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ORIGINAL ARTICLE

Association of *Lasiodiplodia theobromae* with die-back and decline of nutmeg as revealed through phenotypic, pathogenicity and phylogenetic analyses

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Abstract
Lasiodiplodia theobromae is a cosmopolitan pathogen geographically widespread in tropics and subtropics inciting economically important diseases on diverse plant genera. In the present study, *Lasiodiplodia theobromae* associated with nutmeg exhibiting die-back and declining symptoms was identified and characterized by adopting a polyphasic approach. The disease was characterized with the symptoms including general decline, water-soaking patches on branches and tree trunk, die-back of branches, necrotic lesions beneath water-soaked lesions and necrosis of vascular tissues. The isolates representing diverse nutmeg growing tracts were initially identified as *Lasiodiplodia* species based on macro- and micro-morphological characteristics. Subsequent analyses of internal transcribed spacer (ITS), partial elongation factor 1-alpha (*EF1-α*) and *β-tubulin* (*β-tub*) genes identified the pathogen as *Lasiodiplodia theobromae*. Pathogenicity studies were proved on nutmeg twigs and branches (in vitro) as well as on saplings (in vivo). The present investigation enunciated the association of *Lasiodiplodia theobromae* with die-back and decline of nutmeg employing a polyphasic approach which warrants further investigations on its spatio-temporal distribution, pathogen diversity, weather–host–pathogen interaction and formulating prospective disease management strategies.

Keywords Botryosphaeriaceae · Die-back · Decline · Endophyte · Internal transcribed spacer · Stress

Introduction

Nutmeg (*Myristica fragrans* Houtt.), a native of Moluccas Islands, Indonesia is a dioecious, evergreen tropical tree spice representing the family Myristicaceae widely grown in Indonesia, Grenada, Sri Lanka, India, China, Malaysia, Western Sumatra, Zanzibar, Mauritius and the Solomon Islands. The dried seed as well as seed aril of the tree are widely traded as high value spices. Pharmacological research revealed various activities of nutmeg such as antioxidant, antibacterial, antidiabetic, hypolipidemic, hepatoprotective and analgesic (Krishnamoorthy and Rema 2006). The pericarp, mace and seed have been widely used in traditional Ayurveda, Chinese and Thai medicines. Major constituents in the essential oil of nutmeg are myristicin, elemicin, safrole and sabenene which comprise 80% of the oil (Maya et al. 2004). Nutmeg oleoresins are often used in flavouring soft drinks, canned foods and cosmetics. Indonesia is the world's biggest nutmeg producer accounting to 75% of the global production. In India, nutmeg is cultivated in Kerala, Karnataka as well as Andaman and Nicobar islands. Ernakulam, Idukki, Kottayam, Kozhikode and Thrissur are the major nutmeg growing districts in Kerala (Anonymous 2015).

Lasiodiplodia theobromae, a member of Botryosphaeriaceae is a pleomorphic, plurivorous, cosmopolitan Ascomycetous fungus widely distributed in tropical and subtropical regions. It was described first time around 1890 by Saccardo, affecting cocoa in Ecuador. *Lasiodiplodia theobromae* and other species representing Botryosphaeriaceae are often designated as opportunistic or latent pathogens colonizing apparently healthy plant tissues. The latency period might be extended until certain external stimuli like drought stress triggers their transformation into potential

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pathogens which ultimately debilitates the host (Rubini et al. 2005; Mohali et al. 2005). *Lasiodiplodia theobromae* is considered as a potential pathogen inciting cankers, die-back and fruit as well as root rots in more than 500 species including perennial fruit and nut trees, vegetable crops and ornamentals (Punithalingam 1980). It is one among the economically important pathogen with worldwide distribution with a wide host range (Punithalingam 1976) causing shoot blight, die-back, twig blight, cankers in woody plants including fruit and tree species such as pear, apple, silk tree, peach, mango, avocado, citrus, eucalyptus, neem, pinus (Sharma et al. 1984; Verma and Cheema 1984; Mattos and Ames 1986; Sharma and Sankaran 1987; Darvas and Kotze 1987; Britton et al. 1990; Sangchote 1991; Cedeno and Palacios-Pru 1992; Cedeno et al. 1995; Mohali et al. 2005). Die-back and bark canker are reported to be extremely destructive under conducive weather conditions. The bark of infected parts shrinks considerably resulting in depressed lesions. Consequently, longitudinal and transverse cracks appear on the cankered bark of older branches (Verma and Cheema 1984) wherein, the pathogen causes distension, disrupts the cell walls thereby deteriorating strength and toughness of the wood (Shah et al. 2010).

Nutmeg is vulnerable to several obnoxious phytopathogenic microbes. However, in the recent surveys undertaken, symptoms manifested as die-back of young twigs, branches and general decline of the trees were noticed in certain nutmeg cultivating tracts which prompted to explore the possible association of a pathogen with this disease. The present study was undertaken with the objectives to conduct surveys in major nutmeg growing tracts of Kerala, to study symptomatology of the disease, identify and characterize the pathogen associated employing morphological and molecular tools as well as pathogenicity.

Materials and methods

Survey and symptomatology

Field surveys were undertaken during June to August (monsoon) and December to February (post-monsoon) in major nutmeg growing tracts of Kerala consecutively for 2 years *i.e*., 2018 and 2019. Representative samples (branches and bark samples exhibiting characteristic die-back and water soaking/gummosis symptoms, respectively) were collected during the surveys, sealed in clean polythene bags and brought to laboratory for subsequent processing. Briefly, the symptomatic necrotic tissues along with peripheral healthy zones were incised from advancing margin of the lesions, dissected into bits (1 cm × 1 cm), surface sterilized with ethanol (70%) for 30 s followed by sodium hypochlorite (1%) for 2 min and washed with sterile distilled water consecutively three times. Subsequently, the bits were aseptically transferred to potato dextrose agar (PDA) medium, incubated at 25 ± 2 °C with 12 h photoperiod (alternating light and dark conditions) and observed periodically. The growing edges of hyphal initials emerging from the tissues were aseptically transferred to growth medium in Petri dishes and maintained at 25 ± 2 °C under continuous illumination. The cultures were identified initially by comparing the colony as well as conidial characters with published literature. Pure cultures were transferred to PDA slants supplemented with streptomycin sulphate (100 ppm) and maintained at 4 °C, for subsequent studies.

Phenotypic characterization

Observations on macro-morphological characteristics like colony morphology, growth rate and colour (top and reverse) were recorded by culturing the isolates on PDA medium. For morphological characterization, three 5 mm plugs were aseptically punched from actively growing area near the edges of 7-day-old cultures of each isolates using a sterile cork borer. Each plug was subsequently transferred to the growth medium (PDA) and maintained in triplicate at temperature range of 25 ± 2 °C for 7 days. The mean radial mycelial growth (mm per day) of each isolate was recorded daily and colony diameter and colour were recorded. Furthermore, dimensions and shape of microscopic structures like conidia and pycnidia were also recorded. For the examination of conidial morphology, the isolates were cultured on PDA at 25 °C. The conidia harvested from the culture plates were mounted in water, stained with lactophenol cotton blue, examined under research microscope (LEICA DM 5000B), dimensions were recorded and photomicrographs were documented.

Molecular characterization

PCR amplification and sequencing

Pure cultures of the isolates were grown in potato dextrose broth at 25 ± 2 °C for 8 days. The mycelia were harvested by filtering through sterile Whatman No.1 filter paper and washed with sterile distilled water. The DNA was isolated from the mycelium using the method described earlier with slight modifications (Sheji et al. 2009).

The ITS region of ribosomal DNA was amplified using primers ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'-GGAAGCAACAAAAAGG-3') (White et al. 1990). Partial elongation factor 1-alpha (*EF1- α*) and *β-tubulin* genes were amplified using EF1-728F (5'-CAT CGAGAAGTTCGAGAA-3')/EF1-986R (5'- TACTTG AAGGAACCCTTACC-3') (Carbone and Kohn 1999) and

Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′)/Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass and Donaldson 1995) primers, respectively. The amplification conditions were as follows: an initial denaturation step at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min; annealing at 58 °C for the ITS region and *EF1 α*—gene, and 55 °C for *β-tubulin* gene for 1 min; extension at 72 °C for 1 min and a final extension step at 72 °C for 10 min. The PCR assays were performed in a ProFlex™ PCR system (Applied Biosystems, USA). The PCR products were separated by electrophoresis in 1% agarose gel stained with ethidium bromide and viewed under ultraviolet light, photographed and documented with an Syngene G:Box XR5: chemiluminescence and fluorescence imaging system (Integrated scientific solutions, San Diego, CA). The amplified PCR products were purified using the Nucleospin gel and PCR cleanup kit (Macherey Nagel Inc, USA) following manufacturer instructions and sequenced by AgriGenome Labs Private Ltd (Kochi, Kerala, India) using respective primers. The nucleotide sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) under the accession numbers MW219509 to MW219520.

Phylogenetic analyses

Sequences of the reference strains and isolates of *Lasiodiplodia* species of each locus were downloaded from GenBank and used along with the sequences of the present study (Table 1). Multiple sequence alignments for each locus were performed separately using the MEGA 7.0.14 (Kumar et al. 2016), and manual adjustments were done wherever necessary. Alignment of each locus was loaded in Sequence Matrix v.1.8 (Vaidya et al. 2011) to build the concatenated matrix. The phylogeny for the concatenated matrix was inferred under the Maximum Likelihood (ML) and Bayesian Inference (BI) criterion. The ML analyses were done in RAxML-HPC2 on XSEDE (Stamatakis 2014), and Bayesian phylogenetic estimates were done in MrBayes 3.2.6 (Ronquist et al. 2012) on XSEDE implemented on the CIPRES Science Gateway portal (https://www.phylo.org/portal2/home.action; Miller et al. 2010). The ML tree searches were performed under the GTRGAMMA model with 1000 pseudo-replicates. The BI trees were constructed using the GTR + G (ITS), HKY + G (*EF1-α*), and GTR (*β-tubulin*) nucleotide substitution models, with four runs and four MCMC chains per run; Markov chains were run over 100,000,000 generations, and trees and parameter values were sampled every 10,000 generations (Bautista-Cruz et al. 2019). The tree was rooted with *Neoscytalidium hyalinum* CBS145.78. The phylogenetic trees were viewed and edited using FigTree v 1.4.3.

Pathogenicity

The isolates were cultured on PDA for 7 days at 25 °C. After 7 days, the mycelial plugs were punched out with a cork borer from the advanced margins (edges) of the colonies. The pathogenicity tests were performed on (a) nutmeg twigs (b) nutmeg branches and (c) nutmeg saplings. For inoculation, the mycelial plugs were placed with the mycelial region facing the wounds over which moistened cotton was placed. In all the cases, presence of moisture was ensured by periodical spraying of sterile distilled water at the inoculation site using an atomizer.

(a) Nutmeg twigs

Nutmeg twigs of pencil thickness were cut from main branches of nutmeg tree. The outer skin of the twig (size 1 cm × 1 cm) was peeled with knife. The twigs were placed in conical flask (100 ml) containing 5% sucrose solution. The inoculated twigs were maintained at ambient room temperature 25 ± 2 °C until symptoms were developed.

(b) Nutmeg branches

Nutmeg branches of 3 cm diameter and 10 cm long were prepared and soaked in sterile distilled water overnight before inoculation. The outer skin (size 2 cm × 1 cm) was peeled off and the branches were placed in a humid chamber after inoculation which was maintained at 25 ± 2 °C.

(c) Nutmeg saplings

One-year-old nutmeg saplings established in polybags were used for the experiment. The outer bark (size 2 cm × 1 cm) of the saplings was peeled off at nodes as well as internodes. After inoculation, the saplings were covered with a polythene bag to maintain humidity. The inoculum was prepared by deriving mycelial plugs (5 mm diameter) from 7 days old colony of the pathogen. The mycelial plugs were placed on the point of injury and covered with moist cotton. The saplings were observed daily for the development of symptoms, if any.

Results

Survey and symptomatology

Fixed plot surveys facilitated by the contact farmers were carried out in three districts viz., Ernakulam, Palakkad, and Kozhikode of Kerala, India during 2018 and 2019. In each identified locations, observations were recorded in five plots (n = 5) with a population of 50–100 trees

Table 1 Details of isolates of *Lasiodiplodia* spp. used for phylogenetic analysis and their GenBank accession numbers.

Species	Isolate No.	Host	Country	ITS	*EF1- α*	*β-tubulin*
Lasiodiplodia theobromae	LT-C1*	*Myristica fragrans*	India	MW219517	MW219513	MW219509
Lasiodiplodia theobromae	LT-C2*	*Myristica fragrans*	India	MW219518	MW219514	MW219510
Lasiodiplodia theobromae	LT-C3*	*Myristica fragrans*	India	MW219519	MW219515	MW219511
Lasiodiplodia theobromae	LT-C4*	*Myristica fragrans*	India	MW219520	MW219516	MW219512
Lasiodiplodia theobromae	CBS164.96	Fruit along coral reef coast New Guinea	New Guinea	AY640255	AY640258	KU887532
Lasiodiplodia theobromae	CDA 465	*Cocos nucifera*	Brazil	KP244701	KP308465	KP308535
Lasiodiplodia theobromae	ZWLT 481	*Cocos nucifera*	China	MK051003	MK051099	MK051097
Lasiodiplodia theobromae	ZWLT 482	*Cocos nucifera*	China	MK051004	MK051100	MK051098
Lasiodiplodia pseudotheobromae	UACH258	*Citrus latifolia*	Mexico	MH277917	MH286514	MH279929
Lasiodiplodia pseudotheobromae	UACH260	*Citrus latifolia*	Mexico	MH277921	MH286518	MH279924
Lasiodiplodia pseudotheobromae	UACH274	*Citrus latifolia*	Mexico	MH277923	MH286520	MH279922
Lasiodiplodia pseudotheobromae	UACH278	*Citrus latifolia*	Mexico	MH277927	MH286524	MH279918
Lasiodiplodia pseudotheobromae	CMM3887	*Jatropha curcas*	Brazil	KF234559	KF226722	KF254943
Lasiodiplodia pseudotheobromae	CBS116459	*Gmelina arborea*	Costa Rica	EF622077	EF622057	EU673111
Lasiodiplodia brasiliense	UACH257	*Citrus latifolia*	Mexico	MH277910	MH286537	MH279903
Lasiodiplodia brasiliense	UACH267	*Citrus latifolia*	Mexico	MH277912	MH286536	MH279906
Lasiodiplodia brasiliense	CMM4015	*Mangifera indica*	Brazil	JX464063	JX464049	…
Lasiodiplodia brasiliense	COAD 1784	*Cocos nucifera*	Brazil	KP244693	KP308469	KP308524
Lasiodiplodia brasiliense	CDA 431	*Cocos nucifera*	Brazil	KP244694	KP308472	KP308525
Lasiodiplodia citricola	CBS124707	*Citrus* sp.	Iran	GU945354	GU945340	KU887505
Lasiodiplodia citricola	UACH262	*Citrus latifolia*	Mexico	MH277948	MH286541	MH279934
Lasiodiplodia hormozganensis	CBS124708	*Mangifera indica*	Iran	GU945356	GU945344	KU887514
Lasiodiplodia hormozganensis	IRAN1500C	*Olea* sp.	Iran	GU945355	GU945343	…
Lasiodiplodia mahajangana	CMW27818	*Terminalia catappa*	Madagascar	FJ900596	FJ900642	FJ900631
Lasiodiplodia mahajangana	CMW27801	*Terminalia catappa*	Madagascar	FJ900595	FJ900641	FJ900630
Lasiodiplodia egyptiacae	CBS130992	*Mangifera indica*	Egypt	JN814397	JN814424	KU887508
Lasiodiplodia egyptiacae	COAD 1791	*Cocos nucifera*	Brazil	KP244687	KP308462	KP308522
Lasiodiplodia egyptiacae	BOT29	*Mangifera indica*	Egypt	JN814401	JN814428	…
Lasiodiplodia euphorbiicola	CMM3652	*Jatropha curcas*	Brazil	KF234554	KF226715	KF254938
Lasiodiplodia euphorbiicola	CMM3609	*Jatropha curcas*	Brazil	KF234543	KF226689	KF254926.
Lasiodiplodia iraniensis	IRAN1517C	*Citrus* sp.	Iran	GU945349	GU945337	
Lasiodiplodia iraniensis	IRAN1519C	*Mangifera indica*	Iran	GU945350	GU945338	
Lasiodiplodia mediterranea	CBS 137,783 Quercus ilex	*Quercus ilex*	Italy	KJ638312	KJ638331	…
Lasiodiplodia mediterranea	CBS 137,784 Vitis vinifera	*Vitis vinifera*	Italy	KJ638311	KJ638330	…
Lasiodiplodia parva	CBS456.78	Cassava-field soil	Colombia	EF622083	EF622063	…
Lasiodiplodia parva	CBS495.78	Cassava-field soil	Colombia	EF622085	EF622065	…
Neoscytalidium hyalinum	CBS145.78	*Homo sapiens*	United Kingdom	KF531816	KF531795	KF531796.

*Isolates of present study

of age ranging from 15 to 25 years. The population in the surveyed locations encompassed seedling progenies, farmer's as well as released varieties. The symptoms observed in different locations were general decline of the tree compared to healthy trees (Fig. 1), water-soaking symptoms on branches and tree trunk, die-back of twigs/branches, formation of necrotic lesions on the bark beneath water-soaked lesions and necrosis of vascular

Fig. 1 Nutmeg trees **a** Healthy, **b** With die-back and declining symptoms

tissues (Fig. 2). The incidence and severity of the disease and manifestation of symptoms were invariably higher and pronounced on trees in plots which either experienced water logging during monsoon or drought during summer.

Phenotypic characterization

Isolation of the pathogen was carried out following the standard procedures. The hyphal initials were observed after 2 days of incubation and the colonies were dull white in colour initially. Four isolates designated as LT C1 (Angamali, Ernakulam), LT C2 (Peruvannamuzhi, Kozhikode), LT C3 (Kozhinjampara, Palakkad) and LT C4 (Kakkadampoyil, Kozhikode) shortlisted based on topography and climatic conditions of the locations of origin of samples were used for subsequent studies. The isolates based on macro-morphological features like colour and growth pattern of the colony and micro-morphological characters of conidia, pycnidia were tentatively identified as *Lasiodiplodia* species.

In the present investigation, texture of the colony with respect to all the isolates were raised and cottony/wooly with greyish-black top (Fig. 3a) and olivaceous green to black reverse/bottom which attained 90 mm diameter within four days of incubation at 25 ± 2 °C at 12:12 h photoperiod. The immature conidia were initially hyaline, aseptate, ovoid and unmelanized, whereas the mature conidia were uniseptate, melanized, brownish with longitudinally running prominent striations with dimension varying from 20 to 28 (length) X 12 to 18 (breadth) µm (Fig. 3b) The pycnidia were produced on PDA after 4 weeks of incubation, borne solitary, immersed or semi-immersed, grey to black, uniloculate, globose thick walled, central ostiole with a dimension of 255–500 µm (Fig. 3c). In the present study, phenotypic characteristics of the isolates did not exhibit considerable variations which may be due to their low genetic plasticity.

Molecular characterization

Phylogenetic analyses

The nucleotide sequences of ITS, *EF1-α* and *β-tubulin* genes of the *Lasiodiplodia* cultures isolated in this study were generated and used to identify their phylogeny based on ML and BI phylogenetic analyses. The combined ITS, *EF1-α* and *β-tubulin* datasets consisted of 37 taxa, including 1 outgroup taxon (4 from this study and 33 from GenBank). The alignment contained 1,183 characters, of which 281, 399 and 503 are from *EF1-α*, *β-tubulin gene*, and ITS region, respectively. The resultant phylogenetic tree (Fig. 4) is presented with bootstrap support and posterior probability values above the branches. The combined datasets resulted in eleven moderate to well-supported clades corresponding to previously described *Lasiodiplodia* species. The sequences of the isolates obtained in this study clustered within the clade corresponding to *L. theobromae* with a moderate bootstrap value of 81. This clade also included *L. theobromae* ex-type strain CBS164.96 and two other *L. theobromae* isolates (ZWLT 481 and ZWLT 482) of *Cocos nucifera* from China. In BLASTn analysis, the nucleotide sequences of the nutmeg isolates showed 99–100% identity with the sequences of ex-type isolate (CBS 164.96) of *L. theobromae* previously deposited in GenBank (Phillips et al. 2013).

Fig. 2 Symptomatology of die-back and decline **a** Die-back of twig, **b** Gummosis on branches, **c** Gummosis on trunks, **d** Bark exhibiting water soaking, **e** Necrosis of tissues beneath bark, **f** Necrosis of vascular tissues

Pathogenicity

The pathogenicity experiment was performed on nutmeg twigs and branches (in vitro) as well as saplings of 1 year old (in vivo) as outlined in materials and methods. On twigs, the symptoms developed as necrotic lesions 10–12 days after inoculation (Fig. 5a). Whereas, on branches, the symptoms were manifested only after 16–18 days after inoculation (Fig. 5b). On nutmeg saplings, the symptoms developed were similar to that observed under field conditions. Necrotic

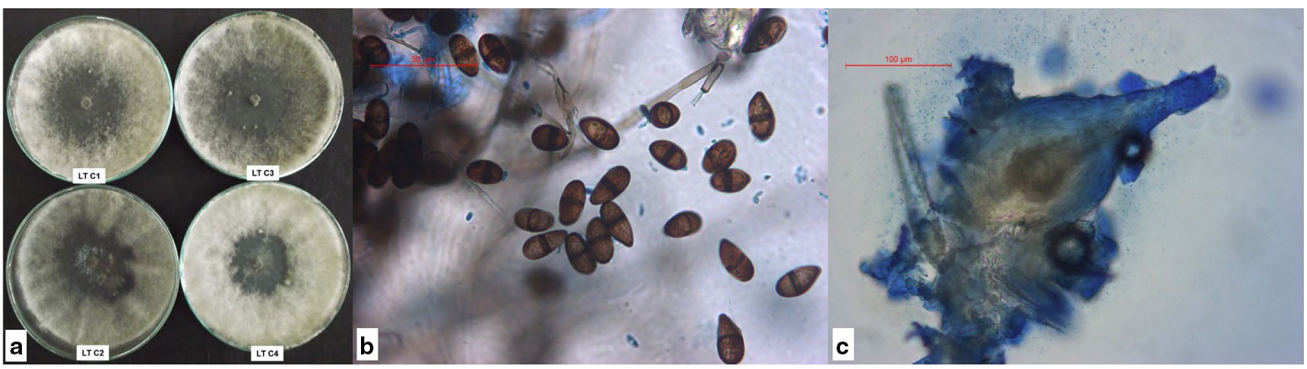

Fig. 3 **a** Colony, **b** Conidia, **c** Pycnidium (formative stage) of *Lasiodiplodia*

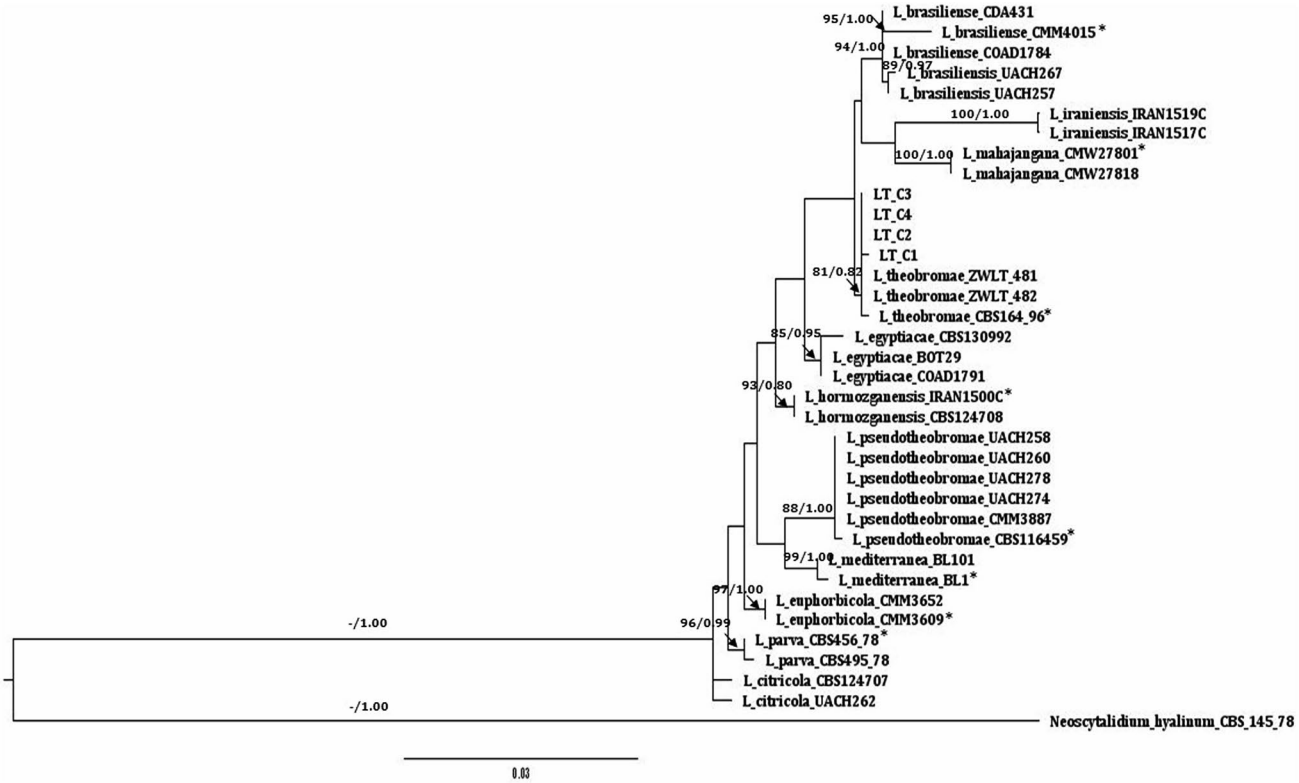

Fig. 4 Phylogenetic tree of *Lasiodiplodia* species inferred from a concatenated alignment of an ITS, *EF-1α* and *β-tubulin* gene sequence alignments. Bootstrap values by the Maximum Likelihood method and probabilities by the Bayesian Inference analyses are shown on the respective branches. Bootstrap values below 80% and posterior probabilities below 0.80 are not shown. The tree is rooted to *Neoscytalidium hyalinum* CBS 145.78. Epi- and ex-type strains are indicated with star. The scale bar indicates the average number of substitutions per site

lesions were formed 20–22 days after inoculation and the necrosis was found to extend beyond the point of inoculation beneath the bark (Fig. 5c). The pathogen was subsequently isolated from the infected regions and compared with the original culture thereby proving Koch's postulates.

Discussion

In the present study, surveys undertaken in Kerala, India, symptoms including die-back of branches and general decline were noticed on nutmeg trees in certain tracts. Subsequent investigations based on morphology, molecular tools and pathogenicity revealed the association of *Lasiodiplodia theobromae* with the disease.

In the present investigation, *L. theobromae* was found inducing diverse symptoms in nutmeg which included, die-back of twigs, water soaking on bark tissues, gummosis and necrosis of internal vasculature. The symptoms were manifested on trees representing wide range of age groups and the most characteristic and conspicuous symptoms were die-back of the twigs, dispersed throughout the entire canopy in severe cases and water soaking on tree trunks. *Lasiodiplodia theobromae* is reported to induce symptoms like general decline in guava (Safdar et al. 2015), die-back in mango (Rodriguez-Galvez et al. 2017) and cashew (Cardoso et al. 2002), canker of grapevine (Leavitt and Munnecke 1987; Urbez-Torres et al. 2008) and stem-end rot of mango (Johnson et al. 1992). *Lasiodiplodia theobromae* is reported in nutmeg causing premature fruit rot in Ghana (Amoako and Ahiatsi 2010). As observed in the present investigation, the symptoms were more pronounced on the trees experiencing/experienced abiotic stress conditions like water stagnation or drought and hence it is hypothesized that, *L. theobromae* colonizing the internal niche as an endophyte in nutmeg might have switched its life style as a pathogen when the host was debilitated under stress conditions. Aberrant alterations in the environmental factors like temperature modulate dynamics of host–microbe interactions consequently activating the dormant virulence factors thereby impacting the bio-geographical profile and pathogenicity as revealed through multi-omics analysis of *L. theobromae* infecting grapevine (Felix et al. 2019). Members of Botryosphaeriaceae represent a prominent component of

Fig. 5 Pathogenicity: development of lesions on **a** Twig, **b** Branch, **c** Sapling

several diverse endophytic communities and are reported to be stress-associated pathogens wherein the disease expression is often correlated with abiotic stress conditions or climatic extremes like frost, drought, waterlogging, unsuitable growing conditions and physical damage (Marsberg et al. 2017). Drought stress is implicated as a major pre-disposing factor for significant and consistent development of cankers due to infection of *L. theobromae*, a stress-influenced pathogen on dogwood seedlings (Mullen et al. 1991).The ability of different *Lasiodiplodia* species to switch their nutritional mode between endophytic and pathogenic phases is reported in several host-pathosystems, reinforcing its designation as latent pathogens (Slippers and Wingfield 2007; Marsberg et al. 2017).

The genus *Lasiodiplodia* is taxonomically characterized principally based on the presence of pycnidial paraphyses and longitudinal striations on matured conidia that are considered as unique features for distinguishing from other genera of Botryosphaeriaceae as well as to delineate different species within *Lasiodiplodia* (Slippers et al. 2017). In nature, the asexual morphs of *L. theobromae* are generally observed which serves as propagules for rapid dissemination under conducive environmental conditions. It is difficult to identify *Lasiodiplodia* at species level using morphological traits and hence, it is imperative to employ DNA sequence data, preferably combining sequences from multiple loci (Phillips et al. 2013; Slippers et al. 2014). In *Lasiodiplodia*, ITS, *EF-1α* and *β-tub gene* sequences are widely used to discriminate between species, especially the cryptic species frequent in the genus (Burgess et al. 2006; Slippers et al. 2014; Rosado et al. 2016; Bautista-Cruz et al. 2019). In recent years, new species of *Lasiodiplodia* have been proposed based on molecular analyses, indicating the existence of a species complex. A combination of morphological and phylogenetic analyses were employed to establish the etiology of the post-harvest stem-end rot of immature coconut and four species were identified *L. brasiliense, L. egyptiacae, L. pseudotheobromae* and *L. theobromae* to be pathogenic to coconut in Brazil (Rosado et al. 2016). Rodriguez-Galvez et al. (2017) used *ITS* and *tef1-a* sequence information to explicitly confirm the association of five species of *Lasiodiplodia* with die-back of mango in Peru which otherwise was earlier thought as a single species viz., *L. theobromae*. Six species of *Lasiodiplodia*; *L. pseudotheobromae, L. theobromae, L. brasiliense, L. subglobosa, L. citricola* and *L. iraniensis* were reported from Persian lime exhibiting canker and die-back symptoms employing multilocus phylogeny (Bautista-Cruz et al. 2019). Similarly, Zhang and Niu (2019) reported that *L. theobromae* causes post-harvest stem-end rot on coconut in China using the same approach. Relatively similar tree topologies were observed with single gene phylogenetic trees based on ITS, *EF1-α* and *β-tubulin* genes (trees not shown). However, we

could find some conflicting nodes and differences in statistical support. Changes in the position of some isolates within the main clades were also noticed. Combined data set analysis resolved these conflicts and improved phylogenetic resolution. In the present study, the *Lasiodiplodia* isolates from nutmeg were identified as *Lasiodiplodia theobromae* based on combined phylogenetic analyses of ITS, *EF1-α* and *β-tub* gene sequence datasets. These isolates were found in a clade along with type strain CBS164.96 and two other *L. theobromae* isolates (ZWLT 481 and ZWLT 482) of *Cocos nucifera* from China. The results of the study indicated that multilocus phylogeny could clearly identify the pathogens as *Lasiodiplodia theobromae*.

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Author contributions Conceptualization of the research approach and experimental designs were developed by C. N. Biju (Principal investigator) with the assistance of M. F. Peeran, A. Jeevalatha, R. Suseela Bhai and Fadla Basima. V. A. Muhammed Nissar, V. Srinivasan, R. Suseela Bhai and Lijo Thomas coordinated the surveys to various locations. Sample preparations, data collection and analyses of data collected from DNA extracts were completed by Fadla Basima, M. F. Peeran and A. Jeevalatha. C. N. Biju, M. F. Peeran and A. Jeevalatha wrote the manuscript and formatted the draft. Reviewing and revision of the manuscript were completed by all authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Ethical approval This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

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