ANDRÉ WILSON CAMPOS ROSADO

PHYLOGENY, IDENTIFICATION AND PATHOGENICITY OF *Lasiodiplodia* ASSOCIATED WITH POSTHARVEST STEM-END ROT OF COCONUT IN BRAZIL

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, para obtenção do título de *Magister Scientiae*.

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APROVADA: 25 de fevereiro de 2014

Prof. Maria Catarina Megumi Kasuya	Prof. Robert Weingart Barreto

Prof. Olinto Liparini Pereira
(Orientador)

“*O destino não é uma questão de sorte, é uma questão de escolha. Não é algo pelo que se espera, mas algo a alcançar*”.

Willian Jennings Bryan

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Biografia

ANDRÉ WILSON CAMPOS ROSADO, filho de Sebastião Mario Lopes Rosado e Raimunda Ferreira Campos Rosado, nasceu em Viçosa, Minas Gerais, no dia 07 de julho de 1985.

Em 2006 ingressou no curso de Agronomia na Universidade Federal de Viçosa (UFV), graduando-se em julho de 2011.

Em março de 2012, iniciou no Programa de Pós-graduação, em nível de Mestrado em Fitopatologia na UFV, concentrando seus estudos nas áreas de etiologia de doenças fúngicas de plantas e micologia (taxonomia e filogenia molecular de fungos fitopatogênicos).

Sumário

Resumo

ROSADO, André Wilson Campos, M.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Filogenia, identificação e patogenicidade de *Lasiodiplodia* associada à podridão-peduncular do coco em pós-colheita no Brasil.** Orientador: Olinto Liparini Pereira.

O coqueiro (*Cocos nucifera* L.) é uma das mais importantes culturas tropicais perenes, por possibilitar a geração de um sistema sustentável de exploração comercial. O Brasil é o quarto maior produtor mundial de coco, atrás da Indonésia, Filipinas e da Índia. A produção do coqueiro é limitada por vários fatores, dentre eles, destacam-se as doenças. A podridão-peduncular é a principal doença em pós-colheita a atingir a cultura, causando grandes perdas em todas as regiões produtoras do país. Essa doença ocorre em frutos após a colheita e caracteriza-se como uma podridão negra no pedúnculo, progredindo para todo o fruto. O fungo *Lasiodiplodia theobromae* é a única espécie relatada associada a essa doença. Entretanto, um estudo acurado desse patógeno como o único agente causal nunca foi realizado. Nos últimos anos novas espécies de *Lasiodiplodia* têm sido propostas a partir de estudos moleculares, evidenciando a existência de um complexo de espécies. Assim, os objetivos deste trabalho foram estudar a etiologia da podridão-peduncular do coco baseada na combinação de características morfológicas e moleculares, estabelecer o posicionamento filogenético das espécies encontradas, bem como comprovar a patogenicidade dos isolados. Foram realizadas coletas de cocos verdes com sintomas da doença em mercados no município de Viçosa/MG. Em adição a esses, foram obtidos isolados dos estados da Bahia, Ceará e Paraíba. Isolados monospóricos foram obtidos e armazenados. Esses tiveram o DNA extraído e a região TEF1-α sequenciada. A partir dos resultados das análises filogenéticas, foi separado um isolado de cada espécie para a caracterização morfológica e teste de patogenicidade. Seis espécies de *Lasiodiplodia* foram identificadas, sendo elas *L. egyptiacae, L. pseudotheobromae*, *L. theobromae*, *L. viticola* e duas a serem propostas como novas (*Lasiodiplodia* sp. 1 e *Lasiodiplodia* sp. 2). Todas as espécies distinguiram-se morfologicamente e filogeneticamente das demais e tiveram a patogenicidade comprovada. Apesar da sua importância, a etiologia da podridão-peduncular do coco foi tradicionalmente negligenciada pela ciência e permaneceu obscura por muito tempo. Neste trabalho são relatadas três espécies diferentes de *Lasiodiplodia* associadas à podridão-peduncular do coco e também relata-se a

ocorrência de outras duas espécies que são aqui descritas e serão posteriormente propostas como novas. Os resultados deste trabalho serão fundamentais para futuros estudos envolvendo medidas de manejo da doença, programas de quarentena e especialmente, para o desenvolvimento de variedades de coco resistentes à podridão-peduncular.

Abstract

ROSADO, André Wilson Campos, M.Sc., Universidade Federal de Viçosa, february, 2014. **Phylogeny, identification and pathogenicity of *Lasiodiplodia* associated with postharvest stem-end rot of coconut in Brazil.** Adviser: Olinto Liparini Pereira.

The coconut palm (*Cocos nucifera* L.) is one of the most important perennial tropical crops. Brazil is the fourth largest producer of coconut, after Indonesia, Philippines and India. Coconut production is limited by several factors, including diseases. Stem-end rot is the major postharvest disease of coconut in Brazil, causing great losses in all producing regions. This disease occurs in fruits after harvest and starts as a black rot in the peduncle that progresses to the entire fruit. The fungus *Lasiodiplodia theobromae* is the only species reported associated with this disease. However, an accurate study of this pathogen as the sole causal agent has never been carried out. In recent years, new species of *Lasiodiplodia* have been proposed after molecular studies, have demonstrated the existence of a species complex behind *L. theobromae*. Here the etiology of stem-end rot of *C. nucifera* was studied based on a combination of morphological characteristics with TEF1-α sequence data, aimed at re-evaluating the phylogenetic position of *Lasiodiplodia* involved in this disease and to assess the pathogenicity of the taxa found. Green coconuts with stem-end rot symptoms and signs of the pathogen were collected in Viçosa, state of Minas Gerais. In addition, isolates were obtained from Bahia, Ceará and Paraíba states. Single-spore cultures were obtained and stored. These had their DNA extracted and their TEF1-α region was sequenced. From the results of the phylogenetic analyses, one isolate of each species was separated for the morphological characterization and pathogenicity tests. Six species were identified: *Lasiodiplodia egyptiacae, L. pseudotheobromae*, *L. theobromae*, *L. viticola* and two will be proposed as new species (*Lasiodiplodia* sp. 1 and *Lasiodiplodia* sp. 2). All species were distinguished morphologically and phylogenetically and were proven to be pathogenic to coconut. Despite its importance, the etiology of stem-end rot of coconut has been traditionally neglected by science and remained somewhat obscure. This work resulted the first reports worldwide of the presence of three different species of *Lasiodiplodia* as causal agents of stem-end rot of coconut. Additionally, two previously undescribed species, which will be proposed as new, were found. Little has been investigated about the *Lasiodiplodia* complex in Brazil. The results of this study may be helpful for further

studies on management measures, quarantine programs or on development of stem-end rot resistant coconut varieties.

Introdução geral

O coqueiro (*Cocos nucifera* L.), pertencente à família Arecaceae*,* é uma planta tropical cultivada comercialmente em cerca de 90 países (Martins e Júnior 2011). A água de coco é um isotônico natural com várias propriedades desejáveis, tais como, reidratante, energética, anti-helmíntica e diurética. Pode ser utilizada no tratamento da nefrite, ser empregada em tratamento de emergência como plasma sanguíneo, substituir o soro reidratante e ainda servir como meio de cultura natural (Viana *et al.*2001). Além da água e do consumo do coco seco, o fruto é usado para produzir fibra, a partir do processamento da casca e óleo extraído do endocarpo (Rosa *et al.* 2002). A planta pode ser utilizada como ornamental (Martins e Júnior 2011) e possui potencial para produção de biodiesel (Neto 2007).

O coqueiro é uma das mais importantes culturas tropicais perenes, por possibilitar a geração de um sistema sustentável de exploração comercial, como ocorre em vários países asiáticos, no qual é importante para a economia e uma das principais fontes de proteínas para populações locais. O continente asiático destaca-se na produção e comercialização do produto *in natura* e nos subprodutos (Pires 2004). A Indonésia é a maior produtora do continente e, em 2012, teve uma produção de 18.000.000 t, em uma área plantada de 3 milhões de ha (FAO 2014).

O Brasil é o quarto maior produtor de coco, atrás da Indonésia, Filipinas e da Índia (FAO 2014). O coqueiro encontra-se disperso por todo o país, mas a região nordeste destaca-se como maior produtora. Em 2012, o país produziu quase 2 bilhões de frutos em uma área de aproximadamente 260.000 ha e exportou 1.207 t do fruto, gerando cerca de US$ 686.000,00. Desses, 69 % dos frutos foram produzidos no nordeste, em cerca de 209.000 ha. O estado da Bahia destaca-se como o principal produtor e consumidor, seguido de Ceará e Sergipe (IBGE 2014; Agrianual 2014).

Ressalta-se que aproximadamente 90 % da produção mundial de coco provém de pequenos agricultores, com áreas de até 5 ha, cuja produção é quase totalmente consumida internamente nos países produtores. No Brasil aproximadamente 70 % da exploração do coqueiro ocorre em propriedades de até 10 ha (Martins e Júnior 2011).

A cocoicultura é limitada por vários fatores, dentre eles, destacam-se as doenças. Várias doenças ocorrem no coqueiro pelo mundo, algumas são encontradas no Brasil e variam de importância de uma região para outra. Entre essas, as mais importantes estão a lixa-pequena [*Camarotella torrendiella* (Batista) Bezerra & Vitória], lixa-grande [*Camarotella acrocomiae* (Mont.) K.D. Hyde & P.F. Cannon], anel-vermelho [*Bursaphelenchus cocophilus* (Cobb) Baujard], murcha-de-fitomonas [*Phytomonas staheli* McGhee & McGhee], podridão-seca (etiologia ainda indefinida, mas acredita-se que o agente seja um fitoplasma), queima-das-folhas [*Botryosphaeria cocogena* Subileau, Renard & Lacoste] e podridão-peduncular (*Lasiodiplodia* spp.).

A podridão-peduncular é a principal doença em pós-colheita a atingir a cultura, causando grandes perdas em todas as regiões produtoras do Brasil (Viana *et al.* 2007). Durante a colheita, os sintomas iniciais de infecção não são visíveis, pois o fungo encontra-se quiescente abaixo das brácteas. Mas, quando o coco é armazenado em condições ambientais, o patógeno inicia sua ação colonizadora dos tecidos do mesocarpo causando a podridão. No início da colonização pode-se observar anasarca e a formação de uma nítida linha divisória entre o tecido doente e o sadio. Aproximadamente 48 h após o aparecimento da anasarca a área escurece, tornando-se marrom clara, depois negra e necrótica, enquanto a anasarca avança para os tecidos sadios. Ao remover as brácteas, podem-se observar sinais do patógeno na forma de um micélio branco-acinzentado. Em lesões velhas, é possível encontrar numerosos cirros conídiais formados sobre os picnídios (Viana *et al.* 2001).

A podridão-peduncular está associada ao fungo *Lasiodiplodia theobromae* (Patouillard) Griffon & Maublanc, que é um membro da família Botryosphaeriaceae (Botryosphaeriales, Dothideomycetes, Ascomycota), cosmopolita, embora mais comum em regiões tropicais e subtropicais, em ampla gama de hospedeiros (Alves *et al.* 2008). Esse fungo é uma ameaça para as culturas, pois pode viver endofiticamente em material vegetal assintomático e não ser detectado pela quarentena. Além disso, é capaz de rapidamente causar doença quando seus hospedeiros estão sob estresse, causando grandes perdas (Slippers e Wingfield 2007).

Lasiodiplodia theobromae é caracterizada por produzir picnídios escuros, ostiolados, estromáticos; conidióforos simples e delgados; conídios imaturos hialinos, unicelulares e de parede celular delgada e conídios maduros elipsoides, escuros, bicelulares e estriados (Viana *et al.* 2001). As principais características que distinguem o gênero de outros relacionados são a presença de paráfises nos picnídios e estrias longitudinais em conídios maduros, devido à deposição de grânulos de melanina na superfície interna da parede (Sutton 1980; Phillips *et al.* 2013).

Apesar da associação de *L. theobromae* com a podridão-peduncular do coco ser conhecida, até o momento não foi realizado um estudo acurado, envolvendo uma diversidade de isolados de várias procedências, buscando a caracterização precisa do agente etiológico dessa doença. Nos últimos anos novas espécies de *Lasiodiplodia* têm sido propostas a partir de estudos moleculares, evidenciando a existência de um complexo de espécies (Pavlic *et al.* 2004; Burgess *et al.* 2006; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Abdollahzadeh *et al.* 2010; Begoude *et al.* 2010; Ismail *et al.* 2012; Liu *et al.* 2012; Urbez-Torres *et al.* 2012; Marques *et al.* 2013; Machado *et al.* 2014; Netto *et al.* 2014). Tradicionalmente, a taxonomia de espécies em Botryosphaeriaceae tem sido baseada na morfologia do estádio anamórfico. Mas, utilizar apenas caracteres morfológicos pode subestimar a verdadeira diversidade da família (Taylor *et al.* 2000). Assim, é muito importante a realização de estudos moleculares associados à caracterização morfológica em estudos etiológicos.

Nos últimos anos, técnicas moleculares baseadas em sequências de DNA têm sido cada vez mais utilizadas para distinguir taxa em Botryosphaeriaceae. Diferenças em sequências de DNA também foram combinadas com sucesso com características morfológicas para identificar e descrever taxa em Botryosphaeriaceae. Em alguns casos, essa abordagem revelou surpreendente diversidade (Slippers e Wingfield 2007).

Devido às elevadas perdas geradas pela podridão-peduncular do coco, surgiu o interesse em pesquisar-se a sua etiologia e a identidade do agente etiológico envolvido, bem como estabelecer o posicionamento filogenético das espécies encontradas. A identificação precisa dos patógenos é fundamental para permitir o desenvolvimento apropriado de medidas de manejo, programas de quarentena e especialmente para o desenvolvimento de variedades resistentes a essa doença.

Sendo assim, objetivou-se com este trabalho: a) estudar a etiologia da podridão-peduncular do coco baseada na combinação de características morfológicas e moleculares; b) estabelecer o posicionamento filogenético das espécies encontradas; c) disponibilizar as sequências de DNA em banco de dados públicos para auxiliar na sua identificação; d) realizar testes de patogenicidade para comprovação da infectividade dos isolados.

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Artigo

According to the guidelines of Fungal Diversity

Phylogeny, identification and pathogenicity of *Lasiodiplodia* associated with postharvest stem-end rot of coconut in Brazil

Phylogeny, identification and pathogenicity of *Lasiodiplodia* associated with postharvest stem-end rot of coconut in Brazil

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Abstract

The coconut palm (*Cocos nucifera* L.) is one of the most important perennial tropical crops. Stem-end rot is the major postharvest disease of coconut in Brazil. The fungus *Lasiodiplodia theobromae* is the only species reported associated with this disease. However, an accurate study elucidating the true identity of this pathogen with molecular tools has never been carried out. In recent years new species of *Lasiodiplodia* have been proposed after molecular studies were carried out, indicating the existence of a species complex behind *L. theobromae*. The aims of this research were: To study the etiology of the postharvest stem-end rot of immature coconut based on a combination of morphological characteristics with TEF1-α sequence data, to establish the phylogenetic position of such taxa and to assess the pathogenicity of each taxon. Six species were identified: *Lasiodiplodia egyptiacae, L. pseudotheobromae*, *L. theobromae*, *L. viticola* and two will be proposed as new (*Lasiodiplodia* sp. 1 and *Lasiodiplodia* sp. 2). All species were distinguished morphologically and phylogenetically and were proven to be pathogenic to coconut. *Lasiodiplodia theobromae* is the most common and also one of the most aggressive species. This work represents the first reports worldwide of the presence of three different species of *Lasiodiplodia* as causal agents of postharvest stem-end rot of immature coconut. Additionally, two previously undescribed species, which will be proposed as new, were found. Such results are critical for further studies on management measures, quarantine programs or on development of stem-end rot resistant coconut varieties.

Keywords: Botryosphaeriaceae, etiology, postharvest pathology, taxonomy, TEF1-α, *Cocos nucifera* L.

Introduction

Cocos nucifera L. is a member of the family Arecaceae and one of the most important perennial tropical crops. The coconut palm is native from the coast of Southeast Asia (Malaysia, Indonesia, Philippines) and Melanesia (Chan and Elevitch 2006). Coconut trees are important sources of food, biofuel, fiber, oil, medicines and construction material (Huang *et al.* 2013) and also widely used as an ornamental (Martins e Júnior 2011).

Brazil is the fourth largest producer of coconut, after Indonesia, Philippines and India (FAO 2014). The coconut palm is grown widely in Brazil, but the Northeast states are the largest producers. In 2012, Brazil produced almost 2 billion of coconuts, in an area of approximately 260,000 ha, and exported 1,207 t of fruit, generating about US$ 686 thousand. These, 69 % of fruits were produced in the northeast region, occupying 208,977 ha (IBGE 2014; Agrianual 2014). It is noteworthy that approximately 90 % of world production of coconut comes from small farmers, with areas up to 5 ha. This production is almost entirely consumed internally in producing countries. In Brazil, approximately 70 % of the holding coconut occurs in properties up to 10 ha (Martins e Júnior 2011).

Coconut production is limited by several factors, among them, diseases have a major role. Stem-end rot is the major postharvest disease of coconut in Brazil, causing great losses in all producing regions (Viana *et al.* 2007). The fungus *Lasiodiplodia theobromae* (Patouillard) Griffon & Maublanc is the only species reported associated with this disease (Mendes *et al.* 1998; Taylor and Hyde 2003; Piepenbring 2006). Despite this association, an accurate study, involving a diversity of isolates of various origins, elucidating the true identity of this pathogen with molecular tools has never been carried out.

Lasiodiplodia theobromae (Botryosphaeriaceae: Botryosphaeriales: Dothideomycetes: Ascomycota) has a cosmopolitan distribution, however it is more commonly found in tropical and subtropical regions (Punithalingam 1980; Marques *et al.* 2013). It has a wide host range, including gymnosperms and angiosperms, and can occur in nature as parasites, saprophytes or endophytes (Alves *et al.* 2008; Machado *et al.* 2014). The main characteristics that distinguish *Lasiodiplodia* of the other related genus are the presence of paraphyses within the pycnidia, and the conidia are initially hyaline and aseptate, but became brown and 1-septate with age, with the formation of longitudinal striations due to the deposition of melanin granules on the inner surface of the wall (Sutton 1980; Phillips *et al.* 2013; Machado *et al.* 2014). This fungus is a threat to crops, because it can live endophytically in asymptomatic plant material and not be detected by quarantine. Moreover, it is able of rapidly cause disease when their hosts are under stress, causing great losses (Slippers and Wingfield 2007).

Traditionally, the taxonomy of species in the Botryosphaeriaceae has been based on the morphology of the anamorph states. But, taxonomy based on morphological characteristics alone may underestimate the true diversity of the family (Taylor *et al.* 2000). Currently, morphological characteristics combined with molecular techniques based on DNA sequences have been used to differentiate taxa of Botryosphaeriaceae and has revealed surprising diversity (Slippers and Wingfield 2007). Molecular studies have revealed several novel species of *Lasiodiplodia*, demonstrating the existence of a species complex behind *L. theobromae* (Pavlic *et al.* 2004; Burgess *et al.* 2006; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Abdollahzadeh *et al.* 2010; Begoude *et al.* 2010; Ismail *et al.* 2012; Liu *et al.* 2012; Urbez-Torres *et al.* 2012; Marques *et al.* 2013; Machado *et al.* 2014; Netto *et al.* 2014). More cryptic species may be found through of molecular studies associated with morphological characterization of *Lasiodiplodia* sp. pathogenic.

The high losses generated by stem-end rot of coconut justified the interest in studying their etiology and phylogeny of their etiological agents. The accurate identification of pathogens is essential to allow proper development of management measures, quarantine programs and especially for the development of stem-end rot resistant coconut varieties.

The present study combined morphological characteristics with TEF1-α sequence data to investigate the diversity of *Lasiodiplodia* species associated with postharvest stem-end rot of immature coconut in Brazil and to assess the pathogenicity of the taxa found involved in this disease.

Materials and methods

Sample collection and isolation

From July to August 2012, fruits presenting postharvest stem-end rot symptoms were collected from local markets in Viçosa, State of Minas Gerais, Brazil and sent to the Laboratório de Patologia de Sementes e de Pós-colheita (Departamento de Fitopatologia, Universidade Federal de Viçosa). Samples were first examined for possible presence of fungal fruiting structures.

Direct isolations were performed from naturally infected coconuts. A mass of conidia was transferred to a Petri dish with 3 % Water Agar (WA - Agar Agar, type I Himedia®), spread with a Drigalski spatula, and a single conidium was transferred to a Petri dish with Potato Dextrose Agar (PDA - Acumedia®) under a stereomicroscope [Motic® SMZ-168 (20X)]. The plates were incubated at 25 ºC.

Additionally, isolates from the states of Bahia (municipality of Juazeiro), Ceará [municipalities of Paraipaba, Pentecoste, Sobral (two isolates), Trairi] and Paraíba were also obtained from symptomatic fruits. These isolates were grown on Petri dishes with 2 % WA overlaid with double-sterilized maize straw and incubated at 25 °C with a 12 h light-dark regime for 3-4 weeks to induce sporulation. Single-spore cultures were obtained as mentioned above.

The isolates with dark mycelium, typical characteristic of Botryosphaeriaceae, were stored in tubes slants on PDA at 10 °C and by the method described by Castellani (1967).

DNA extraction, sequencing and phylogenetic studies

Single-spore isolates were grown on PDA at 25 °C for one week. Approximately 40 mg of mycelia were scraped from the agar surface and placed in a sterile 1.5 mL microcentrifuge tube. The extraction was performed by grinding the mycelium in liquid nitrogen into a fine powder using a microcentrifuge tube pestle. The crushing was continued after adding 100 µL of Nuclei Lysis Solution of the

Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.). After the first grinding, another 500 µL of the above mentioned solution was added. The extraction was continued as described by Pinho *et al.* (2012).

PCR was performed with 12.5 µL of Dream Taq TM PCR Master Mix 2X (MBI Fermentas, Vilnius, Lithuania), 1 µL of 10 µM of respectivily the foward and reverse primer, which were synthesized by Invitrogen (Carlsbad, U.S.A), 1 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, U.S.A.), 5 µL of 100× (10 mg/mL) Bovine Serum Albumin (BSA, Sigma–Aldrich, St. Louis, MO, U.S.A.), 2 µL of genomic DNA (25 ng/µl) and 2.5 µL of nuclease-free water. Target sequences of the Transcription Elongation Factor 1-α (TEF1-α) were amplified using primers EF1F (5'-TGCGGTGGTATCGACAAGCGT-3') and EF2R (5'-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs *et al.* 2004).

The thermal cycle consisted of 95 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min (denaturation), 55 ºC for 1 min (annealing), 72 ºC for 2 min (elongation), and 72 ºC for 10 min (final extension). PCR products were analyzed by electrophoresis on 2 % agarose gels stained with GelRed™ (Biotium Inc., Hayward, CA, U.S.A.) in a 1× TAE buffer and visualized under UV light to check for amplification size and purity. The amplicons were purified and sequenced by Macrogen Inc., Korea (http://www.macrogen.com). The nucleotide sequences were edited with the BioEdit software (Hall 2012). All sequences were checked manually, and nucleotides with ambiguous positions were clarified using both primer direction sequences. Sequences of selected *Lasiodiplodia* species from GenBank were included in this study (Table 1). Consensus sequences were compared against GenBank's database using their Mega BLAST program. The closest hit sequences were then downloaded in FASTA format and aligned using the multiple sequence alignment program MUSCLE® (Edgar 2004), built in MEGA v. 5 software (Tamura *et al.* 2011). Alignments were checked and manual adjustments were made when necessary. Gaps were treated as missing data.

Bayesian inference (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences. Before launching the BI, the best nucleotide substitution model for the gene was determined with MrMODELTEST 2.3

(Posada and Buckley 2004). Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC). The HKY+G model of evolution was used for TEF1-α. The phylogenetic analysis of the alignment was performed with the CIPRES web portal (Miller *et al.* 2010) using MrBayes v.3.1.1 (Ronquist and Huelsenbeck 2003). Four MCMC chains were run simultaneously, starting from random trees for 10 000 000 generations. Trees were sampled every 1000th generation for a total of 10 000 trees. The first 2 500 trees were discarded as burn-in phase of each analysis. Posterior probabilities (Rannala and Yang 1996) were determined from a majority-rule consensus tree generated from the remaining 7 500 trees. Trees were visualized in FigTree (Rambaut 2009) and exported to graphic programs. The tree was rooted with *Spencermartinsia viticola* CBS117009.

Morphological studies

A representative isolate from each clade identified in the phylogenetic analysis was used for morphological characterization. The isolates were grown on plates containing 2 % WA overlaid with double-sterilized twigs of *Pinus* and corn straw and incubated at 25 °C under a photoperiod of 12 h with near-ultraviolet light to induce the formation of fruiting bodies and sporulation. Sections of the fruiting bodies were made manually and mounted in lactophenol. Thirty measurements of all relevant morphological characteristics (conidia, paraphyses and conidiogenous cells) were made using a light microscope OLYMPUS CX31. Images were obtained with an OLYMPUS BX 51 light microscope fitted with a digital camera (OLYMPUS EVOLT330). A representative specimen of each taxon will be deposited in the local herbarium at the Universidade Federal de Viçosa (Herbarium VIC), and all isolates will be deposited in the Culture Collection of Phytopathogenic Fungi “Prof. Maria Menezes” (CMM) at the Universidade Federal Rural de Pernambuco (Recife, Brazil).

Pathogenicity tests

The isolates used in the morphological characterization were tested for pathogenicity. Three coconuts, without visible symptoms of disease, were inoculated with each fungal taxon isolated. For inoculation, isolates were grown on PDA plates for 7 d at 25 °C. The fruits were washed in running water with soap, surface disinfested in 1 % sodium hypochlorite, rinsed in sterile distilled water, then dried at room temperature. Three fragments of the region near to the bracts were removed with a scalpel. A 6-mm-diameter disk containing mycelia from the margins of the growing culture was immediately placed over the wound. As control, pure PDA plugs (6 mm diameter) were placed on wounded surfaces. The inoculated fruits were placed over a Gerbox and incubated at 28 °C. Three paper towel layers, wetted with distilled water, were placed at the bottom of each box and the boxes were wrapped in plastic bags to maintain humidity levels high during incubation. The fruits were kept in moist chamber for 48 h. The aggressiveness of the isolates was assessed by measuring the length (L) and width (W) of the lesion in each fruit at 15 days after inoculation. The lesion area was determined using the equation $A=\pi lw$ (Sakalidis *et al.* 2011; Netto *et al.* 2014), where A is the area of an oval, l is the radius of the length (L/2) and w is the radius of the width (W/2). The experiment was conducted in a completely randomized design with three replications per treatment (isolate) and one fruit per replicate. Data were analyzed by ANOVA and means were compared by Tukey test at the 5% probability using SAS (*Statistical Analysis System*, version 9). From the symptomatic inoculated fruits, the fungi were reisolated in pure culture.

Results

Symptomatology and fungus isolation

In symptomatic fruit, a black rot begins on the peduncle, below the bracts and progresses to whole fruit, including the endosperm. In older lesions, numerous cirri formed on pycnidia (Fig. 1).

A total of 60 isolates of *Lasiodiplodia* spp. were obtained from coconuts. In addition, some *Ceratocystis* and *Pestalotiopsis* isolates were also obtained. Nevertheless, due to the predominance and diversity of *Lasiodiplodia* species associated with stem-end rot of coconut these were not included in this work.

PCR amplification and phylogeny

Isolates were compared based on the sequences of the elongation factor region (TEF1-α) by means of Bayesian analyses (data not shown). PCR was successfully performed for all isolates, except for isolate 472. This isolate had to be amplified using 4 μL of genomic DNA, instead of 2 μL. Amplicons of approximately 700 bp were generated for TEF1-α region.

Phylogenetic analysis was performed with 109 taxa and the alignment of the sequences resulted in a total of 288 characters, of which 82 were parsimony-informative, 126 were variable and 151 were conserved. The phylogenetic analysis revealed six species of *Lasiodiplodia* to be present: *L. egyptiacae* (A.M. Ismail, L. Lombard & Crous), *L. pseudotheobromae* (A.J.L. Phillips, A. Alves & Crous), *L. theobromae* [(Pat.) Griffon & Maubl.], *L. viticola* (J.R. Urbez-Torres, F. Peduto & W.D. Gubler), *Lasiodiplodia* sp. 1 and *Lasiodiplodia* sp. 2. These grouped into six clades (Fig. 2). The majority of isolates (50) formed a large clade with *L. theobromae* by a Bayesian posterior probability (BPP) value of 0.9. A second well-supported clade (BPP: 0.99), with four isolates, was formed together with *L. viticola,* and a species recently described from papaya, *L. brasiliense*. One isolate clustered together with *L. pseudotheobromae,* and another with *L. egyptiacae* (BPP: 1; 0.95, respectively). Four isolates formed two sub-clades (with 2 isolates each) with high Bayesian support (0.82; 0.99), probably representing two new species, *Lasiodiplodia* sp. 1 and *Lasiodiplodia* sp. 2.

Lasiodiplodia theobromae was the most frequently isolated species (83 %), followed by *L. viticola* (7 %), *Lasiodiplodia* sp. 1, *Lasiodiplodia* sp. 2 (3 %), *L. egyptiacae* and *L. pseudotheobromae* (2 %), as shown in Fig. 3.

Taxonomy

All isolates induced to sporulate in culture medium showed typical *Lasiodiplodia* morphology. Paraphyses were present within the pycnidial conidiomata. The conidia were initially hyaline and aseptate, and became brown and 1-septate with age, with the formation of longitudinal striations. Based on phylogenetic analysis and morphological characteristics, six species were distinguished among which four are known taxa (*L. egyptiacae*; *L. pseudotheobromae*; *L. theobromae*; *L. viticola*) and two will be proposed as new (*Lasiodiplodia* sp. 1 and *Lasiodiplodia* sp. 2). Dimensions of each *Lasiodiplodia* species are available in Table 2.

Lasiodiplodia egyptiacae A.M. Ismail, L. Lombard & Crous, Australasian Plant Pathology 41:649-660 (2012) (Fig. 5)

Mycelium immersed or superficial, branched, septate, initially white to grey, becoming dark brown. *Conidiomata* pycnidial, stromatic, carbonaceous, mostly solitary, semi-immersed but becoming erumpent when mature, globose to subglobose, dark brown, mostly uniloculate, with a central ostiole, often covered with dense mycelium, formed superficially on twigs of *Pinus* or corn straw in culture. Wall dark brown, thick-walled, textura angularis, followed by a paler inner region of thin-walled, next to conidiogenous cells. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, determinate, formed from cells lining the inner pycnidial walls, 7–20 × 2–5 µm. *Paraphyses* hyaline, subcylindrical, aseptate, not branched, with rounded apex, appearing between the conidiogenous cells, up to 75.5 µm long, 2–3 µm wide. *Conidia* often oozing from pycnidia in cirri, acrogenous, hyaline, aseptate, obovoid to ellipsoid, granular, somewhat tapered at apex and rounded at base when young, later becoming medially one-septate, dark brown, thick-walled, ellipsoid to ovoid/ sub-ovoid, with longitudinal striations, 21.5–29 × 11–17.5 µm.

Substrate: On *Cocos nucifera* fruits

Material examined: BRAZIL, Viçosa, Minas Gerais, on *Cocos nucifera* fruits, 2012, A. R. Machado, Isolate 166.

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous, Fungal Diversity 28:9 (2008) (Fig. 6)

Mycelium immersed or superficial, branched, septate, initially white to grey, becoming dark brown. *Conidiomata* pycnidial, stromatic, carbonaceous, solitary, superficial, globose, dark brown, uniloculate, with a central ostiole, often covered with dense mycelium, formed superficially on twigs of *Pinus* or corn straw in culture. Wall dark brown, thick-walled, textura angularis, followed by a paler inner region of thin-walled, next to conidiogenous cells. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, determinate, formed from cells lining the inner pycnidial walls, 8–17 × 3–4 µm. *Paraphyses* hyaline, cylindrical, septate, sometimes branched, with rounded apex, appearing between the conidiogenous cells, up to 65 µm long, 2–3 µm wide. *Conidia* often oozing from pycnidia in cirri, acrogenous, hyaline, aseptate, obovoid to ellipsoid, granular, somewhat tapered at apex and rounded at base when young, later becoming medially one-septate, dark brown, thick-walled, ellipsoid, with rounded apex, truncate base, with longitudinal striations, measuring 25–32 × 14–18 µm.

Substrate: On *Cocos nucifera* fruits

Material examined: BRAZIL, Viçosa, Minas Gerais, on *Cocos nucifera* fruits, 2012, A.W.C. Rosado, Isolate 459.

Lasiodiplodia theobromae (Pat.) Griffon & Maubl., Bull. trimest. Soc. Mycol. Fr. 25:57 (1909) (Fig. 7)

Mycelium immersed or superficial, branched, septate, initially white to grey, becoming dark brown. *Conidiomata* pycnidial, stromatic, carbonaceous, mostly solitary, superficial, separate, globose, dark brown, uni- or multiloculate, with a central ostiole, often covered with dense mycelium, formed superficially on twigs of *Pinus* or corn straw in culture. Wall dark brown, thick-walled, textura angularis, followed by a paler inner region of thin-walled, next to conidiogenous cells. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, determinate, formed from cells lining the inner pycnidial walls, 7–14 × 2–4 µm. *Paraphyses* hyaline, cylindrical, aseptate, not branched, with rounded apex, appearing between the conidiogenous cells, up to 55 µm long, 2–4 µm wide. *Conidia* often oozing from pycnidia in cirri, acrogenous, hyaline, aseptate, ellipsoid to obovoid, granular, somewhat tapered at apex and rounded at base when young, later becoming medially one-septate, dark brown, thick-walled, ellipsoid, with rounded apex, truncate base, with longitudinal striations, measuring 18.5–30.5 × 12.5–18 µm.

Substrate: On *Cocos nucifera* fruits

Material examined: BRAZIL, Viçosa, Minas Gerais, on *Cocos nucifera* fruits, 2012, A.W.C. Rosado, Isolate 450. Other isolates examined are listed in Table 1.

Lasiodiplodia viticola J.R. Urbez-Torres, F. Peduto & W.D. Gubler, Fungal Diversity 52:183 (2011) (Fig. 8)

Mycelium immersed or superficial, branched, septate, initially white to grey, becoming dark brown. *Conidiomata* pycnidial, stromatic, carbonaceous, solitary, superficial, globose to ovoid, dark brown, uniloculate, with a central ostiole, often covered with moderate mycelium, formed superficially on twigs of *Pinus* or corn straw in culture, up to 900 µm wide. Wall dark brown, thick-walled, textura angularis, followed by a paler inner region of thin-walled, next to conidiogenous cells. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, determinate, formed from cells lining the inner pycnidial walls, 9–17 × 2–4 µm. *Paraphyses* hyaline, cylindrical, aseptate, not branched, with rounded apex, appearing between the conidiogenous cells, up to 61 µm long, 2–3 µm wide. *Conidia* often oozing from pycnidia in cirri, acrogenous, hyaline, aseptate, ellipsoid to obovoid, granular, somewhat tapered at apex and rounded at base when young, later becoming medially one-septate, dark brown, thick-walled, ellipsoid to subglobose, with rounded apex, truncate base, with longitudinal striations, measuring 20–26 × 12–18 µm.

Substrate: On *Cocos nucifera* fruits

Material examined: BRAZIL, Paraipaba, Ceará, on *Cocos nucifera* fruits, 2012, F.C.O. Freire, Isolate 484. Other isolates examined are listed in Table 1.

***Lasiodiplodia* sp. 1** (To be proposed as new species) (Fig. 9)

Mycelium immersed or superficial, branched, septate, initially white to grey, becoming dark brown. *Conidiomata* pycnidial, stromatic, carbonaceous, solitary, superficial, globose, dark brown, uniloculate, with a central ostiole, often covered with dense mycelium, formed superficially on twigs of *Pinus* or corn straw in culture. Wall dark brown, thick-walled, textura angularis, followed by a paler inner region of thin-walled, next to conidiogenous cells. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, determinate, discrete, formed from cells lining the inner pycnidial walls, 9–18 × 3–4 µm. *Paraphyses* hyaline, cylindrical, aseptate, not branched, with rounded apex, appearing between the conidiogenous cells, up to 65 µm long, 2–3 µm wide. *Conidia* often oozing from pycnidia in cirri, acrogenous, hyaline, aseptate, ellipsoid to obovoid, granular, somewhat tapered at apex and rounded at base when young, later becoming medially one-septate, dark brown, thick-walled, ellipsoid to ovoid, with rounded apex, truncate base, with longitudinal striations, measuring 20–26.5 × 12–15.5 µm.

Substrate: On *Cocos nucifera* fruits

Material examined: BRAZIL, Viçosa, Minas Gerais, on *Cocos nucifera* fruits, 2012, A.W.C. Rosado, Isolate 448. Other isolates examined are listed in Table 1.

***Lasiodiplodia* sp. 2** (To be proposed as new species) (Fig. 10)

Mycelium immersed or superficial, branched, septate, initially white to grey, becoming dark brown. *Conidiomata* pycnidial, stromatic, carbonaceous, solitary, superficial, globose, dark brown, uniloculate, with a central ostiole, often covered with dense mycelium, formed superficially on twigs of *Pinus* or corn straw in culture. Wall dark brown, thick-walled, textura angularis, followed by a paler inner region of thin-walled, next to conidiogenous cells. *Conidiophores* absent. *Conidiogenous cells* holoblastic, hyaline, smooth, thin-walled, cylindrical, determinate, discrete, formed from cells lining the inner pycnidial walls, 10–23 × 3–4 µm. *Paraphyses* hyaline, cylindrical, aseptate, not branched, with rounded apex, appearing between the conidiogenous cells, up to 70.5 µm long, 2–4 µm wide. *Conidia* often oozing from pycnidia in cirri, acrogenous, hyaline, aseptate, ellipsoid to obovoid, granular, somewhat tapered at apex and rounded at base when young, later becoming medially one-septate, dark brown, thick-walled, ellipsoid to ovoid, with rounded apex, truncate base, with longitudinal striations, measuring 20–26.5 × 13–16.5 µm.

Substrate: On *Cocos nucifera* fruits

Material examined: BRAZIL, Viçosa, Minas Gerais, on *Cocos nucifera* fruits, 2012, A.W.C. Rosado, Isolate 455. Other isolates examined are listed in Table 1.

Pathogenicity tests

All isolates were pathogenic to coconut, resulting in visible lesions 4 d after inoculation (Fig. 11). Symptoms were dark brown necrotic lesions with grayish mycelial growth around the inoculation sites, progressing to overcome the whole fruit. During the development of the disease numerous conidial cirri formed on pycnidia were observed, and the fungus invaded internal tissues including the endosperm.

There were differences ($P \leq 0.05$) in aggressiveness among *Lasiodiplodia* species. *Lasiodiplodia* sp. 1 and *L. theobromae* were the most aggressive of all the species tested, causing large lesions (24.74 cm^2, 28.93 cm^2; respectively). The other species had lesions ranging from 14.07 cm^2 to 19.8 cm^2 and differed significantly from the control (Fig. 4). It was possible to reisolate the inoculated fungus from diseased tissues.

Discussion

This work is the first study, involving a diversity of isolates of various origins, in which the diversity of *Lasiodiplodia* species associated with postharvest stem-end rot of immature *Cocos nucifera* was studied, based on phylogenetic and morphological analysis. Six species of *Lasiodiplodia* were identified in association with the disease. Among these, two new species of *Lasiodiplodia* will be proposed. For many years the etiology of postharvest stem-end rot of coconut was neglected, and although the importance of this disease, only *L. theobromae* had been reported on coconut.

Studies on the application of molecular phylogenetic techniques in *Lasiodiplodia* demonstrated that it is possible to separate the species using only the TEF1-α gene (Marques *et al.* 2013; Machado *et al.* 2014; Netto *et al.* 2014). The four known species identified in this study clustered in a well-supported clade with other isolates examined in previously published studies (Fig. 2). Additionally to the TEF1-α sequences generated and analyzed here other genes (ITS and βt) are being included aimed at increasing the phylogenetic support, need for confirmation of the distinction of the putative new species described herein.

Species in *Lasiodiplodia* have been differentiated based on analysis of phylogenetic and morphological characteristics. Most frequently described morphological characteristics are conidial and paraphyses morphology and, to a lesser extent, cultural characteristics (growth and pigment production) (Pavlic *et al.* 2004; Burgess *et al.* 2006; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Abdollahzadeh *et al.* 2010; Begoude *et al.* 2010; Ismail *et al.* 2012; Liu *et al.* 2012; Urbez-Torres *et al.* 2012; Marques *et al.* 2013; Machado *et al.* 2014; Netto *et al.* 2014).

Characteristics pycnidial paraphyses have to be carefully interpreted. The fungus can show aseptate paraphyses when young, but these may develop septa as they mature (Abdollahzadeh *et al.* 2010). *Lasiodiplodia theobromae* has been reported with septate paraphyses (Alves *et al.* 2008), but in this study no septate paraphyses was found. Perhaps this is a variable characteristic. *Lasiodiplodia pseudotheobromae* has been previously described as having aseptate paraphyses (Alves *et al.* 2008), but here its paraphyses were septate, in agreement with the observation of Machado *et al.* (2014). The other morphological characteristics analyzed for these two species were similar to found in previous studies (Tab. 2).

For *L. egyptiacae* conidia, paraphyses and conidiogenous cells were bigger than described by Ismail *et al.* (2012), but similar to the dimensions found by Machado *et al.* (2014).

The conidia of *L. viticola* of this study are longer and wider than previously described by Urbez-Torres *et al.* (2012), but are similar to the species recently described from papaya, *L. brasiliense*. It is not possible to distinguish these two species solely based on the TEF1-α gene (Netto *et al.* 2014). Therefore, other genes have to be sequenced in order to better clarify the phylogenetic position of the isolates of this study.

Lasiodiplodia sp.1 is closely related to *L. theobromae*, but can be distinguished based on TEF1-α sequence, the shape of conidia (conidia ovoid were observed) and the larger paraphyses.

Lasiodiplodia sp.2 is clearly separated from *L. theobromae* based on TEF1-α sequence. Inclusion of other sequences in the phylogenetic analysis may support the distinction of this species. Moreover, the conidia of *Lasiodiplodia* sp.2 can be ovoid and the dimensions of the paraphyses and conidiogenous cells are larger than *L. theobromae*.

Some studies used the cultural characteristics (growth and pigment production in culture) to differentiate *Lasiodiplodia* species (Pavlic *et al.* 2004; Burgess *et al.* 2006; Alves *et al.* 2008; Pavlic *et al.* 2008; Abdollahzadeh *et al.* 2010; Begoude *et al.* 2010; Ismail *et al.* 2012; Marques *et al.* 2013; Netto *et al.* 2014). These characteristics can vary widely among isolates of the same species. Therefore, they have limited value in species determination (Abdollahzadeh *et al.* 2010; Marques *et al.* 2013; Machado *et al.* 2014; Netto *et al.* 2014). Thus, these characteristics were not included in the present study.

Lasiodiplodia theobromae was the most frequent species (83 %), as also found by Marques *et al.* (2013) and Netto *et al.* (2014). This confirms the wide distribution of *L. theobromae* in Brazil. The second most frequent was *L. viticola* (7 %), followed by *Lasiodiplodia* sp. 1 (3 %), *Lasiodiplodia* sp. 2 (3 %). Only one isolate of *L. egyptiacae* and *L. pseudotheobromae* was found (2 %). Similar results were found by Marques *et al.* (2013) where *L. egyptiacae* was the second least frequent isolated species and by Netto *et al.* (2014) where *L. pseudotheobromae* was less isolated species (Fig. 3).

All isolates were pathogenic to coconut. Besides the devaluation due to esthetic damage, *Lasiodiplodia* can also progress into the endosperm rendering the water unfit for consumption, resulting in large losses. *Lasiodiplodia* sp. 1 and *L. theobromae* were the most aggressive of all tested species. Thus, *L. theobromae* is the most common and one of the most aggressive species, which indicates the importance of further studies on this fungal species. Information on this species and its aggressiveness is scarce and further research is need.

This work represents the first report of the presence of three different species of *Lasiodiplodia* (*L. egyptiacae*, *L. pseudotheobromae* and *L. viticola*) as causal agents of postharvest stem-end rot of immature coconut in the world. Two new species were also detected and represent additions to the pathogenic *Lasiodiplodia* complex. These will be proposed as new species (*Lasiodiplodia* sp. 1 and *Lasiodiplodia* sp. 2). These results show the great diversity of *Lasiodiplodia* species associated with coconut stem-end rot.

Despite its importance, the etiology of stem-end rot of coconut has been traditionally neglected by science and remained somewhat obscure. The results of this study may be helpful for further studies on management measures, quarantine programs or on development of stem-end rot resistant coconut varieties.

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Conclusões gerais

A podridão-peduncular do coco verde em pós-colheita no Brasil é causada por um complexo de espécies, incluindo *Lasiodiplodia egyptiacae, L. pseudotheobromae*, *L. theobromae*, *L. viticola* e duas novas espécies *Lasiodiplodia* sp. 1 e *Lasiodiplodia* sp. 2;

À exceção de *L. theobromae*, todas as demais espécies são pela primeira vez relatadas causando podridão-peduncular em coco;

Todas as espécies encontradas são patogênicas, com destaque para *L. theobromae* e *Lasiodiplodia* sp. 1 que são as mais agressivas.

Anexos

Table 1 GenBank accession numbers of DNA sequences of *Lasiodiplodia* and this study used in the phylogenetic analyses.

Species	Isolates	Host/Substrate	Origin	Genbank acession n°
				EF1-α
Lasiodiplodia theobromae	161	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	164	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	165	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	424	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	427	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	428	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	429	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	430	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	432	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	433	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	443	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	444	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	445	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	446	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	447	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	448	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	450	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	451	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	452	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	453	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	454	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	456	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	457	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	458	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	460	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	461	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	462	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	463	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	464	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	466	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	467	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	468	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	469	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	470	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	471	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	472	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	474	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	475	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	476	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	478	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	479	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	480	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	481	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	482	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	483	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	485	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	486	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	487	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	488	*Cocos nucifera*	Brazil	-
Lasiodiplodia theobromae	492	*Cocos nucifera*	Brazil	-
Lasiodiplodia viticola	426	*Cocos nucifera*	Brazil	-
Lasiodiplodia viticola	431	*Cocos nucifera*	Brazil	-

Lasiodiplodia viticola	473	*Cocos nucifera*	Brazil	-
Lasiodiplodia viticola	484	*Cocos nucifera*	Brazil	-
Lasiodiplodia viticola	UCD2604M	*Vitis vinifera*	Unknown	HQ288270
Lasiodiplodia viticola	UCD2553A	*Vitis vinifera*	Unknown	HQ288269
Lasiodiplodia brasiliense	CMM4015	*Mangifera indica*	Brazil	JX464049
Lasiodiplodia brasiliense	CMM2186	*Carica papaya*	Brazil	KC481542
Lasiodiplodia brasiliense	CMM2255	*Carica papaya*	Brazil	KC481523
Lasiodiplodia brasiliense	CMM2313	*Carica papaya*	Brazil	KC481524
Lasiodiplodia egyptiacae	166	*Cocos nucifera*	Brazil	-
Lasiodiplodia egyptiacae	BOT29	*Mangifera indica*	Egypt	JN814428
Lasiodiplodia egyptiacae	BOT10	*Mangifera indica*	Egypt	JN814424.1
Lasiodiplodia marypalme	CMM2173	*Carica papaya*	Brazil	KC481563
Lasiodiplodia marypalme	CMM2272	*Carica papaya*	Brazil	KC481566
Lasiodiplodia marypalme	CMM2275	*Carica papaya*	Brazil	KC481567
Lasiodiplodia marypalme	CMM2289	*Carica papaya*	Brazil	KC481564
Lasiodiplodia euphorbicola	CMM3651	*Jatropha curcas*	Brazil	KF226711
Lasiodiplodia euphorbicola	CMM3652	*Jatropha curcas*	Brazil	KF226715
Lasiodiplodia euphorbicola	CMM3609	*Jatropha curcas*	Brazil	KF226689
Lasiodiplodia subglobosa	CMM3872	*Jatropha curcas*	Brazil	KF226721
Lasiodiplodia subglobosa	CMM4046	*Jatropha curcas*	Brazil	KF226723
Lasiodiplodia pseudotheobromae	459	*Cocos nucifera*	Brazil	-
Lasiodiplodia sp. 1	425	*Cocos nucifera*	Brazil	-
Lasiodiplodia sp. 1	448	*Cocos nucifera*	Brazil	-
Lasiodiplodia sp. 2	455	*Cocos nucifera*	Brazil	-
Lasiodiplodia sp. 2	465	*Cocos nucifera*	Brazil	-
Botryosphaeria rhodina	CBS 164.96	Unknown	Unknown	AY640258
Botryosphaeria rhodina	CMW9074	*Pinus* sp.	Unknown	AY236901
Lasiodiplodia venezuelensis	WAC12539	*Acacia mangium*	Australia	DQ103568
Lasiodiplodia venezuelensis	CMW13513	*Acacia mangium*	Venezuela	DQ103570
Lasiodiplodia rubropurpurea	WAC12536	*Eucalyptus grandis*	Australia	DQ103572
Lasiodiplodia gonubiensis	CBS115812	*Syzygium cordatum*	South	DQ458877
Lasiodiplodia crassispora	CBS110492	Unknown	Unknown	EF622066
Lasiodiplodia crassispora	CMW22653	*Pterocarpus*	South	FJ888452
Lasiodiplodia margaritacea	CBS122519	*Adansonia gibbosa*	Australia	EU144065
Lasiodiplodia pseudotheobromae	CBS116459	*Gmelina arborea*	Costa Rica	EF622057
Lasiodiplodia pseudotheobromae	CMM3887	*Jatropha curcas*	Brazil	KF226722
Lasiodiplodia macrospora	CMM3833	*Jatropha curcas*	Brazil	KF226718
Lasiodiplodia parva	CBS456.78	Cassava-field soil	Unknown	EF622063
Lasiodiplodia parva	CBS495.78	Cassava-field soil	Unknown	EF622065
Lasiodiplodia citricola	IRAN1521C	*Citrus* sp.	Iran	GU945339
Lasiodiplodia citricola	IRAN1522C	*Citrus* sp.	Iran	GU945340
Lasiodiplodia hormozganensis	IRAN1500C	*Olea* sp.	Iran	GU945343
Lasiodiplodia hormozganensis	IRAN1498C	*Mangifera indica*	Iran	GU945344
Lasiodiplodia plurivora	STEU5803	*Vitis vinifera*	South	EF445395
Lasiodiplodia missouriana	UCD2193M	*Vitis vinifera*	Unknown	HQ288267
Lasiodiplodia missouriana	UCD2199M	*Vitis vinifera*	Unknown	HQ288268
Lasiodiplodia gilanensis	IRAN1523C	*Unknown*	Iran	GU945342
Lasiodiplodia gilanensis	IRAN1501C	*Unknown*	Iran	GU945341
Lasiodiplodia iraniensis	IRAN1517C	*Citrus sp.*	Iran	GU945337
Lasiodiplodia iraniensis	IRAN1519C	*Mangifera indica*	Iran	GU945338
Lasiodiplodia jatrophicola	CMM3610	*Jatropha curcas*	Brazil	KF226690
Lasiodiplodia mahajangana	CMW27801	*Terminalia catappa*	Madagasca	FJ900641
Lasiodiplodia mahajangana	CMW27820	*Terminalia catappa*	Madagasca	FJ900643
Lasiodiplodia theobromae	CMW28571	*Terminalia ivorensis*	Cameroon	GQ469897
Lasiodiplodia theobromae	CBS124.13	Unknown	USA	DQ458890.1
Lasiodiplodia theobromae	CBS111530	Unknown	Unknown	EF622054
Spencermartinsia viticola	CBS117009	*Vitis vinifera*	Spain	AY905559

Table 2 Main morphological characteristics of *Lasiodiplodia* spp..

Species	Conidia (μm)	Paraphyses (μm)		Conidiogenous Cells (μm)	Reference
L. abnormis	25–28 × 13–15	–	–	–	Saccardo 1913
L. brasiliense	22.7–29.2 × 11.7–17	–	aseptate	–	Netto *et al.* 2014
L. citricola	22–27 × 12–17	125 × 3–4	1–5 septa	11–16 × 3–5	Abdollahzadeh *et al.* 2010
L. crassispora	27–30 × 14–17	30–62 × 2–3.5	septate	8–16 × 3–7	Burgess *et al.* 2006
L. egyptiacae	20–24 × 11–12	57 × 2–3	aseptate	5–11 × 3–5	Ismail *et al.* 2012
	21.5–29 × 11–17.5	75.5 × 2–3	aseptate	7–20 × 2–5	**This study**
L. euphorbicola	15–23 × 9–12	76 × 2–4	septate	5–15 × 3–4	Machado *et al.* 2014
L. fiorii	24–26 × 12–15	–	–	–	Saccardo 1913
L. gilanensis	28–35 × 15–18	95 × 2–4	1–3 septa	11–18 × 3–5	Abdollahzadeh *et al.* 2010
L. gonubiensis	32–36 × 16–18.5	26.5–47 × 2–2.5	aseptate	10–15 × 2–4	Pavlic *et al.* 2004
L. homozganensis	18–24 × 11–14	83 × 2–4	1–7 septa	9–15 × 3–5	Abdollahzadeh *et al.* 2010
L. iraniensis	17–23 × 11–14	127 × 2–4	1–6 septa	9–16 × 3–5	Abdollahzadeh *et al.* 2010
L. jatrophicola	22–26 × 14–17	70 × 3	septate	7–15 × 2–5	Machado *et al.* 2014
L. macrospora	28–35 × 15–17	105 × 3–4	septate	8–20 × 2.5–4	Machado *et al.* 2014
L. mahajangana	15.5–19 × 11.5–13	66 × 2–5	aseptate	10.5–18×3–5.5	Begoude *et al.* 2010
L. margaritacea	14–17 × 11–12	28–46 × 2–2.5	1–2 septa	10–11× 3–4	Pavlic *et al.* 2008
L. missouriana	17.4–19.6 × 8.9–10.6	55 × 2–3	aseptate	–	Urbez-Torres *et al.* 2012
L. paraphysaria	30–32 × 15–16	90–100 × 3	1–septum	–	Saccardo 1899
L. parva	16–23.5 × 10.5–13	105 × 3–4	septate	–	Alves *et al.* 2008
L. plurivora	26.5–32.5 × 14.5–17	130 × 2–5	2–7 septa	8–13 × 4–7	Damm *et al.* 2008
L. pseudotheobromae	23.5–32 × 14–18	58 × 3–4	aseptate	-	Alves *et al.* 2008
	26–31 × 13–16	65 × 2–3	septate	5–15 × 2–5	Machado *et al.* 2014
	25–32 × 14–18	65 × 2–3	septate	8–17 × 3–4	**This study**
L. ricini	16–19 × 10–11	25–35 × 2	1–septum	–	Saccardo 1915
L. rubropurpurea	24–33 × 13–17	32–52 × 1.5–3.5	aseptate	7–13 × 3–5	Burgess *et al.* 2006
L. subglobosa	16–23 × 11–17	41 × 2–3	aseptate	8–20 × 2.5–4	Machado *et al.* 2014
L. theobromae	21–31 × 13–15.5	55 × 3–4	septate	–	Alves *et al.* 2008
	20 –30 × 10–15	55 × –	–	5–15 × 3	Sutton 1980
	23–31 × 13–15	45 × 2	aseptate	5–11 × 2–4	Machado *et al.* 2014
	18.5–30.5 × 12.5–18	55 × 2–4	aseptate	7–14 × 2–4	**This study**
Lasiodiplodia sp. 1	20–26.5 × 12–15.5	65 × 2–3	aseptate	9–18 × 3–4	**This study**
Lasiodiplodia sp. 2	20–26.5 × 13–16.5	70.5 × 2–4	aseptate	10–23 × 3–4	**This study**
L. thomasiana	28–30 × 11–12	80–90 × 1.5	–	–	Saccardo 1913
L. undulata	20–32 × 13.5–19.2	–	–	8–20 × 3.2–4.8	Abbas *et al.* 2004
L. venezuelensis	26–33 × 12–15	16–41 × 2–5	septate	7–14 × 3–4.5	Burgess *et al.* 2006
L. viticola	18.2–20.5 × 8.8–10.1	60 × 2–3	aseptate	–	Urbez-Torres *et al.* 2012
	20–26 × 12–18	61 × 2–3	aseptate	9–17 × 2–4	**This study**

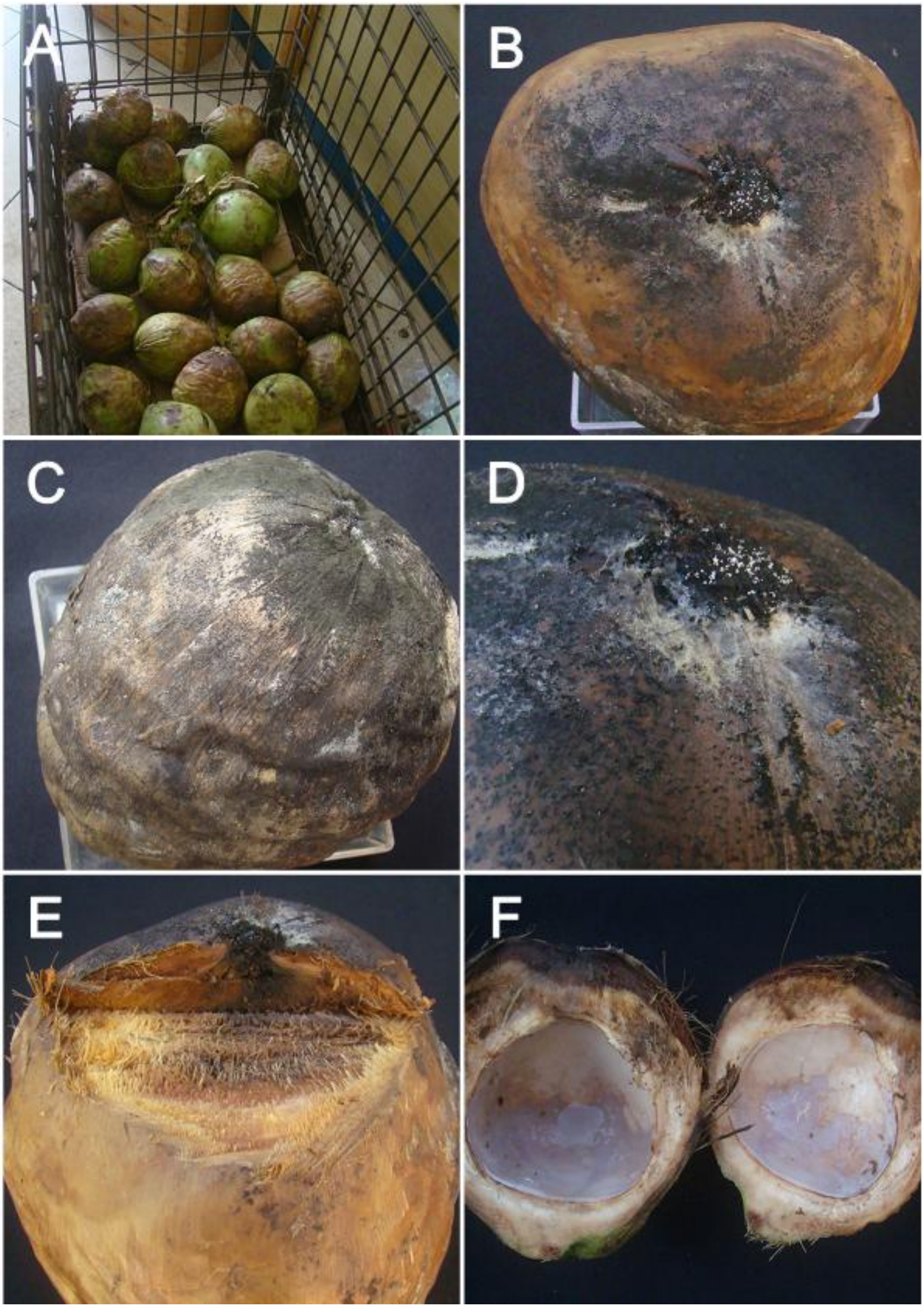

Fig. 1 Symptons of stem-end rot in coconut fruits: **A**, fruits presenting stem-end rot symptoms for sale in local market. **B-C**, symptomatic fruit, the disease beginning on the peduncle and progressing to the whole fruit. **D**, fruit with numerous cirri formed on pycnidia. **E-F**, longitudinal sections showing the fungus progress into the endosperm.

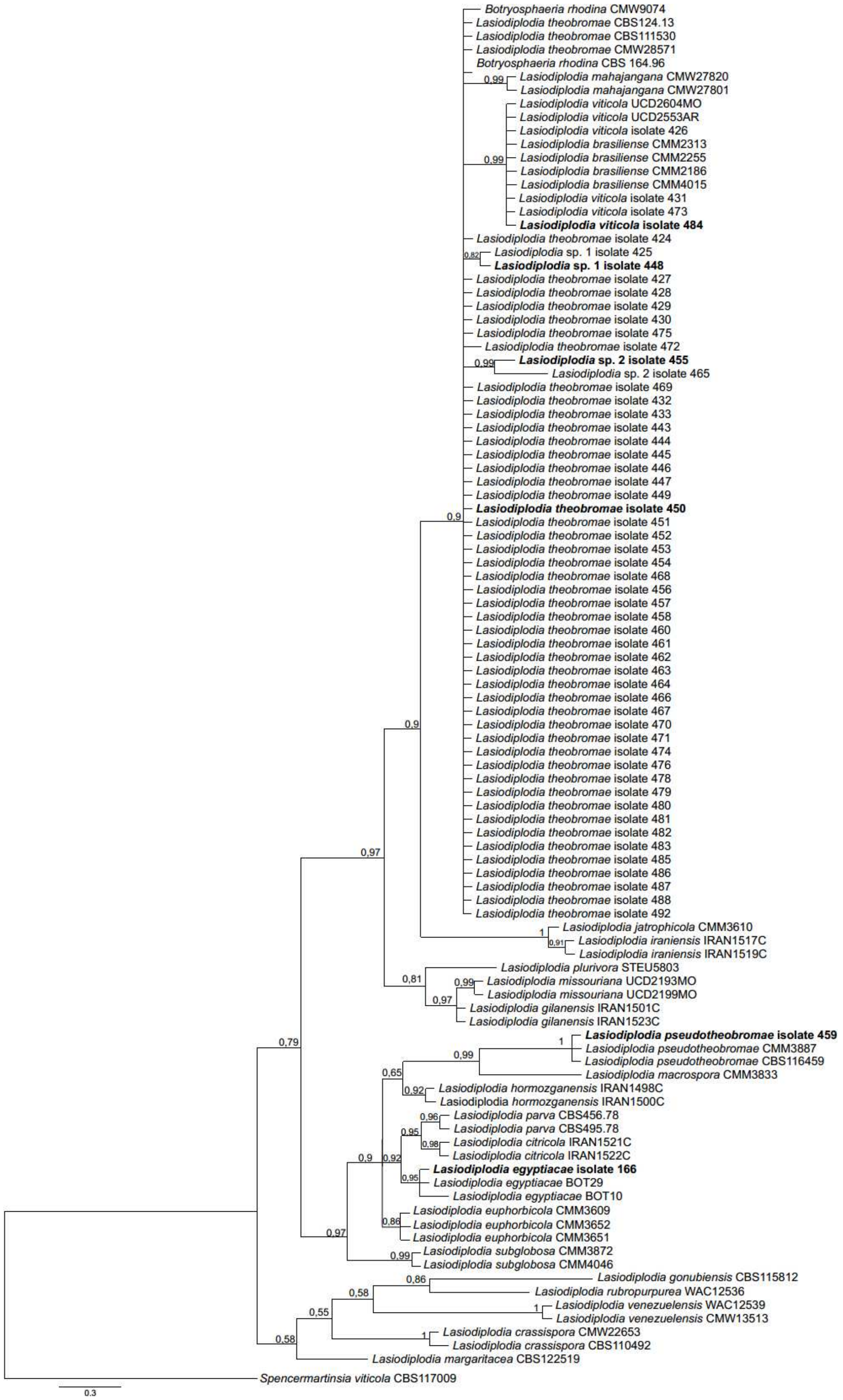

Fig. 2 Phylogenetic tree inferred from Bayesian analysis based in sequences of the TEF1-α. Bayesian posterior probabilites are indicated above the nodes. The tree was rooted to *Spencermartinsia viticola* CBS117009. The representative species selected for this study are highlighted in bold.

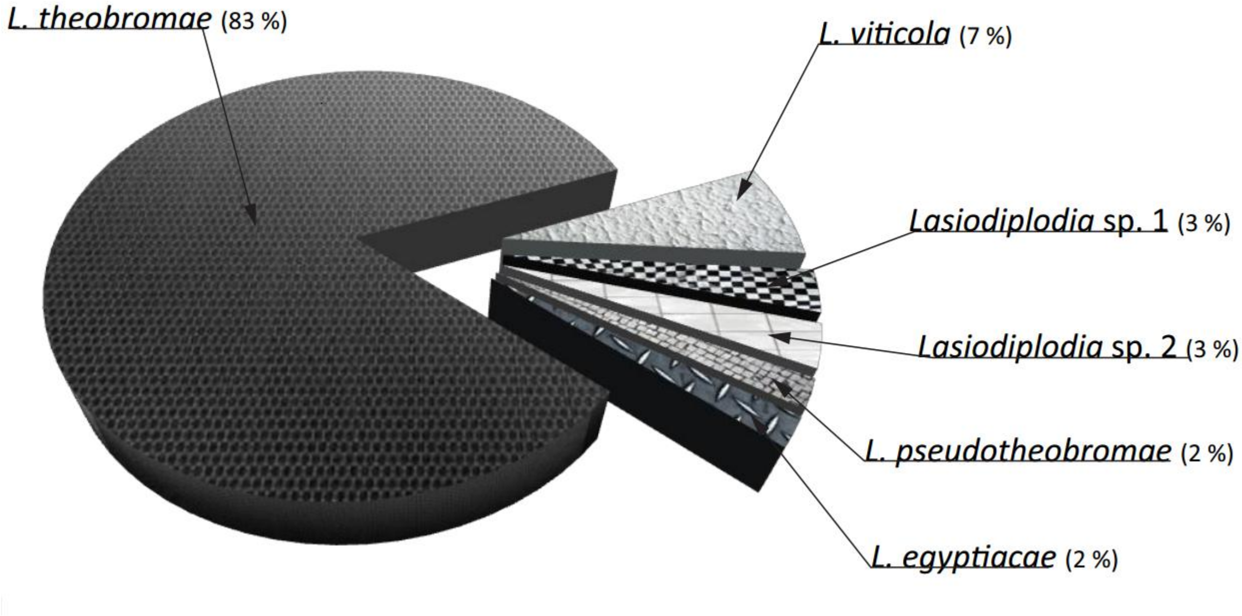

Fig. 3 Frequency (%) of *Lasiodiplodia* species associated with postharvest stem-end rot of immature coconut in Brazil.

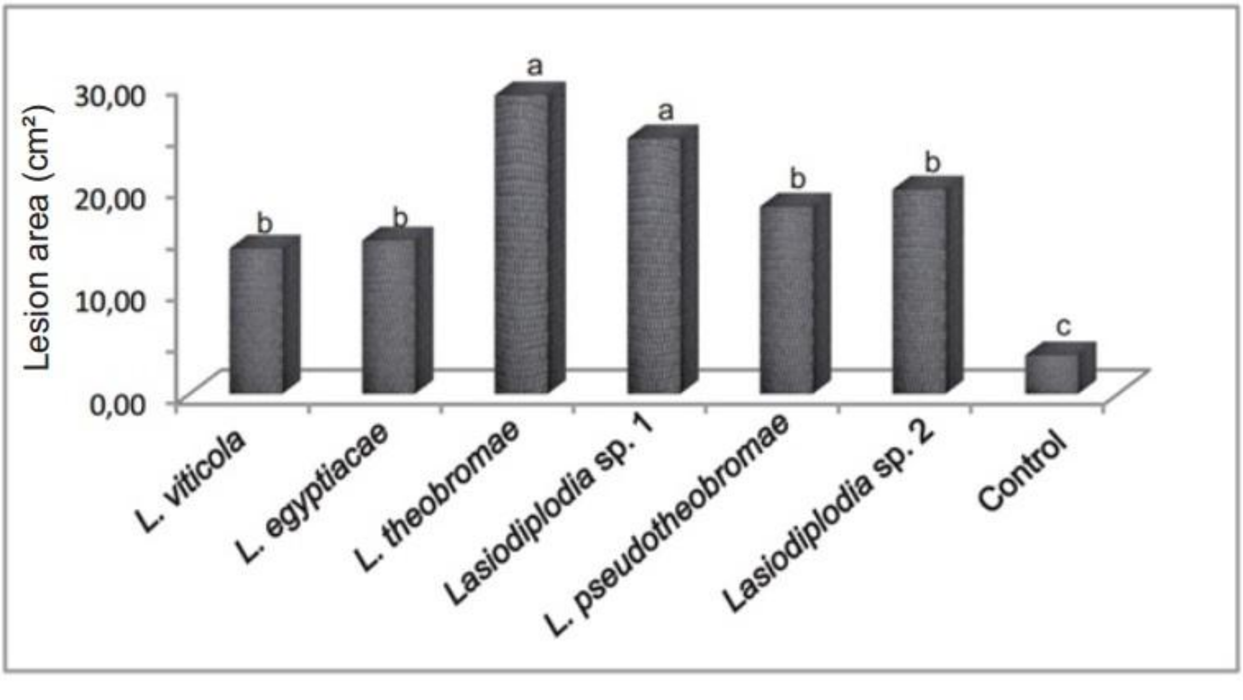

Fig. 4 Mean lesion areas caused by *Lasiodiplodia* species associated with postharvest stem-end rot of immature coconut in Brazil. Columns with same letter do not differ significantly according to Tukey test ($P \leq 0.05$).

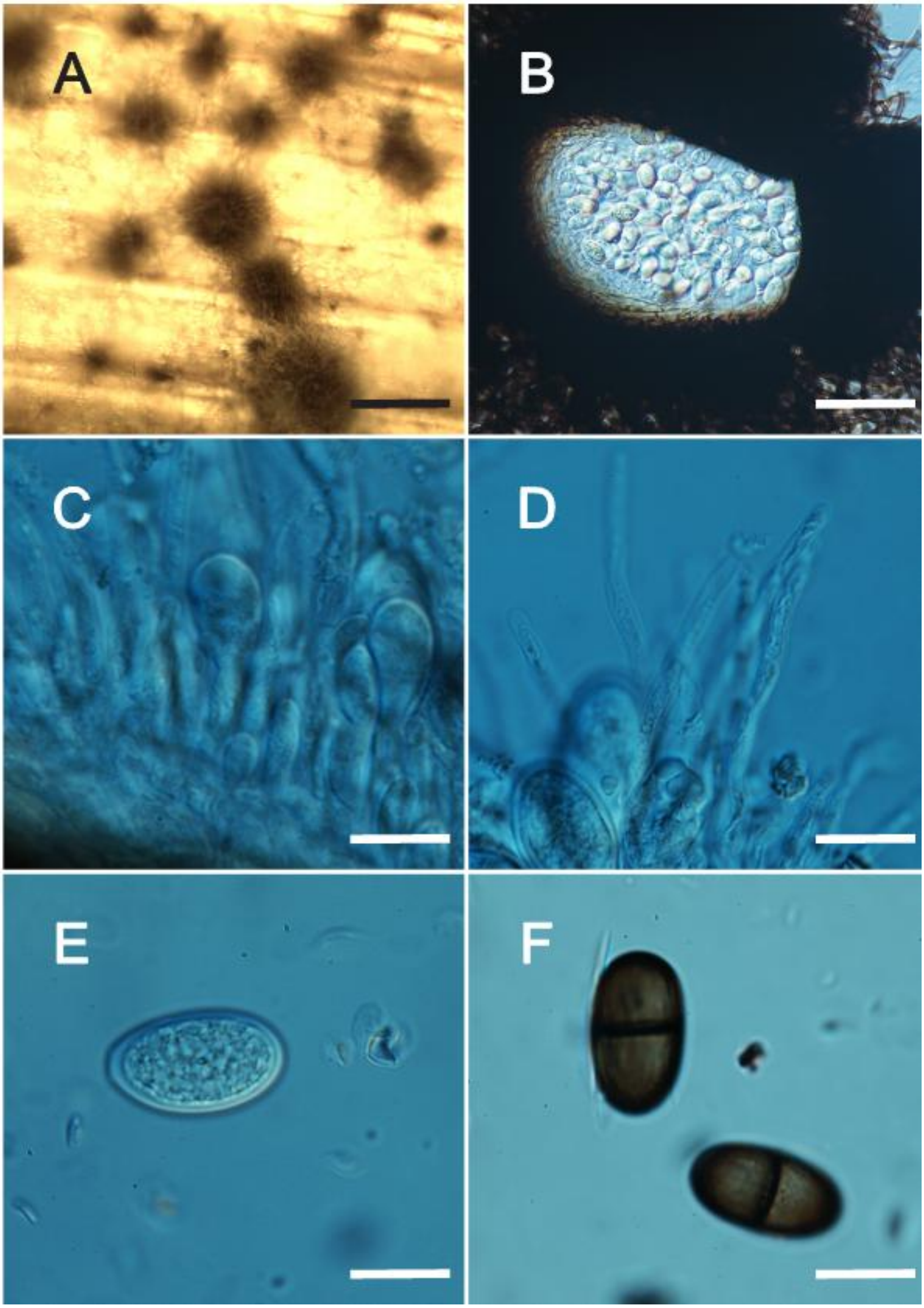

Fig. 5 *Lasiodiplodia egyptiacae* isolate 166. **A**, conidiomata on corn straw in culture. **B**, section of conidioma. **C**, conidia developing on conidiogenous cells. **D**, aseptate paraphyses. **E**, hyaline, immature conidia. **F**, mature conidia. Scale bars: **A**= 1,000 µm; **B**= 50 µm; **C=** 5 µm; **D=** 10 µm; **E-F=** 15 µm.

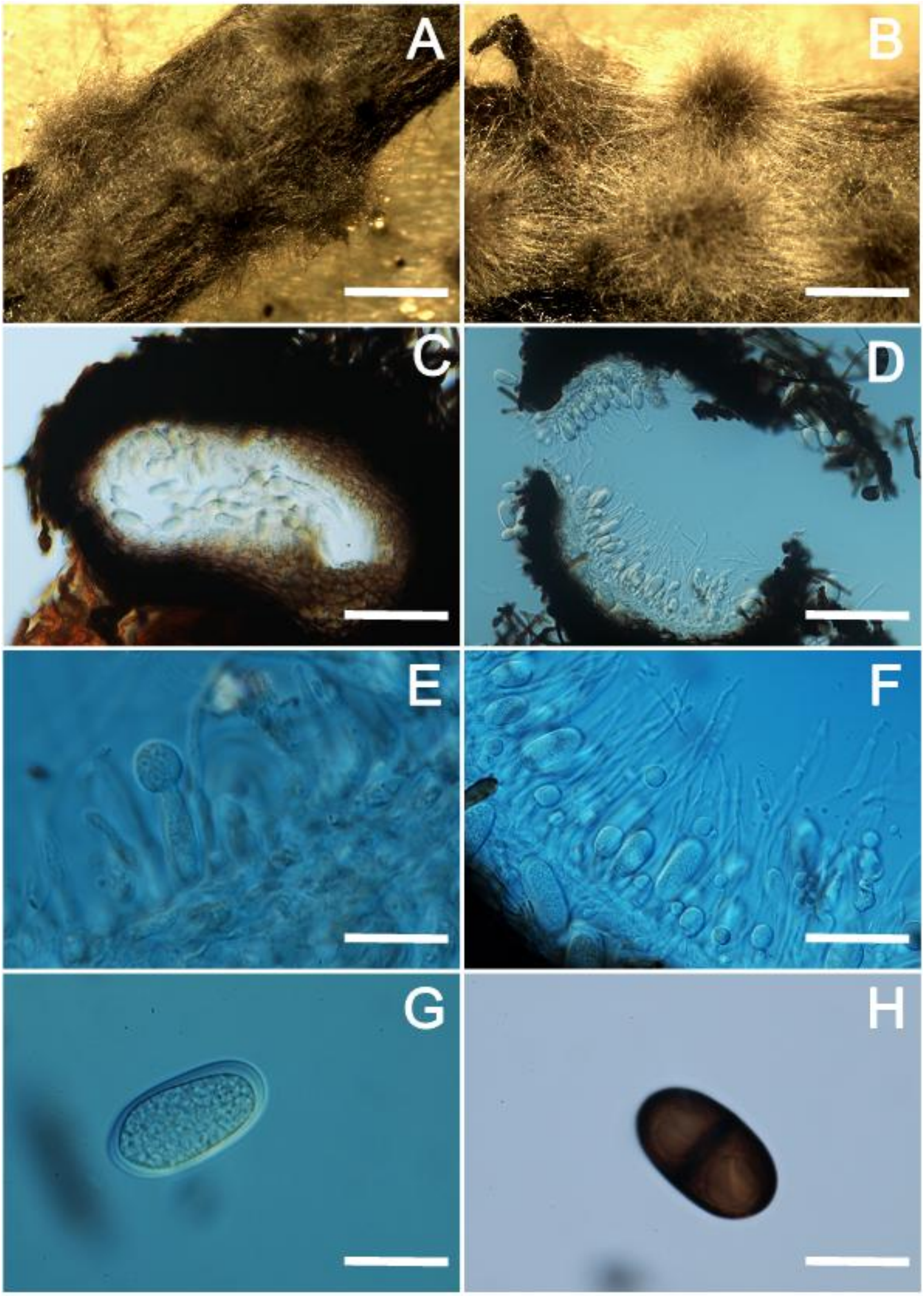

Fig. 6 *Lasiodiplodia pseudotheobromae* isolate 459. **A-B**, conidiomata on *Pinus* twigs in culture. **C-D**, section of conidioma. **E**, conidia developing on conidiogenous cells. **F**, septate and branched paraphyses. **G**, hyaline, immature conidia. **H**, mature conidia. Scale bars: **A**= 1,000 µm; **B**= 500 µm; **C-D**= 50 µm; **E** = 5 µm; **F-H**= 20 µm.

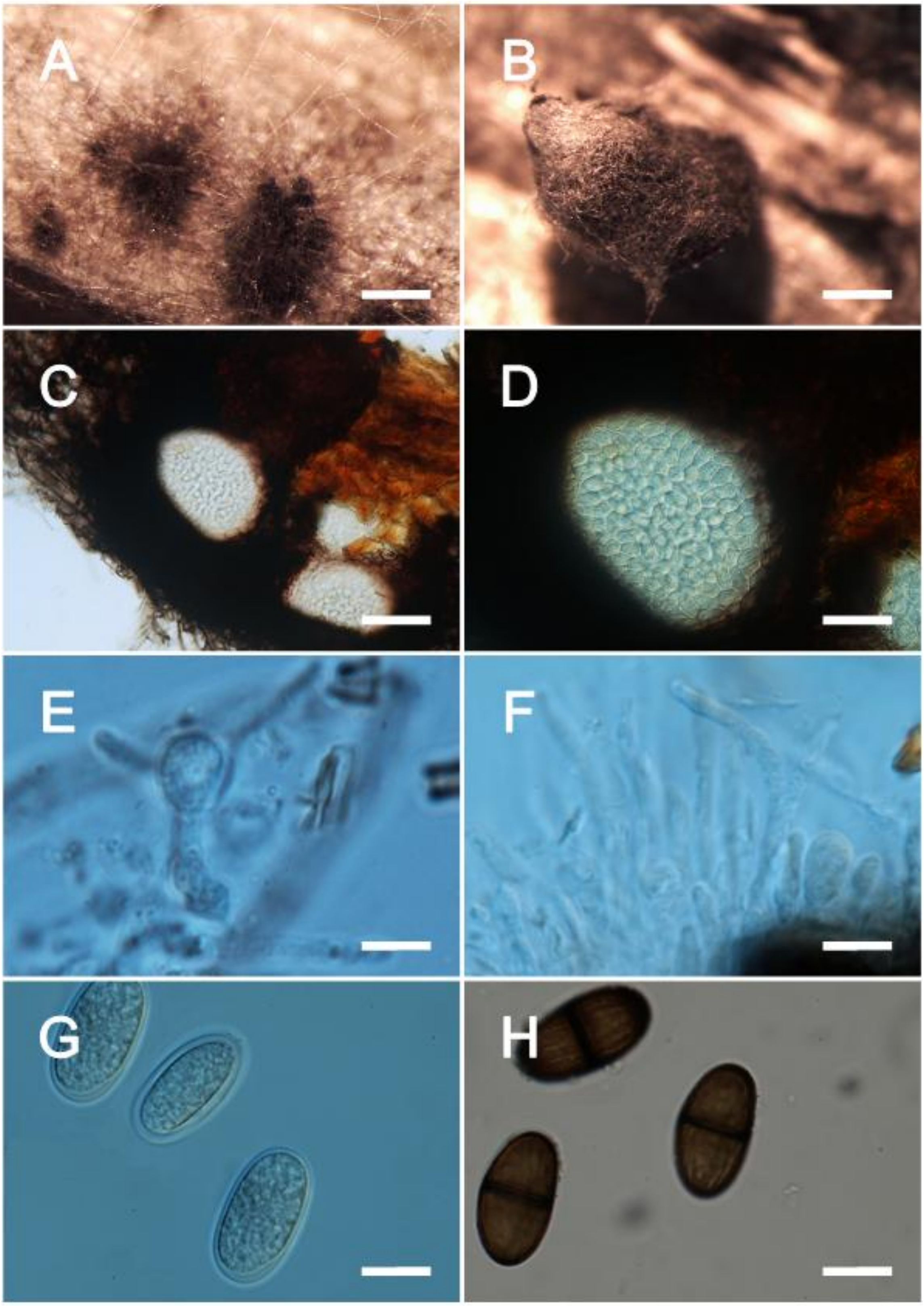

Fig. 7 *Lasiodiplodia theobromae* isolate 450. **A-B**, conidiomata on corn straw in culture. **C,** conidiomata on *Pinus* twigs in culture. **D**, section of conidioma. **E**, conidia developing on conidiogenous cells. **F**, aseptate paraphyses. **G**, hyaline, immature conidia. **H**, mature conidia, with longitudinal striations. Scale bars: **A**= 1,000 µm; **B**= 500 µm; **C**= 250 µm; **D**= 50 µm **; E=** 5 µm; **F**= 10 µm; **G-H**= 15 µm.

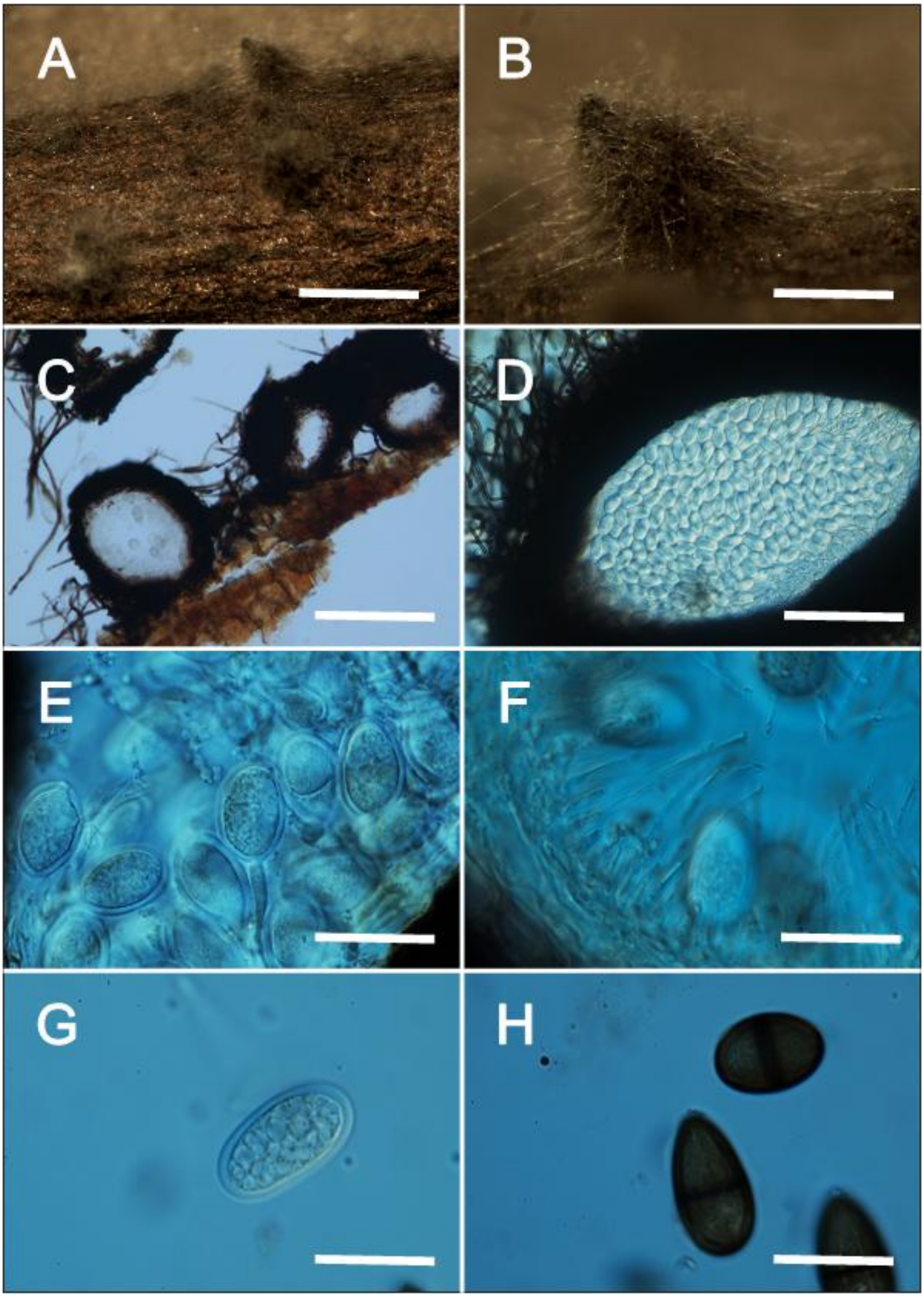

Fig. 8 *Lasiodiplodia viticola* isolate 484. **A-B**, conidiomata on *Pinus* twigs in culture. **C-D**, section of conidioma. **E**, conidia developing on conidiogenous cells. **F**, aseptate paraphyses. **G**, hyaline, immature conidia. **H**, mature conidia, with longitudinal striations. Scale bars: **A**= 1,000 µm; **B**= 500 µm; **C-D**= 50 µm; **E-F**= 15 µm; **G-H**= 20 µm.

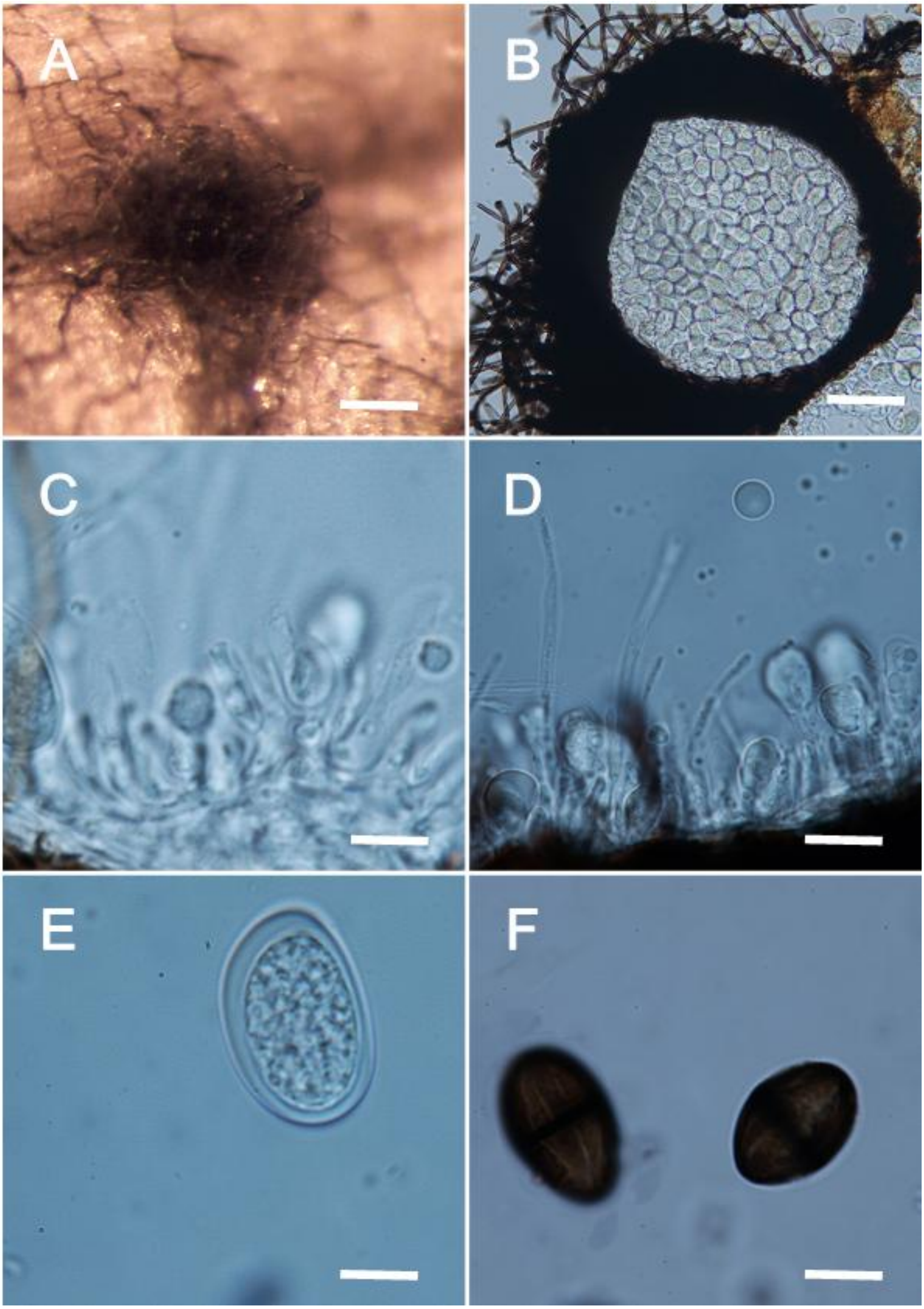

Fig. 9 *Lasiodiplodia* sp. 1 isolate 448. **A**, conidiomata on corn straw in culture. **B**, section of conidioma. **C**, conidia developing on conidiogenous cells. **D**, aseptate paraphyses. **E**, hyaline, immature conidia. **F**, mature conidia, with longitudinal striations. Scale bars: **A**= 1,000 µm; **B**= 500 µm; **C=** 15 µm; **D**= 20 µm; **E**-**F**= 10 µm.

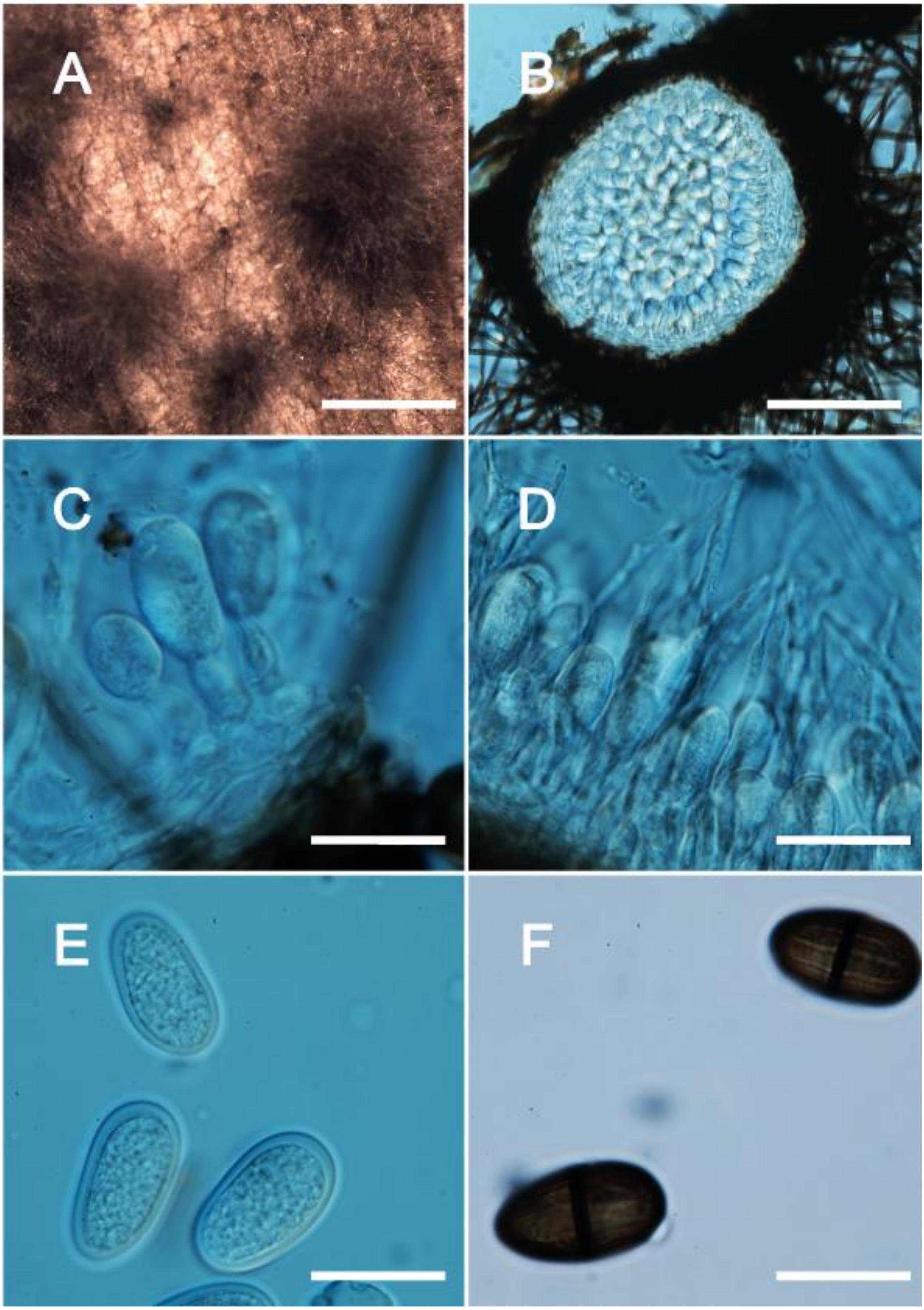

Fig. 10 *Lasiodiplodia* sp. 2 isolate 455. **A**, conidiomata on corn straw in culture. **B**, section of conidioma. **C**, conidia developing on conidiogenous cells. **D**, aseptate paraphyses. **E**, hyaline, immature conidia. **F**, mature conidia, with longitudinal striations. Scale bars: **A**= 1,000 µm; **B**= 500 µm; **C=** 15 µm; **D**= 20 µm; **E-F**= 20 µm.

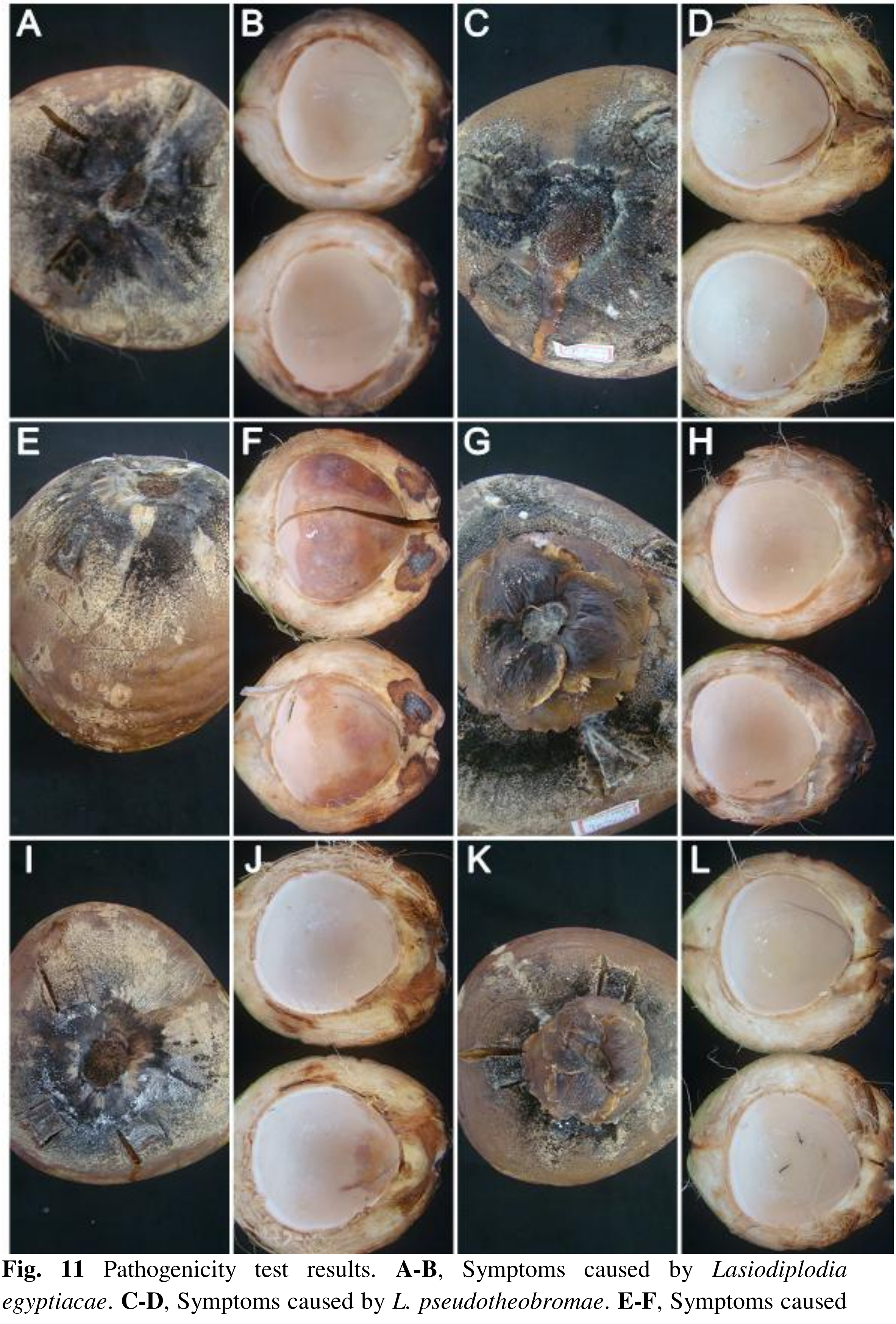

Fig. 11 Pathogenicity test results. **A-B**, Symptoms caused by *Lasiodiplodia egyptiacae*. **C-D**, Symptoms caused by *L. pseudotheobromae*. **E-F**, Symptoms caused by *L. theobromae*. **G-H**, Symptoms caused by *L. viticola.* **I-J**, Symptoms caused by *Lasiodiplodia* sp.1. **K-L**, Symptoms caused by *Lasiodiplodia* sp.2.