in diameter for all the isolates (Fig. 4). The spores were released through ostiole only after drying of exudates.

Affected palm **Affected leaflets** **Cross section of petiole**

Petiole **Buttons** **Nuts**

Fig. 2. Symptoms of leaf blight disease

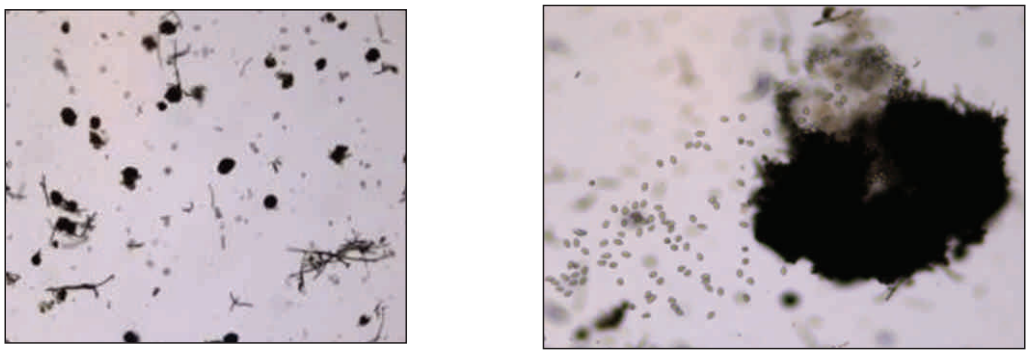
Pycnidia were scatteredon artificial medium in the isolates of LT1 (TN), LT 3 (TN) while, it was arranged in the periphery in LT 2 (TN) isolate. The immature conidia were single celled, hyaline, thick walled and oval shaped. However, the matured one were septate, oval in shape, dark brown in colour with irregular longitudinal striationson the spores. The length and breadth of the conidia ranged from 24.064 to 26.425 µm and 12.827 to 14.354 µm, respectively (Table 1 and Fig. 5).

A **B** **C** **D**

Fig. 3. Cultural characteristics of the fungus isolated from coconut leaflets. Colony was initially white and become greyish to dark grey and mycelia covered the whole PDA surface. (A) Day 4; (B) Day 7; (C) Day 11; (D) Day 14

Fruiting bodies containing water droplets | **Matured fruiting body (Scattered)** | **Matured fruiting body (Periphery)**

Fig. 4. Developing fruiting bodies



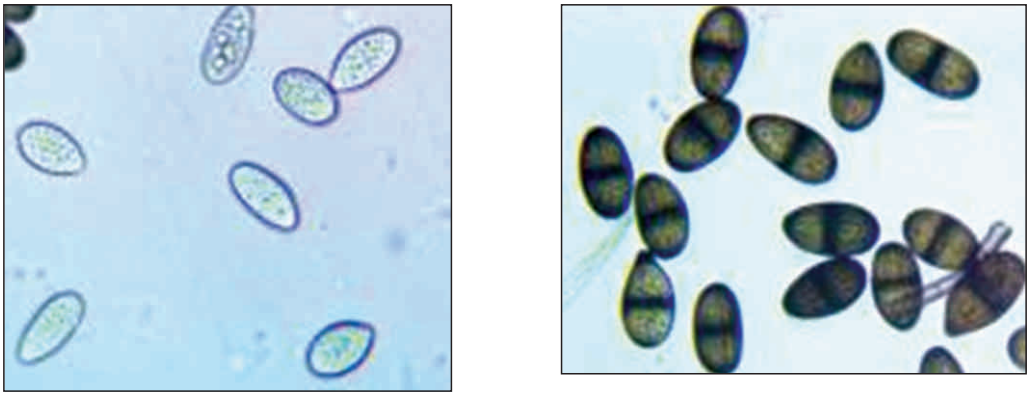
Pycnidia | **Releasing of spores from Pycnidia**

Fig. 5. Structure of fruiting body

Table 1. Morphological characters of *L. theobromae* isolates

Isolate name	Location	Colony texture and color	**Pycnidial character			*Conidia			
			Time taken in days	Arrange-ment	Diameter (µm)	Colour	Size (µm)	Shape	Time taken in days
LT1(TN)	Coimbatore	Raised, uniform, cottony white changing to black	23	Scattered	125-204	Dark Brown	26.425x14.354	Oval	27
LT2(TN)	Tirupur	Raised, uniform, cottony white changing to black	24	Periphery	82-179	Dark Brown	24.064x13.684	Oval	29
LT3(TN)	Krishnagiri	Raised, uniform, cottony white changing to dark grey	22	Scattered	118-184	Dark Brown	24.567x12.827	Oval	27

*Observations on 25 days after incubation; ** Observations on 30 days after incubation



Immature conidia **Matured conidia**

Fig. 6. Conidia was observed under light microscope (40X). Mature conidia with thin cell wall, oval-shaped and dark colour

ITS-rDNA sequence analysis

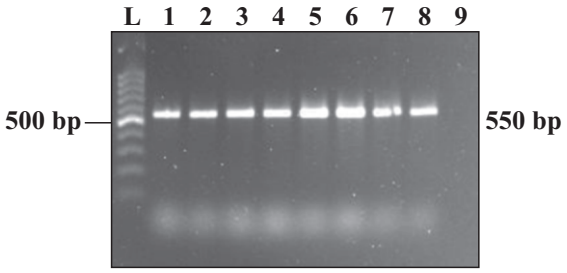
An amplicon of 550 bp fragment was obtained using the primer pair ITS1-F/ITS4 for all the three isolates of *L. theobromae* (Fig. 6). The amplicons were sequenced and analyzed through NCBI blast search domain. The ITS sequence of all the isolates were found to be identical and confirmed as *L. theobromae* (Table 2).

Pathogenicity of isolates

Pathogenicity of the *L. theobromae* isolates was established by artificial inoculation of the isolates on tall and dwarf varieties of coconut seedlings with different methods of inoculation. The *L. theobromae* fungus isolate (CRS LT1) was found to be more virulent and pathogenic on all the coconut seedlings irrespective of varieties and inoculation methods. Inoculated seedlings showed small necrotic spots with yellow halo on 5th day after inoculation during summer at an average the temperature of 34.5 ºC and RH of 76.14 per cent (March, 2017) while, it took 14 days after inoculation during winter, at the temp of 31.8 ºC and RH of 88.22 per cent (December, 2016). The spots enlarge and coalesce together resulting in larger lesions. Size of the lesions on all the 10 seedlings of each variety was measured at 25 days after inoculation and data are presented in Table 3. The maximum lesion size was observed in pinpricked along with spot application of mycelial mat (5 mm disc) inoculated seedlings at 18.89 x 5.48 cm followed by pinprick along with spore suspension spray at 14.62 x 3.12 cm during summer months in West Coast Tall seedlings. The same trend was observed during winter months. No symptom development was observed when inoculation was done without pinprick. The seedlings that received water spray also did not develop any symptoms (Fig. 7).

Table 2. GenBank data comparison of *Lasiodiplodia theobromae* isolates sequence

Isolate Name	GenBank accession no.	Percent Identity	Query length (%)	Isolates accession (GenBank) and reference
LT 1 (TN)	MG685854	100	100	KY052959; Mehl *et al*. (un-published)
LT 2 (TN)	MG 685855	99	100	HM46695; Sulaiman *et al*. (2012)
LT 3 (TN)	MG 697234	99	100	KY052959; Mehl *et al*. (un-published)



L - 100 bp ladder; Lanes 1-2: Infected samples from Coimbatore; Lanes 3-4: Infected samples from Tirupur; Lanes 5-6: Infected samples from Krishnagiri; Lane 7: Positive control (coconut leaf let); Lane 8: Positive control (Cocoa); Lane 9: Negative control (water)

Fig. 7. PCR amplification of *L. theobromae* by using ITS primers

Table 3. Influence of inoculation methods on leaf blight disease expression

Inoculation methods	Winter season				Summer season			
	Arasampatti Tall	WCT	CGD	COD	Arasampatti Tall	WCT	CGD	COD
	Average lesion size in cm (length x breath)							
Spore suspension spray	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00
Spotting of mycelial mat	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x0.00	0.00 x 0.00	0.00 x 0.00
Pinprick + Spore suspension spray	2.56 x 1.26	5.64 x 2.68	1.54 x 1.18	2.12 x 2.14	6.69 x 3.56	14.62 x 3.12	2.51 x 2.38	4.64 x 2.76
Pinprick + Mycelial mat	5.36 x 2.72	7.68 x 3.34	3.28 x 2.24	3.64 x 2.96	13.90 x 4.98	18.89 x 5.48	6.40 x 3.60	9.64 x 4.26
Untreated control	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00	0.00 x 0.00

WCT- West Coast Tall; CGD- Chowghat Green Dwarf; COD- Chowghat Orange Dwarf

Discussion

L. theobromae, causing leaf blight in coconut is a serious disease in southern India especially Tamil Nadu, which has limited the production and productivity under severe conditions. The described symptoms were in accordance with similar blighting pattern in the leaf let reported by Abad *et al*. (1975). Lakshmanan and Jegadeesan (2004) reported nut rot of coconut which leads to 10 to 25 per cent yield loss in adult palms during severe incidence of leaf blight. The identification of *L. theobromae* isolate has proven to be monotonous due to absence of constant morphological and physiological characteristics, which are influenced by the environment.

Previously, pathogens under Botryosphaeriaceae family have been reported on several plants in all climatological regions (Pavlic *et al*., 2007). *Botryosphaeria ribis*, *B. parva* and *B. dothidea* have been reported from temperate regions, while and *B. rhodina* (anamorph: *L. theobromae*) was isolated from tropical locations (Punithalingam, 1980). In the present study, the morphological characters of the pathogen isolated from coconut infected leaflets, fronds and nuts revealed the identity as *L. theobromae*.

The morphological characterizations of *L. theobromae* isolates collected from different coconut growing areas of Tamil Nadu revealed that these isolates were fast to moderately growing raised colonies, with pycnidia of dark black colour. The pycnidia were present along the periphery or scattered in the culture plate. Similarly, variations were observed on pycnidial characters as well as on pathogenic ability. In the present study, the immature conidia were single celled, thick walled, oval in shape and hyaline in nature, while the

matured conidia were thin walled with vertical striations, dark brown to black in colour with single septation. The same observation was made by Kivati (1984), the drought wilt of coconut palm incited by *Botryodiplodia theobromae*. This fungus also produced by buff white mycelial mat, numerous pycnidia and immature conidia as hyaline and aseptate, while matured one was thick walled with longitudinal striation, dark brown and two celled. Cedeno *et al.* (1995) confirmed that *L. theobromae* was the cause of passion fruit vine die-back and studied the cultural characters. The conidia were initially hyaline, single celled with thick cell walls, but later on became thin walled with dark brown colour and single septate with longitudinal striations on the outer wall and measured 23.4-26.1 x12.3-13.9 mm.

The conidia of *Lasiodiplodia* was more obovoid in nature (Burgess *et al*., 2006). Presence of vertical striation on mature conidia of *Lasiodiplodia* spp. is the only character that differentiates other fungi in the same genus (Denman *et al*., 2000). The morphological characters of *L. theobromae* from diverse hosts like grapevines, pear, apple, *Albizia*, peach, mango, avocado, citrus *etc*., are also the same (Denman *et al*., 2000; Mohali *et al*., 2005; Slippers and Wingfield, 2007; Pavishah *et al*., 2010; Sulaiman *et al*., 2012; Bharani Deepan and Ebenezar, 2017). However, the molecular techniques would offer precised identification and characterization of the pathogen up to species level (Punithalingam, 1980).

Some of the overlapping morphological characters of the fungi could be overcome with the intervention of molecular techniques (Pavlic *et al*., 2004; Burgess *et al*., 2006). The main differentiation of the genus *Botryosphaeria* was identified by using DNA sequence of 18S rRNA from fungi (van Nienerk *et al*., 2004; Mohali *et al*., 2005). In this study, the partial sequence of ITS regions confirmed that the fungus was *L. theobromae* which was supported by many workers (Sulaiman *et al*., 2012; Norhayati *et al*., 2016). Thus, the morphology combined with molecular sequence analysis is helpful for clearly define the taxonomy *L. theobromae*.

The pathogenecity test confirmed that the fungus has the ability to incite an infection in coconut seedlings with pinprick method along with spraying of spore suspension and placing of mycelia mat. This result was supported by Kedar Nath (2011), who observed that, pinprick method is the best method for development of fruit rot symptoms in banana caused by *L. theobromae*, but no symptoms on without pinprick method for artificial inoculation. Infected seedlings showed to reduction in photosynthetic activity leading to poor growth while nut infection showed to decay of endosperm completely, reducing the marketable value.

Muhammad Shahbaz *et al*. (2009) observed that the moist condition favours the initial establishment of *L. theobromae* and the existence of favourable temperature and high humidity during February-March and August-September aggravates the disease development. With regard to environment, the condition which is favourable for the development of infection by *L. theobromae* to proliferate is high moisture, temperature and abundant nutrients (Semangun, 2007). But in the present study, the infection starts from 5 days after inoculation during summer months when the temperature (34.5 ºC) was high and RH (76.14%) was low (March, 2017). But during winter months, the infection started 10 days after inoculation when the temperature (31.8 ºC) was low and humidity was high (88.22 %) compared to summer months. In summer months, the symptom develops as a small necrotic spots which coalesces together and the lesion spreads to maximum leaf are at a faster rate than during winter months.

Maximum concentration of conidia was observed in the mycelial mat compared to conidial suspension. Hence, size of the necrotic lesion was observed maximum with pinprick method then with spotting of mycelial mat technique. Based on the study, higher spore concentration, high temperature and low RH favored the development of the disease. The healthy palms may also develop infection subsequently though the appearance is expressed severely during summer season. The incidence was noticed throughout the year and maximum incidence was observed during summer months. Early reports on *Botryosphaeria* spp. revealed that they were seed-borne affects seed germination (Owolade *et al*., 2009), nut rot in physic nut (Sulaiman *et al*., 2012). But in coconut palm, this pathogen is not lethal restricting its severity to reduced photosynthetic activity causing indirect loss it in terms of productivity under

extreme intensity. Based on the above observation, any wound or any injury to the fronds/leaflets is needed for the initial establishment of plant pathogen for further establishment and development of the disease.

Conclusion

Studies on morphological and molecular characteristics of coconut leaf blight pathogen revealed it as *L. theobromae*. Although, the pathogen developed symptoms on the matured fronds and nuts, the level of infection is not at critical stage. Therefore, integrated disease management measures are needed to prevent further spread of this disease. As the pathogenecity proved that, wounds/injuries to leaf lets are the main predisposing factor for initial establishment, avoiding injuries to plant parts can go a long way in reducing the incidence and spread of the disease.

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