ALEXANDRE REIS MACHADO

PHYLOGENY, IDENTIFICATION AND PATHOGENICITY OF THE BOTRYOSPHAERIACEAE ASSOCIATED WITH COLLAR AND ROOT ROT OF THE BIOFUEL PLANT *Jatropha curcas* IN BRAZIL, WITH A DESCRIPTION OF NEW SPECIES

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: 19 de julho de 2012.

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Prof. Maria Catarina Megumi Kasuya Dr. Harold Charles Evans

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Prof. Olinto Liparini Pereira
(Orientador)

"*Não há conquistas fáceis. São as estradas sinuosas que levam ao caminho certo. O profissional, em qualquer ofício, alcançará o triunfo a partir de um espírito tenaz, forte, obstinado*".

Afonso Opazo

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BIOGRAFIA

ALEXANDRE REIS MACHADO, filho de Paulo Renato Machado e Mary de Souza Reis Machado, nasceu na cidade de Montes Claros, Minas Gerais, no dia 11 de novembro de 1986.

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SUMÁRIO

RESUMO

MACHADO, Alexandre Reis, M.Sc., Universidade Federal de Viçosa, julho de 2012. **Phylogeny, identification and pathogenicity of the Botryosphaeriaceae associated with collar and root rot of the biofuel plant *Jatropha curca*s in Brazil, with a description of new species**. Orientador: Olinto Liparini Pereira. Coorientador: Robert Weingart Barreto.

A partir da iniciativa do Governo Federal em introduzir o biodiesel na matriz energética brasileira, surgiu a necessidade de se pesquisar plantas oleaginosas potenciais para produção de matéria-prima para este biocombustível. O pinhão manso (*Jatropha curcas*) tem se destacado por ser uma planta perene, de fácil manejo, além de produzir sementes com alto teor de óleo. A grande expansão das áreas de cultivo tem sido acompanhada pelo surgimento de diversas enfermidades, do qual pouco se conhece sobre os reais agentes etiológicos. Atualmente, em diversas áreas do Brasil, tem-se relatado a ocorrência de uma nova doença que não apenas reduz a produtividade, como tem causado a morte das plantas. Esta doença está associada a uma podridão das raízes e do colo das plantas. Alguns patógenos já foram relatados para essa doença, sendo mais frequente a ocorrência de fungos da família Botryosphaeriaceae, grupo conhecido pela dificuldade de separação das espécies utilizando características morfológicas. Assim, o objetivo deste trabalho foi identificar os possíveis agentes etiológicos, investigar a diversidade de Botryosphaeriaceae associado a essa doença, utilizando características morfológicas aliadas às ferramentas moleculares, bem como provar a potogenicidade dos isolados. Foram realizadas coletas nos estados de Minas Gerais e Espírito Santo. Em adição a estes, foram obtidas amostras dos estados do Piauí e São Paulo. Isolados monospóricos foram obtidos e armazenados. Estes tiveram o DNA extraído e as regiões ITS e TEF1-α sequenciadas. A partir dos resultados das análises filogenéticas, foram separados dois a três isolados de cada espécie para a caracterização morfológica. Nove espécies de Botryosphaeriaceae foram identificadas, sendo *Lasiodiplodia theobromae*, *L. parva*, *L. pseudotheobromae*, *L. iraniensis*, *Neoscytalidium dimidiatum*, *Macrophomina phaseolina* e três a serem propostas como novas espécies (*Lasiodiplodia* sp.1, *Lasiodiplodia* sp.2 e *Macrophomina* sp.1). Todas as espécies distinguiram morfologicamente e filogeneticamente, com exceção de *Macrophomina* sp.1 que não esporulou em meio de cultura. Até o momento, apenas *Lasiodiplodia theobromae, Lasiodiplodia* sp.1 e *Neoscytalidium dimidiatum* tiveram a patogenicidade comprovada.

Pelos testes de Blotter foram encontrados associados às sementes: *Lasiodiplodia theobromae*, *Macrophomina phaseolina* e *Macrophomina* sp.1. Espécies de Botryosphaeriaceae ocorrem em uma ampla gama de hospedeiros e ambientes, e são frequentemente referidos como endofíticos, patógenos latentes ou oportunistas, devido a manifestação da doença estar diretamente associada à ocorrência de estresse do hospedeiro. A expansão das áreas de pinhão manso no mundo tem contribuído para o surgimento de várias doenças que até o momento não tinham seus agentes etiológicos conhecidos. Este estudo fornece informações novas para futuros estudos de manejo da doença, programas de quarentena e especialmente para o desenvolvimento de variedades resistentes à podridão do colo e raiz do pinhão-manso.

ABSTRACT

MACHADO, Alexandre Reis, M.Sc., Universidade Federal de Viçosa, july, 2012. **Phylogeny, identification and pathogenicity of the Botryosphaeriaceae associated with collar and root rot of the biofuel plant *Jatropha curcas* in Brazil, with a description of new species**. Adviser: Olinto Liparini Pereira. Co-adviser: Robert Weingart Barreto.

The introduction of biodiesel in the Brazilian energy matrix by initiative of the Federal Government, encouraged the search for potential oleaginous crops to supply raw material for biofuel production. The physic nut (*Jatropha curcas*) has been highlighted since it is a perennial plant, easy to manage, and produces seeds with a high oil content. The expansion of areas of *Jatropha* in the world has contributed to the emergence of various diseases. Currently in Brazil, the occurrence of a new disease that not only reduces the productivity, since it causes death of the plants. This disease is associated with a collar and root rot of plants. A number of pathogens have been associates with this disease, the occurrence of Botryosphaeriaceae fungi being the most frequent, which is a group known to be difficult to delimit species based on morphological characters. Thus, the purposes of this work was to investigate the diversity of Botryosphaeriaceae associated with collar and root rot of *J. curcas*, with the aid of morphology and molecular tools and to asses the pathogenicity of the species involved. Samples were colleted in states of Minas Gerais and the Espirito Santo. In addition, samples were obtained from Piauí and São Paulo states. Single spore cultures were obtained and stored. These had their DNA extracted and the ITS and TEF1-α regions were sequenced. From the results of the phylogenetic analyses, two to three isolates of each species were separated for the morphological characterization and pathogenicity tests. With the purpose of investigating the association of the pathogens in seeds, Blotter tests were performed. Nine Botryosphaeriaceae species were identified: *Lasiodiplodia theobromae*, *L. parva*, *L. pseudotheobromae*, *L. iraniensis*, *Neoscytalidium dimidiatum*, *Macrophomina phaseolina* and three to be proposed as new species (*Lasiodiplodia* sp.1, *Lasiodiplodia* sp.2 and *Macrophomina* sp.1). All species were distinguished morphologically and phylogenetically, except *Macrophomina* sp.1 that failed sporulate in culture.Currently, only *Lasiodiplodia theobromae*, *Lasiodiplodia* sp.1 and *Neoscytalidium dimidiatum* have proven to be pathogenic. *Lasiodiplodia theobromae*, *Macrophomina phaseolina* and *Macrophomina* sp.1 were associated with seeds. Botryosphaeriaceae species occur in a wide range of hosts and environments, and

are often referred to as endophytes and latent or opportunistic pathogens, because manifestation of the disease is directly linked with host stress. The great expansion of *Jatropha* areas in the world have contributed to the emergence of several diseases, which so far, the etiological agent have remained unknown. This study will provide new information for future studies of disease management, quarantine programs and especially, the development of resistant varieties for collar and root rot of *J. curcas*.

INTRODUÇÃO GERAL

A partir da iniciativa do Governo Federal em introduzir o biodiesel na matriz energética brasileira, surgiu a necessidade de se pesquisar plantas oleaginosas com potencial para produção de matéria-prima para este biocombustível. Dentre as várias culturas com potencial para esse fim, o pinhão manso (*Jatropha curcas*) tem se destacado especialmente em áreas com déficit hídrico recorrente.

O pinhão manso pertence à família *Euphorbiaceae*, com provável origem na América Central, mas já se encontra amplamente distribuído na América do Sul, África e Ásia (Heller 1996). É uma cultura perene, de fácil manejo (Saturnino et al. 2005) e que não requer alto investimento financeiro durante o seu cultivo, tornando-se uma opção para a agricultura familiar (Arruda et al. 2004).

Produz sementes com alto teor de óleo, aproximadamente 47,25% (Akintayo 2004) e com excelentes propriedades, sendo que, em mistura de até 50% com o óleo diesel, pode ser utilizado em motores sem qualquer modificação (Pramanik 2003), contribuindo para a redução da emissão de gases poluentes produzidos pela queima de combustíveis fósseis. Além disso, o seu cultivo seria uma forma de se estocar carbono da atmosfera (Harinder e Makkar 2009).

Além das diversas vantagens de se cultivar o pinhão manso, a maioria dos trabalhos o consideram uma cultura resistente à pragas e doenças (Saturnino et al. 2005), e isso têm contribuído muito para o aumento da área plantada no Brasil. Entretanto, essa grande expansão das áreas de cultivo tem sido acompanhada pelo surgimento de diversas enfermidades, do qual pouco se conhece sobre os reais agentes etiológicos.

A maior parte das doenças descritas na cultura do pinhão manso são causadas por fungos, com destaque para: *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk, *Psathyrella subcorticalis* Speg., *Schizophyllum alneum* (L.) Kuntze, *Aecidium cnidoscoli* Henn., *Ramulariopsis cnidoscoli* Speg., *Uromyces jatrophicola* Henn. (Viégas 1961), *Pestalotiopsis versicolor* (Speg.) Steyaert (Phillips 1975), *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Colletotrichum capsici* (Syd. &

P. Syd.) E.J. Butler & Bisby, *Passalora ajrekari* (Syd. & P. Syd.) U. Braun (Freire e Parente 2006), *Phakopsora arthuriana* Buriticá & J.F. Hennen (Hennen et al. 2005), *Cochliobolus spicifer* R.R. Nelson (Mendes et al. 1998), *Cercospora jatrophicola* (Speg.) Chupp, *Cercospora jatrophigena* U. Braun, *Pseudocercospora jatrophae-curcas* (J.M.Yen) Deighton, *Pseudocercospora jatrophae* (G.F. Atk.) A.K. Das & Chattopadh. e *Pseudocercospora jatropharum* (Speg.) U. Braun (Crous e Braun 2003) e *Elsinoë jatrophae* Bitanc. & Jenkins (Bitancourt e Jenkins 1951).

A grande maioria dos patógenos citados são causadores de manchas foliares e outras doenças que ainda não representam uma limitação à produtividade do pinhão manso. Entretanto, em diversas áreas do Brasil, tem-se relatado a ocorrência de uma nova doença que não apenas é capaz de reduzir a produtividade, como tem causado a morte súbita de plantas, inviabilizado áreas de cultivo. Esta doença está associada a uma podridão do colo e radicular, nas quais os primeiros sintomas são a murcha repentina e amarelecimento das folhas, que em estágio mais avançado caem, finalizando com a morte da planta. Existem trabalhos de várias regiões do mundo que associam alguns patógenos a esses sintomas, a exemplo de: *Nectria haematococca* Berk.& Br. [*Haematonectria haematococca* (Berk. & Broome) Samuels & Nirenberg] e seu anamorfo *Fusarium solani* (Mart.) Sacc. na China (Yue-kai et al. 2011), *Clitocybe tabescens* (Scop.) Bres. nos EUA (USDA 1960) e *Phytophthora palmivora* var. *palmivora* (E.J. Butler) E.J. Butler (Erwin e Ribeiro 1996) em diversos países.

A primeira constatação da referida doença no Brasil e comprovação de sua etiologia foi feita por Pereira et al. (2009) no estado de São Paulo. O agente etiológico foi identificado morfologicamente como *Lasiodiplodia theobromae* e a doença foi considerada como de baixa incidência ou esporádica (Pereira et al. 2009).

Entretanto, recentemente, cultivos de pinhão-manso têm sido abandonados em diferentes regiões do país por conta da podridão do colo associado também ao surgimento de uma nova sintomatologia de podridão radicular. Adicionalmente, a identificação somente morfológica de *L. theobromae* por Pereira et al. (2009) é limitada, uma vez que a taxonomia de *Lasiodiplodia* (e outros Botryosphaeriaceae) foi profundamente alterada por recentes trabalhos envolvendo filogenia molecular (Alves

et al. 2008; Abdollahzadeh et al. 2010) e também pelo fato de nenhum estudo sistemático de coleta de materiais de diferentes regiões do Brasil ter sido feito até o momento.

O controle da doença pode ser futuramente alcançado pelo uso de variedades ou porta-enxertos resistentes. Entretanto, tal possibilidade encontra forte entrave fitopatológico pelo desconhecimento do complexo de espécies envolvidas na referida sintomatologia no país. Em qualquer cultura, o conhecimento dos agentes etiológicos associados a doenças de plantas é pré-requisito crucial para o estudo de possíveis práticas de manejo visando o controle dessas doenças de plantas.

Sendo assim, objetivou-se com esse trabalho: a) Descrever as espécies de fungos associados à podridão do colo e de raiz do pinhão-manso com auxílio de métodos morfológicos 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 e estabelecer medidas de biossegurança; d) Realização de testes de patogenicidade para comprovação etiológica dos isolados.

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ARTIGO

According to the guidelines of Fungal Diversity

Phylogeny, identification and pathogenicity of the Botryosphaeriaceae associated with collar and root rot of the biofuel plant *Jatropha curca*s in Brazil, with a description of new species

Phylogeny, identification and pathogenicity of the Botryosphaeriaceae associated with collar and root rot of the biofuel plant *Jatropha curca*s in Brazil, with a description of new species

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Abstract:

The expansion of areas of *Jatropha* in the world, have contributed to the emergence of various diseases. Currently in Brazil, the occurrence of a new disease has been reported that not only reduces the productivity, but also causes the death of plants. This disease is associated with a collar and rot root of plants. From morphological and phylogenetic studies nine species of Botryosphaeriaceae were identified. These include *Lasiodiplodia theobromae*, *Lasiodiplodia parva*, *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia iraniensis*, *Neoscytalidium dimidiatum*, *Macrophomina phaseolina* and three to be proposed as new species (*Lasiodiplodia* sp.1, *Lasiodiplodia* sp.2 and *Macrophomina* sp.1). Of these *Lasiodiplodia theobromae*, *Macrophomina phaseolina* and *Macrophomina* sp.1 were encountered in seeds. Until now, only *Lasiodiplodia theobromae*, *Lasiodiplodia* sp.1 and *Neoscytalidium dimidiatum* have proven to be pathogenic. The results show that root rot of physic nut is not associated with a single pathogen. Perhaps the emergence of this disease has been favored by stress conditions that the plant has encountered in the field. This study provides new information for future studies of disease management, quarantine programs and especially the development of resistant varieties for the collar and root rot disease of *J. curcas*.

Keywords: *Lasiodiplodia*, *Macrophomina*, *Neoscytalidium*, physic nut, taxonomy.

INTRODUCTION

Jatropha curcas L., popularly known as Physic nut, is a plant of the family Euphorbiaceae, that currently has been cultivated widely in the world with the main purpose supplying raw material for biofuel production. Among oil plants, this species has gained importance for being considered as easy to cultivation (Saturnino et al. 2005), which produces seeds with high oil content (47.25%) (Akintayo 2004) and with excellent fuel properties (Pramanik 2003).

Several research papers have also described *J. curcas* as resistant to pests and diseases, and this has been an additional factor that encouraged its cultivation. However, the great expansion of cultivated areas has been accompanied by the emergence of various diseases of unknown etiology.

Recently in Brazil, a new disease that not only is able to reduce productivity, but also causes the sudden death of plants whas been observed. This disease is associated with root and collar rot, in which, the first symptoms are wilt and yellowing of the leaves, ending with leaf fall and death of the plant. According to Pereira et al. (2009), this disease is associated with *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. in Brazil, as also reported by Latha et al. (2009) in India.

Lasiodiplodia theobromae is a fungus of the family Botryosphaeriaceae (Botryosphaeriales, Ascomycetes), that has great phytopathological importance. Currently, 1060 hosts species are reported for *L. theobromae* (Farr and Rossman 2012). In recent years, this fungus has been the target of many controversial taxonomic studies. Denman et al. (2000) proposed the synonymy of the genus *Lasiodiplodia* with *Diplodia* and *Dothiorella*. However, several other studies showed that they are different genera (Phillips et al. 2005; Burgess et al. 2006; Alves et al. 2008; De Wet et al. 2008).

Others Botryosphaeriaceae have been reported causing the same disease in *J. curcas*, such as *Macrophomina phaseolina* (Patel et al. 2008), pathogen of great phytopathological importance, due to its wide host range and ability of survivability by sclerotia (Dhingra and Sinclair 1978), and *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers, a genus proposed by Crous et al. (2006) for a fungus that

form *Scytalidium*-like synanamorphs in the aerial mycelia and *Fusicoccum*-like conidia in the pycnidia which has recently been reported in Brazil (Machado et al. in press).

Members of the family Botryosphaeriaceae occur in a wide range of hosts, including Gymnosperms and Angiosperms, and can be found as saprophytic, parasites or endophytes. The survival of these fungi as endophytes can represents a great danger and a problem for quarantine barriers, due to possibility of these being introduced into new environments through asymptomatic propagative material. Due to the emergence of unfavorable conditions for the plant, the pathogen can induce the symptoms and cause extensive losses (Slippers and Wingfield 2007). The identification and description of the new species of fungus was based on an analysis of morphological characters, but to undertake this task without a polyphasic approach, can underestimate the true diversity of the species (Taylor et al. 2000).

The species *L. theobromae* was distinguished from others basically on conidia and paraphyses morphology. However, in recent years, various species have emerged from phylogenetic studies, showing the existence of a species complex (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; Urbez-Torres et al. 2012).Thus, the importance of combining and applying morphological and molecular tools to this group of fungi is clear.

In view of the importance that physic nut has acquired in the world, the lack of studies related to the etiology of root and collar rot disease is critical for future plant breeding programs, as well as to assist the establishment of phytosanitary measures and quarantine, in order to avoid its spread.

The purposes of this work were to investigate the diversity of Botryosphaeriaceae associated with collar and root rot of *J. curcas*, with aid of morphology and molecular tools, and to establish the pathogenicity of the species associated.

MATERIALS AND METHODS

Sample collection and isolation

Field surveys were carried out during October 2010 and January-February 2011 in *J. curcas* plantations, with the purpose of finding and collecting plants with symptoms of wilt, leaf fall and yellowing due to root or collar rot. The areas visited belong to the States of Minas Gerais (Biojan; SADA Bioenergia; EPAMIG-URENM) and Espírito Santo (NOVABRA). In addition, samples from the States of Piauí and São Paulo were also obtained from symptomatic plants. The samples were sent to the Laboratório de Patologia de Sementes e de Pós-colheita (Departamento de Fitopatologia, Universidade Federal de Viçosa) where they were first examined for the possible presence of fungal fruiting structures. Longitudinal sections of the stem and roots were made manually for observation of vascular necrosis, from which small fragments of areas of transition between the healthy tissue and the symptomatic tissue were obtained for fungal isolations. These fragments were disinfected in 70% ethanol for 1 min, followed by 1% sodium hypochlorite for 3 min and washed in sterile distilled water. Later, these were placed in Petri dishes with Potato Dextrose Agar (PDA - Acumedia®) and incubated at 25°C.

The isolates with dark mycelium and lack sporulation, typical of *Botryosphaeriaceae*, were grown in Petri dishes with 2% Water Agar (WA - Agar Agar, type I Himedia®) overlaid with double-sterilized maize straw and incubated at 25°C in 12 hours light-dark regime for 3-4 weeks for induction of sporulation. From these, single-spore cultures was obtained and stored in tubes on PDA at 10°C.

With the purpose of detecting these fungi in seeds, Blotter tests were performed as described by Alfenas et al. (2007). The isolates obtained from seeds, were processed in the same manner as described for the other isolates.

After identification, representative isolates of each species were deposited in the herbarium of the Universidade Federal de Viçosa (Herbarium VIC).

Morphological studies

The isolates were grown on Petri dishes containing 2% WA overlaid with double-sterilized twigs of *Pinus* and corn straw and incubated at 25°C with a photoperiod of 12 hours to induce the formation of fruit bodies and sporulation. Sections of the fruiting bodies were manually made and mounted in lactophenol. Thirty measurements of all relevant morphological characters (conidia, paraphyses and conidiogenous cells) were made using a light microscope OLYMPUS CX31 for identification of the species. The images were obtained with an OLYMPUS BX 51 light microscope fitted with a digital camera (OLYMPUS EVOLT330).

DNA extraction, Sequencing and Phylogenetic studies

Single spore isolates were grown on PDA at 25 °C for one week. Approximately 40 mg of fungus mycelia were scraped from the agar surface and placed in a sterile 1.5 mL microcentrifuge tube. The extraction was processed by freezing with liquid nitrogen and grinding into a fine powder using a microcentrifuge tube pestle. The crushing continued to add 100 µL of Nuclei Lysis Solution of the Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.). After, an additional 500 µL of the previous solution was added. The extraction continued as described by Pinho et al. (in press).

PCR reactions were set-up using the following ingredients for each 25 µL reaction: 12.5 µL of Dream Taq TM PCR Master Mix 2X (MBI Fermentas, Vilnius, Lithuania), 1 µL of 10 µM of each forward and reverse primer synthesised 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 nuclease-free water to complete the total volume.

Target regions of the Internal Transcribed Spacer regions 1 and 2 including the 5.8S rRNA gene (ITS), and Transcription Elongation Factor 1-α (TEF1-α) were amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for ITS (White et al. 1990), EF1F (5'-

TGCGGTGGTATCGACAAGCGT-3') and EF2R (5'-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs et al. 2004), EF1-688F (5'-CGGTCACTTGATCTACAAGTGC-3') (Alves et al. 2008) and EF1-986R (5'-TACTTGAAGGAACCCTTACC-3') (Carbone et al. 1999) for partial TEF1-α. 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 (for TEF1-α) or 52 ºC for 1 min (for ITS) (annealing), 72 ºC for 2 min (elongation), and 72 ºC for 10 min (final extension). PCR products were analyzed by 2 % agarose electrophoresis 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. PCR products were purified and sequenced by Macrogen Inc., Korea (http://www.macrogen.com). The nucleotide sequences were edited with the DNA Dragon software (Hepperle 2011). All sequences were checked manually and nucleotides with ambiguous positions were clarified using both primer direction sequences. New sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov). Sequences of ITS and TEF1-α of additional species were retrieved from GenBank (Table 1).

Consensus regions 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. All the ambiguously aligned regions within dataset were excluded from the analyses. Gaps (insertions/deletions) were treated as missing data. The resulting alignment was deposited into TreeBASE (http://www.treebase.org/).

Bayesian inference concatenated (BI) analyses employing a Markov Chain Monte Carlo method were performed with all sequences, first with each gene/locus separately, and then with the concatenated sequences (ITS and TEF1-α). Before launching the BI, the best nucleotide substitution models was determined for each gene 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 SYM+G model of evolution was used for ITS and HKY+G was used for TEF1-α. The phylogenetic analysis of the concatenated alignment was

performed using MrBayes v.3.1.1 (Ronquist and Huelsenbeck, 2003). In MrBayes, data were partitioned by locus and the parameters of the nucleotide substitution models for each partition were set as described above. 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 the burn-in phase of each analysis. Posterior probabilities (Rannala & Yang, 1996) were determined from a majority-rule consensus tree generated with the remaining 7 500 trees. Trees were visualized in FigTree (Rambaut 2009) and exported to graphics programs. The species *Spencermartinsia viticola* CBS117009 were used as an outgroup in these analyses.

Pathogenicity tests

Five *J. curcas* plants of six months old obtained from disinfested seeds, were utilized in the pathogenicity test for each fungal species isolated. The plants were grow in pots with 5kg of sterilized soil, maintained in a greenhouse, and watered once a week until the appearance of symptoms. For inoculation, one representative isolate of each species was grown in a Petri dish with PDA for seven days at 25°C. Six millimeters diameter disks of bark (from the collar regions of healthy plants) were removed with a sterile cork borer and replaced with 6 mm diameter disks containing mycelia from the margins of the growing culture, just below a portion of moistened cotton was placed and subsequently covered with parafilm. On control plants, only PDA plugs were placed on wounded stems. After 2 weeks, the parafilme and cotton were removed. The inoculated plants were maintained in a greenhouse at 25°C for 60 days. From the symptomatic inoculated plants, the fungi were reisolated in culture.

RESULTS

Symptomatology and isolations

On diseased plants, ranging from 6 months to 5 years of age, symptoms of wilting, leaf yellowing with subsequent leaf fall, cracks in the collar region and proliferation of adventitious roots could be observed. Specially on the collar region,

the appearance of black fungal structures in the bark of the plant could be observed. Upon being removed from the soil, plant roots were found to be rotted and the vascular system were necrotic, ranging from light brown to black. Due to loss of support, the plants were often already toppled by wind action (Fig 1) or invaded by termites.

A total of 55 plants were sent to the laboratory and from these, 35 isolates belonging to Botryosphaeriaceae were obtained. Of these, 20 isolates belong to *Neoscytalidium*, 13 to *Lasiodiplodia* and 2 to *Macrophomina*. In addition to these fungal genera, some *Fusarium* spp., *Colletotrichum* spp. and *Chaetomium* spp., were also isolated. However, due to the prevalence and diversity of species of Botryosphaeriaceae associated with root and collar rot of *J. curcas*, the other genera were not included in this work. The *Neoscytalidium* was not detected in the State of the Espírito Santo. In the State of Piauí was detected only *Neoscytalidium*. In São Paulo State only one isolate of *Lasiodiplodia* was obtained. However, in Minas Gerais State, it was possible to detect all genera. From seeds from Minas Gerais and Espírito Santo, five isolates of *Macrophomina* and one isolate of *Lasiodiplodia* were obtained. *Neoscytalidium* was not associated with seeds.

DNA extraction, PCR amplification and Phylogeny

After DNA of the all isolates was extracted, the elongation factor (TEF1-α) was amplified for an indication of possible identity of each isolate by means of Bayesian analyses (data not shown). Subsequently, 2-3 isolates of each previously identified species were selected and the ITS region was amplified for the phylogenetic analyses. DNA extraction and PCR was conducted successfully for all gens regions used, except for the isolates 67 and 125, that did not amplify with the pair of primers EF1F and EF2R (Jacobs et al., 2004). For these, reactions were performed with primers EF1-688F (Alves et al. 2008) and EF2-986R (Carbone et al. 1999). PCR fragments for the ITS had approximately 500 bp in size, while those for TEF1-α had 700bp (EF1F and EF2R) and 300bp (EF1-688F and EF2-986R). All sequences used were deposited in GenBank. The accession numbers are available in Table 1.

The combined analyzes of ITS and TEF1-α dataset included 52 taxa and contained 718 characters with 193 parsimony-informative, 237 variable and 481 conserved.

From the phylogenetic analysis, it was possible to identify nine different species of Botryosphaeriaceae: one species of *Neoscytalidium*, two species of *Macrophomina* and six species of *Lasiodiplodia*. The consensus tree generated with Bayesian analyses is shown (Fig 2).

Taxonomy

The isolates previously identified as *Lasiodiplodia* had common characteristics, such as the presence of paraphyses within the pycnidial conidiomata, conidia initially hyaline and aseptate, but becoming brown and 1-septate with age, with the formation of longitudinal striations due the deposition of melanin granules on the inner surface of the wall. Based on phylogenetic analysis, followed by the morphological descriptions, six species were delimited, in which four are known species and two are to be proposed as new. Dimensions of each *Lasiodiplodia* species are available in Table 2.

Lasiodiplodia theobromae (Pat.) Griffon & Maubl., Bull. trimest. Soc. Mycol. Fr. 25: 57 (1909) (Fig 3)

Mycelium immersed or superficial, branched, septate, dark brown. *Conidiomata* pycnidial, stromatic, superficial, separate, globose, dark brown, uni- or multilocular, often covered by aerial mycelium, formed superficially on twigs of *Pinus* or corn straw in culture. Wall dark brown, thick-walled textura angularis, paler and thinner towards the conidiogenous region. *Conidiophores* absent. *Conidiogenous cells* holoblastic, determinate, discrete, cylindrical, hyaline, smooth and thin-walled, formed from cells lining the inner pycnidial walls, 5–11 × 2–4 μm. *Paraphyses* hyaline, cylindrical, aseptate, not branched, ends rounded, up to 45 μm long, 2 μm wide. *Conidia* acrogenous, aseptate, ellipsoid to ovoid, hyaline when young, later becoming medianly one-septate, dark brown, thick-walled, ellipsoid, frequently with rounded apex, truncate base, 23–31 × 13–15 μm and longitudinal striations.

Habitat: On Jatropha curcas

Known distribution: São Paulo, Espírito Santo and Minas Gerais States, Brazil

Material utilized for morphological description: BRAZIL, Colatina, Espírito Santo, collar and root rot of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 114. Other isolates examined are listed in Table 1.

Lasiodiplodia parva A.J.L. Phillips, A. Alves & Crous, Fungal Diversity 28:9 (2008) (Fig 4)

Mycelium immersed or superficial, branched, septate, dark brown. *Conidiomata* pycnidial, stromatic, superficial, separate, globose, dark brown, unilocular, often covered by aerial mycelium, formed superficially on twigs of *Pinus* or corn straw in culture. Wall dark brown, thick-walled textura angularis, paler and thinner towards the conidiogenous region. *Conidiophores* absent. *Conidiogenous cells* holoblastic, determinate, discrete, cylindrical, hyaline, smooth and thin-walled, formed from cells lining the inner pycnidial walls, 5–15 × 2–5 µm. *Paraphyses* hyaline, cylindrical, septate, occasionally branched, ends rounded, up to 62 µm long, 2–4 µm wide. *Conidia* acrogenous, aseptate, ellipsoid to ovoid hyaline when young, later becoming medianly one-septate, dark brown, thick-walled, ellipsoid, frequently with rounded apex, truncate base, 15–23 × 10–13 µm and longitudinal striations.

Habitat: On Jatropha curcas

Known distribution: Espírito Santo and Minas Gerais States, Brazil

Material utilized for morphological description: BRAZIL, Colatina, Espírito Santo, collar and root rot of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 66. Other isolates examined are listed in Table 1.

Lasiodiplodia pseudotheobromae A.J.L. Phillips, A. Alves & Crous, Fungal Diversity 28:9 (2008) (Fig 5)

Mycelium immersed or superficial, branched, septate, dark brown. *Conidiomata* pycnidial, stromatic, superficial, separate, globose, dark brown, unilocular, often covered by aerial mycelium, formed superficially on twigs of pinus or corn straw in culture. Wall dark brown, thick-walled textura angularis, paler and thinner towards the conidiogenous region. *Conidiophores* absent. *Conidiogenous cells* holoblastic, determinate, discrete, cylindrical, hyaline, smooth and thin-walled, formed from cells lining the inner pycnidial walls, 5–15 × 2–5 µm. *Paraphyses* hyaline, cylindrical, septate, not branched, ends rounded, up to 65 µm long, 2–3 µm wide. *Conidia* acrogenous, aseptate, ellipsoid, hyaline when young, later becoming medianly one-septate, dark brown, thick-walled, ellipsoid, frequently with rounded apex, truncate base, 26–31 × 13–16 µm and longitudinal striations.

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Habitat: On Jatropha curcas

Known distribution: Espírito Santo State, Brazil

Material utilized for morphological description: BRAZIL, Colatina, Espírito Santo, Collar and root rot of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 163.

Lasiodiplodia iraniensis Abdollahzadeh, Zare & A.J.L. Phillips, Persoonia 25:8 (2010) (Fig 6)

Mycelium immersed or superficial, branched, septate, dark brown. *Conidiomata* pycnidial, stromatic, superficial, separate, globose, dark brown, unilocular, often covered by aerial mycelium, formed superficially on twigs of pinus or corn straw in culture. Wall dark brown, thick-walled textura angularis, paler and thinner towards the conidiogenous region. *Conidiophores* absent. *Conidiogenous cells* holoblastic, determinate, discrete, cylindrical, hyaline, smooth and thin-walled, formed from cells lining the inner pycnidial walls, 7–15 × 2–5 µm. *Paraphyses* hyaline,

cylindrical, septate, occasionally branched, ends rounded, up to 70 µm long, 3 µm wide. *Conidia* acrogenous, aseptate, subglobose to subcylindrical, hyaline when young, later becoming medianly one-septate, dark brown, thick-walled, ovoid to subcylindrical, frequently with rounded apex, truncate base, 22–26 × 14–17µm and longitudinal striations.

Habitat: On Jatropha curcas

Known distribution: Espírito Santo State, Brazil

Material utilized for morphological description: BRAZIL, Colatina, Espírito Santo, Collar and root rot of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 67.

***Lasiodiplodia* sp. 1** (To be proposed as new species) (Fig 7)

Mycelium immersed or superficial, branched, septate, dark brown. *Conidiomata* pycnidial, stromatic, superficial, separate, globose, dark brown, uni- or multilocular, often covered by aerial mycelium formed superficially on twigs of pinus or corn straw in culture. Wall dark brown, thick-walled textura angularis, paler and thinner towards the conidiogenous region. *Conidiophores* absent. *Conidiogenous cells* holoblastic, determinate, discrete, cylindrical, hyaline, smooth and thin-walled, formed from cells lining the inner pycnidial walls, 5–19 × 3–5 µm. *Paraphyses* hyaline, cylindrical, aseptate, not branched, ends rounded, up to 90 µm long, 2–3 µm wide. *Conidia* acrogenous, aseptate, ellipsoid, hyaline when young, later becoming dark brown, thick-walled, ellipsoid to obpyriform, frequently with rounded apex, truncate base, forming up to two septa, 26–32 × 14–19 µm and longitudinal striations.

Habitat: On Jatropha curcas

Known distribution: Espírito Santo State, Brazil

Material utilized for morphological description: BRAZIL, Colatina, Espírito Santo, Collar and root rot of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 84. Other isolates examined are listed in Table 1.

***Lasiodiplodia* sp. 2** (To be proposed as new species) (Fig 8)

Mycelium immersed or superficial, branched, septate, dark brown. *Conidiomata* pycnidial, stromatic, immersed or superficial, separate or aggregated, globose, dark brown, uni- or multilocular, often covered by aerial mycelium, formed superficially on twigs of pinus or corn straw in culture. Wall dark brown, thick-walled textura angularis, paler and thinner towards the conidiogenous region. *Conidiophores* absent. *Conidiogenous cells* holoblastic, determinate, discrete, cylindrical, hyaline, smooth and thin-walled, formed from cells lining the inner pycnidial walls, 8–20 × 2.5–4 µm. *Paraphyses* hyaline, cylindrical, septate, not branched, ends rounded, up to 105 µm long, 3–4 µm wide. *Conidia* acrogenous, up to three-septate, ellipsoid to ovoid, hyaline when young, later becoming medianly one-septate, dark brown, thick-walled, ellipsoid, frequently with rounded apex, truncate base, 28–35 × 15–17 µm and longitudinal striations.

Habitat: On Jatropha curcas

Known distribution: Espírito Santo and Minas Gerais States, Brazil

Material utilized for morphological description: BRAZIL, Colatina, Espírito Santo, Collar and root rot of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 116. Other isolates examined are listed in Table 1.

The isolates previously identified as *Neoscytalidium*, had common characteristics: the formation of chains of arthroconidia on the aerial mycelium and on twigs of *Pinus* or corn straw in culture, pycnidial conidiomata, immersed in or superficially on a stroma, with release of *Fusicoccum*-like conidia.

From the phylogenetic analysis, as well as from the morphology, it was possible to separate one *Neoscytalidium* species. The species could be identified as *Neoscytalidium dimidiatum* (Penz.) Crous & Slippers. Dimensions of the species are given in Table 3.

Neoscytalidium dimidiatum (Penz.) Crous & Slippers Stud. Mycol., 55:244 (2006) (Fig 9)

The aerial mycelia formed chains of 0–1 septate *arthroconidia*, oblong to globose, initially hyaline becoming brown thick-walled with age, 4–12 × 2.5–8 µm. *Pycnidia* dark, globose, base up to 250 µm and a neck up to 810 µm, immersed in or superficially on a stroma. *Conidiogenous cells* holoblastic, lageniform to ampulliform, hyaline, 6–10 × 1.5–2.5 µm. *Conidia* hyaline, ellipsoid to nearly fusiform, 8–12 × 4–5 µm. Dark septate conidia not observed.

Habitat: On Jatropha curcas

Known distribution: Piauí and Minas Gerais States, Brazil

Material utilized for morphological description: BRAZIL, Piauí, Collar and root rot of *Jatropha curcas*, 2010, A. R. Machado & O. L. Pereira, Isolate 77. Other isolates examined are listed in Table 1.

The isolates previously identified as *Macrophomina* had common characteristics of the genus, such as the formation of dark mycelia and sclerotia on PDA. On twigs of *Pinus* in culture the formation of pycnidial conidiomata was observed, with release of hyaline conidia with apical mucoid appendages. Only a single isolate sporulated in culture.

Based on morphology and the phylogenetic analyses, it was possible to identify two *Macrophomina* species. *Macrophomina phaseolina* was found only on roots, however, on seeds a new phylogenetic species was found. Dimensions of species are given in Table 4.

Macrophomina phaseolina (Tassi) Goid., Annali della Sperimentazione Agaria, 1: 457 (1947) (Fig 10)

Mycelium superficial or immersed, light to dark brown, branched, septate. *Conidiomata* pycnidial, separate, globose, dark brown, unilocular, thick-walled. *Conidiophores* absent. *Conidiogenous cells* holoblastic, phialidic, determinate, ampulliform, hyaline, smooth, formed from the inner cells of the pycnidial wall.

Conidia hyaline, aseptate, obtuse at each end, fusiform, thin-walled, smooth, with apical mucoid appendages. Brown, 1-septate mature conidia were present.

Habitat: On Jatropha curcas

Known distribution: Espírito Santo and Minas Gerais States, Brazil

Material utilized for morphological description: BRAZIL, Nova Porteirinha, Minas Gerais, Collar and root rot of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 70. Other isolates examined are listed in Table 1.

***Macrophomina* sp.1** (To be proposed as new species)

It was not possible to induce sporulation of the isolates obtained from seeds. However, the phylogenetic studies show that it is a possible new species, due to the formation of a monophyletic group with in the clade, and well supported by posterior probability.

Habitat: On Jatropha curcas

Known distribution: Espírito Santo and Minas Gerais States, Brazil

Material utilized for morphological description: BRAZIL, Jaíba, Minas Gerais, from seeds of *Jatropha curcas*, 2011, A. R. Machado & O. L. Pereira, Isolate 113. Other isolates examined are listed in Table 1.

Pathogenicity tests

Sixty days after inoculation, the plants were evaluated for presence or absence of symptoms. The fungus *Neoscytalidium dimidiatum* reproduced the symptoms observed in the field, which caused collar rot that progressed to the roots. From the lesion, it was possible to isolate and retrieve the inoculated fungus (Fig.11 a–c). The species *Lasiodiplodia theobromae* and *Lasiodiplodia* sp.1 caused localized necrotic lesions, limited to the inoculated region, and not progressing to the roots (Fig.11 d–i). The species *Lasiodiplodia parva* and *Macrophomina phaseolina* did not

produce symptoms in the conditions under which the test was conducted. For the newly found species *Lasiodiplodia* sp. 2, *L. iraniensis* and *L. pseudotheobromae*, the pathogenicity tests are on going.

DISCUSSION

In this work, through phylogenetic analysis supported by morphological studies, nine species of Botryosphaeriaceae were identified associated with the collar rot disease of *J. curcas*. Among these, two new species of *Lasiodiplodia* and one of *Macrophomina* will be proposed.

As with previous studies (Damm et al. 2007; Alves et al. 2008; Abdollahzadeh et al. 2010), the *Lasiodiplodia* species, except for *Lasiodiplodia* sp.2., cannot be distinguished based only on sequences of ITS, however when TEF1-α is also included in the analysis, the species are clearly separated and with strong support. This occurs because the newly described taxa belong to a complex of species within what was previously known as *L. theobromae*.

Utilizing only the TEF1-α sequences for phylogenetic analysis, it was possible to distinguish the species in separate groups, with a similar topology to the combined data, but without sufficient support to propose new species (data not shown).

Allied to the phylogenetic analyses, several studies have used morphological parameters, such as conidial morphology, size, shape and septation of paraphyses, growth and pigment production in culture to differentiate *Lasiodiplodia* species (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; Urbez-Torres et al. 2012). However, Abdollahzadeh et al. (2010) make case against differentiating species on paraphyses septation, due to the fact that this parameter can give erroneous results and should be interpreted with caution. For example, paraphyses can be aseptate when young and become septate with age. In this study, *L. pseudotheobromae* produced septate paraphyses, while Alves et al. (2008) described the presence of aseptate paraphyses for this species. However, conidia size was similar.

In the material of *L. parva* studied by Alves et al. (2008), the paraphyses are longer than the isolates from the same species retrieved in the present study. But, as in the above situation, the dimensions of the conidia are the same.

Alves et al. (2008) also used pigment production in culture at 35 ° C and growth at 10 ° C for the differentiation of *L. parva* and *L. pseudotheobromae* from *L. theobromae*. However, Abdollahzadeh et al. (2010) concludes that this parameter has limited value in species determination. Thus, this parameter was not used in the present study.

The conidia of *L. iraniensis* were larger than reported for Abdollahzadeh et al. (2010). However, they still differ from *L. theobromae* because they were often smaller and can be sepatared from *L. parva* because the conidial shape were often subglobose to subcylindrical.

Lasiodiplodia sp.1 is phylogenetically closer to *L. citricola* Abdollahzadeh, Javadi & A.J.L. Phillips, but conidia are longer and wider than those of *L. citricola*. In addition, conidia up to two-septate and obpyriform were observed. Paraphyses and conidiogenous cells are smaller than in *Lasiodiplodia*. sp.1.

Lasiodiplodia sp.2 is phylogenetically distant from the other species and can be distinguished solely with ITS sequences. The dimensions of the paraphyses are similar to those of *L. parva*, however, the conidia are larger and hyaline and up to three-septate.

L. theobromae analyzed in this study had the same dimensions of conidia and paraphyses as cited by Sutton (1980) and Alves et al. (2008).

These results show the great diversity of *Lasiodiplodia* species associated with *J. curcas*. This is the first description of the occurrence of *L. pseudotheobromae*, *L. parva* and *L. iraniensis* in Brazil, and also from this host. As shown by Begoude et al. (2010), these fungi have a wide geographical distribution and a broad host range that has been increased substantially with these studies.

The isolates of *Neoscytalidium dimidiatum* formed one well supported monophyletic group, being different from *N. novaehollandiae* in contrast to the results shown by Pavlic et al. (2008). The *Neoscytalidium dimidiatum* in this study had

dimensions of conidia, arthroconidia and conidiogenous cells similar to the same species in previous studies and of the species *N. novaehollandiae* Pavlic, T.I. Burgess & M.J. Wingf.. Pavlic et al. (2008) reached the same conclusion, but according to these authors, the presence of *Dichomera*-like conidia in *N. novaehollandiae* is the main character that differentiates this species from *N. dimidiatum*.

The fungus *Neoscytalidium dimidiatum* is cosmopolitan, occurring in a wide range of hosts, which includes apple, banana, citrus, fig, grapevine, potato, walnut, yam and mango, sometimes causing canker and rot or branch wilt (Sutton 1980; Polizzi et al. 2009; Ray et al. 2010). Recently, it has been described causing collar and rot root of *J. curcas* in Brazil (Machado et al. in press).

In this study it was observed that this fungus is present in regions with a semi-arid climate (North of Minas Gerais and Piauí) and was not found in more humid regions (Espírito Santo). It is known that climatic variations can influence the distribution of different Botryosphaeriaceae (Úrbez-Torres et al. 2006; Begoude et al. 2010). But, for *Neoscytalidium*, more studies should be conducted with a broader survey to confirm this hypothesis.

Two species of *Macrophomin*a are reported in this paper. *Macrophomina phaseolina* formed a monophyletic group with other sequences from the same species. The conidia had dimensions similar to those described by Sutton (1980), however, the conidiogenous cells were longer and narrower.

Based on the phylogenetic analysis, we obtained a new species of *Macrophomina* isolated from seeds, but to confirm this information, a morphological differentiation from the other species previously reported will be required. So far, these isolates did not sporulate in culture using the method tested.

This study shows that the Botryosphaeriaceae species isolated exhibit different levels of pathogenicity on *J. curcas*. *Lasiodiplodia theobromae* and *N. dimidiatum* reproduced the symptoms observed in the field, and can be considered as primary pathogens. Other species of *Lasiodiplodia* and *Macrophomina* tested, possibly act as secondary pathogens, due to the fact that they not produced the disease symptoms. However, we believe that these fungi can also be a primary pathogens,

since factors such as the age of the plant, inoculation method or environmental conditions can influence the inoculations results. Future studies should be realized with the purposes of investigating the possible existence of the severity levels between species and to test the pathogenicity of isolates in plants under environmental stress.

Botryosphaeriaceae species occur in a wide range of hosts and environments, and are often referred to as endophytes, latent or opportunistic pathogens, because the manifestation of the disease is directly linked with the occurrence of stress of the host (Slippers and Wingfield 2007).

The great expansion of *Jatropha* areas in the world has contributed to the emergence of several diseases for which, so far, the etiological agents are unknown. This study provides new information for future studies of disease management, quarantine programs and especially the development of resistant varieties for collar and root rot of *J. curcas*.

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CONCLUSÃO GERAL

Foram identificadas nove espécies de *Botryosphaeriaceae* associados a podridão do colo e da raiz do pinhão manso, sendo *Lasiodiplodia theobromae*, *Lasiodiplodia pseudotheobromae*, *Lasiodiplodia parva*, *Lasiodiplodia iraniensis*, *Lasiodiplodia* sp.1, *Lasiodiplodia* sp.2, *Neoscytalidium dimidiatum*, *Macrophomina phaseolina* e *Macrophomina* sp.1.

ANEXOS

Table 1 Isolates from GenBank and this study used in the phylogenetic analyses.

Species	Isolates	Host/Substrate	Origin	Genbank acession n°	
				ITS	EF1-α
Neoscytalidium dimidiatum	CBS 499.66	*Mangifera indica*	Mali	AY819727	EU144063
Neoscytalidium dimidiatum	PD104	*Ficus carica*	USA	GU251107	GU251239
Neoscytalidium dimidiatum	88	*Jatropha curcas*	Brazil	-	-
Neoscytalidium dimidiatum	86	*Jatropha curcas*	Brazil	-	-
Neoscytalidium dimidiatum	77	*Jatropha curcas*	Brazil	-	-
Neoscytalidium dimidiatum	65	*Jatropha curcas*	Brazil	-	-
Neoscytalidium	CBS122072	*Adansonia gibbosa*	Australia	EF585535	EF585581
Neoscytalidium	CBS122610	*Acacia synchronicia*	Australia	EF585536	EF585578
Macrophomina phaseolina	PD112	*Prunus dulcis*	USA	GU251105	GU251237
Macrophomina phaseolina	MUCC531	*Sesbania formosa*	Australia	EF585505	EF585560
Macrophomina phaseolina	70	*Jatropha curcas*	Brazil	-	-
Macrophomina phaseolina	90	*Jatropha curcas*	Brazil	-	-
Macrophomina phaseolina	162	*Jatropha curcas*	Brazil	-	-
Macrophomina sp1.	113	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia venezuelensis	WAC12539	*Acacia mangium*	Australia	DQ103547	DQ103568
Lasiodiplodia venezuelensis	CMW13513	*Acacia mangium*	Venezuela	DQ103549	DQ103570
Lasiodiplodia rubropurpurea	WAC12536	*Eucalyptus grandis*	Australia	DQ103554	DQ103572
Lasiodiplodia gonubiensis	CBS115812	*Syzygium cordatum*	South	DQ458892	DQ458877
Lasiodiplodia crassispora	CBS110492	Unknown	Unknown	EF622086	EF622066
Lasiodiplodia crassispora	CMW22653	*Pterocarpus*	South	FJ888465	FJ888452
Lasiodiplodia margaritacea	CBS122519	*Adansonia gibbosa*	Australia	EU144050	EU144065
Lasiodiplodia margaritacea	CBS122065	*Adansonia gibbosa*	Australia	EU144050	EU144065
Lasiodiplodia	CBS116459	*Gmelina arborea*	Costa Rica	EF622077	EF622057
Lasiodiplodia	IRAN1518C	*Citrus* sp.	Iran	GU973874	GU973866
Lasiodiplodia	163	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia parva	CBS356.59	*Theobroma cacao*	Sri Lanka	EF622082	EF622062
Lasiodiplodia parva	91	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia parva	112	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia parva	66	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia citricola	IRAN1521C	*Citrus* sp.	Iran	GU945353	GU945339
Lasiodiplodia citricola	IRAN1522C	*Citrus* sp.	Iran	GU945354	GU945340
Lasiodiplodia sp.1	84	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia sp.1	68	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia hormozganensis	IRAN1500C	*Olea* sp.	Iran	GU945355	GU945343
Lasiodiplodia hormozganensis	IRAN1498C	*Mangifera indica*	Iran	GU945356	GU945344
Lasiodiplodia sp.2	125	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia sp.2	116	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia plurivora	STEU5803	*Vitis vinifera*	South	EF445362	EF445395
Lasiodiplodia gilanensis	IRAN1523C	*Unknown*	Iran	GU945351	GU945342
Lasiodiplodia gilanensis	IRAN1501C	*Unknown*	Iran	GU945352	GU945341
Lasiodiplodia iraniensis	IRAN1517C	*Citrus sp.*	Iran	GU945349	GU945337
Lasiodiplodia iraniensis	IRAN1519C	*Mangifera indica*	Iran	GU945350	GU945338
Lasiodiplodia iraniensis	67	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia mahajangana	CMW27801	*Terminalia catappa*	Madagascar	FJ900595	FJ900641
Lasiodiplodia mahajangana	CMW27820	*Terminalia catappa*	Madagascar	FJ900597	FJ900643
Lasiodiplodia theobromae	CMW28571	*Terminalia ivorensis*	Cameroon	GQ469924	GQ469897
Lasiodiplodia theobromae	CMW10130	*Vitex donniana*	Uganda	AY236951	AY236900
Lasiodiplodia theobromae	115	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia theobromae	114	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia theobromae	69	*Jatropha curcas*	Brazil	-	-
Lasiodiplodia theobromae	83	*Jatropha curcas*	Brazil	-	-
Spencermartinsia viticola	CBS117009	*Vitis vinifera*	Spain	AY905554	AY905559

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

Species	Conidia (µm)	Paraphyses (µm)		Conidiogenous Cells (µm)	Reference
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	This study
L. pseudotheobromae	23.5–32 × 14–18	58 × 3–4	asseptate	-	Alves et al. 2008
	26–31 × 13–16	65 × 2–3	septate	5–15 × 2–5	This study
L. parva	16–23.5 × 10.5–13	105 × 3–4	septate	-	Alves et al. 2008
	15–23 × 10–13	62 × 2–4	septate	5–15 × 3–4	This study
Lasiodiplodia sp.2	28–35 × 15–17	105 × 3–4	septate	8–20 × 2.5–4	This study
L. iraniensis	17–23 × 11–14	127 × 2–4	1–6 septate	9–16 × 3–5	Abdollahzadeh et al. 2010
	22–26 × 14–17	70 × 3	septate	7–15 × 2–5	This study
L. plurivora	26.5–32.5 × 14.5–17	130 × 2–5	2–7 septate	8–13 × 4–7	Damm et al. 2008
L. citricola	22–27 × 12–17	125 × 3–4	1–5 septate	11–16 × 3–5	Abdollahzadeh et al. 2010
Lasiodiplodia sp.1	26–32 × 14–19	90 × 2–3	aseptate	5–19 × 3–5	This study
L. crassispora	27–30 × 14–17	30–62 × 2–3.5	septate	8–16 × 3–7	Burgess et al. 2006
L. gilanensis	28–35 × 15–18	95 × 2–4	1–3 septate	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 septate	9–15 × 3–5	Abdollahzadeh et al. 2010
L. margaritacea	14–17 × 11–12	28–46 × 2–2.5	1–2 septate	10–11× 3–4	Pavlic et al. 2008
L. rubropurpurea	24–33 × 13–17	32–52 × 1.5–3.5	aseptate	7–13 × 3–5	Burgess et al. 2006
L. venezuelensis	26–33 × 12–15	16–41 × 2–5	septate	7–14 × 3–4.5	Burgess et al. 2006
L. undulata	20–32 × 13.5–19.2	–	–	8–20 × 3.2–4.8	Abbas et al. 2004
L. missouriana	17.4–19.6 × 8.9–10.6	55 × 2–3	aseptate	–	Úrbez-Torres et al. 2012
L. viticola	18.2–20.5 × 8.8–10.1	60 × 2–3	aseptate	–	Úrbez-Torres et al. 2012
L. fiorii	24–26 × 12–15	–	–	–	Saccardo 1913
L. paraphysaria	30–32 × 15–16	90–100 × 3	1–septate	–	Saccardo 1899
L. ricini	16–19 × 10–11	25–35 × 2	1–septate	–	Saccardo 1915
L. thomasiana	28–30 × 11–12	80–90 × 1.5	–	–	Saccardo 1913
L. abnormis	25–28 × 13–15	–	–	–	Saccardo 1913

Table 3 Main morphological characteristics of *Neoscytalidium* spp..

Species	Conidia (µm)	Arthroconidia (µm)	Conidiogenous Cells (µm)	Reference
N. dimidiatum	12–13.5 × 4–5.5	Up to 10 × 4	8–10 × 4	Nattrass 1933
N. dimidiatum	8–12 × 4–5	4–12 × 2.5–8	6–10 × 1.5–2.5	This study
N. novaehollandiae	10.5–12.5 × 4–5	5.5–7.5 × 3.5–4.5	7–10 × 2–3	Pavlic et al. 2008

Table 4 Main morphological characteristic of *Macrophomina* spp..

Species	Conidial dimensions (µm)	Conidiogenous Cells (µm)	Reference
M. limbalis	22–28 × 8–10	10–20 × 1–2	Sydow 1924
M. philippinensis	16–24 × 6–7.5	6–12 × 1.5	Petrak 1923
M. pseudeverniae	16–22 × 6–9	7–10 × 6–7	Etayo & Diederich 1996
M. phaseolina	14–30 × 5–10	5–13 × 4–6	Sutton 1980
	12–30 × 6–10	8–20 × 2.5–4	This study

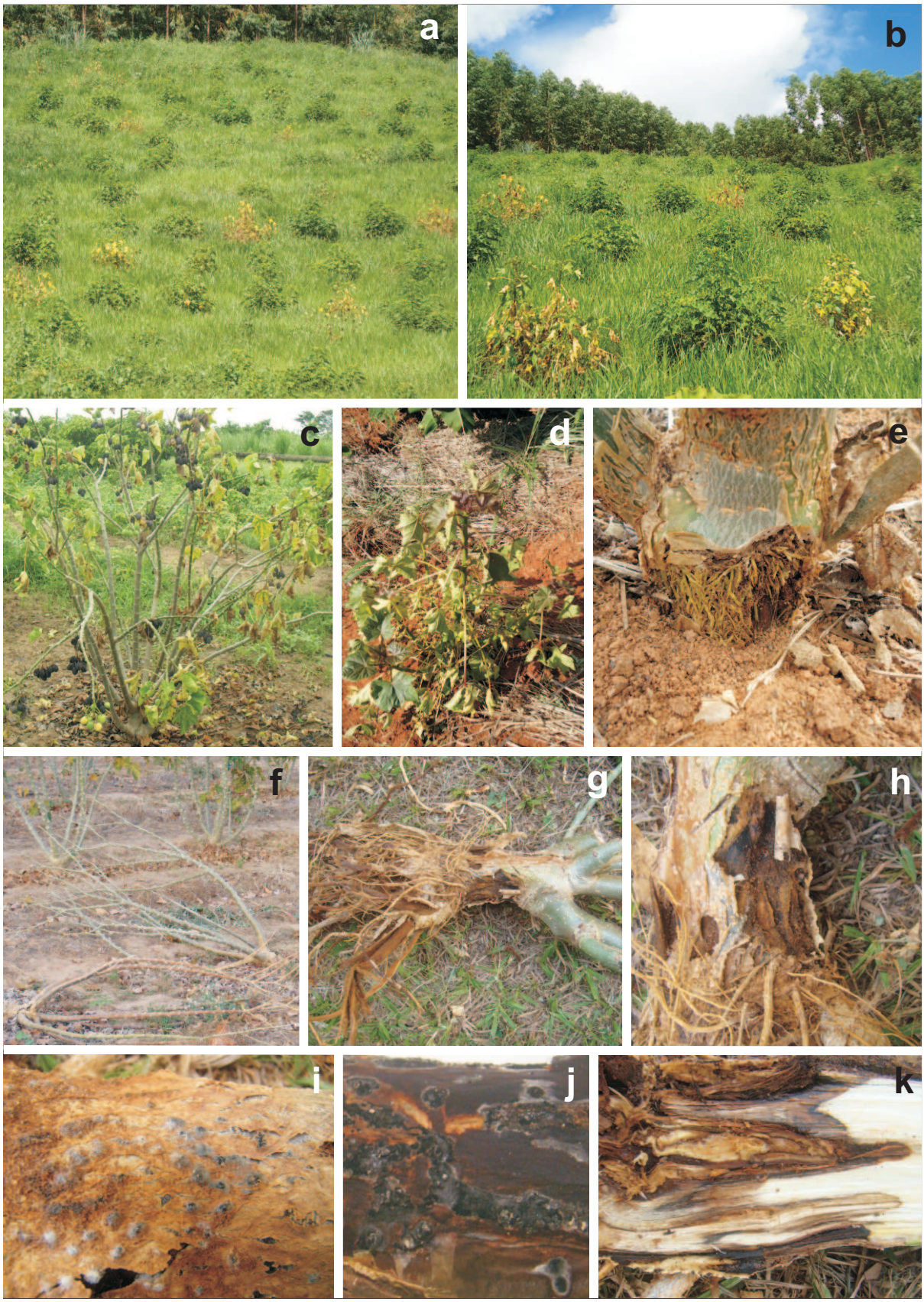

Fig.1 Symptoms of collar and root rot in Jatropha curcas. **a–b,** diseased plants in the field. **c–d,** plants wilting. **e,** proliferation of adventitious roots and collar rot. **f,** death plants. **g–h,** collar and root rot. **i–j,**black fungal structures rupting from the bark of the plants. **K,** necrotic symptoms on vascular system.

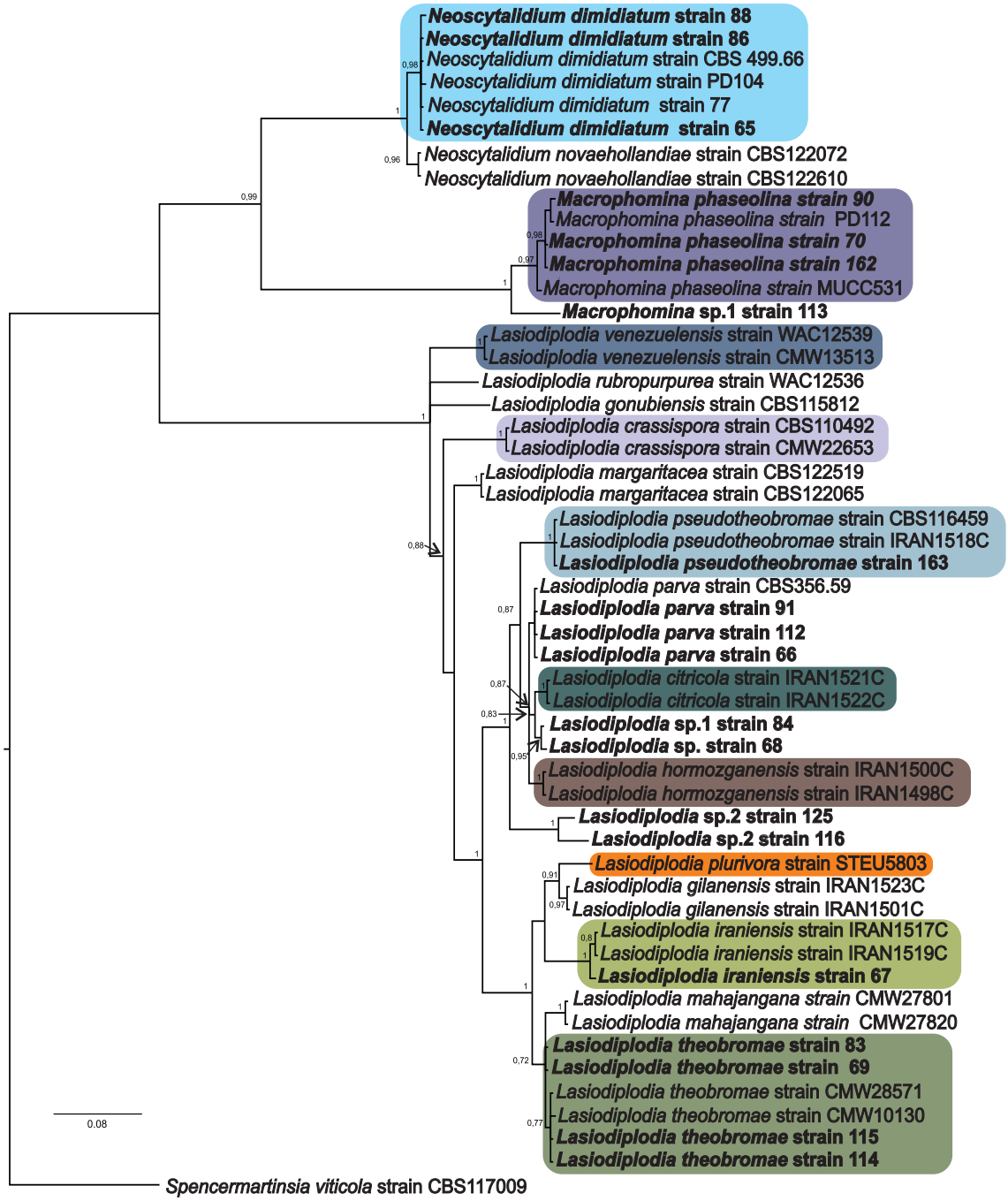

Fig.2 Multilocus phylogenetic tree inferred from Bayesian analysis based on the combined regions of the ITS and EF-1α. Bayesian posterior probability of >75% are indicated above the nodes. *Spencermartinsia viticola* represents the outgroup taxon. The species in this study are highlighted in bold.

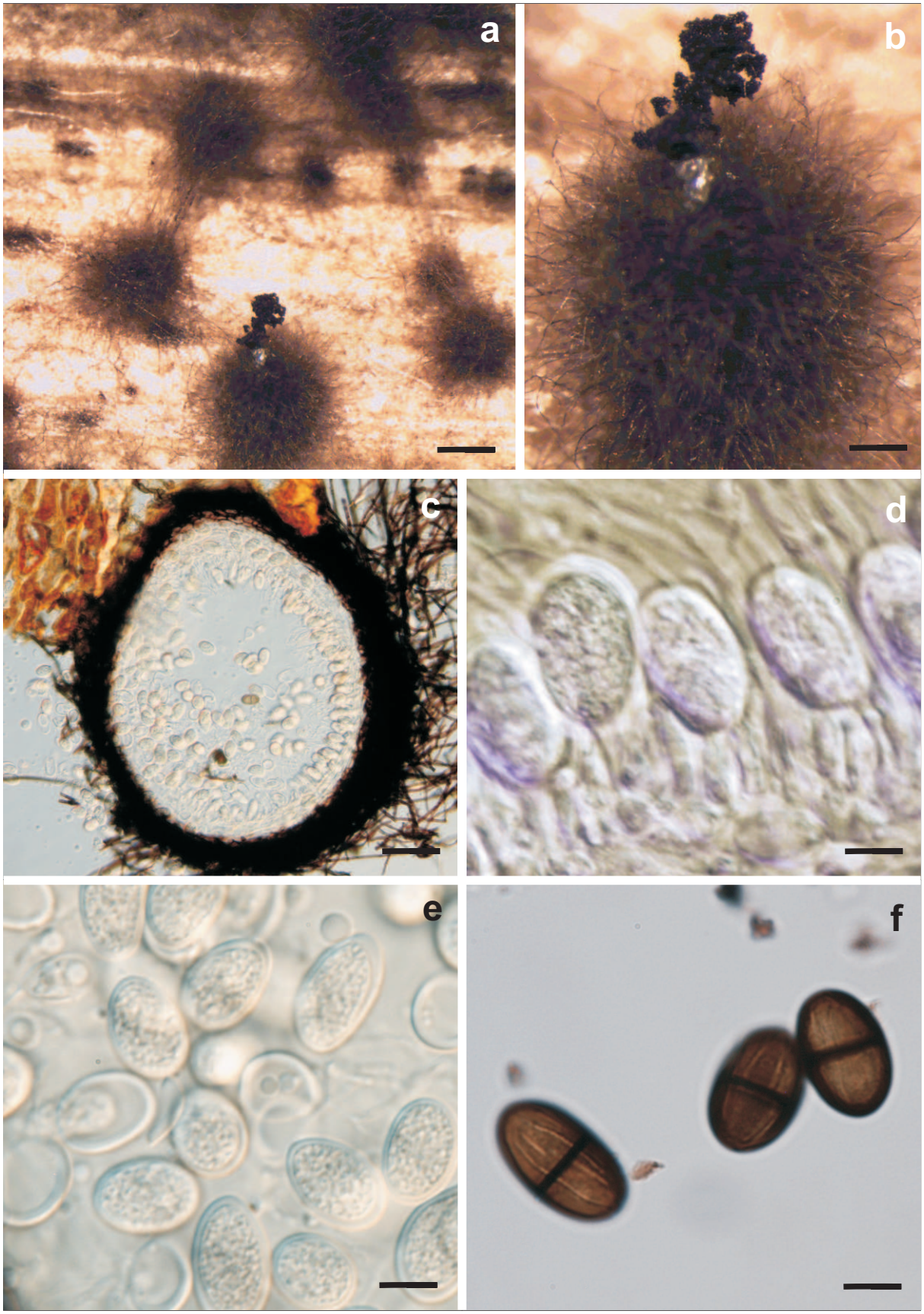

Fig.3 a–f *Lasiodiplodia theobromae* strain 83. **a–b,** conidiomata on corn straw in culture. c, section of conidioma. **d,** conidia developing on conidiogenous cells. **e,** immature conidia. **f,** mature conidia. Scale bars: a =1000µm; b – c =100µm; d= 10µm; e – f= 15µm.

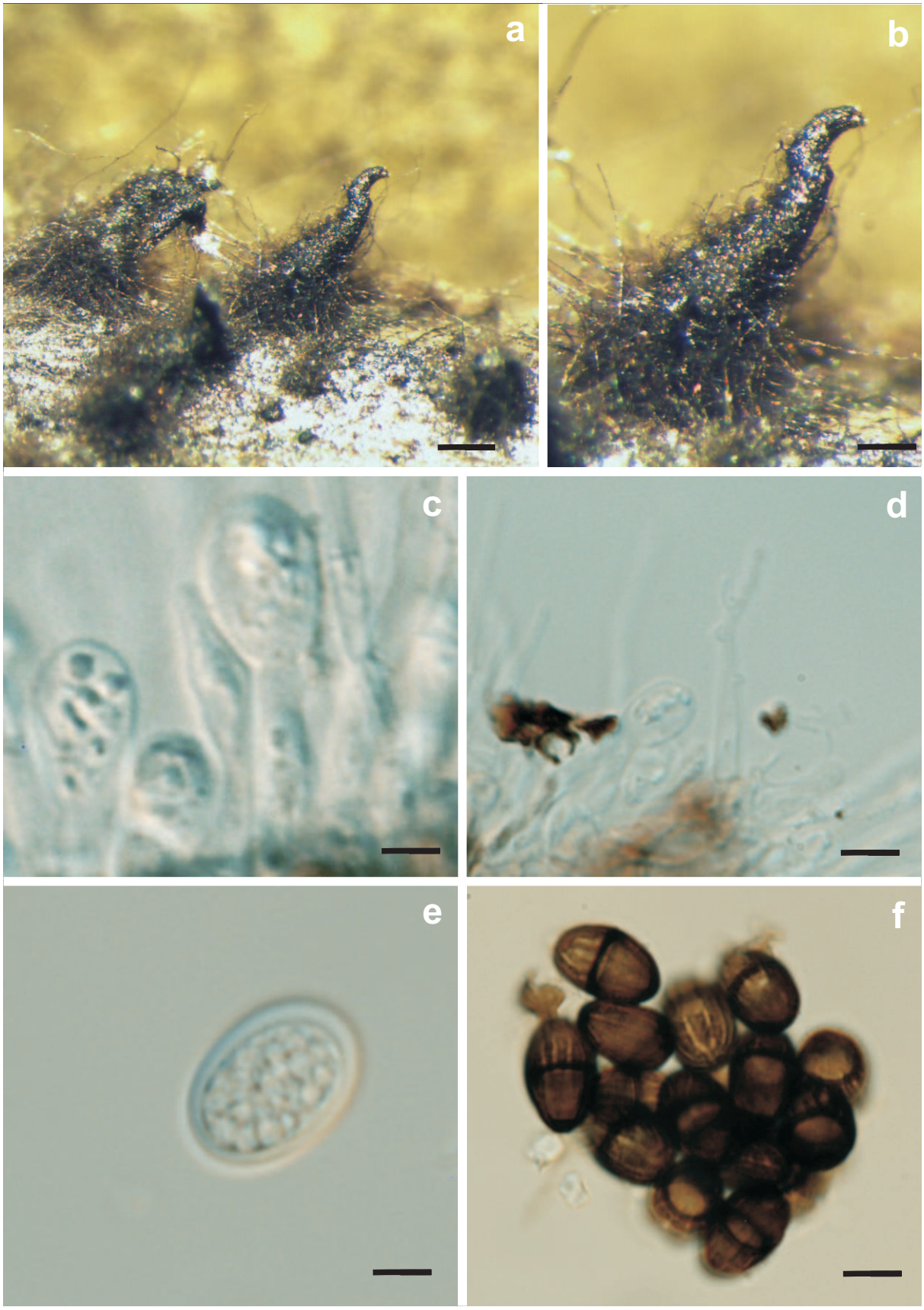

Fig.4 a–f *Lasiodiplodia parva* strain 66. **a–b,** conidiomata on *Pinus* twigs in culture. **c,** conidia developing on conidiogenous cells and septate paraphyses. **d,** paraphyses branched. **e,** immature conidia. **f,** mature conidia. Scale bars: a =1000µm; b =500µm; c – d= 4µm; e – f= 15µm.

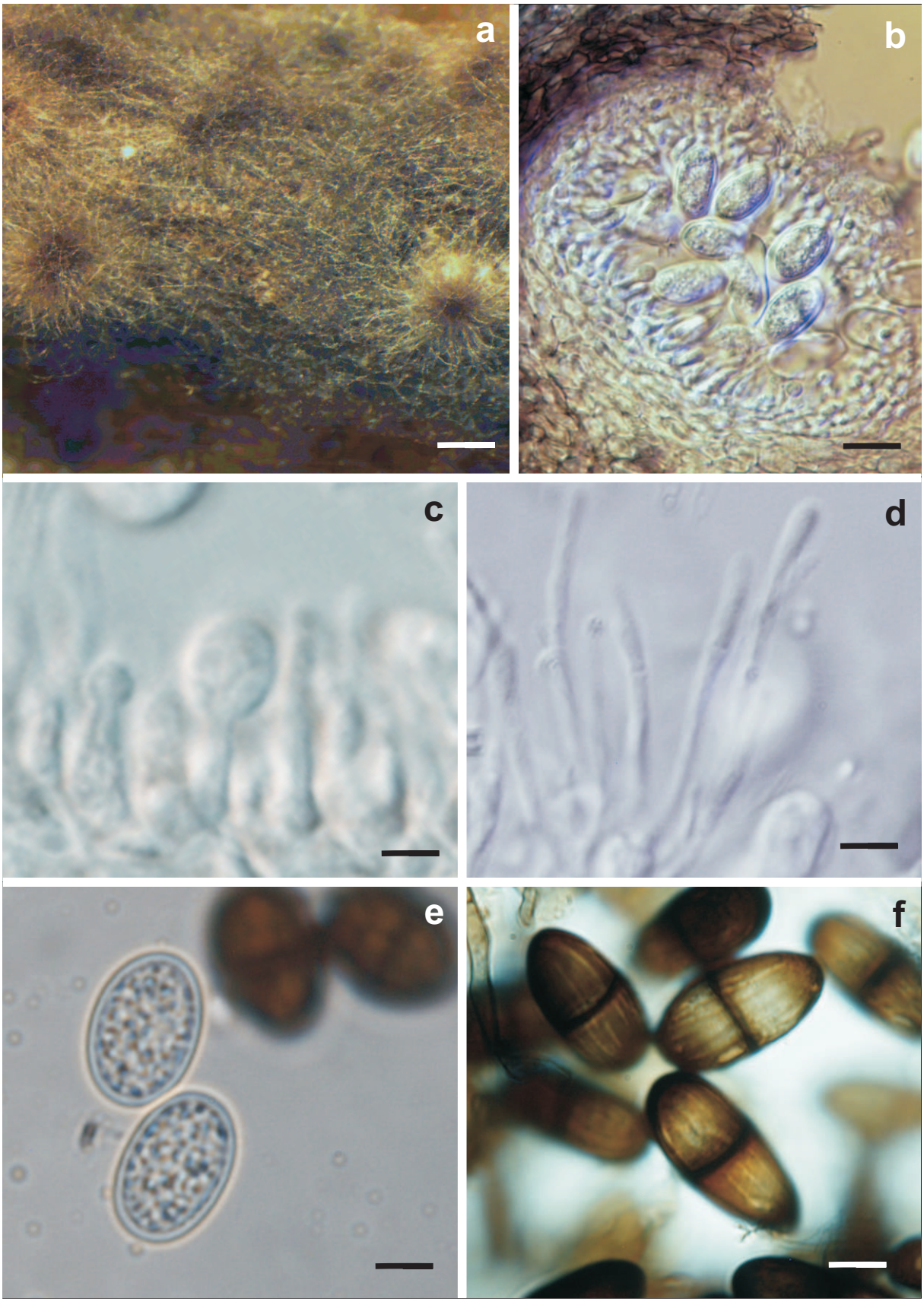

Fig.5 a–f *Lasiodiplodia pseudotheobromae* strain 163. **a,** conidiomata on *Pinus* twigs in culture. **b,** section of conidioma. **c,** conidia developing on conidiogenous cells. **d,** septate paraphyses. **e,** immature conidia. **f,** mature conidia. Scale bars: a =1000µm; b =20µm; c – d= 4µm; e – f= 15µm.

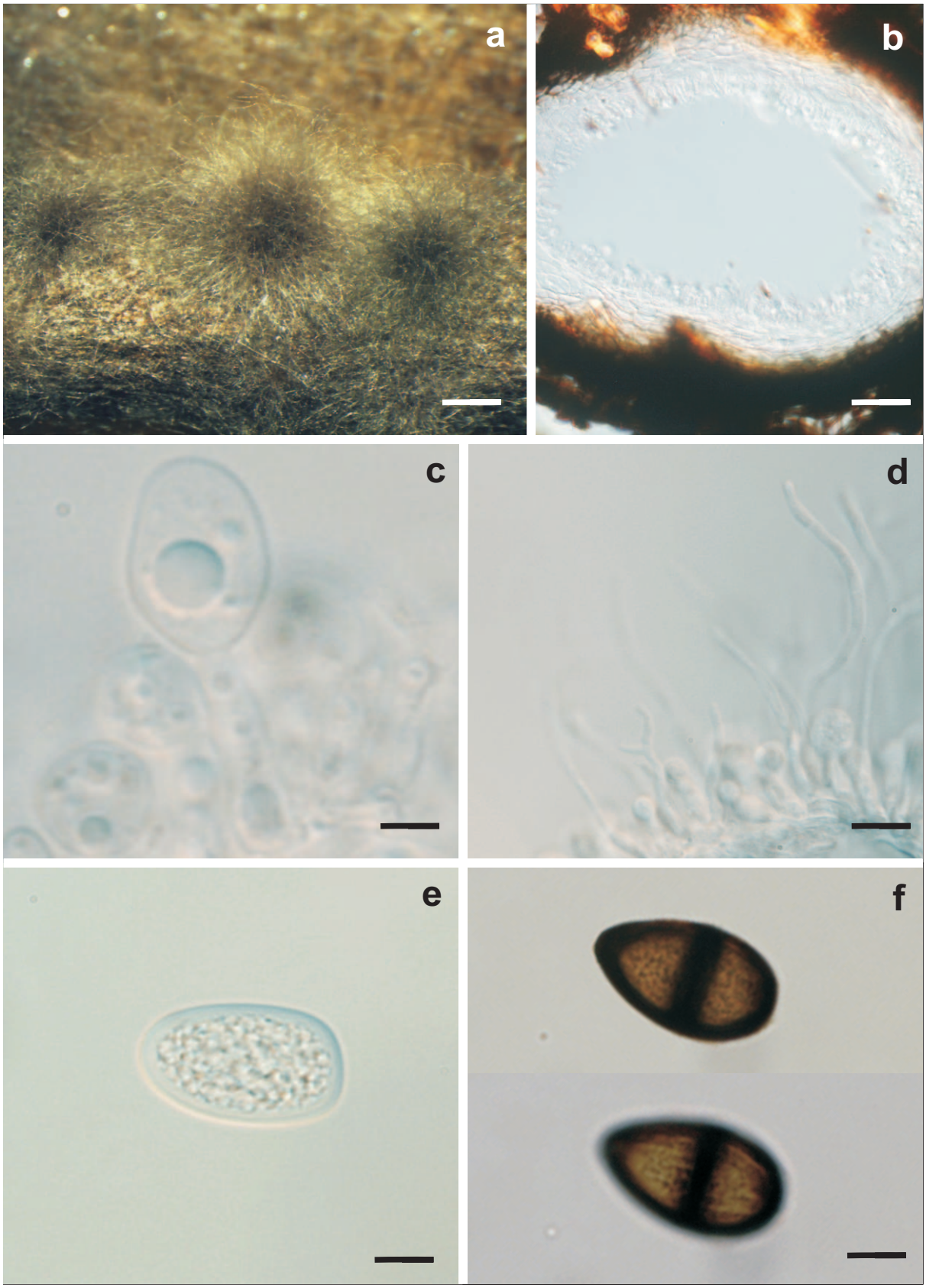

Fig.6 a–f *Lasiodiplodia iraniensis* strain 67. **a,** conidiomata on *Pinus* twigs in culture. **b,** section of conidioma. **c,** conidia developing on conidiogenous cells. **d,** branched paraphyses. **e,** immature conidia. **f,** mature conidia in two different focal planes to show the longitudinal striations. Scale bars: a =1000µm; b =50µm; c – d= 4µm; e – f= 15µm.

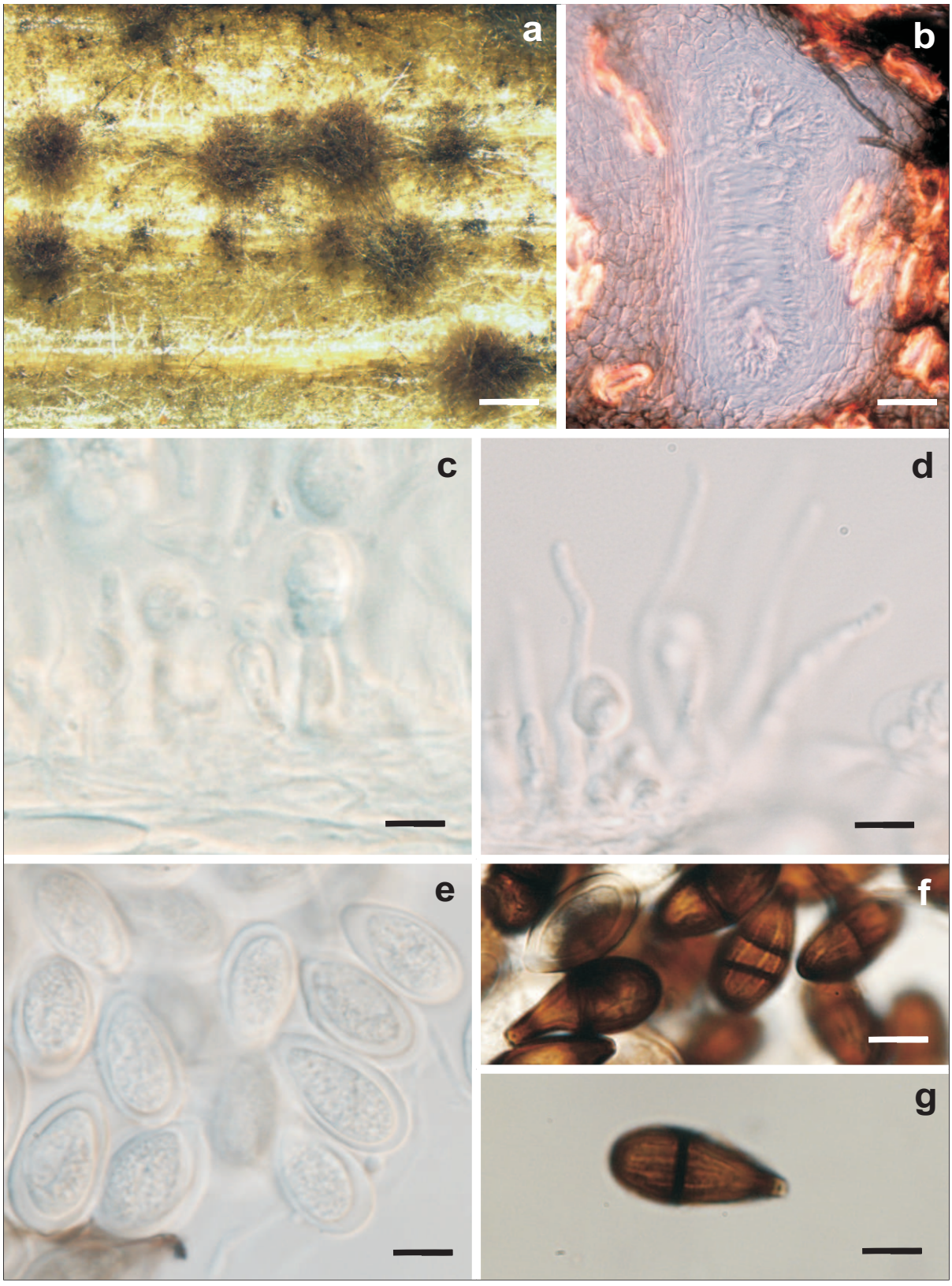

Fig.7 a–g *Lasiodiplodia* sp.1 strain 84. **a,** conidiomata on corn straw in culture. **b,** Section of conidioma. **c,** conidia developing on conidiogenous cells. **d,** paraphyses. **e,** hyaline conidia. **f**, mature conidia with two septa. **g,** mature and obpyriform conidia. Scale bars: a =1000µm; b =100µm; c – d= 4µm; e – g = 15µm.

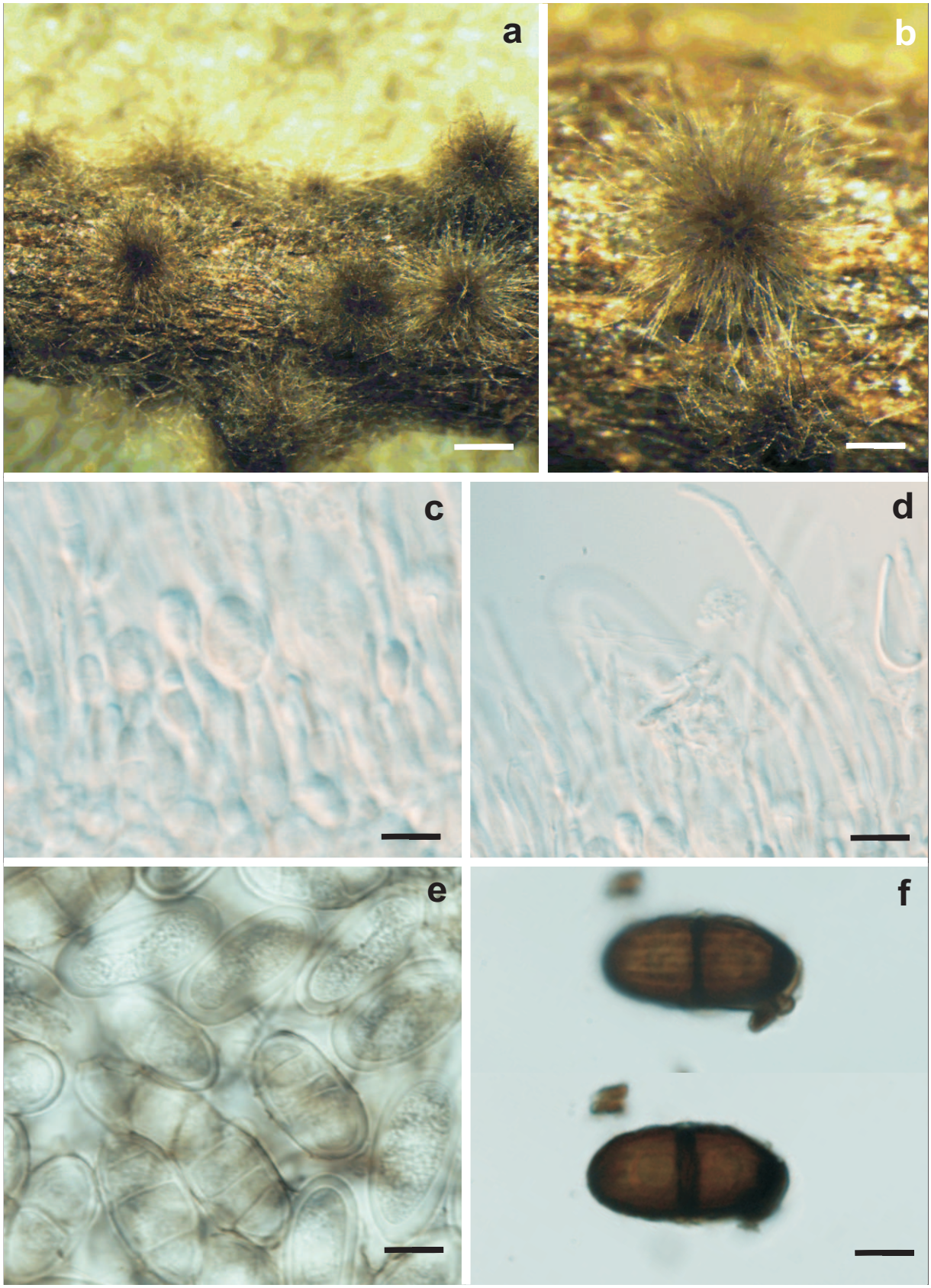

Fig.8 a–f *Lasiodiplodia* sp.2 strain 116. **a–b,** conidiomata on *Pinus* twigs in culture. **c,** conidia developing on conidiogenous cells. **d,** paraphyses. **e,** immature three-septate conidia. **f,** mature conidia in two different focal planes to show the longitudinal striations. Scale bars: a =1000µm; b =500µm; c – d= 4µm; e – f= 15µm.

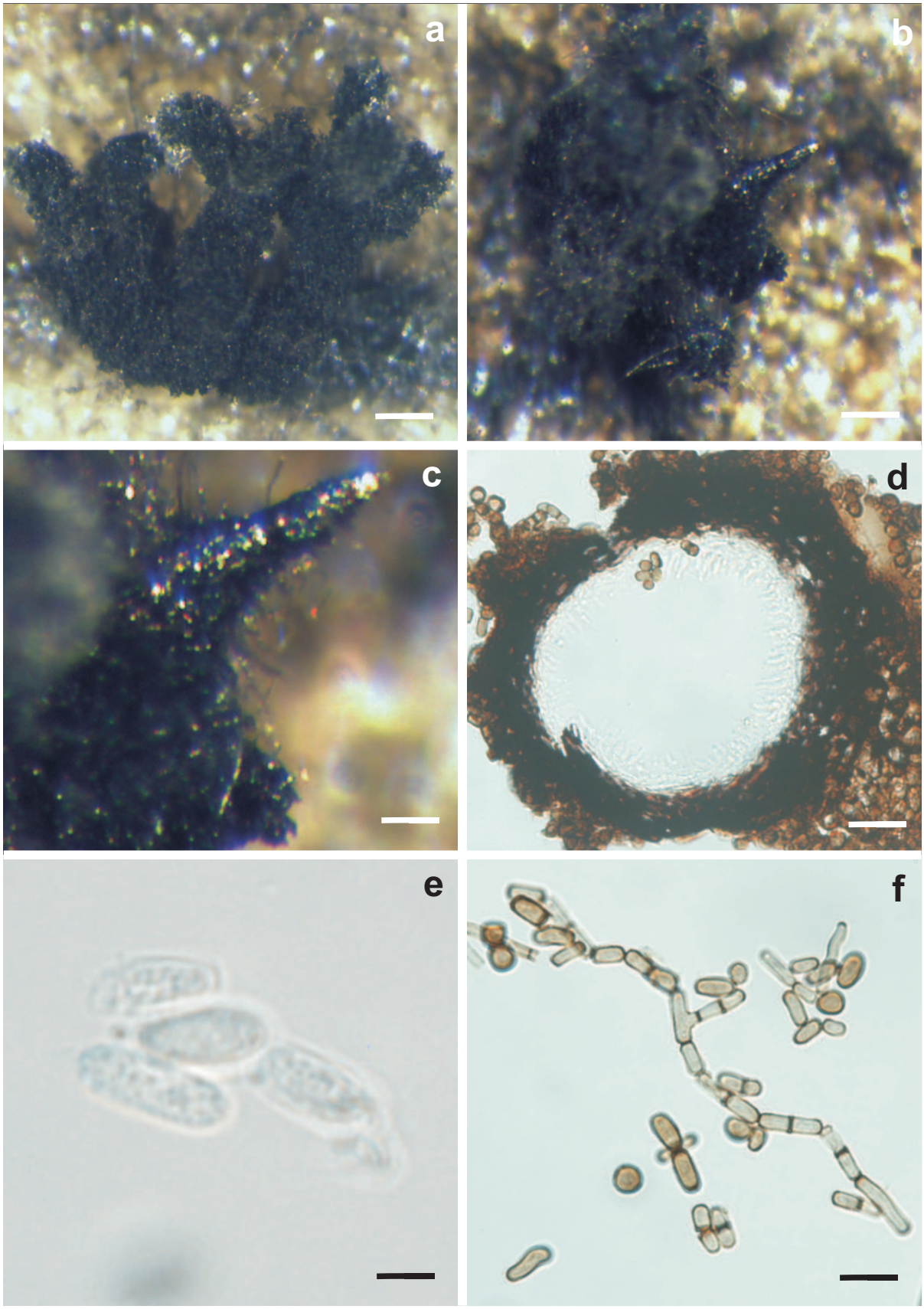

Fig.9 a–f *Neoscytalidium dimidiatum* strain 77. **a,** stroma on corn straw in culture. **b,** conidiomata forming on the stroma. **c,** conidioma. **d,** section of conidioma. **e,** hyaline conidia. **f,** arthroconidia. Scale bars: a – b =1000µm; c =20µm; d – f = 10µm.

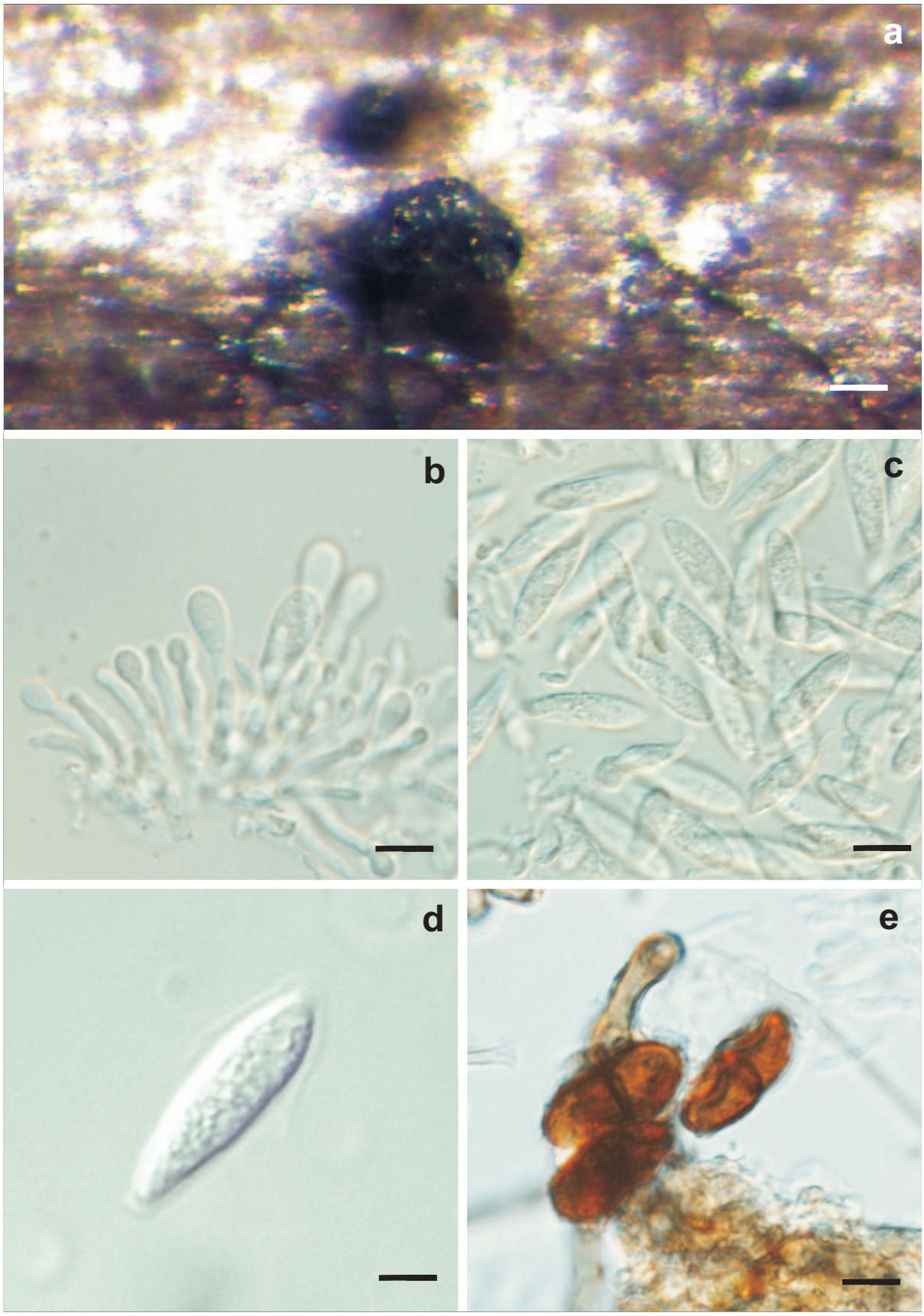

Fig.10 a–e *Macrophomina phaseolina* strain 70. **a,** conidiomata on *Pinus* twigs in culture. **b,** conidia developing on conidiogenous cells. **c – d,** immature conidia. **e,** mature conidia. Scale bars: a =100µm; b–c =10µm; d – e = 5µm.

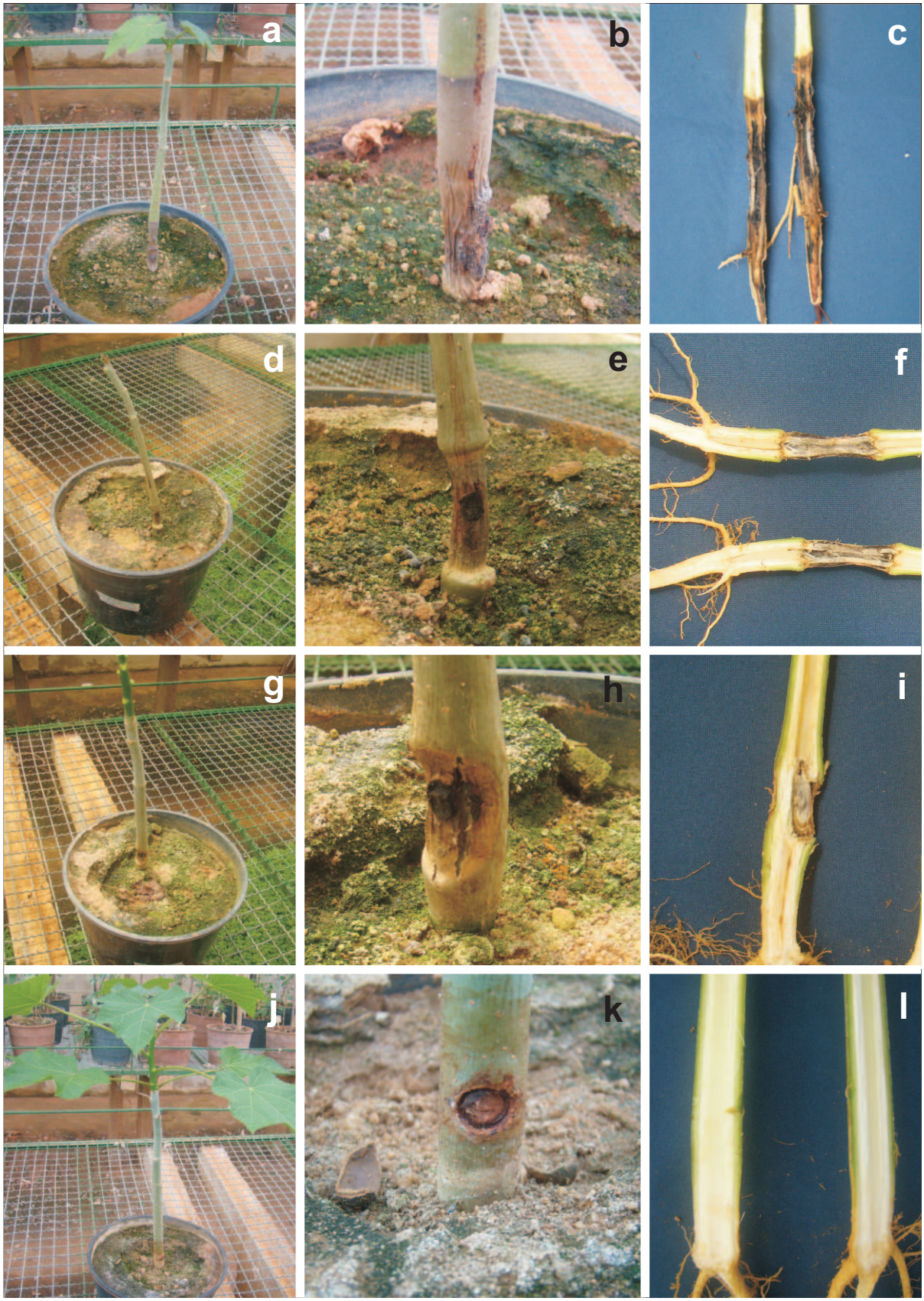

Fig.11 Pathogenicity tests results. **a–c,** Symptoms caused by *Neoscytalidium dimidiatum*. **d–f,** Symptoms caused by *Lasiodiplodia theobromae*. **g–i,** Symptoms caused by Lasiodiplodia sp.1. **j–l,** control plants inoculated with PDA plugs.