RAFAEL FERREIRA ALFENAS

TAXONOMY AND BIOLOGY OF *Calonectria* IN BRAZIL

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

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APROVADA: 29 de maio de 2013

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Prof. Luis Cláudio Vieira da Cunha

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Dr. Fabiano Branco Rocha

Prof. Olinto Liparini Pereira
(Orientador)

To my parents, Acelino Couto Alfenas and Rita de Cássia Ferreira Alfenas

to my wife, Gabriela Piccolo Maitan-Alfenas

I DEDICATE

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Special thanks to my father Prof. Acelino Couto Alfenas for his friendship, enthusiasm, patience, for the uncountable opportunities, and for teaching me the exciting science of Forest Pathology.

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BIOGRAFIA

RAFAEL FERREIRA ALFENAS, filho de Acelino Couto Alfenas e Rita de Cássia Ferreira Alfenas, nasceu em 14 de junho de 1983, em Viçosa, Minas Gerais.

Em 2003, iniciou o Curso de Engenharia Florestal da Universidade Federal de Viçosa (UFV), quando foi bolsista de Iniciação Científica no Departamento de Fitopatologia, na área de Patologia Florestal, sob a orientação do Professor Acelino Couto Alfenas.

Em março de 2008, iniciou o curso de Mestrado em Fitopatologia na mesma Universidade, sob a orientação do Professor Olinto Liparini Pereira, concluindo sua dissertação intitulada "Produção de inóculo de Cylindrocladium pteridis sob condições controladas", em outubro de 2009.

Em 25 de agosto de 2009, foi aprovada a sua mudança de nível a partir do mês de setembro de 2009 passando do mestrado para o doutorado com defesa de dissertação. Assim, a partir de setembro, ingressou no Programa de Pós-Graduação, em nível de Doutorado em Fitopatologia da UFV, sob a orientação do Professor Olinto Liparini Pereira. Em maio de 2012 foi realizar parte (1 ano) do doutorado no instituto de pesquisa CBS-KNAW fungal biodiversity centre sob orientação do Prof. Pedro Crous. Regressou ao Brasil em maio de 2013 para submeter à defesa da tese.

SUMÁRIO

RESUMO

ALFENAS, Rafael Ferreira, D. Sc., Universidade Federal de Viçosa, maio de 2013. **Taxonomia and Biologia de Calonectria no Brazil**. Orientador: Olinto Liparini Pereira. Co-Orientadores: Pedro W. Crous e Acelino Couto Alfenas.

Espécies do gênero Calonectria (= Cylindrocladium) são importantes patógenos em uma ampla gama de plantas hospedeiras, principalmente em regiões de climas tropical e subtropical. A maioria das doenças causadas por espécies de Calonectria ocorre em plantas das famílias Fabaceae (Acacia spp.), Myrtaceae (Eucalyptus spp.) e Pinaceae (Pinus spp.). Atualmente, 76 espécies de Calonectria são reconhecidas com base, principalmente, em morfologia e inferência filogenética. Estudos filogenéticos com este grupo de fungos foram baseados em sequências do gene β-tubulina (TUB2). Entretanto fragmentos da região gênica calmodulina (CAL) e fator de elongação 1-alpha (TEF-1α) tem apresentado melhor resolução para a identificação de novas espécies em Calonectria. Baseado em análise filogenética de sequências de TEF-1α de 1017 isolados obtidos de amostras de plantas e solo em diferentes regiões do Brasil, demonstrou-se que as espécies de Calonectria estudadas pertencem a seis complexos. Dentre esses, C. pteridis, C. brassicae, C. morganii, C. scoparia e C. naviculata contem espécies novas, sendo que o primeiro (87%) predomina nas plantações de eucalipto no Brasil. De acordo com as análises filogenéticas multigênicas de β-tubulina (TUB), histona H3 (HIS3), calmodulina (CAL) e fator de elongação (TEF-1α) e com características morfológicas foram descritas 28 novas espécies de Calonectria. Além disso, 17 espécies de eucalipto (Eucalyptus e Corymbia) foram avaliadas quanto à resistência à mancha de calonectria. Eucalyptus aglomerata, E. brassiana, E. saligna e E. scias foram as mais resistentes, e as espécies E. tereticornis, E. pilularis, C. maculata, E. grandis, E. dunii e C. citriodora foram as mais suscetíveis.

ABSTRACT

ALFENAS, Rafael Ferreira, D. Sc., Universidade Federal de Viçosa, May 2013. **Taxonomy and Biology of Calonectria in Brazil**. Adviser: Olinto Liparini Pereira. Co-Advisers: Pedro W. Crous and Acelino Couto Alfenas.

Species of the genus Calonectria (= Cylindrocladium) are important pathogens in a wide range of host plants, particularly in tropical and subtropical climates. Most of the diseases caused by species of Calonectria are associated mainly with plants of Fabaceae (Acacia spp.), Myrtaceae (Eucalyptus spp.) and Pinaceae (Pinus spp.). Nowadays there are about 76 species of Calonectria that are recognized based mainly on morphology and phylogenetic inference. Taxonomic studies on these fungi have chiefly relied on DNA sequences comparisons of the β-tubulin gene region. However calmodulin (CAL) and translation elongation factor 1-alpha (TEF-1α) gene regions have been shown best resolution for the identification of new species in Calonectria. Based on TEF-1α sequence analysis of 1017 isolates collected from samples of plants and soils in different regions of Brazil, it was demonstrated that Calonectria spp. studied belong to six complexes. Among these C. pteridis, C. brassicae, C. morganii, C. scoparia, and C. naviculata complexes contain 28 new species here described. Calonectria pteridis complex predominates (87%) on eucalypt plantations in Brazil. According to morphological features and multigenic phylogenetic analysis of β-tubulin (TUB2), histone H3 (HIS3), calmodulin (CAL) and the elongation factor (TEF-1α) genes 28 new Calonectria species were described. Moreover, in this study 17 species of eucalypt (Eucalyptus and Corymbia) were evaluated for resistance to calonectria leaf blight (CLB). Eucalyptus aglomerata, E. brassiana, E. saligna, and E. scias were the most resistant and E. tereticornis, E. pilularis, C. maculata, E. grandis, E. dunii, and C. citriodora the most susceptible species.

GENERAL INTRODUCTION

The genus Calonectria was first described in 1867 as the Calonectria daldiniana species by De Notari; however, based on its morphological features, this species was renamed Calonectria pyrochroa (Desmazières) by Saccardo in 1878 (Rossman, 1979). The genus Calonectria is characterized as bright perithecia, warty, with asci clavate, multi-septate ascospores, hyaline and fusiform (Figure 1). On certain occasions, but not rarely, the perithecia in the host plant tissue can be observed (Crous, 2002 Lombard et al. 2010a). Its anamorph, Cylindrocladium, was first described by Morgan (1892) and is characterized by bright sporulation, penicilate conidiophores in a stipe that ends at a characteristically shaped vesicle, and uni- or multi-septate cylindrical conidia (Figure 2) (Crous & Wingfeld 1994). Cylindrocladium (anamorph) is the most common genus in nature and is crucial to species-level identification (Peerally 1991).

Although several species of Calonectria have been described using the characteristics of the anamorph Cylindrocladium, currently use only the name Calonectria, regardless of whether the teleomorph was observed or not. Phylogenetic inferences suggest that all species are connected to Calonectria, though Cylindrocladium is its oldest name (Lombard et al., 2010a, Wingfield et al. 2012).

Calonectria species are widely distributed around the world and cause disease in a wide range of host plants in tropical and subtropical climates. The genus Calonectria is pathogenic to numerous agronomic species, such as peanuts, potatoes, peas and soybeans; forest species, such as Eucalyptus, Pinus and Acacia; and certain ornamental species (Crous, 2002, Lombard et al. 2010a)

In Brazil, different Calonectria species produce damping-off or induce leaf blight and defoliation in Eucalyptus spp. (Alfenas et al. 1979, Alfenas, 1986). Given favorable conditions in the forest economy and a great expansion in eucalypts toward hot and humid regions in the recent years, leaf blight followed by defoliation due to Calonectria in Eucalyptus spp. became the primary fungal leaf disease for eucalyptus plants in north and northeast Brazil (Alfenas et al. 2009).

This disease was first observed in commercial plants in 1970, in a large crop of Eucalyptus grandis Hill ex Maid. (Australian origin), New Era, MG, more than 80% of trees showed severe defoliation (Alfenas & Ferreira 1979). The causal agent was identified as Calonectria morganii Crous, Wingfield & Alfenas (as Cylindrocladium scoparium). Nevertheless, additional species, such as C. ovata D. Victor & Crous (= Cylindrocladium ovatum El-Gholl, Alfenas, Crous & TS Schubert), C. scoparia Peerally (= Cylindrocladium candelabrum Viégas), C. ilicicola Boedijn & Reitsma (= Cylindrocladium parasiticum by Crous, Wingfield

& Alfenas) and Cylindrocladium gracile (Bugnicourt) Boesewinkel, were also associated with eucalyptus leaf blight and defoliation in Brazil (Almeida & Bolkan 1981, Alfenas et al. 1979, Alfenas, 1986).

In the 1990s, Eucalyptus leaf blight and defoliation caused by Calonectria pteridis Crous, MJ Wingf. & Alfenas (Cylindrocladium pteridis Wolf, FA), was identified in southeast Bahia and Para causing as the basis for severe defoliation in E. grandis crops (Ferreira et al. 1995). Since then, C. pteridis has become the most common species in commercial crops, primarily in Eucalyptus camaldulensis (Dehnh.), Eucalyptus cloeziana (F. Muell.), Eucalyptus grandis (W. Hill ex. Maiden), Eucalyptus saligna, Eucalyptus tereticornis (Smith), Eucalyptus urophylla (ST Blake) and the hybrid E. grandis x E. urophylla (Alfenas et al. 2009).

Under field conditions, for most Eucalyptus species, the disease is characterized by spots that are initially small, circular or elongated and light-gray to light brown but progress and extend throughout the leaf blade and induce intense defoliation (Alfenas & Ferreira, 1979). It is believed that peeling caused by the fungus decreases timber volume due to a reduced photosynthetic area (Berger et al. 2007, Domiciano et al. 2009) and that weed growth is promoted due to light in the understory, which subjects the plants to weed competition.

Given the impact of this disease on eucalyptus crops, various control methods have been proposed to minimize loss, and, the planting of resistant genotypes is the most effective and economic way to control this disease in the field (Fonseca et al. 2010, Santos et al. 2008). However, selecting resistant genotypes is difficult because several Calonectria species may be associated with the disease. Moreover, such species were identified based solely on the anamorph's morphological characteristics (conidial dimensions and vesicle shape), which can generate taxonomic errors.

Although morphological characteristics play an important role in fungal species' descriptions (Taylor et al. 2000) and form the basis for additional fungi descriptions, which is required by the International Code of Botanical Nomenclature - ICBN (McNeill et al. 2005), DNA sequence analyses have identified various species complexes morphologically similar in Calonectria (Chen et al. 2011, Lombard et al. 2010b).

The aim of this study was to survey and identify Calonectria species in Brazil through DNA sequence analyses. This study was based on a collection of isolates obtained from plant and soil samples in different regions of Brazil. Furthermore, was also aim of this study identify and select new resistant sources to Calonectria-leaf-blight in the genera Corymbia and Eucalyptus. The aim of this study was to survey and identify Calonectria species in Brazil through DNA sequence analyses. This study was based on a collection of isolates generated from plant and soil samples in different regions of Brazil. An additional aim of this study was to identify and select new resistance sources to spot Calonectria in the genera Corymbia and Eucalyptus.

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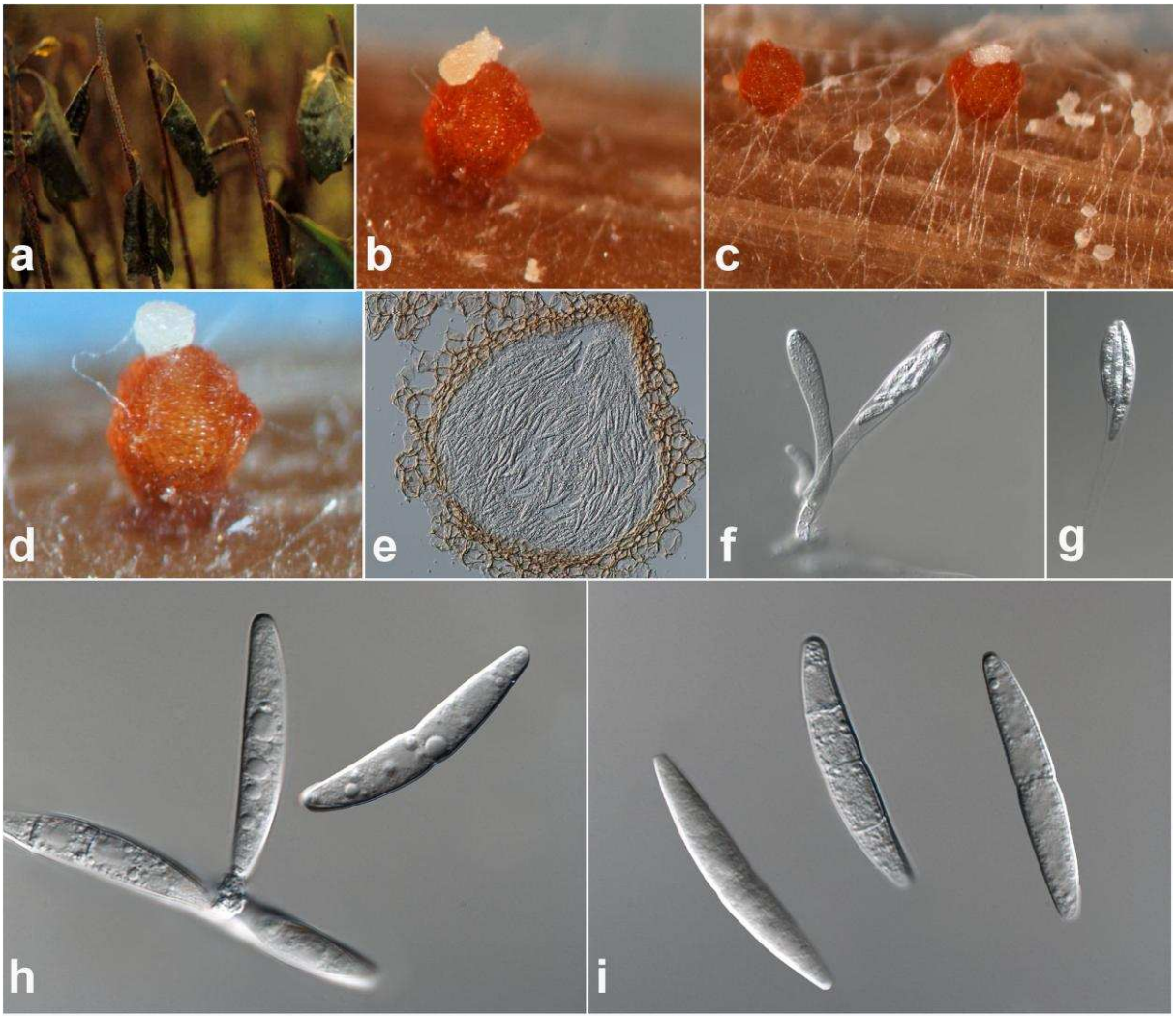

Figure 1: Typical features for Calonectria spp.: A – C: A perithecia in eucalyptus cuttings; D – I: Bright perithecia with asci, clavate hyaline ascospores and fusiform Calonectria spp.

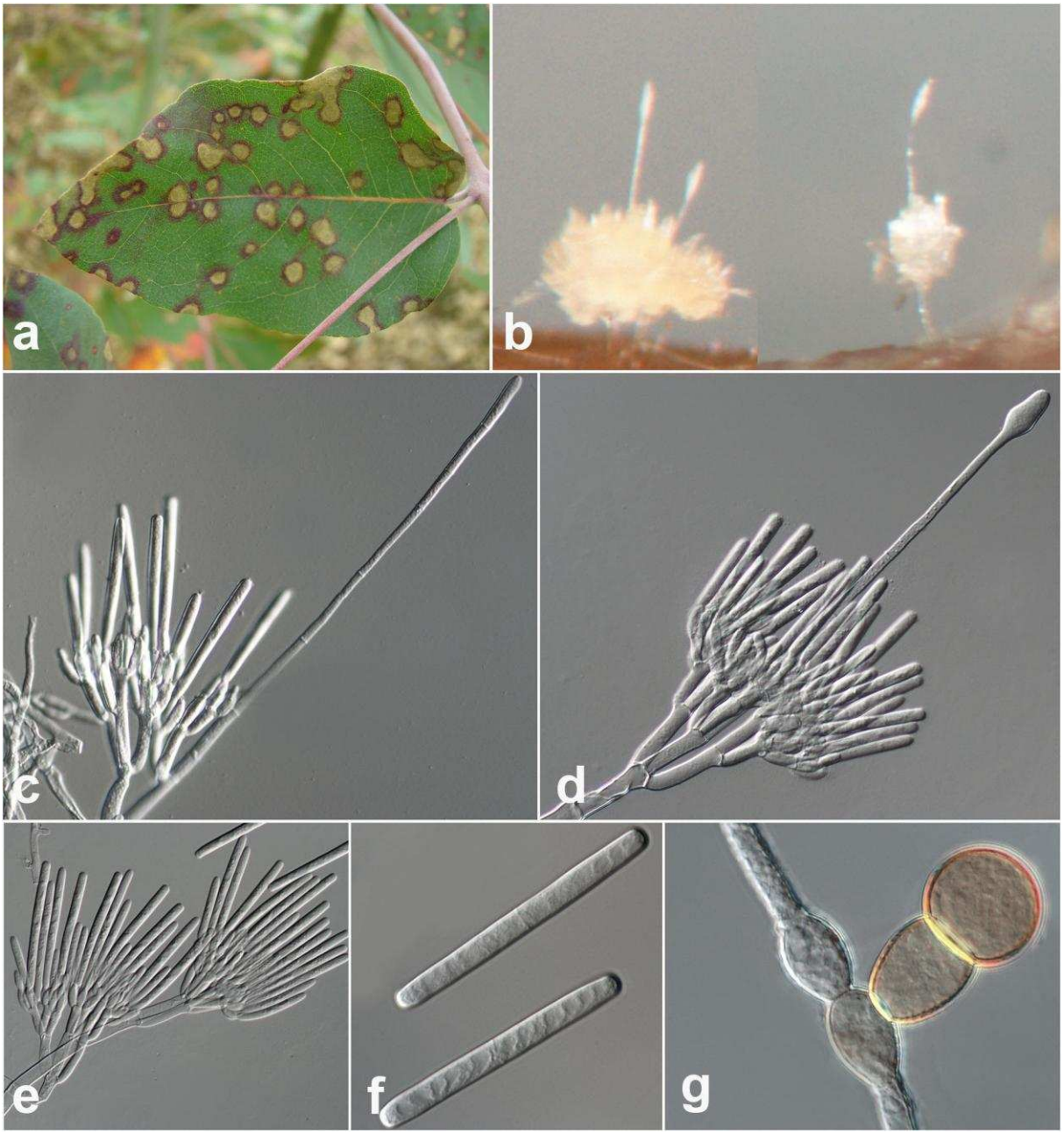

Figure 2: Disease symptoms and typical features for Cylindrocladium spp. A – B: Leaf blight on Eucalyptus dunii with typical bright esporulation; C – F: Conidiophores with a stipe that ends in a vesicle with characteristic shape, and typical cylindrical conidia; and G: Chlamydospore.

CHAPTER 1

Calonectria metrosideri, a highly aggressive pathogen causing leaf blight, root rot, and wilt of Metrosideros spp. in Brazil

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Calonectria metrosideri, a highly aggressive pathogen causing leaf blight, root rot, and wilt of Metrosideros spp. in Brazil

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Summary

The genus Metrosideros includes several tree shrub, and vine species, native to the Pacific Islands. Seedlings from 25 seed lots of Metrosideros polymorpha and two seed lots of M. tremuloides with symptoms of root rot, stem girdling, wilting, and round, purple leaf spots were observed in the Forestry Nursery at the Universidade Federal de Viçosa, Brazil. In the original disease site, seedling mortality reached up to 71 % in M. polymorpha, and 34 % in M. tremuloides. Single conidial cultures obtained from infected leaf, root and stem samples of M. polymorpha were used to identify the fungal species. Morphological characters and DNA sequences of four loci, containing partial sequences of β-tubulin (TUB2), histone H3 (HIS3), calmodulin (CAL) and the elongation factor (tef-1α) genes of three isolates indicated that they belong to a new species, described here as Calonectria metrosideri sp. nov. Potting medium infestation and inoculation of seedlings of M. polymorpha with an inoculum suspension at $1x10^4$ conidia ml^{-1} induced typical symptoms of the disease (leaf spots, root rot and wilt), similar to those observed under natural conditions. Calonectria metrosideri was re-isolated, which fulfilled Koch's postulates, and confirmed its status as a pathogen.

Keywords: Cylindrocladium, forest pathology, Ohia, Hypocreales, pathogenicity, taxonomy.

1 Introduction

Metrosideros is a genus that includes several tree, shrub, and vine species native to the Pacific Islands from the Philippines to New Zealand. Metrosideros polymorpha Gaudich, popularly known as ohia (Figure 1) is the species dominant in Hawaiian ecosystems, occupying a wide variety of habitats (Cordell et al. 1998). This species and others of this genus can be used for medicinal purposes, wood production for energy, poles, and several other uses (Friday & Herbert 2006).

In Hawaii, approximately 80 % of native forests are composed of species of Metrosideros, especially M. polymorpha (Uchida et al. 2006). In April 2005, a rust fungus (Puccinia psidii Winter), a highly damaging pathogen in myrtaceous hosts in South America, was found on plants of Metrosideros spp. in Hawaii, and this rust pathogen is considered a threat to Hawaiian forest ecosystems (Uchida et al. 2006). Subsequently, half-sib families of Hawaiian ohia seeds were germinated and grown in Brazil to assess the genetic resistance to Brazilian strains of P. psidii. However, during a routine inspection at the nursery, seedlings of M. polymorpha showing symptoms of leaf spots, defoliation, young leaf wilt, and seedling death were recorded (Figure 2). In addition, stem necrosis and girdling with root rot were observed. Seedlings with the above disease symptoms kept in a moist chamber showed intense sporulation of a Calonectria sp. on the lesions. Thus, the objective of this study was to characterize the causal agent of this disease through a combination of morphological and molecular data, and pathogenicity tests.

2 Material and methods

2.1 Sampling and fungal isolation

Samples of infected plants of M. polymorpha, containing round, purplish leaf spots, and stem cankers and root rot were collected in the Forest Nursery at the Universidade Federal de Viçosa.

The samples were kept in a moist chamber at 26 °C for 48 h. After incubation, single conidial cultures of the Calonectria sp. were obtained on Malt Extract Agar (MEA) at 26 °C for 10 days. Three selected isolates (LPF 101, LPF 103, and LPF 104), used in this study were deposited at CBS Fungal Biodiversity Institute in the Netherlands (CBS), and nomenclatural data were submitted to MycoBank (Crous et al. 2004b).

2.2 DNA extraction, amplification and purification

Mycelia of the respective isolates were scraped from colonized MEA plates, and placed separately in 2-ml -microtubes for genomic DNA extraction using the Wizard ® Genomic DNA Purification (Promega Corporation, WI, USA) kit. For PCR, the DreamTaq™ Master Mix (MBI Fermentas, Vilnius, Lithuania) was used, following the manufacturer's protocol.

Four loci, including fragments of β-tubulin (TUB2), histone H3 (HIS3), elongation factor (tef-1α) and calmodulin (CAL) gene regions were amplified using the primers T1 (O'Donnell & Cigelnik, 1997) and CYLTUB1R (Crous et al. 2004a) for TUB2, CYLH3F and CYLH3R (Crous et al. 2004a) for HIS3, EF1-728F (O'Donnell et al. 1998) and EF-2 (Carbone & Kohn 1999) for TEF-1α and CAL-228F and CAL-737R (Carbone & Kohn 1999) for CAL. Amplification was performed with an initial denaturing at 96 °C for 5 min followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 52 °C for 30 s, extension initial 72 °C for 1 min and 4 min final extension at 72 °C. The PCR product was visualized on a 2% agarose gel, to determine fragment size and purity. PCR products were purified with an ExoSAP-IT® kit, according to the manufacturer's recommended protocol (2 µL reagent per 5 µL amplified DNA product) and incubated in a thermal cycler for 15 min at 37 °C followed by an additional incubation for 15 min at 80 °C.

2.3 Sequencing and phylogenetic analysis

Sequencing was performed at the Laboratory of Genomics in the Institute of Biotechnology Applied to Agriculture (BIOAGRO) at the Universidade Federal de Viçosa,

Brazil. Sequences quality was checked by means of Sequence Scanner Software v. 1.0 (Applied Biosystems, Foster City, California, United States), and edited using the software package SeqMan from DNAStar Inc. Madison, Wisconsin, USA (www.DNASTAR.com). All sequences were manually corrected and the arrangement of nucleotides in ambiguous positions was corrected using the sequences of primers in the forward and reverse direction. New sequences derived from this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) and other sequences used in phylogenetic analysis were obtained from GenBank (Table 1).

Consensus regions were compared in the GenBank database using the Mega BLAST program. Based on the results of the BLAST, new sequences were added to the alignment of Lombard et al. (2011). All sequences were assembled in the MAFFT v. 6 online version (http://mafft.cbrc.jp/alignment/server/) (Katoh & Toh 2010) and aligned sequences were then manually corrected when necessary using MEGA v. 5 (Tempe, Arizona, USA) (Tamura et al. 2011). Spaces (gaps) (insertions / deletions) were treated as absent.

PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Sunderland, Massachusetts, USA; Swofford 2002) was used to analyze the DNA sequence data sets. A partition homogeneity test (Farris et al. 1994) and a 70 % reciprocal bootstrap method (Gueidan et al. 2007) were applied to determine whether the data sets were consistent and combinable. Phylogenetic relationships were estimated by heuristic searches based on 1,000 random addition sequences and tree bisection-reconnection, with the branch swapping option set on ‘best trees’ only. All characters were weighed equally and alignment gaps were treated as missing data. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). Bootstrap analyses (Hillis & Bull 1993) were based on 1,000 replications.

Analysis of Bayesian Inference (BI) was performed using the algorithm of Markov chain Monte Carlo (MCMC) and the model of nucleotide substitution used was determined using the MrModeltest v. 2.3 (Nylander 2004). The models were estimated separately for each gene region. The likelihood values were calculated and the model was selected according to Akaike Information Criterion (AIC). BI analysis was completed with MrBayes v. 3.1.1 (Ronquist & Heulsenbeck 2003) with 10 million random generations. Trees were sampled at every 1,000 generations, resulting in 10,000 trees. The first 2,500 trees were discarded from the analysis. The posterior likelihood values (Rannala & Yang 1996) were determined using the consensus tree. The convergence of the log likelihood was analyzed using the software

147 TRACER v. 1.5 (Auckland, New Zealand; Rambaut & Drummond 2009) and no indication of
148 lack of convergence was detected. Calonectria colombiensis Crous and Calonectria chinensis
149 (Crous) L. Lombard, M.J. Wingf. & Crous were used as outgroups in the analysis.
150
151 **2.4 Morphological characterization**
152 Single conidial cultures were grown on synthetic nutrient poor agar (SNA) (Nirenburg
153 1981) at 26 °C, following the protocols set for Calonectria by Lombard et al. (2009, 2010 b,
154 c). After 7 days of incubation, the morphological characteristics were determined by
155 mounting fungal structures in clear lactic acid and 30 measurements at ×1,000 magnification
156 were determined for each isolate using a Zeiss Axioscope 2 microscope (Jena, Germany) with
157 differential interference contrast (DIC) illumination. The 95 % confidence levels were
158 determined and extremes of conidial measurements are given in parentheses. For other
159 structures, only extremes are presented.
160
161 **2.5 Pathogenicity**
162 Because the undescribed Calonectria species was isolated from infected M.
163 polymorpha, this plant was selected to confirm pathogenicity. For this test, 10 seedlings were
164 spray-inoculated with a conidial suspension at $1x10^4$ ml^{-1} of each isolate, as described by
165 Graça et al. (2009). Potting medium (Mec Plant®substrate, Telêmaco Borba, Paraná, Brazil)
166 supporting 10 healthy seedlings was also infested by adding 30 ml of the same conidial
167 suspension in each of the four holes made around each plant. Five plants treated with distilled
168 water served as control. The development of symptoms was monitored daily for 10 days.
169
170 **2.6 Source of inoculum**
171 To determine the inoculum source of inoculum, samples of irrigation water and
172 samples of unused/ used substrate were tested for the presence of the pathogen using the
173 castor bean leaf bio-baiting method (Gonçalves et al. 2001).
174
175 **2.7 Disease progress**
176 Disease progress was evaluated on plants growing at the original nursery where the
177 disease was discovered by counting the number of wilted or dead plants at biweekly intervals
178 from April to July, 2010.
179

3 Results

3.1 Phylogenetic analysis

Amplicons of approximately 450 bases for HIS3 and 500 bases each for TUB2, TEF-1α and CAL were generated. Based on preliminary tef-1*α* sequence analyses with 49 taxa including outgroups (Figure 3), the multigene analysis was performed with closely related species, which belong to the Calonectria scoparia complex.

The combined sequence analysis was performed with 18 taxa, including outgroups. Comparing the tree topologies of the 70 % reciprocal bootstrap trees indicated no conflicts. Subsequently, the data sets were combined and this resulted in a data set consisting of 1,899 characters including gaps. Of these 1,634 were constant and parsimony uninformative and 295 were parsimony informative. Analysis of the 295 parsimony informative characters yielded four equally most parsimonious trees (TL = 561, CI = 0.904, RI = 0.911, RC = 0.823). Evolution models HKY + I for TUB2 and CAL, a GTR + G for HIS3 and tef-1*α* were selected and incorporated into the Bayesian analysis.

The preliminary tree performed with tef-1*α* can distinguish Calonectria scoparia complex from the other Calonectria complexes (C. variabilis and C. mexicana), however, it is not useful for separating C. metrosideri from other species within the C. scoparia complex (Figure 3). The newly described C. metrosideri can be distinguished from other Calonectria spp. within the C. scoparia complex using an additional three loci (HIS3, TUB2, and CAL). The multigene analysis formed a distinct and well-supported clade close to but distinct from C. pseudoscoparia and C. scoparia (Figure 4).

3.2 Taxonomy

Based on the DNA sequence data and morphological features of the anamorph, we conclude that the Calonectria isolates from M. polymorpha represent an undescribed new species, described below as follows:

Calonectria metrosideri R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. MycoBank MB 802511 (Figure 5)

Etymology: In reference to the genus Metrosideros, from which the fungus was isolated.

Hosts: Metrosideros polymorpha

Distribution: Brazil.

Specimens examined: Brazil, Minas Gerais state, Viçosa, on Metrosideros polymorpha, April, 2010; Rafael F. Alfenas (CBS H-21146 holotype of Calonectria metrosideri, culture ex-type **CBS 133603**)

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 40–105 × 4–7 µm; stipe extensions septate, straight to flexuous, 90–170 µm long, 2–4 µm wide at the apical septum, terminating in spathulate to obpyriform vesicles, 5–9 µm diam (abnormal bifurcate vesicles frequently observed). Conidiogenous apparatus 40–65 µm long, 60–75 µm wide; primary branches aseptate, 18–30 × 4–5 µm; secondary branches aseptate, 18–22 × 3–4 µm; tertiary and additional branches (–4), aseptate, 8–15 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides elongate-doliiform to reniform, hyaline, aseptate, 8–11 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (40–)44–46(–51) × 3–5 µm (av. = 45 × 4 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Notes: Calonectria metrosideri (conidia av. 45 × 4 µm) can be distinguished from C. scoparia (conidia av. 60 × 4.5 µm) and C. pseudoscoparia (conidia av. 48 × 4 µm) based on smaller macroconidia and on being phylogenetically distinct. Mating tests resulted in no successful matings, suggesting that the fungus is either heterothallic, with no compatible tester strains found, or has lost the ability for sexual mating.

Culture characteristics: Rapid growth (50–55 mm) diam after 10 days at 25 ºC on Malt Extract Agar (MEA), aerial mycelial and sporulation sparse; chlamydospores forming brown, thick-walled microsclerotia.

3.3 Pathogenicity

As observed in the nursery under natural infection, spray-inoculated plants showed leaf spots, and seedlings grown in pathogen-infested substrate exhibited root rot and wilt symptoms, and eventually died. Intense defoliation was also found with spray-inoculated plants.

3.4 Source of inoculum

Irrigation water and potting medium were Calonectria free. However, used substrate produced pathogen colonization in 6.3 % of castor bean leaf baits. These results indicate that neither the irrigation water nor the substrate were the primary inoculum source of the fungus.

3.5 Disease progress

The number of infected plants of M. polymorpha and M. tremuloides increased significantly over time, reaching up 71 % and 34 % of diseased seedlings, respectively, in about four months. Higher disease levels occurred on Meterosideros polymorpha compared to M. tremuloides (Figure 6).

4 Discussion

To characterize the causal agent of the Meterosideros disease, isolates of a Calonectria sp. obtained from infected plants were identified as a phylogenetically undescribed species. This is described here as Calonectria metrosideri sp. nov., which is closely related to the C. scoparia complex (Schoch et al. 1999, 2001). This complex includes C. pauciramosa C.L. Schoch & Crous, C. scoparia Peerally, C. mexicana CL Schoch & Crous, C. spathulata El-Gholl, Kimbr., E.L. Barnard, Alfieri & Schoult, and C. insularis Schoch & Crous (Schoch et al. 1999). More recently, Lombard et al. (2011, 2010b) added a further five species to this complex, namely C. zuluensis Lombard, Crous & MJ Wingf., C. polizzi Lombard, Crous & MJ Wingf., C. colombiana L. Lombard, Crous & MJ Wingf., C. pseudomexicana L. Lombard, G. Polizzi & Crous and C. tunisiana L. Lombard, G. Polizzi & Crous. Species of C. scoparia sensu lato are characterized by having obpyriform (= as spathulate) to ellipsoidal vesicles, as well as uniseptate conidia (Schoch et al. 1999).

Although C. metrosideri is phylogenetically and morphologically close to C. scoparia and C. pseudoscoparia, it grouped in a well-supported, distinct clade. Furthermore, it also has smaller conidia than the latter two species. Currently, identification of species based on phylogenetic inference has shown that many species of plant pathogens represent a species complex (Crous & Groenewald, 2005; Hyde et al. 2010). The problem is that sometimes the phylogenetic species concept is not correlated with morphology, and the boundaries of separation between taxa remains unclear. In some cases, the separation of two or more groups of isolates as distinct taxa may occur, but in fact they could belong to the same species (Summerell et al. 2010). Therefore, before assigning isolates to a new species, it is necessary

to find robust differences by employing additional techniques (Summerell et al. 2010), as done in the present work.

Recent studies describing novel species of Calonectria, have employed a combination of the phylogenetic and morphological species concepts (Lombard et al. 2010a-c). The difficulty of only adopting the biological species concept in Calonectria, is that some isolates of different phylogenetically related species (C. hawksworthii, C. insulare and C. scoparium) can interbreed and produce fertile progeny.

Recently, Lombard et al. (2011) also described two new species of Calonectria from Metrosideros sp. (C. pseudomexicana and C. tunisiana) and underlined the importance of phytosanitary and quarantine measures, to prevent the introduction of these species into Hawaii. Calonectria metrosideri differs phyllogenetically and morphologically from C. pseudomexicana and C. tunisiana, which also have wider conidia and broadly ellipsoidal vesicles.

Although the present description is based on characteristics of the anamorph (Cylindrocladium), the new species from ohia is named in the genus Calonectria, since all species of Cylindrocladium are phylogenetically connected to Calonectria. Moreover, the oldest name prevails (Crous 2002, Crous et al. 2004a, 2006, Schoch et al. 1999) and the use of Calonectria is being adopted in proposals of new species, even when the sexual state is not observed (Lombard et al. 2010a, 2011, Wingfield et al. 2012).

All three isolates (CBS133603, CBS133604 and CBS133605) of C. metrosideri tested were pathogenic and induced disease symptoms in seedlings of M. polymorpha similar to those observed in the nursery under natural infection. However, wilted and dead plants were only observed when the potting medium was infested with inoculum of the pathogen. In this case, the fungus infects the root system and induces seedling wilt. Species of Calonectria are soil-borne pathogens (Crous 2002). In a eucalypt cutting nursery, Calonectria spp. and other pathogens are spread and infect healthy plants mainly from inoculum in contaminated water (Mafia et al. 2008), infested substrate, tubes and scissors, as well as infected shoots used to make cuttings (Alfenas et al. 2009). In this study we confirmed that the irrigation water and the potting medium used for growing ohia plants were pathogen inoculum free at the time of testing. Therefore, contaminated pots were probably the primary inoculum source of C. metrosideri on ohia. This conclusion is based on the fact that once pots were subjected to a hot water treatment (80 °C min^{-1}) (Alfenas et al. 2009), the disease was successfully controlled.

As observed among and within species of Eucalyptus susceptible to leaf blight caused by Calonectria pteridis (Alfenas et al. 2009; Zarpelon et al. 2011), indications of differences in resistance to C. metrosideri infection were also observed between M. polymorpha and M. tremuloides. However, in nurseries, cultural practices aiming to eradicate the sources of inocula and to reduce the environmental conditions favourable to infection are the most important forms of disease control, whereas breeding for resistance is more applicable to the establishment of plantations or replacement of forest trees.

The description of C. metrosideri sp. nov. represents a novel species for Brazil. The rapid progress of this disease indicates the high aggressiveness of this pathogen, and the urgent need for control methods, especially cultural practices, to minimize losses from the disease in forest nurseries.

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Figure 1: Meterosideros polymopha in natural stands: A – Adult trees; B – Flowering plants; C – Typical red flowers. (Photos: Forest & Kim Starr, Starr Environmental, Bugwood.org).

435 Table 1: Accession numbers, Calonectria species, Gene regions sequenced of Calonectria spp., and Host/Substrate Columns.

Isolate number	Species[1]	GenBank accession nr[2]				Host/substrate
		β - tubulin (TUB2)	Histone 3 (HIS3)	Elongation factor (tef-*1α*)	Calmodulin (CAL)	
CBS 230.30	C. brasiliensis	GQ267241	GQ267259	GQ267328	GQ267421	Eucalyptus sp.
CBS 114257	C. brasiliensis	GQ267242	GQ267260	GQ267329	GQ267422	Leaf litter
CBS 123693	C. cerciana	FJ918510	FJ918528	FJ918559	GQ267369	E. grandis × urophylla
CBS 123695	C. cerciana	FJ918511	FJ918529	FJ918560	GQ267370	E. grandis × urophylla
CBS 112744	C. chinensis	AY725618	AY725660	AY725709	AY725746	Soil
CBS 115127	C. colombiana	FJ972423	FJ972442	FJ972492	GQ267455	Soil
CBS 115638	C. colombiana	FJ972422	FJ972441	FJ972491	GQ267456	Soil
CBS 112220	C. colombiensis	GQ267207	AY725662	AY725711	AY725748	Soil
CBS 111870	C. hawksworthii	AF333407	DQ190649	FJ918558	GQ267386	Nelumbo nucifera
CBS 114558	C. insularis	AF210861	FJ918526	FJ918556	GQ267389	Soil
CBS 114559	C. insularis	AF210862	FJ918525	FJ918555	GQ267390	Soil
CBS 109166	C. leucothoës	FJ918508	FJ918523	FJ918553	GQ267392	Leucothoë axillaris
CBS 110918	C. mexicana	AF210863	FJ972460	FJ972526	GQ267396	Soil
CBS 1303533	C. mexicana	JN607280	JN607265	JN607295	-	Dodonaea viscosa
CBS 110666	C. morganii	FJ918509	FJ918527	FJ918557	GQ267423	Ilex vomitoria
CBS 119669	C. morganii	DQ521599	DQ521601	GQ421796	-	Pistacia lentiscus
CMW 5683	C. pauciramosa	FJ918514	FJ918531	FJ918565	GQ267405	E. grandis
CPC 416	C. pauciramosa	FJ918515	FJ918532	FJ918566	GQ267404	E. grandis
CBS 123402	C. polizzii	FJ972419	FJ972438	FJ972488	-	Arbutus unedo
CBS 125270	C. polizzii	FJ972417	FJ972436	FJ972486	GQ267461	Callistemon citrinus
CBS 1303513	C. polizzii	JN607270	JN607255	JN607285	-	Myrtus communis
CBS 1303523	C. polizzii	JN607275	JN607260	JN607290	-	Metrosideros thomasii
DISTEF-TMC2	C. polizzii	JN607269	JN607254	JN607284	-	Myrtus communis
DISTEF-TMEA1	C. polizzii	JN607272	JN607257	JN607287	-	Metrosideros excelsa cv. Aurea
DISTEF-TMN3	C. polizzii	JN607274	JN607259	JN607289	-	Metrosideros sp.
CBS 1303543	C. pseudomexicana	JN607281	JN607266	JN607496	-	Callistemon sp. (rouge)
CBS 1303553	C. pseudomexicana	JN607282	JN607267	JN607497	-	Callistemon sp. (rouge)

436

Table 1: (Continued).

Isolate number	Species[1]	GenBank accession nr.[2]				Host/substrate
		β - tubulin (TUB2)	Histone 3 (HIS3)	Elongation factor (tef-*1α*)	Calmodulin (CAL)	
DISTEF-TCROU4	C. pseudomexicana	JN607283	JN607268	JN607498	-	Callistemon sp. (rouge)
CBS 125256	C. pseudoscoparia	GQ267228	GQ267277	GQ267348	GQ267440	E. grandis
CBS 125257	C. pseudoscoparia	GQ267229	GQ267278	GQ267349	GQ267441	E. grandis
CPC 1675	C. scoparia	FJ972426	FJ972476	FJ972525	GQ267367	Eucalyptus sp.
CPC 1679	C. scoparia	GQ421779	GQ267246	GQ267298	GQ267368	Eucalyptus sp.
CBS 133603	C. metrosideri sp. nov	KC294313	KC294307	KC294310	KC294304	Metrosideros polymorpha (leaf)
CBS 133604	C. metrosideri sp. nov	KC294314	KC294308	KC294311	KC294305	Metrosideros polymorpha (leaf)
CBS 133605	C. metrosideri sp. nov	KC294315	KC294309	KC294312	KC294306	Metrosideros polymorpha (root)
CBS 112689	C. spathulata	AF308463	FJ918524	FJ918554	GQ267426	E. viminalis
CBS 555.92	C. spathulata	GQ267215	GQ267261	GQ267331	GQ267427	Araucaria angustifolia
CBS 125248	C. sulawesiensis	GQ267223	GQ267272	GQ267343	GQ267435	Eucalyptus sp.
CBS 125253	C. sulawesiensis	GQ267220	GQ267269	GQ267340	GQ267432	Eucalyptus sp.
CBS 1303563	C. tunisiana	JN607277	JN607262	JN607292	-	Callistemon sp. (rouge)
CBS 1303573	C. tunisiana	JN607276	JN607261	JN607291	-	Callistemon laevis
DISTEF-TCV1	C. tunisiana	JN607278	JN607263	JN607293	-	Callistemon viminalis
DISTEF-TCROS4	C. tunisiana	JN607279	JN607264	JN607294	-	Callistemon sp. (rosè)
DISTEF-TME1	C. tunisiana	JN607271	JN607256	JN607286	-	Metrosideros excelsa
DISTEF-TMN1	C. tunisiana	JN607273	JN607258	JN607288	-	Metrosideros sp.
CBS 112691	C. variabilis	GQ267240	GQ267264	GQ267335	GQ267458	Eucalyptus sp.
CBS 114677	C. variabilis	AF333424	GQ267263	GQ267334	GQ267457	Eucalyptus sp.
CMW 9188	C. zuluensis	FJ972414	FJ972433	FJ972483	GQ267459	Eucalyptus sp.
CMW 9896	C. zuluensis	FJ972415	FJ972434	FJ972484	GQ267460	Eucalyptus sp.

[1]CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands; CPC: Cultures of Pedro Crous housed at CBS; LPF: Laboratory of Forest Pathology, DFT-UFV, Viçosa, Minas Gerais, Brazil; isolate number in **bold** were sequenced in this study; [2] GenBank Accession Number. – No sequences in Genbank.

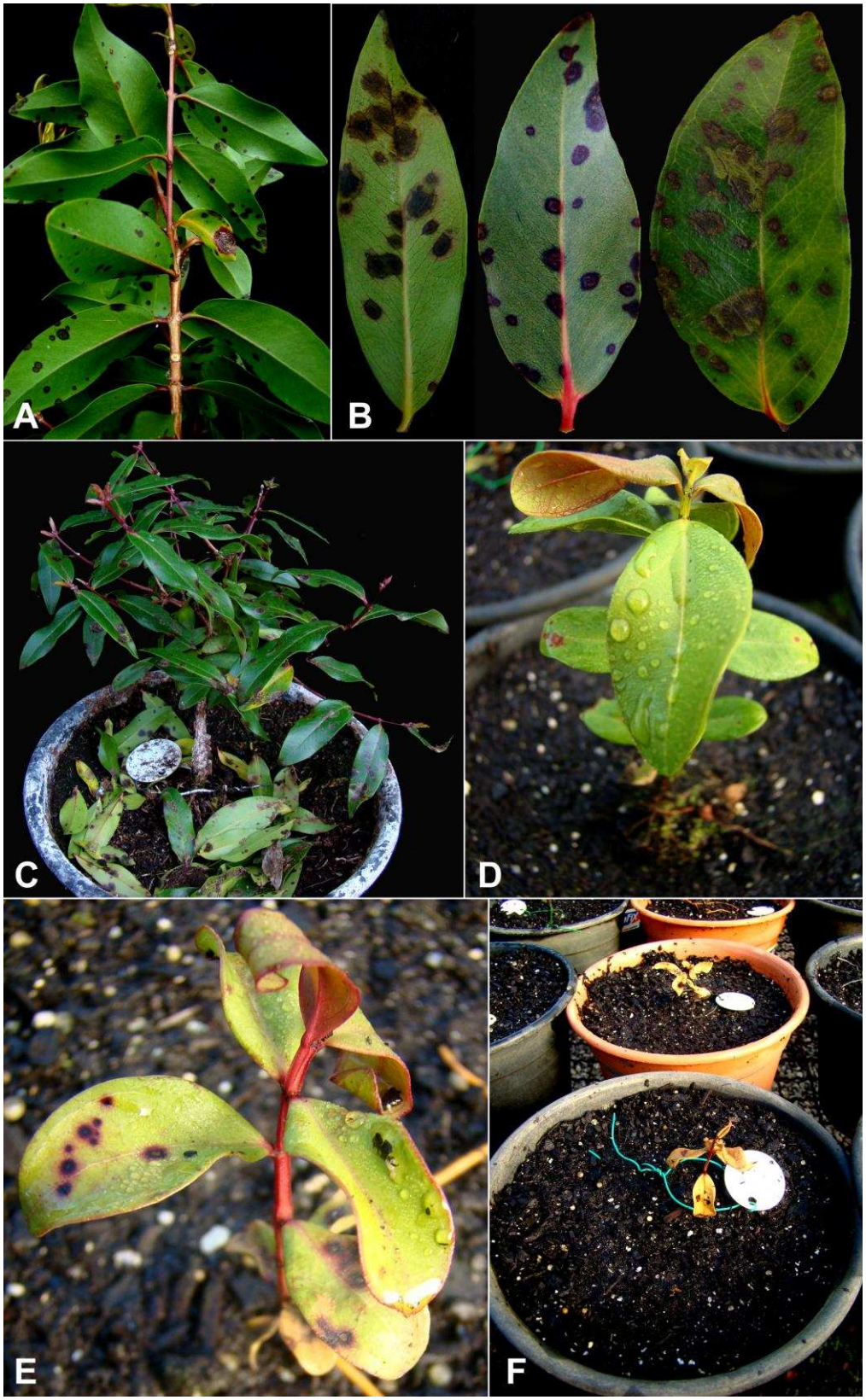

Figure 2: Incidence of Calonectria metrosideri on seedlings of Metrosideros polymorpha in nursery. A – B: Leaf spots; C – Defoliation; D – E: Wilting of young leave on seedlings; F: Dead seedlings.

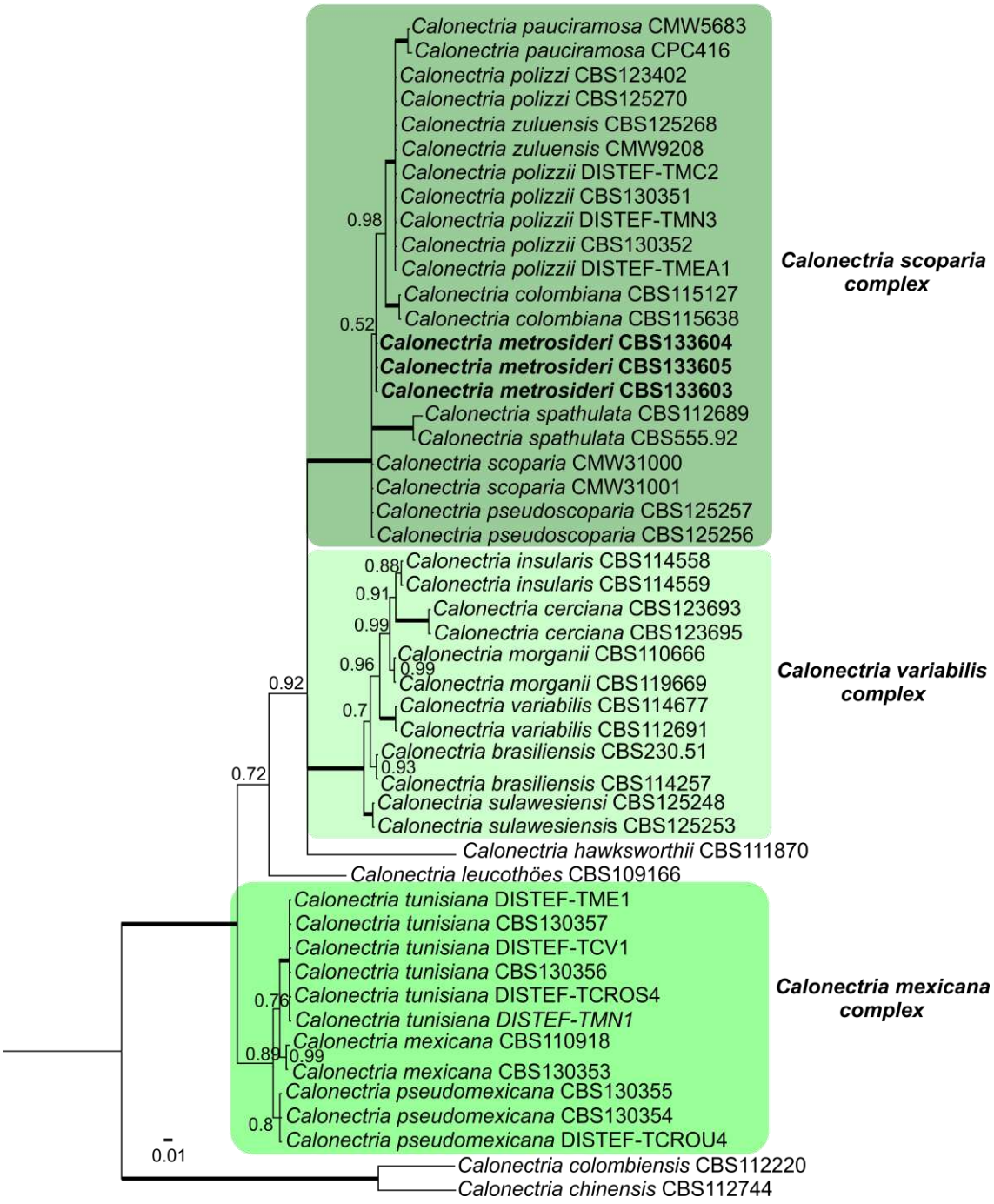

Figure 3: Phylogenetic tree obtained by Bayesian inference using sequences of translation elongation factor 1α sequence alignments of the Calonectria isolates. The bold lines indicate posterior probability values of 1.00. The tree was rooted to C. chinensis (CBS 112744) and C. colombiensis (CBS 112220). Isolates in bold were obtained during the survey.

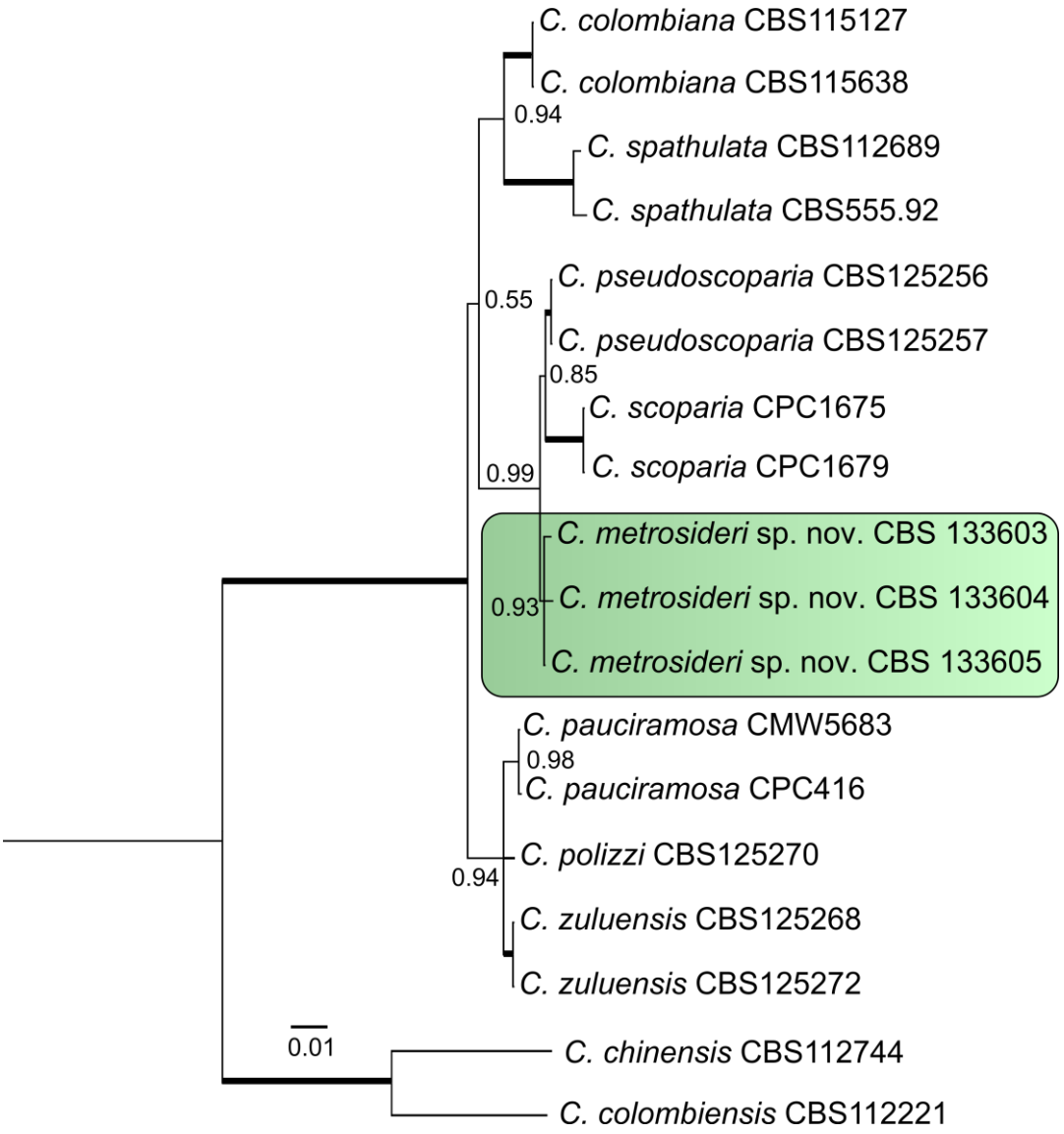

Figure 4: Phylogenetic tree obtained by Bayesian inference using combined sequences of β-tubulin, histone H3, translation elongation factor 1α and calmodulin sequence alignments of Calonectria isolates. The bold lines indicate posterior probability values of 1.00. The tree was rooted to C. chinensis (CBS 112744) and C. colombiensis (CBS 112220). Isolates in bold were obtained during the survey.

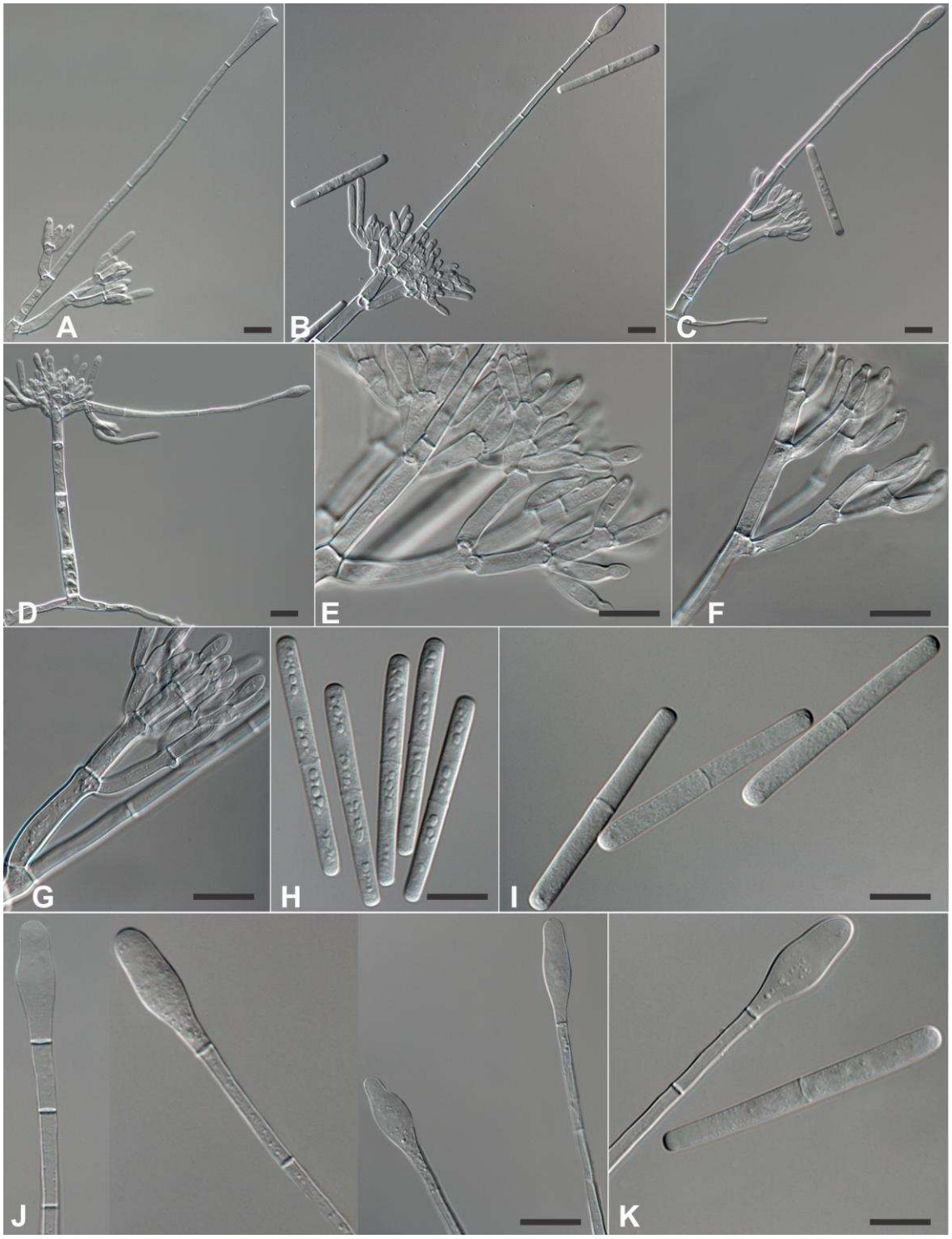

Figure 5: Morphological characteristics of Calonectria metrosideri. A: Macroconidiophores containing an abnormal bifurcate vesicles; B - D: Macroconidiophores containing typical vesicles; E – G: Three branched conidiophores; H - I: 1-septate macroconidia and J – K: Spathulate to obpyriform vesicles. Scale bars= 10 µm.

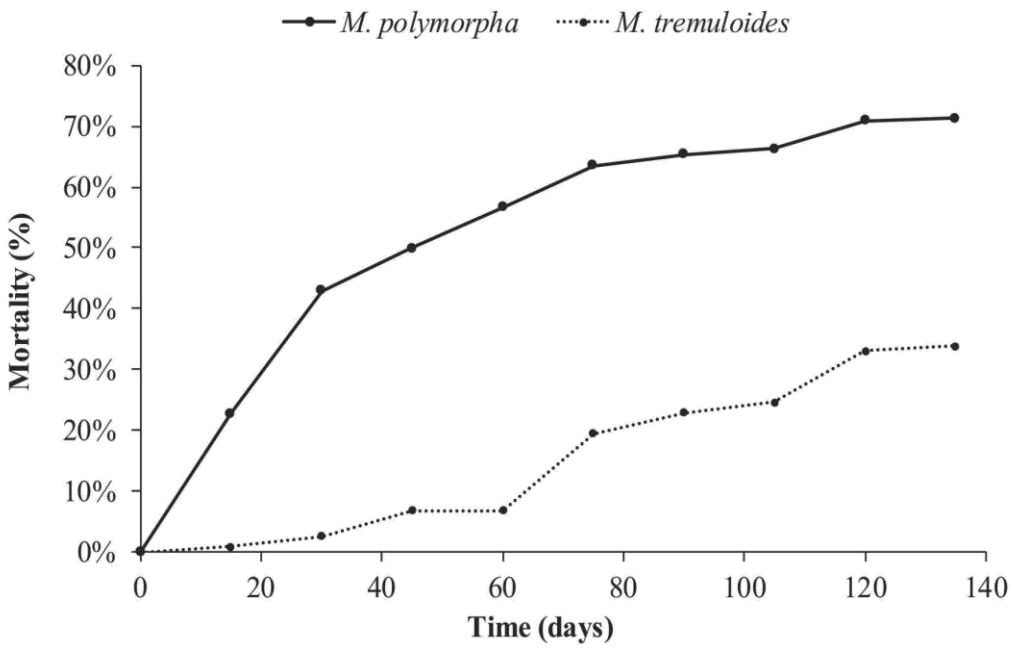

Figure 6: Seedling mortality of Meterosideros polymorpha and M. tremuloides caused by Calonectria metrosideri in a forest nursery with temperatures of 25 °C to 30 °C and irrigation daily.

CHAPTER 2

A new species of Calonectria causing leaf blight and cutting rot of three forest species in Brazil

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A new species of Calonectria causing leaf blight and cutting rot of three forest tree species in Brazil

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ABSTRACT

Several species of Calonectria cause diseases on a wide range of forest tree species that are propagated either via seedlings or rooted cuttings. In nurseries these fungi cause damping-off, cutting and root rots, stem lesions, and leaf blights. Recently a Calonectria sp. was isolated from rooted cuttings of Anadenanthera peregrina (Fabaceae), Piptadenia gonoacantha (Fabaceae), and Azadirachta indica (Meliaceae) exhibiting leaf blight and cutting rot in a forest nursery at the Universidade Federal de Viçosa, Brazil. Morphological comparisons and DNA sequences of three loci containing partial gene sequences of β-tubulin (TUB2), calmodulin (CAL), and elongation factor (TEF-1α) indicated that these isolates represent an unnamed species of Calonectria, described here as C. hodgesii sp. nov. Spray-inoculated plants of all three hosts with a suspension at $1x10^4$ conidia mL^{-1} induced leaf lesions, cutting rot, and intense defoliation as observed under natural conditions. Calonectria hodgesii was re-isolated from infected tissue, which fulfilled Koch's postulates, and confirmed its status as a pathogen with a wide host range.

KEYWORDS: forest pathology, Hypocreales, pathogenicity, phylogeny, taxonomy, tropical fungi.

INTRODUCTION

In recent years there has been an increasing demand for planting forest tree species in Brazil, in part due to the government's initiatives to restore degraded areas and mitigate global warming. Anadenanthera peregrina (L.) Speg. (Angico-Vermelho) and Piptadenia gonoacantha (Mart.) J.F. Macbr. (Pau-Jacaré) are among the most commonly used native species (Carvalho, 1994; Araújo et al., 2006). There has also been an increase in plantations of Azadirachta indica A. Juss. (neem), which is native to India. The increased planting of A. indica is largely due to its multiple applications in the pharmaceutical industry, use in agriculture as a natural insecticide, and more recently for biodiesel production (Mossini & Kemmelmeier, 2005). Nevertheless, propagation of these species either via cuttings or seedlings is generally still done in nurseries with low technology, where appropriate management practices for disease control are not employed (Mafia et al., 2007).

Numerous pathogenic fungal species, especially species of Calonectria, have been described from forest nurseries and have been reported as pathogens of a wide range of plant hosts cultivated via seedlings or vegetative propagation (Crous, 2002). Among the major nursery diseases, damping-off, cutting rot, stem girdling and leaf blight caused by Calonectria spp. are commonly encountered (Hodges & May, 1972; Lombard et al., 2010a). In recent years several new species of Calonectria have been newly described from hosts in forestry nurseries using a polyphasic approach incorporating morphological and molecular data (Lombard et al., 2010c; Alfenas et al., 2013).

Because of the importance of the genus Calonectria as plant pathogen in tropical and subtropical climates, we have for the past 2 years been collecting plant and soil samples from different hosts throughout Brazil to facilitate population biology studies

of Calonectria spp.

During one these collecting in the Forest Nursery at the Universidade Federal de Viçosa, Brazil, in May 2011, we found rooted cuttings of A. peregrina, A. indica and P. gonoacantha exhibiting necrotic leaf blight, defoliation and cutting rot symptoms with brown and necrotic tissues at the stem base, covered by profuse white sporulation typical of Calonectria infection (Figure 1). The same symptoms were observed on all three hosts. The primary aim of this study was to identify the causal agent of this disease through a combination of morphological and molecular characterization, and pathogenicity tests.

MATERIAL AND METHODS

Isolates

Single conidial isolates of a Calonectria sp. were obtained from leaves and cuttings of infected plants of A. peregrina, A. indica, and P. gonoacantha.

To obtain single conidial cultures, pathogen structures observed under a stereoscopic microscope (45 x) were deposited on Petri dishes containing Water Agar Medium [WA, 1.5% (w / v) agar]. Subsequently 2 mL of sterile distilled water were added to each WA dish, and shaken manually. Excess water was removed by inverting the Petri dish, and under a stereomicroscope (45 x) a single conidium was transferred to a Petri dish containing Malt Extract Agar (MEA). Plates were maintained at 26 ºC for 5 days to promote fungal growth. One representative isolate from each host was selected for further studies. To maintain viable isolates strains were stored in a glycerol solution (10 %) at -80 ^{o}C.

570 **DNA extraction and amplification**

571 Mycelia of the respective isolates were scraped from colonized MEA plates, and
572 placed separately in 2 mL microtubes for genomic DNA extraction using the Wizard ®
573 Genomic DNA Purification (Promega Corporation, WI, USA) kit. For PCR, the
574 DreamTaq^{TM} Master Mix (MBI Fermentas, Vilnius, Lithuania) was used, following the
575 manufacturer's protocol.

576 Three loci, including fragments of the β-tubulin (TUB2), calmodulin (CAL), and
577 elongation factor (TEF-1α) gene regions were amplified using the primers T1
578 (O'Donnell & Cigelnik, 1997) and CYLTUB1R (Crous et al., 2004) for TUB2, CAL-
579 228F and CAL-737R (Carbone & Kohn, 1999) for CAL, and EF1-728F (O'Donnell et
580 al., 1998) and EF-2 (Carbone & Kohn, 1999) for TEF-1α. Amplification was performed
581 with an initial denaturing at 96 °C for 5 min followed by 35 cycles of denaturation at 96
582 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min and a final 4 min
583 extension at 72 °C. The PCR product was visualized on a 2 % agarose gel to determine
584 fragment size and purity. PCR products were prepared for sequencing with an ExoSAP-
585 IT ® kit, according to the manufacturer's protocol.

586

587 **Sequencing and phylogenetic analyses**

588 Sequencing was performed at the Laboratory of Genomics of the Instituto de
589 Biotecnologia Aplicada à Agropecuária (BIOAGRO) at the Universidade Federal de
590 Viçosa, Brazil. Sequence quality was checked via Sequence Scanner Software v. 1.0
591 (Applied Biosystems), and edited using the software package Seqman from DNAStar
592 Inc. Consensus regions of edited sequences were compared in the NCBI GenBank
593 nucleotide database (www.ncbi.nlm.nih.gov) using the nucleotide collection (nr/nt)
594 optimised for highly similar sequences (megablast). Calonectria sequences generated in

this study were deposited in GenBank (Table 1). All sequences were assembled in MAFFT v. 6 (Katoh & Toh, 2010), using the FFT-NS-i (Slow; iterative refinement method) alignment strategy with the 200PAM/ K=2 scoring matrix and a gap opening penalty of 1.53 with an offset value of 0.0. Aligned sequences were then manually corrected when necessary using MEGA v. 5 (Tamura et al., 2011).

PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Swofford, 2002) was used to analyse the DNA sequence datasets. A partition homogeneity test (Farris et al., 1994) was applied to determine whether the data sets were consistent and combinable. Phylogenetic relationships were estimated by heuristic searches based on 1,000 random addition sequences and tree bisection-reconnection, with the branch swapping option set on 'best trees' only. All characters were weighed equally and alignment gaps were treated as missing data. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and rescaled consistence index (RC). Bootstrap analyses (Hillis & Bull, 1993) were based on 1,000 replications.

Analysis of Bayesian Inference (BI) was performed with MrBayes v. 3.1.1 (Ronquist & Heulsenbeck, 2003) using the algorithm of Markov Chain Monte Carlo (MCMC) with two sets of four chains (one cold and three heated) with 10 million random generations. The sample frequency was set to 1,000; the first 25 % of trees were removed as burnin. The likelihood values were calculated and the best model of nucleotide substitution for each gene was selected according to Akaike Information Criterion (AIC) using MrModeltest v. 2.3 (Nylander, 2004).

The convergence of the log likelihood was analysed using TRACER v. 1.5 (Auckland, New Zealand; Rambaut & Drummond 2009) and no indication of lack of convergence was detected. Calonectria chinensis (Crous) L. Lombard, M. J. Wingf. & Crous was used as outgroup in the analysis.

620 **Morphological characterization**

621 For morphological characterization single conidial cultures were grown in
622 synthetic nutrient-poor agar (SNA) at 26 °C for 7 days. Fungal structures were mounted
623 in clear lactic acid for morphological examination, and 30 measurements of each
624 structure determined at 1,000 × magnification using a Zeiss Axioscope-2 microscope
625 with differential interference contrast (DIC) illumination. The 95 % confidence levels
626 were determined and extremes of conidial measurements are given in parentheses. For
627 other structures, only extremes are presented.

628

629 **Pathogenicity test**

630 Single conidial cultures were transferred aseptically to Petri dishes (90 mm
631 diam) containing Malt Extract Agar (MEA), and subsequently incubated at 26 °C for 10
632 days for pathogenicity studies. Healthy rooted cuttings (five per species) of *A.*
633 *peregrina*, *A. indica* and *P. gonoacantha* were spray-inoculated with a conidial
634 suspension of 1×10^4 conidia mL^{-1} of each isolate, as described by Graça et al. (2009).
635 Five plants of each host species were treated with distilled water to serve as controls.
636 The inoculated plants were maintained in a greenhouse under controlled conditions (25
637 °C ± 3 °C) and the development of symptoms was monitored daily for 10 days, after
638 which time the fungus was re-isolated from the lesion margins.

639

640 **RESULTS**

641

642 **Phylogenetic analysis**

643 Amplicons of approximately 500 bases each for TUB2, TEF-1α, and CAL were
644 generated. The 70 % reciprocal bootstrap trees showed no conflict in tree topologies for

645 the three gene regions separately, and therefore they were combined in a dataset
646 consisting of 1,531 characters including gaps. Of these 1,100 were constant and
647 parsimony uninformative and 431 were parsimony informative. Analysis of the 431
648 parsimony informative characters yielded three equally most parsimonious trees (TL =
649 1033 CI = 0.691, RI = 0.871, RC = 0.602). Evolution models HKY + G for TUB2, TEF,
650 and CAL were selected and incorporated into the Bayesian analysis. The consensus tree
651 obtained for the Bayesian analyses confirmed the tree topology obtained with
652 parsimony. The isolates of Calonectria from A. peregrina, A. indica and P.
653 gonoacantha formed a distinct, well-supported clade (PP=1,00) (Figure 2).
654
655 **Taxonomy**
656 Based on the DNA sequence data and morphological features, we conclude that
657 the Calonectria isolates from A. peregrina, A. indica and P. gonoacantha represent a
658 novel species, which is described below:
659 **Calonectria hodgesii** R.F Alfenas, O.L. Pereira, Crous & Alfenas, sp. nov.
660 MycoBank MB 803943; Figure 3
661 **Etymology**: Named after Dr. Charles S. Hodges, in honor of his contribution to
662 forest pathology in the tropics.
663 **Hosts**: Anadenanthera peregrina, Azadirachta indica and Piptadenia
664 gonoacantha.
665 **Specimen examined:** Brazil, Minas Gerais state, Viçosa, on Anadenanthera
666 peregrina, May 2011, Rafael F. Alfenas (**Holotype** CBS H-21147, **Culture ex-type**
667 CBS 133609).
668 Conidiophores containing a stipe bearing penicillate suites of fertile branches,
669 stipe extensions, and terminal vesicles; stipe septate, hyaline, smooth, 40–82 × 5–7 µm

µm; stipe extensions septate, straight to flexuous 136–196 µm long, 2–4 µm wide at the apical septum, terminating in pyriform to ellipsoidal or ovoid to sphaeropedunculate vesicles, 6–11 µm diam. Conidiogenous apparatus 45–65 µm long, 61–72 µm wide at apex; primary branches aseptate, 18–27 × 4–5 µm; secondary branches aseptate, 12–24 × 3–4 µm, and tertiary branches aseptate, 9–18 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, 5–10 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (44–)49–51(–55) × 3–5 µm (av. = 50 × 4.5 µm), 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia not seen.

Notes: Calonectria hodgesii is phylogenetically closely related to C. brasiliensis and C. sulawesiensis, but C. hodgesii can easily be distinguished from these species based on the size of its macroconidia, vesicle shape, number of macroconidiophore branches, and DNA sequence data.

Culture characteristics: Colonies sienna to umber on the surface and sepia to brown-vinaceous in reverse, with moderate aerial mycelium; chlamydospores moderate to extensive, occurring throughout the colony, forming microsclerotia; extensive sporulation on the aerial mycelium; moderate to rapid growth (50–65 mm) diam after 7 days at 25 ºC on MEA.

Pathogenicity test

After 10 days spray-inoculated plants showed necrotic leaf blight, cutting rot with brown and necrotic tissues of the basal stem, and intense defoliation as observed under natural conditions in the nursery. Profuse sporulation was also observed on necrotic lesions of inoculated organs of all three host species.

DISCUSSION

In the present study we describe a new species of Calonectria associated with necrotic leaf blight and cutting rot of A. peregrina, A. indica and P. gonoacantha in Brazil based on morphological and molecular data. Calonectria hodgesii formed a distinct and well-supported phylogenetic clade, closely related to C. brasiliensis and C. sulawesiensis, which belong to the C. morganii species complex. This complex, characterised by having uniseptate macroconidia and vesicles varying from pyriform to obpyriform or ovoid to ellipsoidal, includes C. cerciana L. Lombard, M.J. Wingf. & Crous, C. insularis C.L. Schoch & Crous, C. morganii, C. sulawesiensis, C. hawksworthii (Peerally) L. Lombard, M.J. Wingf. & Crous, C. leucothöes (El-Gholl, Leahy & T.S. Schub.) L. Lombard, M.J. Wingf. & Crous, C. variabilis Crous, B.J.H. Janse, D. Victor, G.F. Marias & Alfenas and C. brasiliensis (Peerally) L. Lombard, M.J. Wingf. & Crous (Schoch et al., 2001; Lombard et al., 2010c).

Calonectria hodgesii is characterised by having macroconidia larger than those of C. brasiliensis, C. morganii and C. sulawesiensis, but smaller than C. variabilis (Table 2). Superficially C. hodgesii resembles C. variablilis in having vesicles that vary in shape, and it is quite probable that many isolates previously identified as C. variabilis, were in fact representative of C. hodgesii. The two species can be distinguished, however, in that C. hodgesii has 1-septate conidia, while C. variablilis has (1–)3(–4)-septate conidia. Although the vesicle shape of C. hodgesii is also quite variable, it is mainly obpyriform to ellipsoidal, while those of C. variabilis vary from clavate to ellipsoidal. Interestingly, the new species shares morphological characteristics with phylogenetically distant species, such as Calonectria citri (H.S. Fawc. & Klotz) L. Lombard, M.J. Wingf. & Crous and Calonectria canadiana L. Lombard, M.J. Wingf. & Crous.

Species of the C. morganii complex are well-known pathogens of various hosts worldwide (Crous, 2002), and some, like C. brasiliensis, are known to be highly aggressive to Eucalyptus seedlings (Batista, 1951). Originally, C. brasiliensis was described as a variety of C. morganii Crous, Alfenas & M.J. Wingf. (as Cylindrocladium scoparium var. brasiliensis Batista & Ciferri) based on having macroconidia smaller than those of Calonectria morganii (Batista, 1951; Peerally, 1974). Based on morphological characteristics and total protein banding patterns however, Crous et al. (1993a) reduced C. brasiliensis to synonymy under C. morganii. By employing multigene DNA sequence data, Lombard et al. (2010b) recently showed that the ex-type culture of C. brasiliensis (CBS 230.51) is phylogenetically and morphologically distinct from C. morganii, and therefore reinstated it to species level.

Calonectria sulawesiensis Lombard et al. (2010c), described from Eucalyptus sp. in Indonesia, is another phylogenetically closely related species, but it is morphologically distinct, and presently nothing is yet known regarding its pathogenicity and host range.

In our studies all three selected isolates (CBS133608, CBS133609, and CBS133610) of C. hodgesii tested were pathogenic, and induced leaf blight, defoliation and cutting rot in A. peregrina, A. indica and P. gonoacantha similar to that observed in the nursery under natural conditions.

The occurrence of C. hodgesii sp. nov. causing leaf blight and cutting rot on these hosts represents an alert for nurseries that propagate these forest species either from seedlings or rooted cuttings.

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834 **Table 1:** Details pertaining to Calonectria spp. studied.

Species[1]	Isolates	GenBank accession nr.[2]			Host/substrate	Country	Reference
		β - tubulin (TUB2)	Elongation factor (*TEF1α*)	Calmodulin (CAL)			
C. brasiliensis	CBS 230.51	GQ267241	GQ267328	GQ267421	Eucalyptus sp.	Brazil	Lombard et al. (2010c)
C. brasiliensis	CBS 114257	GQ267242	GQ267329	GQ267422	Eucalyptus sp.	Brazil	Lombard et al. (2010c)
C. canadiana	CBS 110817	AF348212	GQ267297	AY725743	Picea sp.	Canada	Kang et al. (2001)
C. cerciana	CBS 123693	FJ918510	FJ918559	GQ267369	Hybrid "urograndis"	China	Lombard et al. (2010d)
C. cerciana	CBS 123695	FJ918511	FJ918560	GQ267370	Hybrid "urograndis"	China	Lombard et al. (2011)
C. chinensis	CBS 112744	AY725618	AY725709	AY725746	Soil	China	Crous et al. (2004)
C. citri	CBS 186.36	AF333393	GQ267299	GQ267371	Citrus sinensis	U.S.A	Lombard et al. (2010c)
C. colombiana	CBS 115127	FJ972423	FJ972492	GQ267455	Soil	Colombia	Lombard et al. (2010c)
C. colombiana	CBS 115638	FJ972422	FJ972491	GQ267456	Soil	Colombia	Lombard et al. (2010c)
C. colombiensis	CBS 112221	AY725620	AY725712	AY725749	Soil	Colombia	Crous et al. (2004)
C. densa	CMW 31182	GQ267232	GQ267352	GQ267444	Soil	Ecuador	Lombard et al. (2010c)
C. densa	CMW 31184	GQ267230	GQ267350	GQ267442	Soil	Ecuador	Lombard et al. (2010c)
C. hodgesii	**CBS 133608**	**KC491227**	**KC491224**	**KC491221**	**Piptadenia gonoacantha**	**Brazil**	**This study**
C. hodgesii	**CBS 133609**	**KC491228**	**KC491225**	**KC491222**	**Anadenanthera peregrina**	**Brazil**	**This study**
C. hodgesii	**CBS 133610**	**KC491229**	**KC491226**	**KC491223**	**Azadirachta indica**	**Brazil**	**This study**
C. humicola	CMW 31183	GQ267233	GQ267353	GQ267445	Soil	Ecuador	Lombard et al. (2010c)
C. humicola	CMW 31187	GQ267235	GQ267355	GQ267447	Soil	Ecuador	Lombard et al. (2010c)
C. insularis	CBS 114558	AF210861	FJ918556	GQ267389	Soil	Madagascar	Lombard et al. (2010d)
C. insularis	CBS 114559	AF210862	FJ918555	GQ267390	Soil	Madagascar	Lombard et al. (2011)
C. kyotensis	CBS 170.77	GQ267209	GQ267308	GQ267380	Robina pseudoacacia	Japan	Crous (2002)
C. kyotensis	CBS 413.67	GQ267208	GQ267307	GQ267379	Paphiopedilum callosum	Germany	Crous (2002)
C. leucothoës	CBS 109166	FJ918508	FJ918553	GQ267392	Leucothoë axillaris	U.S.A	Lombard et al. (2010c)

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Table 1: Continued.

Species[1]	Isolates	GenBank accession nr.[2]			Host/substrate	Country	Reference
		β - tubulin (TUB2)	Elongation factor (*TEF1α*)	Calmodulin (CAL)			
C. morganii	CBS 110666	FJ918509	FJ918557	GQ267423	Rosa sp.	U.S.A	Lombard et al. (2010c)
C. pauciramosa	CMW 5683	FJ918514	FJ918565	GQ267405	Eucalyptus sp.	Brazil	Lombard et al. (2010b)
C. pauciramosa	CMW 30823	FJ918515	FJ918566	GQ267404	Eucalyptus grandis	South Africa	Lombard et al. (2010b)
C. polizzii	CBS 125270	FJ972417	FJ972486	GQ267461	Callistemon citrinus	Italy	Lombard et al. (2010b)
C. polizzii	CBS 125271	FJ972418	FJ972487	GQ267462	Callistemon citrinus	Italy	Lombard et al. (2010b)
C. pseudospathiphylli	CBS 109165	FJ918513	FJ918562	GQ267412	Soil	Ecuador	Lombard et al. (2010d)
C. scoparia	CMW 31000	FJ972426	FJ972525	GQ267367	Eucalyptus	Brazil	Lombard et al. (2010b)
C. scoparia	CMW 31001	GQ421779	GQ267298	GQ267368	Eucalyptus	Brazil	Lombard et al. (2010b)
C. spathiphylli	CBS 114540	AF348214	GQ267330	GQ267424	Spathiphyllum sp.	U.S.A	Kang et al. (2001)
C. spathiphylli	CBS 116168	FJ918512	FJ918561	GQ267425	Spathiphyllum sp.	U.S.A	Lombard et al. (2010d)
C. spathulata	CBS 112689	AF308463	FJ918554	GQ267426	Eucalyptus viminalis	Brazil	Lombard et al. (2010c)
C. spathulata	CBS 555.92	GQ267215	GQ267331	GQ267427	Araucaraia angustifolia	Brazil	Lombard et al. (2010c)
C. sulawesiensis	CBS 125248	GQ267223	GQ267343	GQ267435	Eucalyptus sp.	Indonesia	Lombard et al. (2010c)
C. sulawesiensis	CBS 125253	GQ267220	GQ267340	GQ267432	Eucalyptus sp.	Indonesia	Lombard et al. (2010c)
C. variabilis	CBS 112691	GQ267240	GQ267335	GQ267458	Theobroma grandiflorum	Brazil	Crous (2002)
C. variabilis	CBS 114677	AF333424	GQ267334	GQ267457	Schefflera morotoni	Brazil	Crous (2002)
C. zuluensis	CBS 125268	FJ972414	FJ972483	GQ267459	Eucalyptus grandis	South Africa	Lombard et al. (2010b)
C. zuluensis	CMW 9896	FJ972415	FJ972484	GQ267460	Eucalyptus grandis	South Africa	Lombard et al. (2010b)

[1]CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW: Cultures of Mike Wingfield, FABI, South Africa; CPC: Cultures of Pedro Crous, maintained at CBS; LPF: Laboratory of Forest Pathology, DFT-UFV, Viçosa, Minas Gerais, Brazil.

[2] GenBank Accession Number.

Figure 1: Leaf blight and cutting rot caused by Calonectria hodgesii on rooted cuttings of forest species in a nursery. A - B: General view containing infected rooted cuttings of Anadenanthera peregrina and Piptadenia gonoacantha; C - E: Cutting rot and leaf blight in A. peregrina; F - H: Cutting rot in P. gonoacantha; I - L: Cutting rot and leaf blight with intense sporulation on seedlings of Azadirachta indica.

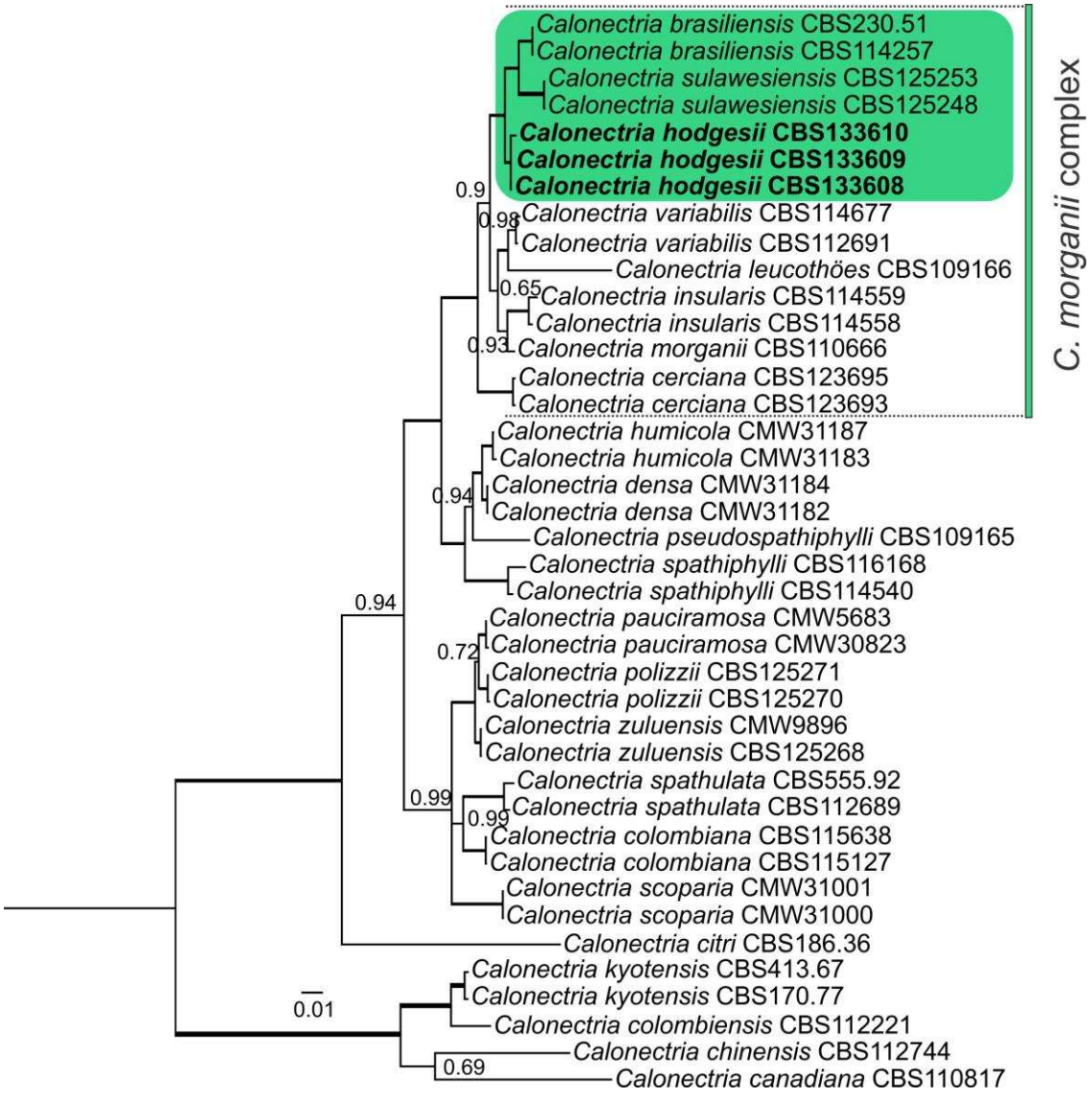

Figure 2: Phylogenetic tree obtained from Bayesian inference using combined sequences of the β-tubulin, translation elongation factor-1α and calmodulin genes of Calonectria isolates. The bold lines indicate posterior probability values of 1.00. The tree was rooted to C. chinensis (CBS 112744). Isolates in bold were obtained during the survey.

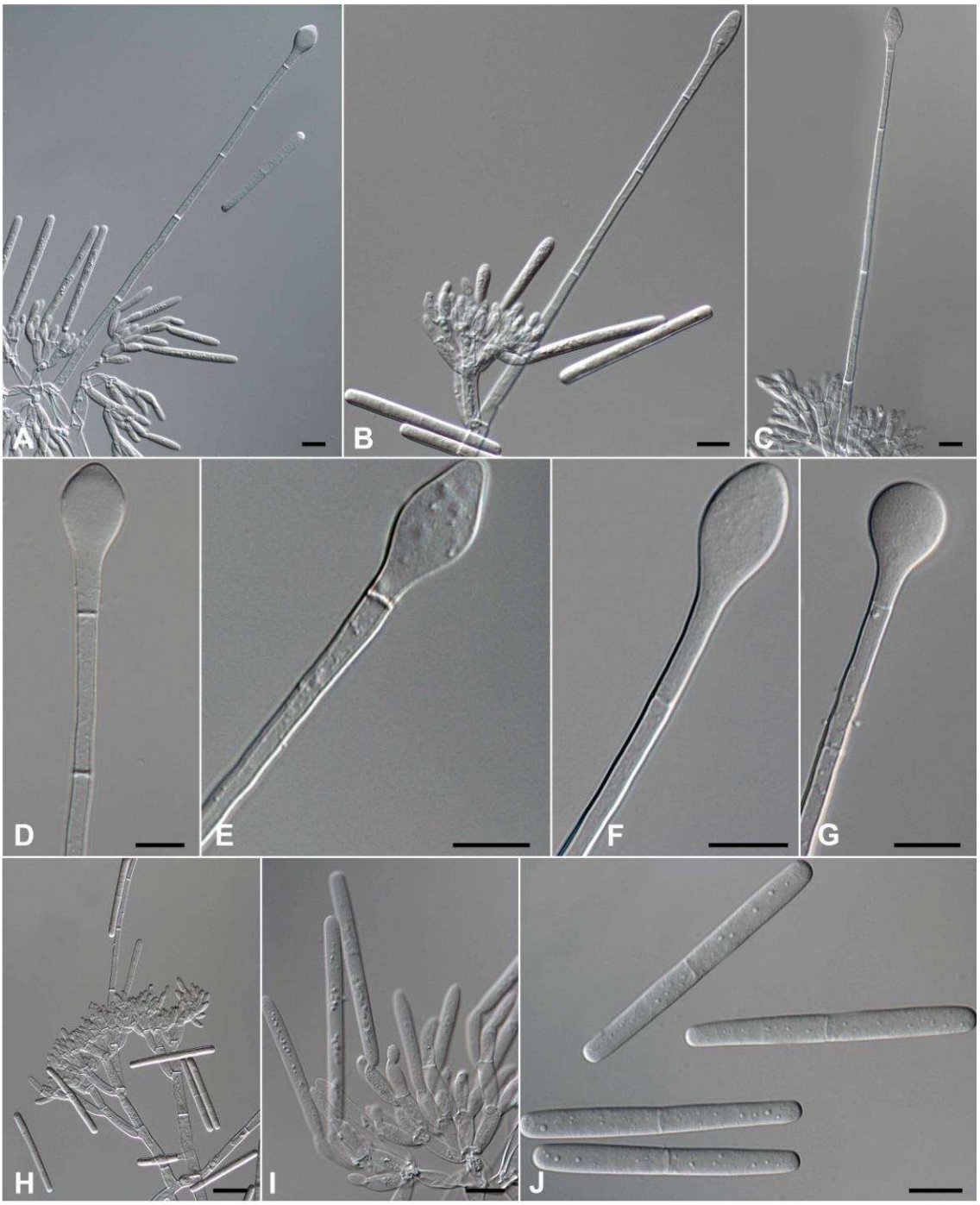

Figure 3: Morphological characteristics of Calonectria hodgesii. A - C: Macroconidiophores containing obpyriform to ellipsoidal or sphaeropedunculate vesicles; D - G: Variation in vesicle shape; H - I: Macroconidiophores; J: Uniseptate macroconidia. Scale bars = 10 µm; H = 20 µm.

858 **Table 2**: Distinctive morphological characters of Calonectria hodgesii and related species.

Species	Vesicle shape	Vesicle diameter	Macroconidial size	Macroconidial septation
C. brasiliensis[a]	ellipsoidal to obpyriform	7–11 µm	(35–)38(–41) × 3–5 µm	1-septate
C. canadiana[d]	pyriform to sphaeropedunculate	6–10 µm	(38–)50(–65) × 4–5 µm	1-septate
C. citri[e]	obovoid to spathulate	6.5–10 µm	(50–)57.5(–65) × 3–4 µm	(1–)3-septate
C. hodgesii	ellipsoidal to pyriform, or ovoid to sphaeropedunculate	6–11 µm	(44–)50(–55) × 3– 5 µm	1-septate
C. morganii[c]	clavate, ellipsoid to pyriform	6–8 µm	(40–)45(–66) × 3–5 µm	1-septate
C. sulawesiensis[b]	broadly clavate to ellipsoidal	5–7 µm	(41–)48(–54) × 3–6 µm	1-septate
C. variabilis[c]	sphaeropedunculate to ovoid, or ellipsoid to clavate	6–11 µm	(48–)73(–85) × 4–6 µm	(1–)3(–4)-septate

859 [a] Lombard et al., 2010b; [b] Lombard et al., 2010c; [c] Crous et al., 1993a; [d] Crous et al., 1993b; [d] Kang et al., 2001; [e] Boedjin & Reitsma, 1950

CHAPTER 3

Taxonomy of Calonectria in Brazil

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Taxonomy of Calonectria in Brazil

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Summary

Species of genus Calonectria (Hypocreales) represent an important group of plant pathogenic fungi that cause serious losses to crops in tropical and subtropical climates. Calonectria leaf blight is currently one of the main leaf diseases of eucalyptus in Brazil and various species of Calonectria have been reported. Nevertheless, these past reports were mainly based on morphologic characteristics, which can have resulted in inaccurate identification. The aim of this study was identify and establish the phylogenetic relationships among species that occurs in Brazil from the main eucalyptus-growing regions, using sequences of the genes β-tubulin, elongation factor 1-α and calmodulin. Prelimary bayesian analyses using TEF-1α was performed on a total data set of 1017 isolates to determine generic relationships, and based on this analysis concludes that C. pteridis complex is the most important complex that occurs in eucalyptus plantation in Brazil. Subsequently, other Bayesian analysis representing unknown Calonectria species was made using a three gene regions (TEF-1α, TUB and CAL), and 26 new species were described.

Keywords: Cylindrocladium, forest pathology, Hypocreales, pathogenicity, taxonomy.

1 Introduction

Calonectria is a member of the order Hypocreales and its species are characterised by the production of Cylindrocladium anamorphs (Crous & Wingfield 1994). Members of this genus are further defined by their brightly coloured ascomata that change colour when placed in a 3% KOH solution, warty peridial structure and darkened stromatic bases (Rossman et al. 1999). The Cylindrocladium anamorph is the form most frequently encountered in nature, and has a considerable number of morphological characteristics for identification at species level (Peerally 1991, Crous & Wingfeld 1994). Consequently, Calonectria species are primarily distinguished by the characteristics of the anamorph, which are identified basically according to the vesicle shape, stipe length, and number of septa and size of the conidia, under standardized conditions (Boesewinkel 1982, Peerally 1991, Crous & Wingfeld 1994, Crous 2002).

Studies using a greater number of isolates of the same species have revealed intraspecific variation for characters such as conidial size and vesicle shape (Crous & Peerally 1996, Crous et al. 1993, 1998). This morphological variation has been the source of much taxonomic confusion in the past, and has resulted in various species being amalgamated (Schoch et al. 1999). Then application of molecular techniques and particularly phylogenetic inference has employed to improve the capacity of identification at species level. However, recently phylogenetic studies on Calonectria have substantially influenced the taxonomy of these genera and it is somewhat shocking, even for mycologist (Lombard et al. 2010b-c, Chen et al. 2011).

The genus Calonectria are known to be a pathogen on approximately with 335 plant hosts (Crous, 2002; Lombard et al. 2010a). The majority of disease reports associated with Calonectria species in forestry include hosts in five plant families, of which the most important are associated with Fabaceae (Acacia spp.), Myrtaceae (Eucalyptus spp.) and Pinaceae (Pinus spp.).

In eucalyptus, species of Calonectria can cause leaf blight and defoliation, and this disease is known as Cylindrocladium leaf blight (CLB) (Sharma & Mohanan 1991). These fungi are also important causal agents of cutting rot and seedling blight in Eucalyptus nurseries (Alfenas et al. 1979, Alfenas, 1986, Sharma et al. 1984).

Nowadays in Brazil, especially in humid and high temperature regions, the Cylindrocladium leaf blight is currently one of the main leaf diseases of eucalyptus, and various species have been reported in Brazil (Alfenas, 1986; Crous & Wingfield, 1994; Crous

et al, 2006). Nevertheless, these past reports were mainly based on morphologic characteristics, which can have resulted in inaccurate identification. Then, the aim of this study was identify and establish the phylogenetic relationships among species that occurs in Brazil from the main eucalyptus-growing regions, using sequences of the genes β-tubulin, elongation factor 1-α and calmodulin that have been provide be the best resolution to distinguish Calonectria spp. (Lombard et al, 2010c).

2 Material and methods

2.1 Sampling and fungal isolation

Samples of eucalyptus leaves were collected from different clones and species, bearing the characteristic symptoms of the disease in the main Brazilian eucalyptus growing regions. Since the planting areas of the forest companies are divided in Management Operacional Units (MOU), according to the characteristics of soil and clima, a sample of 30 leaves per infected clone/species and one soil sample (400 g in the 0-20 cm layer)/MOA and another from the closest native vegetation were randomly collected. The leaves were mantained in paper bags and the soil samples in plastic bags and brougth to the Forest Pathology Laboratory/Bioagro of the Federal University of Viçosa. However, eventually samples from other host were collected as well.

Single spore cultures were obtained by transfering a conidial mass with a cirurgical needle to a Petri dish, containing Malt-Extract-Agar (MEA), to which aproximately 3 ml of sterile distilled water will be added. Subsequently, the Petri dish is inverted to remove the water excess and, under a stereoscopic microscope (45 X), one single conidium is picked up and transferred to another Petri dish, containing MEA, which will be kept at 26 ^{o}C for the mycelial growth of the fungus. The obtained single spore cultures were stored in vials, containing 10% glycerol. The isolates that represent new species were deposited at CBS Fungal Biodiversity Institute in the Netherlands (CBS), and taxonomic novelties were deposited in MycoBank (Crous et al. 2004a).

2.2 DNA extraction, amplification and purification

Genomic DNA was isolated from fungal mycelium grown on the Malt Extract Agar (MEA) plates following the Wizard® Genomic DNA Purification (Promega Corporation, WI, USA) kit. For PCR, the DreamTaq™ Master Mix (MBI Fermentas, Vilnius, Lithuania) was used, following the manufacturer's protocol.

All isolates were sequenced primarily with elongation factor (TEF-1α) using the primers EF1-728F (O'Donnell et al. 1998) and EF-2 (Carbone & Kohn 1999). Subsequently, fragments of β-tubulin (TUB2) and calmodulin (CAL), were amplified using the primers T1 (O'Donnell & Cigelnik, 1997) and Bt2b (Glass & Donaldson, 1995) or CYLTUB1R (Crous et al. 2004b) for TUB2, and CAL-228F and CAL-737R (Carbone & Kohn 1999) or CAL1Rd (Groenewald et al. 2012) for CAL.

The protocols and conditions outlined by Crous et al. (2004b) were followed for standard amplification and subsequent sequencing of the loci.

The PCR product was visualized on a 2% agarose gel, to determine fragment size and purity. PCR products were purified with an ExoSAP-IT® kit, according to the manufacturer's recommended protocol (2 µL reagent per 5 µL amplified DNA product) and incubated in a thermal cycler for 15 min at 37 °C followed by an additional incubation for 15 min at 80 °C.

2.3 Sequencing and phylogenetic analysis

DNA sequencing reactions was performed with the BigDye® Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems Life Technologies, Carlsbad, CA, USA) following the protocol of the manufacturer. DNA sequencing reactions used the same primers as those for the PCR reactions. DNA sequencing amplicons were purified through Sephadex® G-50 Superfine columns (Sigma Aldrich, St. Louis, MO) in MultiScreen HV plates (Millipore, Billerica, MA). Purified sequence reactions were run on an ABI Prism 3730xl DNA Sequencer (Life Technologies, Carlsbad, CA, USA).

The quality of sequences were checked by means of Sequence Scanner Software v. 1.0 (Applied Biosystems), and edited using the software package Seqman from DNAStar Inc. All sequences were manually corrected and the arrangement of nucleotides in ambiguous positions was corrected using the sequences of primers in the forward and reverse direction. In addition to the sequences generated in this study, other sequences were obtained from NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov) and added to the DNA sequence datasets generated in this study (Table 1).

Sequence datasets for the three genomic loci were aligned in MAFFT Online version v. 7.0 (Katoh & Toh 2010), using the FFT-NS-i (Slow; iterative refinement method) alignment strategy with the 200PAM/ K=2 scoring matrix and a gap opening penalty of 1.53 with an offset value of 0.0. Aligned sequences were then manually corrected when necessary using MEGA v. 5 (Tamura et al. 2011).

A phylogenetic re-construction was conducted for the aligned TEF-1α data set with 1017 taxa including outgroup to determine generic relationships using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck 2003).

An initial BI analysis with 156 isolates representing unknown Calonectria species was made using a three gene regions (TEF-1α, TUB and CAL). Subsequently the Calonectria spp. were divided into four separate data sets representing Ca. brassicae and Ca. pteridis complex, Ca. morganii complex, Ca. scoparia complex and Ca. naviculata complex, to reduce the number of gaps in the alignment and consequently to reduce the penalty for improve the resolution of the analysis.

The phylogenetic analysis for each Calonectria complex was performed as follow:

A congruence index trees (de Vienne et al. 2007) and a 70 % reciprocal bootstrap method (Gueidan et al. 2007) was applied among single gene data sets, to determine if it were consistent and combinable. This analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony, v. 4.0b10; Sunderland, Massachusetts, USA; Swofford 2002).

The likelihood values were calculated and the best model of nucleotide substitution for each gene was selected according to Akaike Information Criterion (AIC) using MrModeltest v. 2.3 (Nylander 2004).

The multi-gene Bayesian Inference (BI) was performed on MrBayes v. 3.1.1 (Ronquist & Heulsenbeck, 2003) using the algorithm of Markov chain Monte Carlo (MCMC) with two sets of four chains (one cold and three heated) and the stoprule option, stopping the analysis at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 1000; the first 25 percent of trees were removed as burnin.

The resulting trees were printed with Geneious v. 5.5.4 (Drummond et al. 2011). Sequences derived in this study were deposited in GenBank (Table 1), the alignments in TreeBASE (www.treebase.org/treebase/index.html), and nomenclatural data were submitted to MycoBank (www.MycoBank.org; Crous et al. 2004a).

2.4 Morphological characterization

Single conidial cultures were grown on synthetic nutrient agar (SNA) (Nirenburg 1981) at 26 °C, following the protocols set for Calonectria by Lombard et al. (2009). After 7 days of incubation, the morphological characteristics were determined by mounting fungal structures in clear lactic acid and 30 measurements at ×1,000 magnification were determined for each isolate using a Zeiss Axioscope 2 microscope with interference contrast (DIC)

192 illumination. The 95% confidence levels were determined and extremes of conidial
193 measurements are given in parentheses. For other structures, only extremes are presented.
194
195
196 **3 Results**
197
198 **3.1 Phylogenetic analysis**
199 Amplification products and gene sequences of similar size to that reported previously
200 (Lombard et al. 2010c) were obtained.
201 Based on preliminary TEF-1α sequence analyses with 1017 taxa including outgroup
202 we conclude that C. pteridis complex is the most important complex that occur in eucalyptus
203 plantation in Brazil, and also that have 26 unknown Calonectria spp. (Table 2).
204 A congruence index trees and a 70 % reciprocal bootstrap showed no conflict in tree
205 topologies for the three gene regions. Subsequently the data sets were combined for each of
206 the four separate data sets, and based on the results of MrModeltest the nucleotide substitution
207 models were implemented for Bayesian analyses in MrBayes for the different partitions
208 (Table 3).
209 The initial BI analysis with combined data set contained 156 taxa and a total of 1,573
210 characters. In this analysis we identify five well-defined Calonectria complexes however the
211 larger number of taxa makes it difficult to visualize the interspecific genetic distance between
212 the recognized species. Then, the phylogenetic analysis was perforrmed for each complex
213 separately.
214 For C. brassicae and C. pteridis complex, the final aligned combined data set
215 contained 59 taxa with a total of 1.511 characters, including gaps. The Bayesian analysis
216 lasted 550.000 generations and the consensus trees and posterior probabilities were calculated
217 from the 834 trees (Fig 1). In this complex were identified 11 new phylogenetic Calonectria
218 species somewhat supported by morphological features (Table 4).
219 For C. morganii complex, the final aligned combined data set contained 45 taxa with a
220 total of 1.498 characters, including gaps. The Bayesian analysis lasted 815.000 generations
221 and the consensus trees and posterior probabilities were calculated from the 1224 trees (Fig
222 2). In this complex were identified five new phylogenetic Calonectria species somewhat
223 supported by morphological features (Table 5).
224 For C. scoparia complex, the final aligned combined data set contained 46 taxa with a
225 total of 1.530 characters, including gaps. The Bayesian analysis lasted 1.020.000generations

and the consensus trees and posterior probabilities were calculated from the 1532 trees (Fig 3). In this complex were identified eight new phylogenetic Calonectria species somewhat supported by morphological features (Table 6).

For C. naviculata complex, the final aligned combined data set contained 11 taxa with a total of 1.533 characters, including gaps. The Bayesian analysis lasted 145.000 generations and the consensus trees and posterior probabilities were calculated from the 58 trees (Fig 4). In this complex were identified two new phylogenetic Calonectria species somewhat supported by morphologic features (Table 7).

3.2 Taxonomy

Based on the DNA sequence data and morphological features of the anamorph, we describe 24 new Calonectria species as follows:

1. Calonectria brassicae complex

Calonectria sp. nov. 1 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig. 5).

Hosts/substrate: Soil

Specimens examined: Brazil, Pará state, Monte Dourado, from soil (Eucalyptus plantation), Aug. 2011, Rafael F. Alfenas, culture ex-type **CBS134669 = LPF430;** Brazil, Pará state, Monte Dourado, from soil (tropical forest), Aug. 2011, Rafael F. Alfenas LPF429.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 52–110 × 5–7 µm; stipe extensions septate, straight to flexuous, 120–195 µm long, 3–5 µm wide at the apical septum, terminating in clavate vesicles, 4–6 µm diam. Conidiogenous apparatus 45–55 µm long, 60–75 µm wide; primary branches aseptate, 18–24 × 4–6 µm and secondary branches aseptate, 14–23 × 3–5 µm each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 7–11 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (35–) 40 – 43 (–45) × 3–6 µm (av. = 42 × 5 µm), L/W ratio = 8.85 µm, 1-septate, lacking a visible

abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies buff on the surface and ochraceous to umber in reverse; extensive aerial mycelium; chlamydospores not seen; sparse sporulation on the aerial mycelium. Colonies moderate growing (40–60 mm) diam on MEA, and fast growing (75–85 mm) diam. on OA, after seven days at 25 ºC.

Calonectria sp. nov. 2 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig.6).

Hosts/substrate: Soil

Specimens examined: Brazil, Bahia state, Mucuri, from soil (tropical forest), Oct. 2011, Edival Zauza, culture ex-type **CBS134664 = LPF217;** Brazil, Bahia state, Mucuri, from soil (Eucalyptus plantations), Apr. 2011, Edival Zauza CBS134667 = LPF263.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 55–125 × 5–7 µm; stipe extensions septate, straight to flexuous, 100–225 µm long, 2–4 µm wide at the apical septum, terminating in clavate vesicles, 3–6 µm diam. Conidiogenous apparatus 45–95 µm long, 40–80 µm wide; primary branches aseptate, 20–30 × 5–8 µm; secondary branches aseptate, 15–30 × 4–5 µm; tertiary branches aseptate, 10–20 × 5–6 µm and additional branches (–4), aseptate, 10–15 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 7–11 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (35–) 40 – 42 (–46) × 3–6 µm (av. = 41 × 5 µm), L/W ratio = 9.13 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies buff on the surface and ochraceous to umber in reverse; extensive aerial mycelium; chlamydospores not seen; sparse sporulation on the aerial mycelium. Colonies moderate growing (45–60 mm) diam. at 25 ºC on MEA, and fast growing (76–83 mm) diam. on OA, after seven days at 25 ºC.

Calonectria sp. nov. 3 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 7)

Hosts/substrate: Soil (Eucalyptus plantation)

Specimens examined: Brazil, Pará state, Monte Dourado, from soil (Eucalyptus plantation), Jun. 2011; Rafael F. Alfenas, culture ex-type **CBS134665 = LPF305.**

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 40–170 × 6–8 µm; stipe extensions septate, straight to flexuous, 110–340 µm long, 3–4 µm wide at the apical septum, terminating in clavate vesicles, 4–6 µm diam. Conidiogenous apparatus 30–80 µm long, 40–110 µm wide; primary branches aseptate, 20–30 × 5–7 µm; secondary branches aseptate, 15–25 × 4–6 µm and tertiary branches aseptate, 10–20 × 4–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–10 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (30–) 39–42 (–46) × 3–5 µm (av. = 40 × 4 µm), L/W ratio = 9.78 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies rosy buff to buff on the surface and ochraceous to umber in reverse; extensive aerial mycelium; sparse sporulation on the aerial mycelium; chlamydospores not seen. Colonies moderate growing (59–64 mm) diam on MEA, and fast growing (80–83 mm) on OA, after seven days at 25 ºC.

Calonectria sp. nov. 4 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 8)

Hosts/substrate: Soil

Specimens examined: Brazil, Minas Gerais state, Salinas, from soil (forest), Oct. 2011, Danilo B. Pinho (culture ex-type **CBS134659 = LPF216**)

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–80 × 7–9 µm; stipe extensions septate, straight to flexuous, 175–250 µm long, 2–4 µm wide at the apical septum, terminating in clavate vesicles, 4–6 µm diam. Conidiogenous apparatus 40–70 µm long, 60–95 µm wide; primary branches aseptate, 15–35 × 5–6 µm; secondary branches aseptate, 10–25 × 4–6 µm and tertiary branches aseptate, 7–15 × 4–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8–15 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to narrowly curved, (30–) 41–43 (–50) × 4–6 µm (av. = 42 × 5 µm), L/W ratio = 8.38 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies rosy buff to buff on the surface and ochraceous to umber in reverse; extensive aerial mycelium; sparse sporulation on the aerial mycelium; chlamydospores not seen. Colonies moderate growing (59–64 mm) diam on MEA, and fast growing (80–83 mm) diam on OA, after seven days at 25 ºC.

Calonectria sp. nov. 5 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 9)

Hosts/substrate: Soil

Specimens examined: Brazil, Bahia state, Mucuri, from soil (Eucalyptus plantation), Nov. 2011, Edival Zauza, culture ex-type **CBS134658 = LPF234;** Brazil, Bahia state, Mucuri, from soil (Eucalyptus plantation), Nov. 2011, Edival Zauza, CBS134657 = LPF236.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 55–155 × 6–8 µm; stipe extensions septate, straight to flexuous, 180–300 µm long, 3–4 µm wide at the apical septum, terminating in clavate vesicles, 5–7 µm diam. Conidiogenous apparatus 35–95 µm long, 35–80 µm wide; primary branches aseptate, 15–40 × 3–7 µm; secondary branches aseptate, 10–30 × 3–6 µm and tertiary branches aseptate, 10–20 × 3–6 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 7–15 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight (35–) 41– 44 (–55) × 4–6 µm (av. = 45 × 5 µm), L/W ratio = 9.3 µm, 1-

septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies buff forming rosy buff concentric ring on the surface and ochraceous in reverse; extensive aerial mycelium; chlamydospores and sporulation on the aerial mycelium not seen.

Colonies slow to moderate growing (50–60 mm diam.) on MEA, and fast growing (80–85 mm diam.) on OA, after seven days at 25 ºC.

Calonectria sp. nov. 6 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 10)

Hosts/substrate: Soil

Specimens examined: Brazil, Pará state, Santana, Apr. 2011, Acelino Alfenas, culture ex-type **CBS134662 = LPF280**)

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–125 × 5–8 µm; stipe extensions septate, straight to flexuous, 190–300 µm long, 3–5 µm wide at the apical septum, terminating in clavate vesicles, 5–6 µm diam. Conidiogenous apparatus 50–115 µm long, 60–100 µm wide; primary branches aseptate, 15–30 × 5–7 µm; secondary branches aseptate, 15–25 × 4–6 µm and tertiary branches aseptate, 10–20 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 7–15 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (30–) 39– 42 (–48) × 4–6 µm (av. = 41 × 5 µm), L/W ratio = 8.04 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies light amber forming rosy buff concentric ring on the surface and ochraceous to umber in reverse; extensive aerial mycelium; sparse sporulation on the aerial mycelium; chlamydospores not seen.

Colonies moderate growing (58–61 mm) diam on MEA, and fast growing (80–84 mm) diam on OA, after seven days at 25 ºC.

Calonectria sp. nov 7 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig. 11)

Hosts/substrate: Eucalyptus sp. (leaf)

Specimens examined: Brazil, Maranhão state, Açailândia, on leaves of Eucalyptus sp., May, 2011, Rafael F. Alfenas, culture ex-type **CBS134652 = LPF192**)

Description: leaf blight round or elongated, with a light-gray color progressing to a light-brown coalescing color.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 65–120 × 5–8 µm; stipe extensions septate, straight to flexuous, 125–225 µm long, 3–4 µm wide at the apical septum, terminating in acicular to clavate vesicles, 4–5 µm diam. Conidiogenous apparatus 15–60 µm long, 30–70 µm wide; primary branches aseptate, 18–35 × 4–7 µm; secondary branches aseptate, 10–20 × 3–5 µm; tertiary branches aseptate, 10–20 × 3–5 µm and additional branches (–6), aseptate, 10–15 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 5–10 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (45–) 49–52 (–60) × (3–) 4 (–5) µm (av. = 50 × 4 µm), L/W ratio = 12.6 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies folded, umber to sienna on the surface, and umber to sepia in reverse; sparse to moderate aerial mycelium forming chlamydospores sparse occurring throughout the medium, with moderate to extensive sporulation on the aerial mycelium. Colonies slow growing (34–40 mm) on MEA, and fast growing (70–80 mm) diam OA, after seven days at 25 ºC.

Calonectria sp. nov. 8 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 12)

Hosts/substrate: Soil (Forest).

Specimens examined: Brazil, Pará state, Monte Dourado, Ago, 2010; Rafael F. Alfenas, culture ex-type **CBS134656 = LPF434**)

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 45–95 × 4–7 µm; stipe extensions septate, straight to flexuous, 175–310 µm long, 3–5 µm wide at the apical septum, terminating in acicular to clavate vesicles, 4–6 µm diam. Conidiogenous apparatus 20–60 µm long, 30–50 µm wide; primary branches aseptate, 20–30 × 4–6 µm and secondary branches aseptate, 10–20 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 7–15 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (35–) 44–48 (–55) × (3–) 4 (–5) µm (av. = 46 × 4 µm), L/W ratio = 11.38 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies folded, umber to fawn on the surface and dark brick in reverse; sparse aerial mycelium; chlamydospores sparse occurring throughout the medium, with moderate to extensive sporulation on the aerial mycelium.
Colonies slow growing (33–43 mm) diam MEA, and fast growing (79–83 mm) diam on OA, after seven days at 25 ºC.

Calonectria sp. nov 9 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 13)

Hosts/substrate: Soil (Eucalyptus plantation).

Specimens examined: Brazil, Pará state, Monte Dourado, from soil (Eucalyptus plantation), May, 2011; Rafael F. Alfenas, culture ex-type **CBS134654 = LPF065**; Brazil, Pará state, Monte Dourado, from soil (Eucalyptus plantation), May, 2011; Rafael F. Alfenas, LPF302;)

Perithecia solitary or in groups, orange to red, becoming brown with age; in section apex and body orange to red, base red-brown, pyriform to sub-globose, 160–400 µm high, 115–250 µm diam, body turning dark red, and base dark red-brown (KOH+). Perithecial walls rough consisting of 2 thick-walled layers: outside layer of textura globulosa, 25–85 µm wide; becoming more compressed towards inner layer of textura angularis, 10–30 µm wide; becoming thin-walled and hyaline towards the centre, outer layer cells 10–20 × 10–30 µm;

inner cells 4–6 × 8–15 µm: perithecial base up to 135 µm wide; consisting of dark red, angular cells; merging with an erumpent stroma, cells of the outer wall layer continuing into the pseudoparenchymatous cells of the erumpent stroma. Asci 8-spored, clavate, 50–105 × 10–25 µm, tapering to a long thin stalk. Ascospores aggregated in the upper third of the ascus, hyaline, guttulate, fusoid with rounded ends, straight to curved, (1–)3-septate, slightly constricted at the septum, (25–) 39–42 (–50) × (5–) 6 (–7) µm (av. = 40 × 6 µm). Cultures were homothallic.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–145 × 5–7 µm; stipe extensions septate, straight to flexuous, 170–340 µm long, 2–4 µm wide at the apical septum, terminating in narrowly clavate to clavate vesicles, 3–5 µm diam. Conidiogenous apparatus 30–60 µm long, 35–65 µm wide; primary branches aseptate, 10–35 × 3–6 µm; secondary branches aseptate, 10–30 × 3–5 µm; tertiary branches aseptate, 10–20 × 2–4 µm and additional branches (–5 rarely), aseptate, 10–15 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–18 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (45–) 57–61 (–70) × (4–) 5 (–6) µm (av. = 59 × 5 µm), L/W ratio = 11.57 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies folded, umber to fawn on the surface and dark brick in reverse; sparse aerial mycelium; chlamydospores sparse occurring throughout the medium, with moderate to extensive sporulation on the aerial mycelium.
Colonies moderate growing (57–70 mm) diam on MEA, and fast growing (78–81 mm) diam on OA, after seven days at 25 ºC.

Calonectria sp. R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 14)

Hosts/substrate: Soil (Eucalyptus plantation).

Specimens examined: Brazil, Pará state, Santana, from soil (Eucalyptus plantation), Apr, 2011, Acelino Alfenas, culture ex-type **CBS134655 = LPF281**.

Perithecia solitary or in groups, orange to red, becoming brown with age; in section apex and body orange to red, base red-brown, globose to ovoid, 205–300 µm high, 170–300

µm diam, body turning orange to red, and base dark red-brown (KOH+). Perithecial walls rough consisting of 2 thick-walled layers: outside layer of textura globulosa, 55–90 µm wide; becoming more compressed towards inner layer of textura angularis, 15–30 µm wide; becoming thin-walled and hyaline towards the centre, outer layer cells 20–30 × 15–30 µm; inner cells 10–20 x 4–8 µm: perithecial base up to 120 µm wide; consisting of dark red, angular cells; merging with an erumpent stroma, cells of the outer wall layer continuing into the pseudoparenchymatous cells of the erumpent stroma. Asci 8-spored, clavate, 70–135 × 7–20 µm, tapering to a long thin stalk. Ascospores aggregated in the upper third of the ascus, hyaline, guttulate, fusoid, straight with rounded ends, straight to curved, 1- septate, not or slightly constricted at the septum, (25–) 33–37 (–45) × (4–) 6 (–7) µm (av. = 35 × 6 µm). Culture homothallic.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 80–175 × 6–8 µm; stipe extensions septate, straight to flexuous, 165–250 µm long, 3–4 µm wide at the apical septum, terminating in narrowly clavate to clavate vesicles, 3–5 µm diam. Conidiogenous apparatus 30–70 µm long, 40–80 µm wide; primary branches aseptate, 15–30 × 4–7 µm; secondary branches aseptate, 10–25 × 4–5 µm and tertiary branches aseptate, 10–15 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–11 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (35–) 48–52 (–60) x (4–) 5 (–6) µm (av. = 50 × 5 µm), L/W ratio = 10.95 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Mega- and microconidia were not seen observed.

Culture characteristics: Colonies vinaceous buff to greyish sepia on the surface and dark brick to sepia in reverse; moderate aerial mycelium; chlamydospores sparse occurring throughout the medium, with moderate sporulation on the aerial mycelium.
Colonies slow growing (46–50 mm) diam on MEA, and moderate growing (65–70 mm) diam on OA, after seven days at 25 ºC.

Calonectria sp. nov 10 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 15)

Hosts/substrate: Soil (Eucalyptus plantation).

Specimens examined: Brazil, Pará state, Santana, from soil (Eucalyptus plantation), Brazil; Apr, 2011; Acelino Alfenas (culture ex-type **CBS134674 = LPF267**).

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 35–105 × 5–7 µm; stipe extensions septate, straight to flexuous, 140–280 µm long, 3–6 µm wide at the apical septum, terminating in fusiform, ovate to ellipsoidal vesicles, 8–12 µm diam. Conidiogenous apparatus 55–121 µm long, 75–105 µm wide; primary branches aseptate or 1-septate, 25–75 × 5–8 µm; secondary branches aseptate, 15–35 × 4–7 µm and tertiary branches aseptate, 15–30 × 4–6 µm, each terminal branch producing 2–6 phialides; phialides elongate doliiform to reniform, hyaline, aseptate, 10–25 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight, (55–) 67–70 (–80) × (4–) 5 (–7) µm (av. = 69 × 5 µm), L/W ratio = 13.73 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime.

Microconidiophores comprising a stipe, a stipe elongation and a penicillate or subverticillate arrangement of fertile branches. Stipe elongation septate, thin-walled, terminating in an ellipsoidal to ovoid vesicle 3–5 µm diam. Primary branches aseptate 8–15 × 2–4 and secondary branches aseptate 5–10 × 2–4, terminating in 1–3 phialides; phialides elongate doliiform to reniform, straight to slightly curved, hyaline, aseptate, 7–15 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Microconidia cylindrical, straight to curved, rounded at apex, (10–) 20–23 (–30) × (3–) 4 (–6) µm (av. = 22 × 4 µm), L/W ratio = 5.38 µm 1-septate, held in fascicles by colorless slime. Megaconidia were not observed.

Culture characteristics: Colonies ochraceous to rosy buff on the surface and umber in reverse; moderate to extensive aerial mycelium; sparse sporulation on the aerial mycelium; chlamydospores not seen.

Colonies moderate growing (55–64 mm) diam on MEA and on OA, after seven days at 25 ºC.

Calonectria sp. nov 11 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 16)

Hosts/substrate: Eucalyptus sp. (leaf)

Specimens examined: Brazil, Maranhão state, Imperatriz, Mar, 2011, Rafael Alfenas, culture ex-type **CBS134673 = LPF202**.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 75–165 × 5–8 µm; stipe extensions septate, straight to flexuous, 180–305 µm long, 3–4 µm wide at the apical septum, terminating in clavate vesicles, 3–6 µm diam. Conidiogenous apparatus 25–55 µm long, 30–65 µm wide; primary branches aseptate, 15–35 × 3–5 µm; secondary branches aseptate, 15–30 × 3–5 µm and tertiary branches aseptate, 10–20 × 3–4 µm, each terminal branch producing 2–4 phialides; phialides elongate doliiform to reniform, hyaline, aseptate, 10–20 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (65–) 81–86 (–100) × (4–) 5.5 (–7) µm (av. = 84 × 5.5 µm), L/W ratio = 15.57 µm, 1(–3)-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies cinnamon to light umber on the surface and umber in reverse; sparse to moderate aerial mycelium; extensive sporulation on the aerial mycelium; chlamydospores moderate occurring throughout the medium forming microsclerotia. Colonies slow growing (49–54 mm) diam. on MEA, and moderate growing (60–66 mm) diam. on OA, after seven days at 25 ºC.

2. Calonectria morganii complex

Calonectria sp. nov.12 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Azadirachta indica (leaf)

Specimens examined: Brazil, Minas Gerais state, Viçosa, on leaf of root cuttings of Azadirachta indica, Mar, 2011; Rafael Alfenas (culture ex-type **CBS134818=LPF262**).

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 35–160 × 5–8 µm; stipe extensions septate, straight to flexuous, 160–250 µm long, 2–5 µm wide at the apical septum, terminating in clavate (rarely), ellipsoidal to obpyriform vesicles, 4–10µm diam. (av. = 8 µm) Conidiogenous apparatus 50–90 µm long, 50–95 µm wide; primary branches aseptate, 20–35 × 4–7 µm; secondary branches aseptate, 15–30 × 4.5–6 µm and tertiary branches aseptate,

10–20 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 7–15 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (45–) 53–55 (–65) × (3–) 4.5 (–5) µm (av. = 54 × 4.5 µm), L/W ratio = 11.95 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies fawn to cinnamon, with rosy buff on margin on the surface and sepia in reverse; extensive white aerial mycelium; chlamydospores moderate to extensive occurring throughout the medium, with extensive sporulation on the aerial mycelium. Colonies moderate growing (60–65 mm) diam. on MEA, and fast growing (80–85 mm) diam. on OA, after seven days at 25 ºC.

Calonectria sp. nov. 13 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Etymology: Name refers to

Hosts/substrate: Substrate for Eucalyptus cuttings

Specimens examined: Brazil, Maranhão state, Imperatriz, Jul, 2011; Rafael Alfenas, culture ex-type **CBS134827=LPF389**.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–245 × 6–7 µm; stipe extensions septate, straight to flexuous, 180–250 µm long, 3–6 µm wide at the apical septum, terminating in ellipsoidal, obpyriform to umbonate vesicles, 6–12 µm diam. Conidiogenous apparatus 40–90 µm long, 50–85 µm wide; primary branches aseptate, 19–30 × 4–6 µm; secondary branches aseptate, 15–25 × 4–6 µm and tertiary branches aseptate, 12–15 × 4–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8–12 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (40–) 47–50 (–55) × (3–) 4 (–5) µm (av. = 49 × 4 µm), L/W ratio = 11.5 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies folded vinaceous buff to fawn on the surface and dark brick in reverse; sparse aerial mycelium; chlamydospores sparse occurring throughout the medium, with moderate to extensive sporulation on the aerial mycelium.
Colonies slow growing (40–50 mm) diam. on MEA, and moderate to fast growing (70–75 mm) diam. on OA, after seven days at 25 ºC.

Calonectria sp. nov. 14 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Soil (Eucalyptus plantation)

Specimens examined: Brazil, Maranhão state, Urbano Santos, Jul, 2011; Edival Zauza, culture ex-type **CBS134828 = LPF441**.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 45–185 × 6–7 µm; stipe extensions septate, straight to flexuous, 155–225 µm long, 3–6 µm wide at the apical septum, terminating in ellipsoidal, obpyriform to sphaeropedunculate vesicles, 6–10 µm diam. Conidiogenous apparatus 35–70 µm long, 50–90 µm wide; primary branches aseptate, 20–30 × 4–6 µm; secondary branches aseptate, 10–25 × 3–6 µm and tertiary branches aseptate, 10–12 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 5–12 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (40–) 48–50 (–55) × (3–) 4 (–5) µm (av. = 49 × 4 µm), L/W ratio = 11.97 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies folded (or sectored), vinaceous buff to fawn on the surface and sepia in reverse; moderate white aerial mycelium; chlamydospores extensive to extensive occurring throughout the medium, with extensive sporulation on the aerial mycelium.
Colonies moderate growing (55–60 mm) diam. on MEA, and fast growing (80–85 mm) diam. on OA, after seven days at 25 ºC.

Calonectria sp. R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Eucalyptus sp. (leaf)

Specimens examined: Brazil, Maranhão state, Açailândia, Mai, 2011; Acelino Alfenas, culture ex-type **CBS134812 = LPF143**.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 55–105 × 6–9 µm; stipe extensions septate, straight to flexuous, 125–190 µm long, 3–5 µm wide at the apical septum, terminating in ellipsoidal, obpyriform to sphaeropedunculate vesicles, 7–11 µm diam. Conidiogenous apparatus 45–71 µm long, 45–65 µm wide; primary branches aseptate, 20–45 × 3–6 µm; secondary branches aseptate 15–20 × 3–5 µm and tertiary branches aseptate, 11–16 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 8–15 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (50–) 56–58 (–65) × (3–) 5 (–6) µm (av. = 57 × 5 µm), L/W ratio = 11.85 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies greyish sepia to dark brick on the surface and sepia to umber in reverse; extensive white aerial mycelium; chlamydospores moderate to extensive occurring throughout the medium, with moderate sporulation on the aerial mycelium. Colonies moderate growing (50–55 mm) diam. on MEA, and fast growing (80–85 mm) diam. on OA, after seven days at 25 ºC

Calonectria sp. nov. 15 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Eucalyptus cuttings (stem)

Specimens examined: Brazil, Pará state, Santana, April, 2011; Acelino Alfenas, culture ex-type **CBS134815= LPF220;** Brazil, Pará state, Santana, from Eucalyptus cuttings, April, 2011; Acelino Alfenas, CBS134816=LPF22.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 52–180 × 6–8 µm; stipe extensions septate, straight to flexuous, 130–250 µm long, 2–5 µm wide at the apical septum, terminating in ellipsoidal, obpyriform to sphaeropedunculate vesicles, 5–12 µm diam. Conidiogenous apparatus 31–85 µm long, 40–75 µm wide; primary branches aseptate or 1-septate, 18–30 × 3–7 µm; secondary branches aseptate 10–22 × 3–6, tertiary branches aseptate, 11–20 × 3–5 µm and additional branches (–4), aseptate, 9–15 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 5–12 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (40–) 48–51 (–55) × (3–) 4 (–5) µm (av. = 49 × 4µm), L/W ratio = 12.67 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies buff to light umber on the surface and sepia in reverse; moderate to extensive aerial mycelium; extensive sporulation on the aerial mycelium, especially on the center of colony; chlamydospores moderate occurring throughout the medium forming microsclerotia. Colonies fast growing (65–70 mm) diam. on MEA, and (80–85 mm) diam. on OA, after seven days at 25 ºC.

Calonectria sp. nov. 16 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Substrate for Eucalyptus cuttings

Specimens examined: Brazil, Pará state, Santana, April, 2011; Acelino Alfenas, culture ex-type **CBS134820= LPF287**.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 55–190 × 5–9 µm; stipe extensions septate, straight to flexuous, 145–290 µm long, 3–5 µm wide at the apical septum, terminating in obpyriform to sphaeropedunculate vesicles, 7–14 µm diam. Conidiogenous apparatus 60–115 µm long, 60–105 µm wide; primary branches aseptate or 1-septate, 25–45

× 4–7 µm; secondary branches aseptate or 1-septate (rarely) 17–32 × 3–6, tertiary branches aseptate, 12–20 × 3–5 µm and additional branches (–4), aseptate, 8–13 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–13 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (35–) 42–45 (–50) × (3–) 4.5 (–6) µm (av. = 43 × 4.5 µm), L/W ratio = 9.87 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies buff to rosy buff on the surface and umber to sepia in reverse; extensive white aerial mycelium; sparse sporulation on the aerial mycelium; chlamydospores sparse occurring throughout the medium forming microsclerotia. Colonies fast growing (75–85 mm diam.) on MEA and on OA, after seven days at 25 ºC.

3. Calonectria scoparia complex

Calonectria sp. nov. 17 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Soil (Eucalyptus plantation)

Specimens examined: Brazil, Alagoas state, Maceió, April, 2011; Marcelo Magalhães Coutinho, culture ex-type **CBS134845 = LPF210**.

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 62–220 × 6–8 µm; stipe extensions septate, straight to flexuous, 160–210 µm long, 2–4 µm wide at the apical septum, terminating in ellipsoidal to obpyriform vesicles, 5–7 µm diam. Conidiogenous apparatus 30–76 µm long, 45–65 µm wide; primary branches aseptate or 1-septate (rarely), 21–30 × 5–7 µm; secondary branches aseptate 16–22 × 4–7 and tertiary branches aseptate, 10–17 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides elongated doliiform to reniform, hyaline, aseptate, 9–17 × 3–5 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (40–) 49–52 (–60) × (3–) 4.5 (–5) µm (av. = 51 × 4.5 µm), L/W ratio = 11.34 µm, 1-septate, lacking

a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies folded cinnamon to dark brick on the surface and sepia in reverse; moderate aerial white mycelium; moderate to extensive sporulation on the aerial mycelium, especially on the border of colony; chlamydospores moderate to extensive occurring throughout the medium forming microsclerotia. Colonies slow growing (35–40 mm) diam. on MEA, and moderate growing (45–50 mm) diam. on OA, after seven days at 25 ºC

Calonectria sp. nov. 18 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Eucalyptus sp. seeding (stem)

Specimens examined: Brazil, Minas Gerais state, Santa Bárbara, December, 2010; Acelino Couto Alfenas, culture ex-type **CBS134847 = LPF124**

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–242 × 5–10 µm; stipe extensions septate, straight to flexuous, 145–170 µm long, 2–4 µm wide at the apical septum, terminating in ellipsoidal to obpyriform vesicles, 5–7 µm diam. Conidiogenous apparatus 35–62 µm long, 45–75 µm wide; primary branches aseptate, 20–25 × 4–6 µm; secondary branches aseptate 16–19 × 3–5 and tertiary branches aseptate, 9–16 × 2–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–12 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (43–) 49–52 (–55) × (3–) 4 (–5) µm (av. = 50 × 4 µm), L/W ratio = 12.20 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies cinnamon to dark brick on the surface and sepia in reverse; moderate to extensive sporulation on the aerial mycelium, especially on the border of colony; chlamydospores moderate occurring throughout the medium forming microsclerotia.

Colonies slow growing (40–45 mm) diam. on MEA, and moderate growing (50–55 mm) diam. on OA, after seven days at 25 ºC.

Calonectria sp. nov. 19 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Eucalyptus sp. seeding (stem)

Specimens examined: Brazil, Minas Gerais state, Martinho Campos, July, 2010; Acelino Couto Alfenas, culture ex-type **CBS134852 = LPF406**

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–130 × 5–7 µm; stipe extensions septate, straight to flexuous, 100–165 µm long, 2–4 µm wide at the apical septum, terminating in ellipsoidal to narrowly obpyriform vesicles, 3–5 µm diam. Conidiogenous apparatus 27–45 µm long, 25–40 µm wide; primary branches aseptate, 14–22 × 3–5 µm and secondary branches aseptate 11–15 × 3–5, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 5–13 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (45–) 50–52 (–55) × (3–) 4 (–5) µm (av. = 50 × 4 µm), L/W ratio = 12.06 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies buff on the surface and sepia to umber in reverse; extensive aerial mycelium; chlamydospores sparse occurring throughout the medium forming microsclerotia; moderate sporulation on the aerial mycelium. Colonies moderate growing (45–60 mm) diam on MEA and on OA, after seven days at 25 ºC

Calonectria sp. nov. 20 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Soil (Eucalyptus brassiana plantation)

Specimens examined: Brazil, Piauí state, Teresina, July, 2011; Rafael Alfenas, culture ex-type **CBS134855= LPF378**

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 55–155 × 5–8 µm; stipe extensions septate, straight to flexuous, 90–172 µm long, 2–3 µm wide at the apical septum, terminating in ellipsoidal to narrowly obpyriform vesicles, 3–7 µm diam. Conidiogenous apparatus 50–80 µm long, 50–135 µm wide; primary branches aseptate, 20–30 × 4–6 µm, secondary branches aseptate 15–25 × 3–6, and tertiary branches aseptate, 10–17 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 9–15 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (35–) 50–56 (–65) × (3–) 4 (–5) µm (av. = 53 × 4 µm), L/W ratio = 12.91 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies buff on the surface and sepia in reverse; extensive white aerial mycelium; chlamydospores sparse occurring throughout the medium forming microsclerotia; moderate sporulation on the aerial mycelium. Colonies moderate growing (40–60 mm) diam on MEA and on OA, after seven days at 25 ºC

Calonectria sp. nov. 21 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

Hosts/substrate: Soil (Eucalyptus brassiana plantation)

Specimens examined: Brazil, Piauí state, Teresina, July, 2011; Rafael Alfenas, culture ex-type **CBS134850 = LPF377**

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–110 × 4–6 µm; stipe extensions septate, straight to flexuous, 95–130 µm long, 2–3 µm wide at the apical septum, terminating in ellipsoidal to narrowly obpyriform vesicles, 3–7 µm diam. Abundant lateral stipe extensions also present. Conidiogenous apparatus 20–60 µm long, 35–80 µm wide; primary branches aseptate, 12–20 × 3–5 µm and secondary branches aseptate 8–10 × 3–4, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–12 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (38–) 47–52 (–60) × (3–) 4.5 (–5) µm (av. = 49 × 4.5 µm), L/W ratio = 11.27 µm, 1-septate, lacking a visible

abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies buff on the surface and sepia in reverse; extensive white aerial mycelium; chlamydospores sparse occurring throughout the medium forming microsclerotia; moderate sporulation on the aerial mycelium. Colonies moderate growing (50–75 mm) diam after seven days at 25 ºC on MEA and on OA

Calonectria sp. nov. 22 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 17)

Hosts/substrate: Soil (Tropical Forest)

Specimens examined: Brazil, Bahia state, Mucuri, August, 2011; Edival Zauza, culture ex-type **LPF081**

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–220 × 7–9 µm; stipe extensions septate, straight to flexuous, 130–195 µm long, 3–4 µm wide at the apical septum, terminating in, 7–10 µm diam. Conidiogenous apparatus 35–90 µm long, 45–105 µm wide; primary branches aseptate, 20–30 × 3–6 µm, secondary branches aseptate 13–26 × 3–6, and tertiary branches aseptate 8–15 × 3–5 µm each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–10 × 3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (30–) 40–42 (–50) × (3–) 4.5 (–5) µm (av. = 41 × 4.5 µm), L/W ratio = 9.17 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies folded cinnamon to dark brick on the surface and sepia in reverse; moderate aerial mycelium; extensive sporulation on the aerial mycelium, especially on the center of colony; chlamydospores moderate to extensive occurring throughout the medium forming microsclerotia. Colonies slow growing (30–40 mm) diam. on MEA, and moderate growing (45–50 mm) on OA, after seven days at 25 °C.

Calonectria sp. nov. 23 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov.

881
882 **Hosts/substrate**: Soil (Tropical Forest)
883 **Specimens examined:** Brazil, Minas Gerais state, Araponga (Serra do Brigadeiro),
884 August, 2010; Acelino Alfenas and Pedro Crous, culture ex-type **CBS134837 = LPF085**
885 Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe
886 extension, and terminal vesicle; stipe septate, hyaline, smooth, 50–105 × 6–12 µm; stipe
887 extensions septate, straight to flexuous, 150–205 µm long, 2–4 µm wide at the apical septum,
888 terminating in obpyriform vesicles, 7–13 µm diam. Conidiogenous apparatus 40–60 µm long,
889 50–80 µm wide; primary branches aseptate, 19–25 × 3–7 µm, secondary branches aseptate
890 11–18 × 3–5, tertiary branches aseptate 9–12 × 3–5 µm and rarely additional branches (–4),
891 aseptate 7–10 × 3–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to
892 reniform, hyaline, aseptate, 5–11 × 2–4 µm; apex with minute periclinal thickening and
893 inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly
894 curved, (40–) 44–46 (–50) × (3–) 4 (–5) µm (av. = 45 × 4 µm), L/W ratio = 11.06 µm, 1-
895 septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless
896 slime. Micro and Megaconidia were not observed.
897
898 **Culture characteristics:** Colonies buff on the surface and sepia to umber in reverse;
899 extensive white aerial mycelium; chlamydospores sparse occurring throughout the medium
900 forming microsclerotia; sparse to moderate sporulation on the aerial myceliumColonies fast
901 growing (55–80 mm) diam. on MEA and on OA, after seven days at 25 °C.
902
903 Calonectria sp. nov. 24 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp.
904 nov. (Fig 18)
905
906 **Hosts/substrate**: Soil (Tropical Forest)
907 **Specimens examined:** Brazil, Minas Gerais state, Araponga (Serra do Brigadeiro),
908 August, 2010; Acelino Alfenas and Pedro Crous, culture ex-type **CBS134841 = LPF072**
909 Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe
910 extension, and terminal vesicle; stipe septate, hyaline, smooth, 45–95 × 5–8 µm; stipe
911 extensions septate, straight to flexuous, 145–190 µm long, 2–4 µm wide at the apical septum,
912 terminating in obpyriform vesicles, 7–10 µm diam. Conidiogenous apparatus 30–70 µm long,
913 65–100 µm wide; primary branches aseptate, 15–25 × 4–7 µm, secondary branches aseptate

12–20 × 4–5, and tertiary branches aseptate 10–12 × 3–5 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 5–10 ×3–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (38–) 41–44 (–50) × (3–) 4 (–5) µm (av. = 43 × 4 µm), L/W ratio = 10.46 µm, 1-septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless slime. Micro and Megaconidia were not observed.

Culture characteristics: Colonies folded cinnamon to dark brick on the surface and sepia to umber in reverse; extensive aerial white mycelium; moderate sporulation on the aerial mycelium, chlamydospores moderate occurring throughout the medium forming microsclerotia. Colonies slow to moderate growing (40–60 mm) on MEA and on OA, after seven days at 25 °C.

4. Calonectria naviculata complex

Calonectria sp. nov. 25 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp. nov. (Fig 19)

Hosts/substrate: Soil (Eucalyptus plantation)

Specimens examined: Brazil, Bahia state, Mucuri, August, 2010; Edival Zauza, culture ex-type **CBS134858 = LPF233**

Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe extension, and terminal vesicle; stipe septate, hyaline, smooth, 45–90 × 5–7 µm; stipe extensions septate, straight to flexuous, 75–140 µm long, 2–5 µm wide at the apical septum, terminating in naviculate vesicles, 4–7 µm diam., abundant lateral stipe extension also present. Conidiogenous apparatus 30–65 µm long, 40–70 µm wide; primary branches aseptate, 19–22 × 3–6 µm, secondary branches aseptate 9–18 × 3–6, and tertiary branches aseptate 9–12 × 2–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to reniform, hyaline, aseptate, 6–12 × 2–4 µm; apex with minute periclinal thickening and inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly curved, (40–) 44–49 (–52) × (2–) 3.5 (–4) µm (av. = 46 × 3.5 µm), L/W ratio = 13.72 µm, 1-

946 septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless
947 slime. Micro and Megaconidia were not observed.
948
949 **Culture characteristics:** Colonies buff on the surface and sepia to umber in reverse;
950 extensive white aerial mycelium; chlamydospores not seen; sparse to moderate sporulation on
951 the aerial mycelium. Colonies moderate to fast growing (50–70 mm) diam. on MEA and on
952 OA, after seven days at 25 °C.
953
954 Calonectria sp. nov. 26 R.F. Alfenas, O.L. Pereira, P.W Crous & A.C. Alfenas, sp.
955 nov. (Fig 20)
956
957 **Hosts/substrate**: Soil (Eucalyptus plantation)
958 **Specimens examined:** Brazil, Pará state, Monte Dourado, August, 2008; Rafael
959 Alfenas, culture ex-type **CBS134861 = LPF448**
960 Conidiophores containing a stipe bearing penicillate suites of fertile branches, stipe
961 extension, and terminal vesicle; stipe septate, hyaline, smooth, 25–90 × 4–7 µm; stipe
962 extensions septate, straight to flexuous, 70–145 µm long, 2–5 µm wide at the apical septum,
963 terminating in naviculate vesicles, 3–7 µm diam., abundant lateral stipe extension also
964 present. Conidiogenous apparatus 35–60 µm long, 40–80 µm wide; primary branches
965 aseptate, 15–22 × 2–5 µm, secondary branches aseptate 11–15 × 3–5, and tertiary branches
966 aseptate 8–11 × 2–4 µm, each terminal branch producing 2–6 phialides; phialides doliiform to
967 reniform, hyaline, aseptate, 5–9 × 2–4 µm; apex with minute periclinal thickening and
968 inconspicuous collarette. Macroconidia cylindrical, rounded at both ends, straight to slightly
969 curved, (34–) 36–41 (–45) × (2–) 3 (–4) µm (av. = 38 × 3 µm), L/W ratio = 11.52 µm, 1-
970 septate, lacking a visible abscission scar, held in parallel cylindrical clusters by colourless
971 slime. Micro and Megaconidia were not observed.
972
973 **Culture characteristics:** Colonies buff on the surface and sepia to umber in reverse;
974 extensive white aerial mycelium; chlamydospores not seen; sparse to moderate sporulation on
975 the aerial mycelium. Colonies moderate growing (50–60 mm) diam. on MEA and on OA,
976 after seven days at 25 °C.
977
978
979

4 General discussion

The present study represents the first DNA phylogeny of the genus Calonectria in Brazil. Phylogenetic studies on Calonectria have substantially influencied the taxonomic of these genera (Lombard et al. 2010c). In the recent years at least 20 new Calonectria species were discovered mainly based on phylogenetic species recogntion concept in fungi proposed by Taylor et al. (2000) (Alfenas et al. 2013, Lombard et al. 2010c, Chen et al. 2011, Xu et al. 2012).

In this study were discovered 26 new Calonectria species in Brazil based on morphological characteristics and phylogenetic inference. This result supports the hypotese that many more species of Calonectria could be discovered, particulary from the tropics and Southern Hemisphere (Crous et al. 2006, Lombard et al. 2010c).

For phylogenetic inference was adopted the concept proposed by Cracraft (1983): "smallest diagnosable clade of individual organisms within which there is a pattern of ancestry and descent" with at least 0.90 posterior probabilitie as measures of clade support.

Results of others studies (Schoch et al., 2000, Crous, 2002, Lombard et al., 2010 b,c) as well as the present study support characters such as conidial morphology (length, septation and length/diam ratio), vesicle morphology (shape and width) and, number of branches per conidiophore, as primary characters, at least for species complex recognition.

Calonectria brassicae complex is chacterizated mainly by clavate vesicles and small (< 60 µm), 1-sepatate macroconidia. Belongs to this complex are: C. orienatlis L. Lombard, M.J. Wingf. & Crous, C. pini L. Lombard, M.J. Wingf. & Crous, C. brachiatica L. Lombard, M.J. Wingf. & Crous, C. brassicae (Panwar & Borha) L. Lombard, M.J. Wingf. & Crous, C. clavata Alfieri, El-Gholl & E.L. Bernard, C. ecuadoriae (Crous & M.J. Wingf) L. Lombard, M.J. Wingf. & Crous Crous and C. gracilis Crous, M.J. Wingf & Alfenas. Calonectria pteridis complex here represented by clade 5 and clade 6 also was included in the same phylogenetic analysis, because of morphologic similarity in vesicle shape as well as in phylogenetic relationship.

Crous et al. (2006) studied species with clavate vesicles but contrary to what expected, only two new species could be resolved. However, in the present study 11 new species for this complex could be resolved, and of which only Calonectria sp. 7 and Calonectria sp. 11 were found causing leaf blight in Eucalyptus sp. The others species were collected from soil and the pathogenicity test should be made to understand the biology of these new species.

Calonectria morganii complex is characterised by having 1-septate macroconidia and vesicles varying form pyriform to obpyriform or ovoid to ellipsoidal, including C. cerciana L. Lombard, M.J. Wingf. & Crous, C. insularis C.L. Schoch & Crous, C. morganii, C. sulawesiensis, C. hawksworthii (Peerally) L. Lombard, M.J. Wingf. & Crous, C. leucothöes (El-Gholl, Leahy & T.S. Schub.) L. Lombard, M.J. Wingf. & Crous, C. variabilis Crous, B.J.H. Janse, D. Victor, G.F. Marias & Alfenas, C. brasiliensis (Peerally) L. Lombard, M.J. Wingf. & Crous, C. hodgesii R.F Alfenas, O.L. Pereira, Crous & Alfenas (Lombard et al., 2010c, Alfenas et al. 2013). In this complex were discovered five new species based on phylogenetic inference (Fig. 2) and morphological features (Table 5). Several past studies focused on taxonomy of Calonectria spp. in this complex were initially regarded as either C. morganii (= Cylindrocladium scoparium) or C. scoparia (= Cy. candelabrum) based on their morphological similarities. However, C. morganii was circumscribed as having mainly ellipsoidal to pyriform vesicles and Ca. scoparia having ellipsoidal to obpyriform vesicles by Crous et al. (1993).

Calonectria scoparia complex is characterised by species having ellipsoidal to obpyriform vesicles and producing 1-septate macroconidia (Schoch et al. 1999, Crous 2002). In this complex are includes: C. pauciramosa C. L. Schoch & Crous, C. scoparia Peerally, C. pseudoscoparia L. Lombard, M.J. Wingf. & Crous, C. polizzii L. Lombard, M.J. Wingf. & Crous, C. zuluensis L. Lombard, M.J. Wingf. & Crous, C. colombiana L. Lombard, M.J. Wingf. & Crous, C. spathulata El- Gholl, Kimbr., E.L. Barnard, Alfieri & Schoult and, C. metrosideri R.F Alfenas, O.L. Pereira, Crous & Alfenas (Lombard et al., 2010b, Alfenas et al. 2013). In the present study were discovered eight new Calonectria species. These, Calonectria sp. 19, Calonectria sp. 20 and, Calonectria sp. 21 were found causing diseases in forest nursery on seedlings of Metrosideros polymorpha and of Eucalyptus sp. Calonectria scoparia complex represent an important pathogen complex, has been reported worldwide on numerous plant hosts and this complex is regarded as the dominant pathogen in commercial forest nurseries (Crous 2002, Lombard et al. 2010b, Schoch et al. 1999). This complex has been found on regions that the climatic conditions differs significantly, supporting the view that these species can tolerate a wide range of temperature conditions

In the first first phylogeny study of the genus Calonectria using β- tubulin sequence data, Schoch et al. (2001) separated Calonectria species in two main groups: Prolate and Sphaero-naviculate groups. Recently, this view was supported by Lombard et al. (2010 c), using actin, β-tubulin, calmodulin, histone H3 and translation elongation 1-alpha. The prolate

group includes the majority of the plant pathogenic Calonectria spp., and has some correlation in their distribuition. Calonectria spp. representing C. reteaudii complex have been reported only from Australia, China, Indonesia and New Zealand, while Calonectria spp representing C. brassicae have all been reported from South and Central America, with the exception of C. orientalis. Isolates from other sub-clades in prolate group appeared to have a broad geographic distribuition (Lombard et al. 2010c, Schoch et al. 2001).

In the Sphaero-naviculate Group there were no obvious patterns of distribuition and pathogenicity, and only vesicle morphology appeared consistent. The majority of species in this group belong to C. kyotensis complex and C. naviculata complex. Here, were discovered two new species in C. naviculata complex based on phylogenetic inference and on morphologic characterization. This complex can easily be distinguished from others Calonectria species because of the presence of naviculate vesicle shape.

Results of the present study support the morphological and phylogenetic concepts that are used in taxonomy of Calonectria. However more analysis shoul be made to confirm these recent results, as well as more research is needed on the population level.

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1173 **Table 1:** Isolates of Calonectria species studied

Isolates[1]	Species	City/Estate/Country	Host/substrate	Collector	GenBank accession nr.[2]		
					TEF-1α	TUB	CAL
CBS134652[T]	Calonectria sp.	Açailândia, Maranhão, Brazil	Eucalyptus sp. (leaf)	Rafael Alfenas			
CBS134653	Calonectria sp.	Açailândia, Maranhão, Brazil	Eucalyptus sp. (leaf)	Rafael Alfenas			
LPF190	Calonectria sp.	Açailândia, Maranhão, Brazil	Eucalyptus sp. (leaf)	Rafael Alfenas			
CBS134654[T]	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134863	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134655[T]	Calonectria sp.	Santana, Pará, Brazil	Soil (Eucalyptus plantation)	Acelino Alfenas			
CBS134656[T]	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Forest)	Rafael Alfenas			
LPF453	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF237	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS134657[T]	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
LPF235	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS134658[T]	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS134659[T]	Calonectria sp.	Salinas, Minas Gerais, Brazil	Soil	Danilo Pinho			
CBS134660	Calonectria sp.	Salinas, Minas Gerais, Brazil	Soil	Danilo Pinho			
CBS134661	Calonectria sp.	Santana, Pará, Brazil	Soil (Eucalyptus plantation; Clone 3244)	Acelino Alfenas			
CBS134662[T]	Calonectria sp.	Santana, Pará, Brazil	Soil (Eucalyptus plantation; Clone H-3911)	Acelino Alfenas			
CBS134663	Calonectria sp.	Salinas, Minas Gerais, Brazil	Soil (Forest)	Danilo Pinho			
CBS134664[T]	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Forest)	Edival Zauza			
CBS134665[T]	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134666	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF300	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF032	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF301	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF435	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF306	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF308	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF309	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
LPF429	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Forest)	Rafael Alfenas			
CBS134667	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS134668	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS134669[T]	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation; Clone 2646)	Rafael Alfenas			
CBS134670	Calonectria sp.	Imperatriz, Maranhão, Brazil	Eucalyptus sp. (leaf); Clone MA 2006	Rafael Alfenas			
CBS134671[T]	Calonectria sp.	Monte Dourado, Pará, Brazil	Eucalyptus sp. (leaf); Clone 2646	Rafael Alfenas			
CBS134673[T]	Calonectria sp.	Imperatriz, Maranhão, Brazil	Eucalyptus sp. (leaf); Clone I-144	Rafael Alfenas			

1174 **Table 1:** (Continued)

Isolates[1]	Species	City/Estate/Country	Host/substrate	Collector	GenBank accession nr.[2]		
					TEF-1α	TUB	CAL
CBS134674[T]	Calonectria sp.	Santana, Pará, Brazil	Soil (Eucalyptus plantation; Clone H-1206)	Acelino Alfenas			
CBS134675	Calonectria sp.	Santana, Pará, Brazil	Soil (Eucalyptus plantation; Clone U-1095)	Acelino Alfenas			
LPF286	Calonectria sp.	Santana, Pará, Brazil	Soil (Eucalyptus plantation; Clone U-1095)	Acelino Alfenas			
CBS111284	Ca. gracilis	Brazil	Soil	P. Crous	GQ267324	DQ190567	GQ267408
CBS111299	Ca. ovata	Tucuruí, Pará, Brazil	Eucalyptus tereticornis	P. Crous	GQ267318	GQ267212	GQ267400
CBS111307	Ca. ovata	Tucuruí, Pará, Brazil	Eucalyptus tereticornis	P. Crous	GQ267319	AF210868	GQ267401
CBS111394	Ca. ecuadoriae	Ecuador	Soil	M.J. Wingfield	GQ267304	DQ190599	GQ267376
CBS111406[T]	Ca. ecuadoriae	Ecuador	Soil	M.J. Wingfield	GQ267303	DQ190600	GQ267375
CBS111478	Ca. brassicae	Brazil	Soil	Acelino Alfenas	FJ918568	DQ190611	GQ267383
CBS111793	Ca. pteridis	U.S.A	Arachnoides adiantiformis	P. Crous	FJ918563	DQ190578	GQ267413
CBS111807	Ca. gracilis	Belém, Pará, Brazil	Manilkara zapota	M. Aragaki	GQ267323	AF232858	GQ267407
CBS111869	Ca. brassicae	Indonesia	Argyeia splendens	F. Bugnicourt	FJ918567	AF232857	GQ267382
CBS111871	Ca. pteridis	Spain	Pinus sp	T.L. Krugner	FJ918564	DQ190579	GQ267414
CBS112142	Ca. gordoniae	U.S.A	Gordonia liasanthus	D. Chiappini	GQ267309	AF449449	GQ267381
CBS114557[T]	Ca. clavata	U.S.A	Callistemon viminalis	C.P. Seymour & E.L. Barnard	GQ267305	AF333396	GQ267377
CBS114666	Ca. clavata	U.S.A	root debris in peat	D. Ferrin/ N.E. El-Gholl	GQ267306	DQ190549	GQ267378
CBS125552[T]	Ca. pini	Buga, Colombia	Pinus patula	C.A Rodas	GQ267344	GQ267224	GQ267436
CBS123699	Ca. brachiatica	Buga, Colombia	Pinus tecunumanii	M.J. Wingfield	GQ267295	FJ716708	GQ267365
CBS123700[T]	Ca. brachiatica	Buga, Colombia	Pinus maximinoi	M.J. Wingfield	GQ267296	FJ696388	GQ267366
CBS125253	Ca. pini	Buga, Colombia	Pinus patula	C.A Rodas	GQ267345	GQ267225	GQ267437
CBS125259	Ca. orientalis	Teso East, Indonesia	Soil	M.J. Wingfield	GQ267357	GQ267237	GQ267449
CBS125260[T]	Ca. orientalis	Lagan, Indonesia	Soil	M.J. Wingfield	GQ267356	GQ267236	GQ267448
CBS115674[T]	Ca. gracilipes	La Selva, Colombia	Soil	M.J. Wingfield	GQ267310	AF333406	GQ267384
CBS111141	Ca. gracilipes	La Selva, Colombia	Soil	M.J. Wingfield	GQ267311	DQ190566	GQ267385
CBS134811	Calonectria sp.	Açailândia, Maranhão, Brazil	Eucalyptus sp. (leaf)	Acelino Alfenas			
CBS134812[T]	Calonectria sp.	Açailândia, Maranhão, Brazil	Eucalyptus sp. (leaf)	Acelino Alfenas			
CBS134813	Calonectria sp.	Viçosa, Minas Gerais, Brazil	Eucalyptus sp. seedling (stem)	Rafael Alfenas			
CBS134814	Calonectria sp.	Viçosa, Minas Gerais, Brazil	Eucalyptus sp. seedling (stem)	Rafael Alfenas			
LPF218	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus sp. seedling (leaf)	Acelino Alfenas			
LPF221	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus sp. seedling (stem)	Acelino Alfenas			
CBS134815[T]	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus sp. seedling (stem)	Acelino Alfenas			
CBS134817	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus sp. seedling (leaf)	Acelino Alfenas			
CBS134818[T]	Calonectria sp.	Viçosa, Minas Gerais, Brazil	Azadirachta indica (leaf)	Rafael Alfenas			

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1176 **Table 1:** (Continued)

Isolates[1]	Species	City/Estate/Country	Host/substrate	Collector	GenBank accession nr.[2]		
					TEF-1α	TUB	CAL
CBS134819	Calonectria sp.	Viçosa, Minas Gerais, Brazil	Azadirachta indica (leaf)	Rafael Alfenas			
CBS134820[T]	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus nursery/used substrate	Acelino Alfenas			
CBS134821	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus nursery/used substrate	Acelino Alfenas			
CBS134822	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus sp. seedling (stem)	Acelino Alfenas			
CBS134823	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus sp. seedling (stem)	Acelino Alfenas			
CBS134824[T]	Calonectria sp.	Santana, Pará, Brazil	Eucalyptus sp. seedling (stem)	Acelino Alfenas			
CBS134825	Calonectria sp.	Imperatriz, Maranhão, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134826	Calonectria sp.	Imperatriz, Maranhão, Brazil	Eucalyptus nursery/substrate	Rafael Alfenas			
CBS134827[T]	Calonectria sp.	Imperatriz, Maranhão, Brazil	Eucalyptus nursery/substrate	Rafael Alfenas			
CBS134828[T]	Calonectria sp.	Urbano Santos Maranhão, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS134829	Calonectria sp.	Urbano Santos Maranhão, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS109165	Ca. pseudospathiphylli	Ecuador	Soil	M.J. Wingfield	FJ918562	FJ918513	GQ267412
CBS109166	Ca. leucothoës	U.S.A	Leucothoë axillaris	N.E. El-Gholl	FJ918553	FJ918508	GQ267392
CBS110666	Ca. morganii	U.S.A	Rosa sp.	N.E. El-Gholl	FJ918557	FJ918509	GQ267423
CBS112691	Ca. variabilis	Brazil	Theobroma grandiflorum	F. Carneiro	GQ267335	GQ267240	GQ267458
CBS114257	Ca. brasiliensis	Aracruz nursery, Brazil	Eucalyptus sp.	A. Alfenas	GQ267329	GQ267242	GQ267422
CBS114540	Ca. spathiphylli	U.S.A	Spathiphyllum sp.	S. A. Alfieri	GQ267330	AF348214	GQ267424
CBS114558	Ca. insularis	Tamatave, Madagascar	Soil	P. Crous	FJ918556	AF210861	GQ267389
CBS114559	Ca. insularis	Tamatave, Madagascar	Soil	P. Crous	FJ918555	AF210862	GQ267390
CBS114677	Ca. variabilis	Brazil	Schefflera morotoni	F. C. de Albuquerque	GQ267334	AF333424	GQ267457
CBS116168	Ca. spathiphylli	Switzerland	Spathiphyllum sp.	L. Petrini	FJ918561	FJ918512	GQ267425
CBS123693[T]	Ca. cerciana	Zhanjiang Prov., CERC nursery, China	Hybrid "urograndis"	M.J. Wingfield & X.D. Zhou	FJ918559	FJ918510	GQ267369
CBS123695	Ca. cerciana	Zhanjiang Prov., CERC nursery, China	Hybrid "urograndis"	M.J. Wingfield & X.D. Zhou	FJ918560	FJ918511	GQ267370
CBS125248	Ca. sulawesiensis	Sulawesi, Indonesia	Eucalyptus sp.	M.J.Wingfield	GQ267343	GQ267223	GQ267435
CBS125249	Ca. densa	Pichincha Province, Ecuador	Soil	M.J.Wingfield	GQ267350	GQ267230	GQ267442
CBS125251[T]	Ca. humicola	Pichincha Province, Ecuador	Soil	M.J.Wingfield	GQ267353	GQ267233	GQ267445
CBS125261[T]	Ca. densa	Pichincha Province, Ecuador	Soil	M.J.Wingfield	GQ267352	GQ267232	GQ267444
CBS125269	Ca. humicola	Pichincha Province, Ecuador	Soil	L. Lombard	GQ267355	GQ267235	GQ267447
CBS125277[T]	Ca. sulawesiensis	Sulawesi, Indonesia	Eucalyptus sp.	M.J.Wingfield	GQ267342	GQ267222	GQ267434
CBS133608	Ca. hodgesii	Viçosa, MG	Piptadenia gonoacantha	R. Alfenas	KC491224	KC491227	KC491221
CBS133609[T]	Ca. hodgesii	Viçosa, MG	Anadenanthera peregrina	R. Alfenas	KC491225	KC491228	KC491222
CBS133610	Ca. hodgesii	Viçosa, MG	Azadirachta indica	R. Alfenas	KC491226	KC491229	KC491223
CBS230.51	Ca. brasiliensis	Brazil	Eucalyptus sp.	R. Ciferri	GQ267328	GQ267241	GQ267421

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Table 1: (Continued)

Isolates[1]	Species	City/Estate/Country	Host/substrate	Collector	GenBank accession nr.[2]		
					TEF-1α	TUB	CAL
CBS134836	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
LPF071	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
LPF081^{T}	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Tropical Forest)	Edival Zauza			
LPF096	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
CBS134837^{T}	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
CBS134838	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
CBS134839	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
CBS134840	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
CBS134841^{T}	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
CBS134842	Calonectria sp.	Araponga, Minas Gerais, Brazil	Soil (Tropical Forest)	Acelino Alfenas/ Pedro Crous			
CBS134843	Calonectria sp.	Viçosa, Minas Gerais, Brazil	Metrosideros polymorpha (leaf)	Rafael Alfenas			
CBS134844	Calonectria sp.	Açailândia, Maranhão, Brazil	Eucalyptus sp. (leaf)	Acelino Alfenas			
CBS134845^{T}	Calonectria sp.	Maceió, Alagoas, Brazil	Soil (Eucalyptus plantation)	Marcelo Magalhães Coutinho			
CBS134846	Calonectria sp.	Eunápolis, Bahia, Brazil	Eucalyptus sp. (leaf)	Acelino Alfenas			
CBS134847^{T}	Calonectria sp.	Santa Bárbara, Minas Gerais, Brazil	Eucalyptus sp. seeding (stem)	Acelino Alfenas			
CBS134848	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134849	Calonectria sp.	Serra das Confusões, Piauí	Soil (Tropical Forest)	Olinto Liparini Pereira			
CBS134850^{T}	Calonectria sp.	Teresina, Piauí, Brazil	Soil (Eucalyptus brassiana plantation)	Rafael Alfenas			
CBS134851	Calonectria sp.	Teresina, Piauí, Brazil	Soil (Tropical Forest)	Rafael Alfenas			
CBS134852^{T}	Calonectria sp.	Martinho Campos, Minas Gerais, Brazil	Soil (Eucalyptus plantation)	Acelino Alfenas			
CBS134853	Calonectria sp.	Bico do Papagaio, Tocantins, Brazil	Eucalyptus sp. (leaf)	Rafael Alfenas			
CBS134854	Calonectria sp.	Bico do Papagaio, Tocantins, Brazil	Eucalyptus sp. (leaf)	Rafael Alfenas			
CBS134855^{T}	Calonectria sp.	Teresina, Piauí, Brazil	Soil (Eucalyptus brassiana plantation)	Rafael Alfenas			
CBS134856	Calonectria sp.	Teresina, Piauí, Brazil	Soil (Eucalyptus brassiana plantation)	Rafael Alfenas			
CBS134857	Calonectria sp.	Teresina, Piauí, Brazil	Soil (Eucalyptus brassiana plantation)	Rafael Alfenas			
CBS134858^{T}	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS134859	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134860	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134861^{T}	Calonectria sp.	Monte Dourado, Pará, Brazil	Soil (Eucalyptus plantation)	Rafael Alfenas			
CBS134862	Calonectria sp.	Mucuri, Bahia, Brazil	Soil (Eucalyptus plantation)	Edival Zauza			
CBS125255	Ca. pseudoscoparia	Pichincha Province, Ecuador	Eucalyptus grandis (nursery)	M.J.Wingfield	GQ267347	GQ267227	GQ267439
CBS112689	Ca. spathulata	Brazil	Eucalyptus viminalis	N.E. El-Gholl	FJ918554	AF308463	GQ267426
CBS115127^{T}	Ca. colombiana	La Selva, Colombia	Soil	M.J.Wingfield	FJ972492	FJ972423	GQ267455

Table 1: (Continued)

Isolates[1]	Species	City/Estate/Country	Host/substrate	Collector	GenBank accession nr.[2]		
					TEF-1α	TUB	CAL
CBS115638	Ca. colombiana	La Selva, Colombia	Soil	M.J.Wingfield	FJ972491	FJ972422	GQ267456
CBS125257[T]	Ca. pseudoscoparia	Pichincha Province, Ecuador	Eucalyptus grandis (nursery)	M.J.Wingfield	GQ267349	GQ267229	GQ267441
CBS125268[T]	Ca. zuluensis	Kwa-Zulu Natal, Kwambonambi, South Africa	Eucalyptus grandis	L. Lombard	FJ972483	FJ972414	GQ267459
CBS125270	Ca. pollizii	Sicily, Messina, Italy	Callistemon citrinus	G. Polizzi	FJ972486	FJ972417	GQ267461
CBS125271	Ca. pollizii	Sicily, Messina, Italy	Arbustus unedo	G. Polizzi	FJ972487	FJ972418	GQ267462
CBS133603	Ca. metrosideri	Viçosa, MG	Metrosideros polymorpha	Rafael Alfenas	KC294310	KC294313	KC294304
CBS133604	Ca. metrosideri	Viçosa, MG	Metrosideros polymorpha	Rafael Alfenas	KC294311	KC294314	KC294305
CBS133605	Ca. metrosideri	Viçosa, MG	Metrosideros polymorpha	Rafael Alfenas	KC294312	KC294315	KC294306
CBS555.92	Ca. spathulata	São Paulo, Brazil	Araucaraia angustifolia	C. Hodges	GQ267331	GQ267215	GQ267427
CMW30823	Ca. pauciramosa	Tzaneen, South Africa	Eucalyptus grandis (nursery)	S. de Buisson	FJ918566	FJ918515	GQ267404
CMW31000	Ca. scoparia	Amazonas, Brazil	Eucalyptus sp.	Acelino Alfenas	FJ972525	FJ972426	GQ267367
CMW31001	Ca. scoparia	Amazonas, Brazil	Eucalyptus sp.	Acelino Alfenas	GQ267298	GQ421779	GQ267368
CMW5683[T]	Ca. pauciramosa	Knysna, South Africa	Soil	P. Crous	FJ918565	FJ918514	GQ267405
CBS125272	Ca. zuluensis	Kwa-Zulu Natal, Kwambonambi, South Africa	Eucalyptus grandis x urophylla hybrid cutting	L. Lombard	FJ972484	FJ972415	GQ267460

[1]CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, Netherlands; CMW: Cultures of Mike Wingfield; LPF: Laboratory of Forest Pathology, DFT-UFV, Viçosa, Minas Gerais, Brazil;[2] GenBank Accession Number : TEF-1α = Translation elongation factor 1-alpha, TUB = β-tubulin and CAL = Calmodulin; [T] Ex-type cultures

Table 2: Preliminary results based on phylogenetic analysis using TEF 1α

Species complex	Nb. isolates	New species
C. brassicae complex	119	9
C. morganii complex	100	5
C. naviculata complex	42	2
C. pteridis complex	565	2
C. scoparia complex	191	8
Amount	1017	**26**

Table 3: Nucleotide substitution models used in phylogenetic analyses

Calonectria complex	Evolution model			Nb taxa	Combined character
	TEF-1α	TUB	CAL		
C. brassicae and C. pteridis complexes	HKY+G	HKY+G	GTR+G	59	1511
C. morganii complex	GTR+G	GTR+G	HKY+G	45	1530
C. naviculata complex	GTR+I	HKY+I	HKY+I	11	1533
C. scoparia complex	GTR+G	HKY+G	HKY+G	46	1498
All taxa	GTR+I+G	HKY+I+G	GTR+G	155	1582

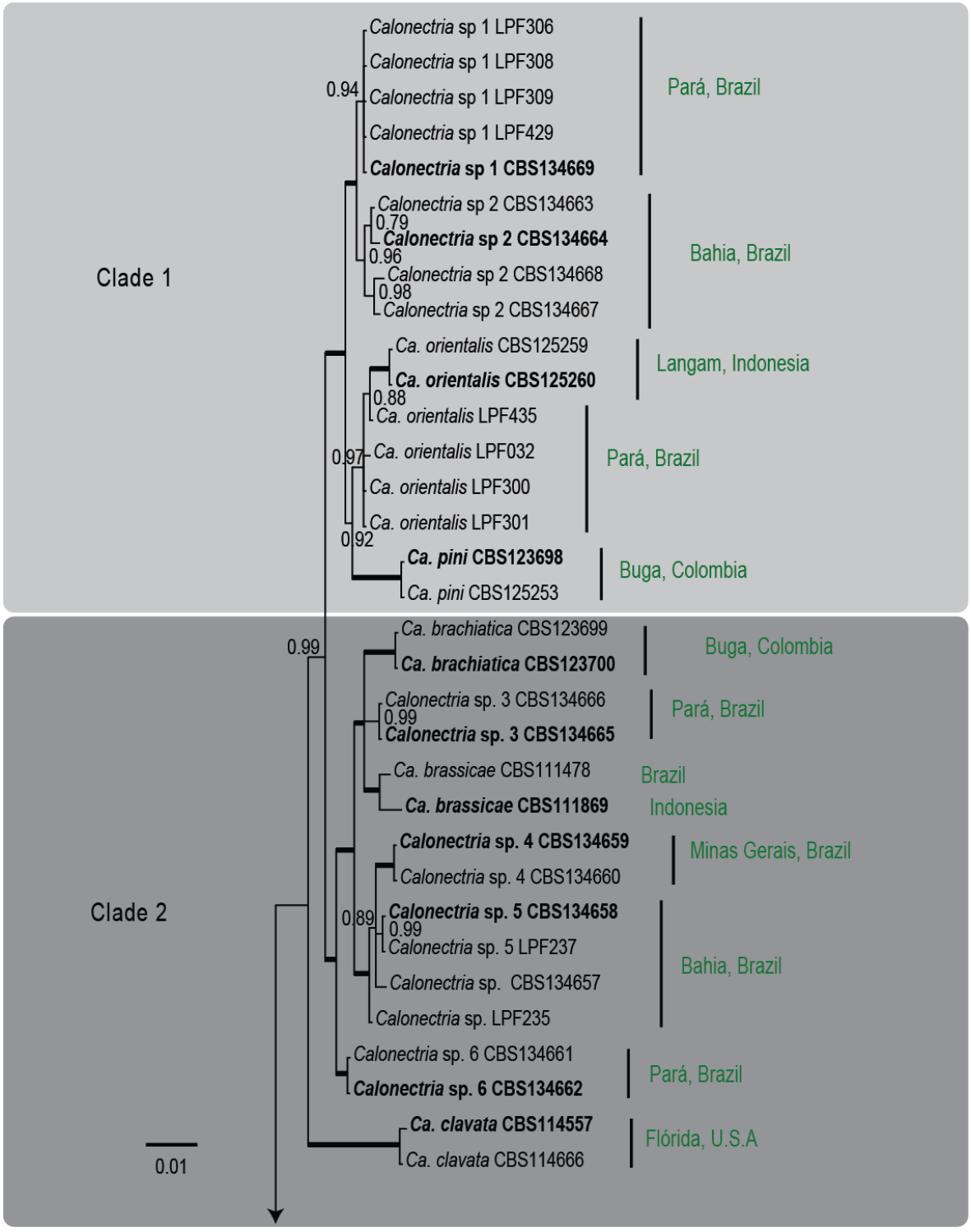

Fig 1: Phylogenetic tree obtained by Bayesian inference using combined sequences of β-tubulin, translation elongation factor 1α and calmodulin sequence alignments of Calonectria brassicae and Ca. pteridis complex The Bayesian posterior probability values are show at the nodes Calonectria colombiensis (CBS 112220) is used as outgroup. Culture accession numbers and place of origin are listed. Ex-type isolates are emphasized in bold. The place where the isolates were collected is also indicate.

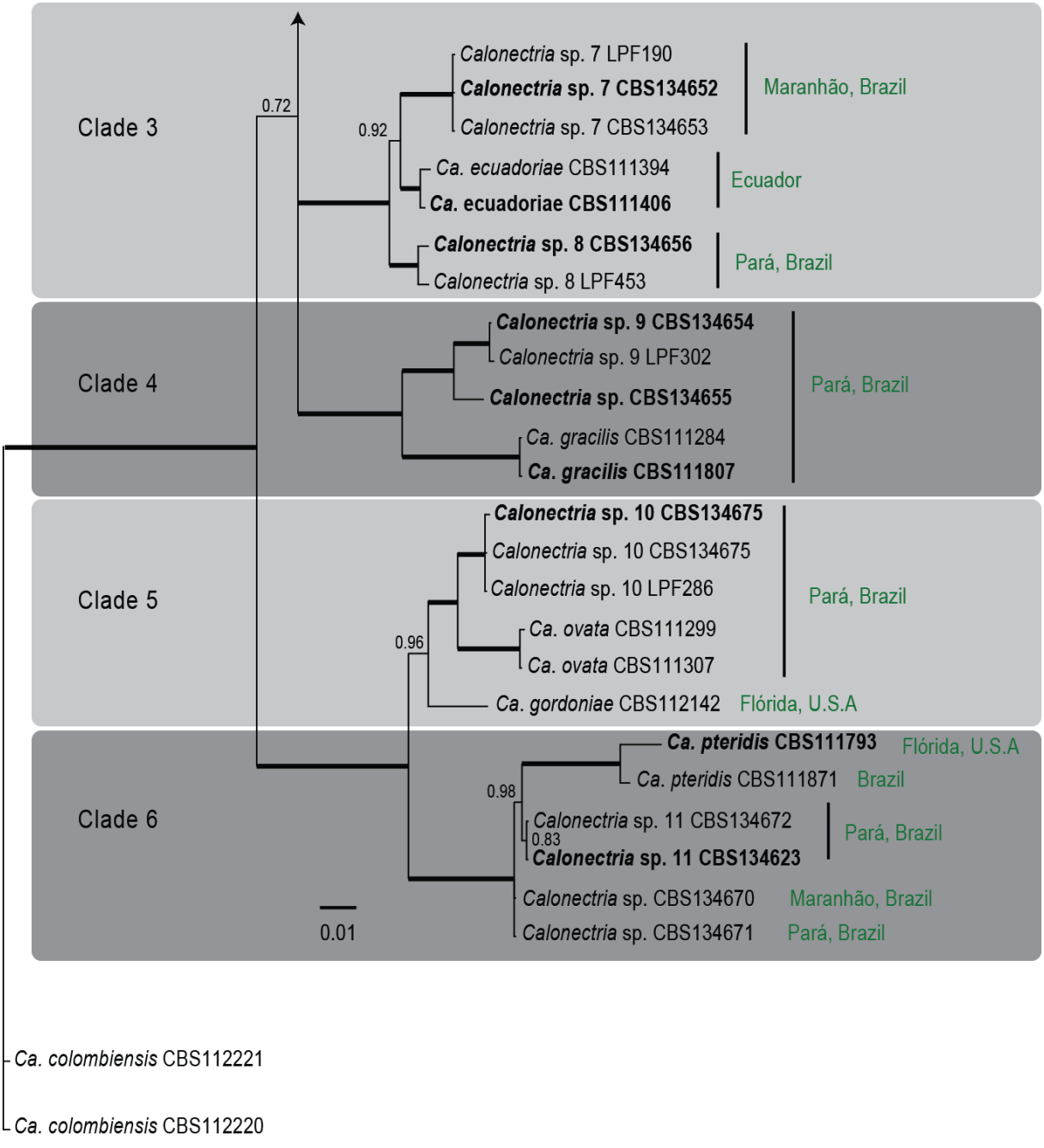

Fig 1: (Continued)

Table 4: Distinctive morphological characters of Calonectria brassicae complex.

Species	Vesicle shape	Vesicle diameter (µm)	Macroconidial size (µm)	Length/Diam. Ratio (µm)	Macroconidial septation	Laterial stipe extension	Nb. branches
C. pini	Clavate	4–6	(37–) 44 (–50) × (4–) 5 (–6)	8.8	1-septate	absent	3
C. orientalis	clavate to broadly clavate	5–10	(43–) 48 (–53) × (4–) 4 (–5)	12.0	1-septate	absent	5
Calonectria sp 1	Clavate	4–6	(35–) 42 (–45 × (3–) 5 (–6)	8.85	1-septate	absent	2
Calonectria sp 2	Clavate	3–6	(35–) 41 (–50) × (3–) 5 (–6)	9.13	1-septate	absent	4
C. brachiatica	Clavate	5–7	(37–) 44 (–50) × (4–) 5 (–6)	8.8	1(–2)-septate	present (90° to the axis)	5
Calonectria sp 3	Clavate	4–6	(30–) 40 (–46) × (3–) 4 (–5)	9.78	1-septate	absent	3
C. brassicae	Clavate	2–6	(38–) 53 (–50) × (3.5–) 4.5 (–6)	11.78	1-septate	absent	5
Calonectria sp 4	Clavate	4–6	(30–) 42 (–50) × (4–) 5 (–6)	8.38	1-septate	absent	3
Calonectria sp 5	Clavate	4–6	(35–) 45 (–55) × (3–) 5(–6)	9.3	1-septate	absent	3
Calonectria sp 6	Clavate	5–6	(30–) 41 (–48) × (4–) 5 (–6)	8.04	1-septate	absent	3
C. clavata	norrowly clavate	2–5	(44–) 65 (–80) × (4–) 5 (–6)	13.0	1(–3)-septate	absent	4
Calonectria sp 7	acicular to clavate	4–5	(45–) 50 (–60) × (3–) 4 (–5)	12.6	1-septate	absent	6
C. ecuadoriae	Clavate	3–5	(45–) 51 (–65) × (4–) 4.5 (–5)	11.33	1(–3)-septate	absent	7
Calonectria sp 8	acicular to clavate	4–6	(35–) 46 (–55) × (3–) 4 (–5)	11.42	1-septate	absent	2
Calonectria sp 9	narrowly clavate to clavate vesicles	3–5	(45–) 59 (–70) × (4–) 5 (–6)	11.57	1-septate	absent	5
Calonectria sp	norrowly clavate to clavate	3–5	(35–) 50 (–60) × (4–) 5 (–6)	10.95	1-septate	absent	3
C. gracilis	narrowly clavate	2–5	(40–) 56 (–65) × (3.5–) 4.5 (–5)	12.44	1(–3)-septate	absent	4
Calonectria sp 10	ovate to ellipsoidal vesicles	8–12	(55–) 69 (–80) × (4–) 5 (–7)	13.73	1-septate	absent	3
C. ovata	Ovate	8–14	(50–) 70 (–110) × (4–) 5 (–6)	14.0	1(–3)-septate	absent	3
Calonectria sp 11	Clavate	3–6	(65–) 84 (–100) × (4–) 5.5 (–7)	15.57	1(–3)-septate	absent	3
C. pteridis	clavate to norrowly ellipsoidal	4–6	(50–) 82 (–100) × (4–) 5.5 (–6)	14.91	1(–3)-septate	absent	5

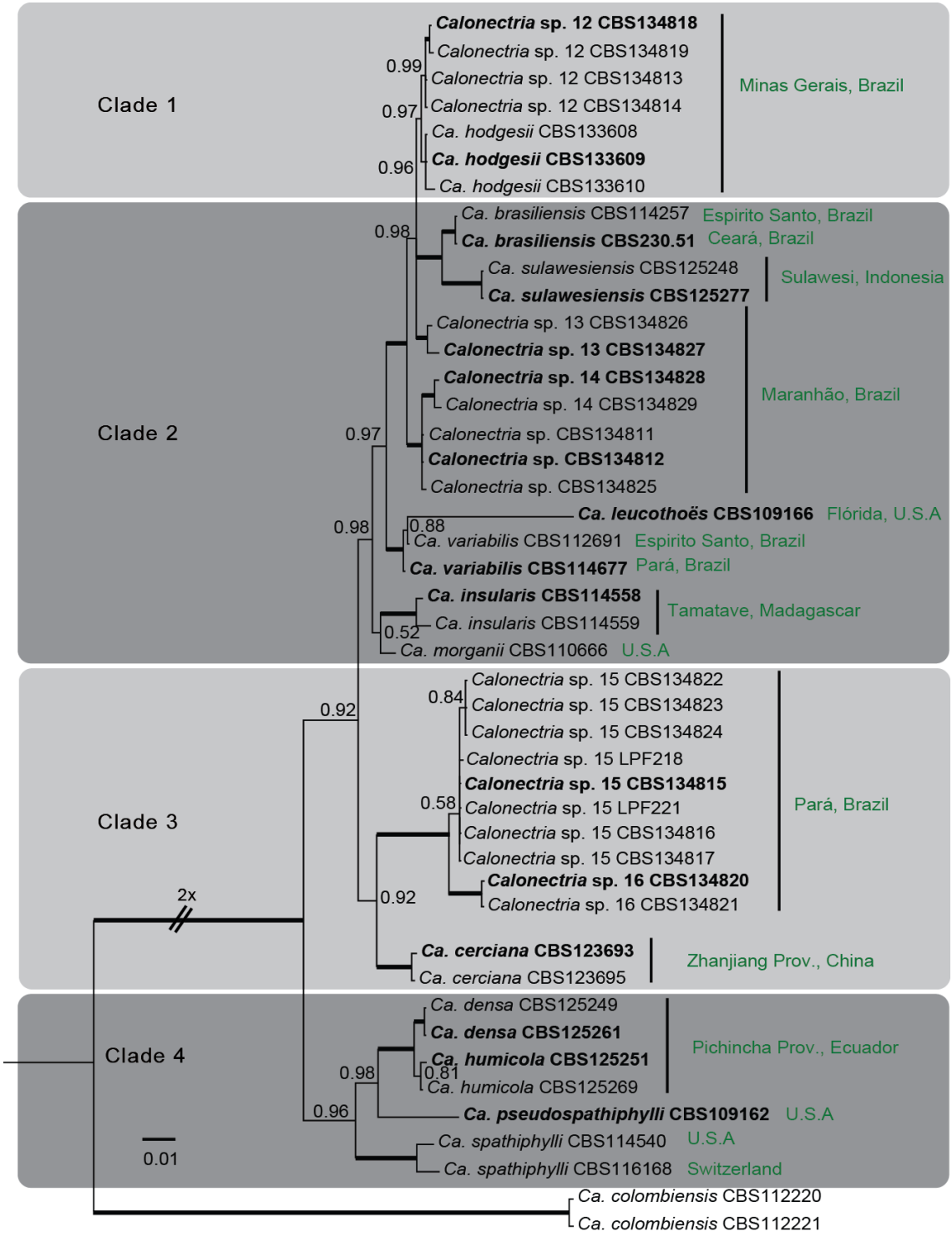

Fig 2: Phylogenetic tree obtained by Bayesian inference using combined sequences of β-tubulin, translation elongation factor 1α and calmodulin sequence alignments of Calonectria morganii complex The Bayesian posterior probability values are show at the nodes Calonectria colombiensis (CBS 112220) is used as outgroup. Culture accession numbers and place of origin are listed. Ex-type isolates are emphasized in bold. The place where the isolates were collected is also indicate.

Table 5: Distinctive morphological characters of Calonectria morganii complex.

Species	Vesicle shape	Vesicle diameter (µm)	Macroconidial size (µm)	Length/Diam. Ratio (µm)	Macroconidial septation	Laterial stipe extension	Nb. branches
Calonectria sp 12	clavate (rarely), ellipsoidal to obpyriform	4–10	(45–) 54 (–65) × (3–) 4.5 (–5)	11.95	1-septate	absent	3
C. hodgesii	pyriform to ellipsoidal or ovoid to sphaeropedunculate	6–11	(44–) 50 (–55) × (3–) 4.5 (–5)	11.50	1-septate	absent	3
Calonectria sp 13	ellipsoidal, obpyriform to umbonate	6–12	(40–) 49 (–55) × (3–) 4 (–5)	11.5	1-septate	absent	3
C. brasiliensis	ellipsoidal to obpyriform	7–11	(35–) 38 (–41) × (3–) 3.5 (–5)	10.4	1-septate	absent	3
C. sulawesiensis	broadly clavate to ellipsoidal	5–7	(41–) 48 (–54) × (3–) 4 (–6)	11.3	1-septate	absent	5
Calonectria sp 14	ellipsoidal, obpyriform to sphaeropedunculate	6–10	(40–) 49 (–55) × (3–) 4 (–5)	12.0	1-septate	absent	3
Calonectria sp	ellipsoidal, obpyriform to sphaeropedunculate	7–11	(50–) 57 (–65) × (3–) 5 (–6)	11.85	1-septate	absent	3
C. leucothöes	ellipsoidal to obpyriform	6–11.5	(45–) 73 (–97) × (4–) 5 (–6.5)	14.6	(1-)3(-6)-septate	absent	6
C. variabilis	sphaeropedunculate to ovoid or ellipsoid to clavate	6–11	(48–) 73 (–85) × (4–) 5 (–6)	14.6	(1-) 3 (-4)-septate	absent	3
C. insularis	obpyriform to broadly ellipsoidal	4–13	(33–) 45 (–60) × (3.5–) 4 (–4)	11.90	1-septate	absent	6
C. morganii	ellipsoidal to pyriform or clavate vesicle	6–8	(40–) 45 (–66) × (3–) 4 (–5)	12.4	1-septate	absent	6
Calonectria sp 15	ellipsoidal, obpyriform to sphaeropedunculate	5–12	(35–) 49 (–55) × (3–) 4 (–5)	12.67	1-septate	absent	4
Calonectria sp 16	obpyriform to sphaeropedunculate	7–13	(35–) 43 (–50) × (3–) 4.5 (–6)	9.87	1-septate	absent	4
C. cerciana	fusiform to obpyriform	8–13	(37–) 44 (–49) × (5–) 5 (–6)	8.8	1-septate	absent	4
C. densa	globose to ovoid to sphaeropedunculate	10–12	(47–) 54 (–62) × (5–) 6 (–6)	9.85	1-septate	present	4
C. humicola	globose to ovoid to sphaeropedunculate	10–12	(45–) 51 (–56) × (4–) 5 (–5)	10.20	1-septate	absent	3
C. pseudospathiphylli	sphaeropedunculate to ellipsoidal	8–12	(40–) 52 (–60) × (4–) 4 (–5)	11.2	1(-3)-septate	absent	4
C. spathiphylli	globoid or ellipsoid to obpyriform	8–15	(45–) 70 (–60) × (5–) 6 (–7)	12.5	1(-3)-septate	absent	4

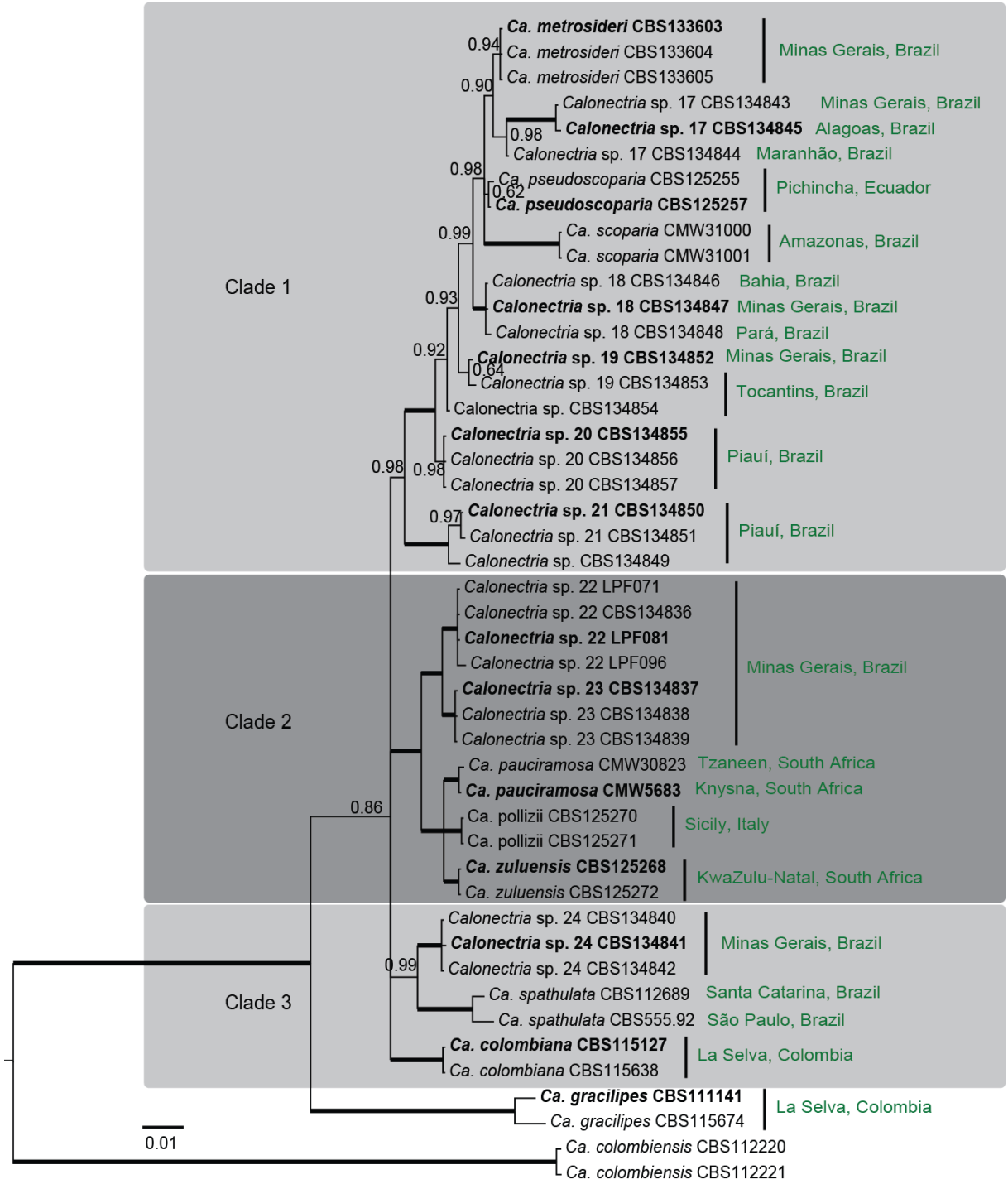

Fig 3: Phylogenetic tree obtained by Bayesian inference using combined sequences of β-tubulin, translation elongation factor 1α and calmodulin sequence alignments of Calonectria scoparia complex The Bayesian posterior probability values are show at the nodes Calonectria colombiensis (CBS 112220) is used as outgroup. Culture accession numbers and place of origin are listed. Ex-type isolates are emphasized in bold. The place where the isolates were collected is also indicate.

Table 6: Distinctive morphological characters of Calonectria scoparia complex.

Species	Vesicle shape	Vesicle diameter (µm)	Macroconidial size (µm)	Length/Diam. Ratio (µm)	Macroconidial septation	Laterial stipe extension	Nb. branches
C. metrosideri	spathulate to obpyriform (abnormal bifurcate vesicles frequently observed)	5–9	(40–) 45 (–51) × (3–) 4 (–5)	11.02	1-septate	absent	4
Calonectria sp 17	ellipsoidal to obpyriform	5–7	(40–) 51 (–60) × (3–) 4.5 (–5)	11.34	1-septate	absent	3
C. pseudoscoparia	obpyriform to elipsoidal	6–10	(41–) 48 (–52) × (3–) 4 (–5)	12.01	1-septate	absent	4
C. scoparia	ellipsoidal to narrowly obpyriform	5–8	(45–) 60 (–80) × (4–) 5 (–6)	12.76	1-septate	absent	5
Calonectria sp 18	ellipsoidal to obpyriform	5–7	(43–) 50 (–55) × (3–) 4 (–5)	12.20	1-septate	absent	3
Calonectria sp 19	narrowly obpyriform to ellipsoidal	3–5	(45–) 50 (–55) × (3–) 4 (–5)	12.06	1-septate	absent	2
Calonectria sp 20	narrowly obpyriform to ellipsoidal	3–7	(35–) 53 (–65) × (3–) 4 (–5)	12.91	1-septate	absent	3
Calonectria sp 21	narrowly obpyriform to ellipsoidal	3–7	(38–) 49 (–60) × (3–) 4.5 (–5)	11.27	1-septate	present	2
Calonectria sp 22	obpyriform	7–10	(30–) 41 (–50) × (3–) 4.5 (–5)	9.17	1-septate	absent	3
Calonectria sp 23	obpyriform	7–13	(40–) 45 (–50) × (3–) 4 (–5)	11.06	1-septate	absent	4
C. pauciramosa	obpyriform to ellipsoidal	5–11	(30–) 50 (–60) × (3.5–) 4.5 (–5)	11.01	1-septate	absent	3
C. polizzii	broadly clavate to pyriform	6–9	(31–) 37 (–49) × (3–) 4 (–5)	9.66	1-septate	absent	3
C. zuluensis	broadly clavate to obpyriform	6–10	(31–) 36 (–40) × (3–) 4 (–5)	9.08	1-septate	absent	3
Calonectria sp 24	obpyriform	7–10	(35–) 43 (–50) × (3–) 4 (–5)	10.46	1-septate	absent	3
C. spathulata	ellipsoid to obpyriform or clavate	6–10	(48–) 80 (–100) × (4–) 5 (–6)	13.33	(1-)3(-6)-septate	absent	3
C. colombiana	obpyriform to fusiform	8–12	(33–) 37 (–40) × (3–) 3 (–4)	11.4	1-septate	absent	4
C. gracilipes	clavate vesicle	3–4	(35–) 45 (–60) × (4–) 5 (–6)	9.08	1-septate	absent	3

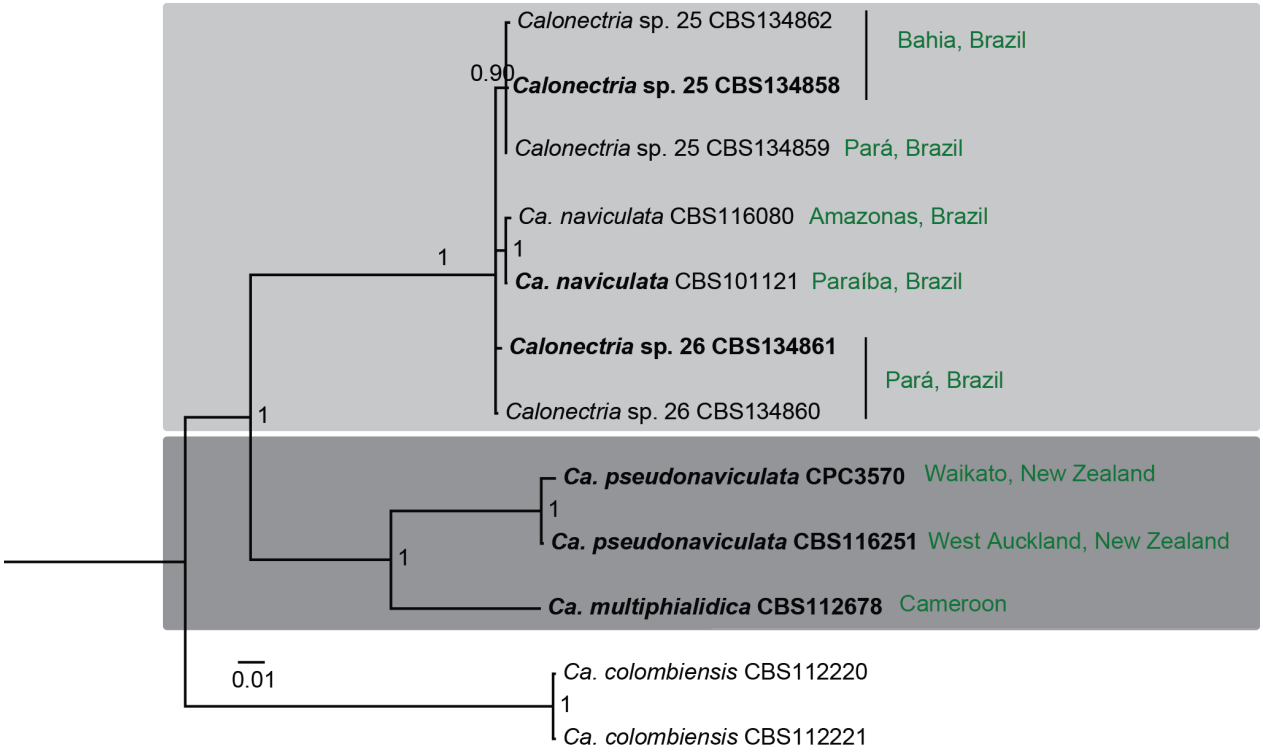

Fig 4: Phylogenetic tree obtained by Bayesian inference using combined sequences of β-tubulin, translation elongation factor 1α and calmodulin sequence alignments of Calonectria naviculata complex The Bayesian posterior probability values are show at the nodes Calonectria colombiensis (CBS 112220) is used as outgroup. Culture accession numbers and place of origin are listed. Ex-type isolates are emphasized in bold. The place where the isolates were collected is also indicate.

Table 7: Distinctive morphological characters of Calonectria naviculata complex.

Species	Vesicle shape	Vesicle diameter (µm)	Macroconidial size (µm)	Length/Diam. Ratio (µm)	Macroconidial septation	Laterial stipe extension	Nb. branches
Calonectria sp 25	Naviculate	4–7	(40–) 46 (–52) × (2–) 3.5 (–4)	13.72	1-septate	present	3
C. naviculata	naviculate to ellipsoidal	5–11	(40–) 45 (–52) × (3–) 3 (–4)	12.83	1-septate	absent	4
Calonectria sp 26	Naviculate	3–7	(34–) 38 (–45) × (2–) 3 (–4)	11.52	1-septate	present	3
C. pseudonaviculata	Naviculate	4–8	(50–) 55–65(–80) × (4–) 5 (–6)	12.45	1(-3)-septate	absent	4
C. multiphialidica	clavate to sphaeropedunculate	8–16	(45–) 53 (–65) × (4–) 4.5 (–5)	11,78	1-septate	absent	8

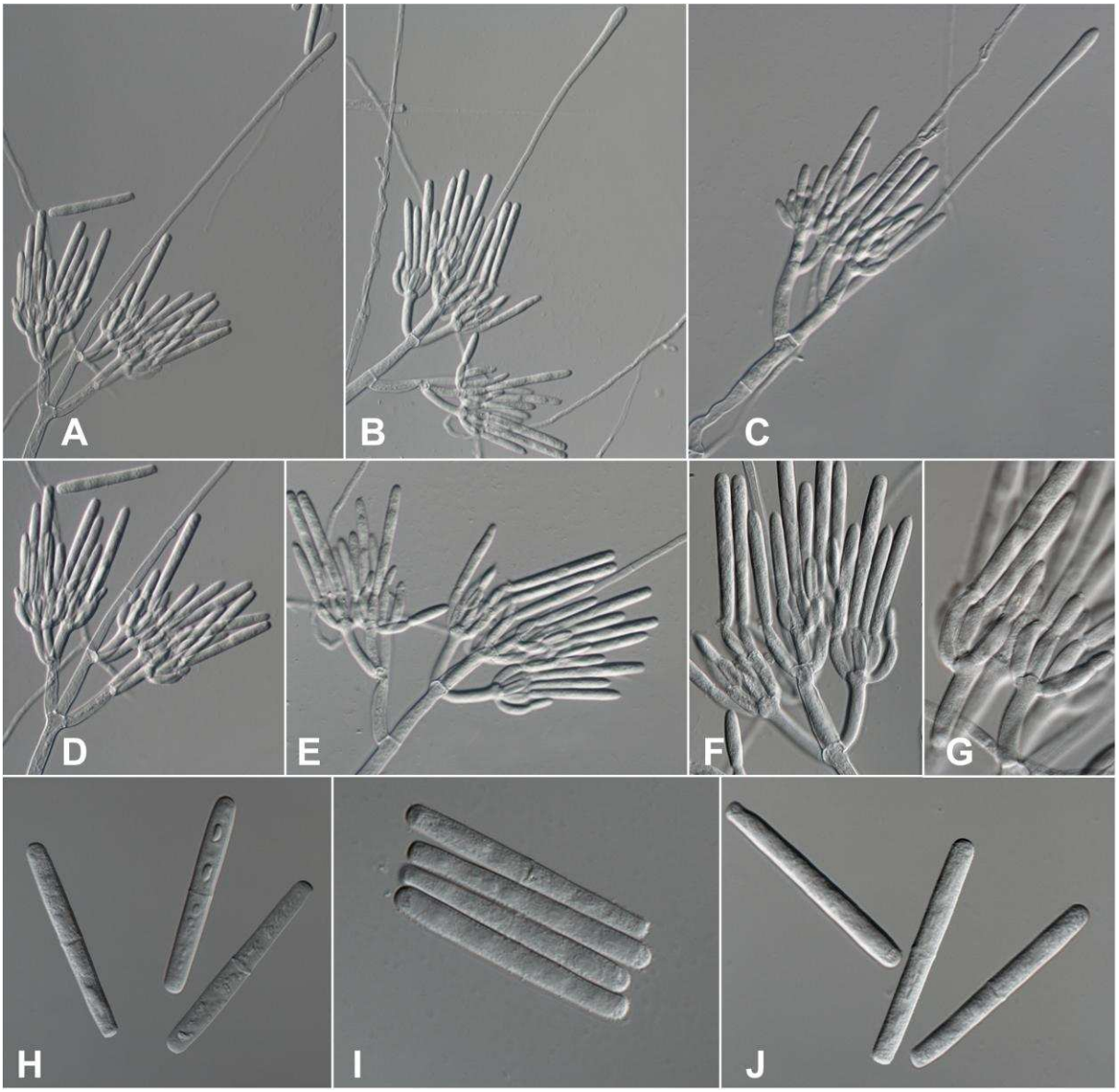

Fig 5: Morphological characteristics of Calonectria sp. 1. A–C: Macroconidiophores containing clavate vesicles; D–E: Macroconidiophores; F–G: Phialide doliiform to reniform; H–J: Uniseptate macroconidia.

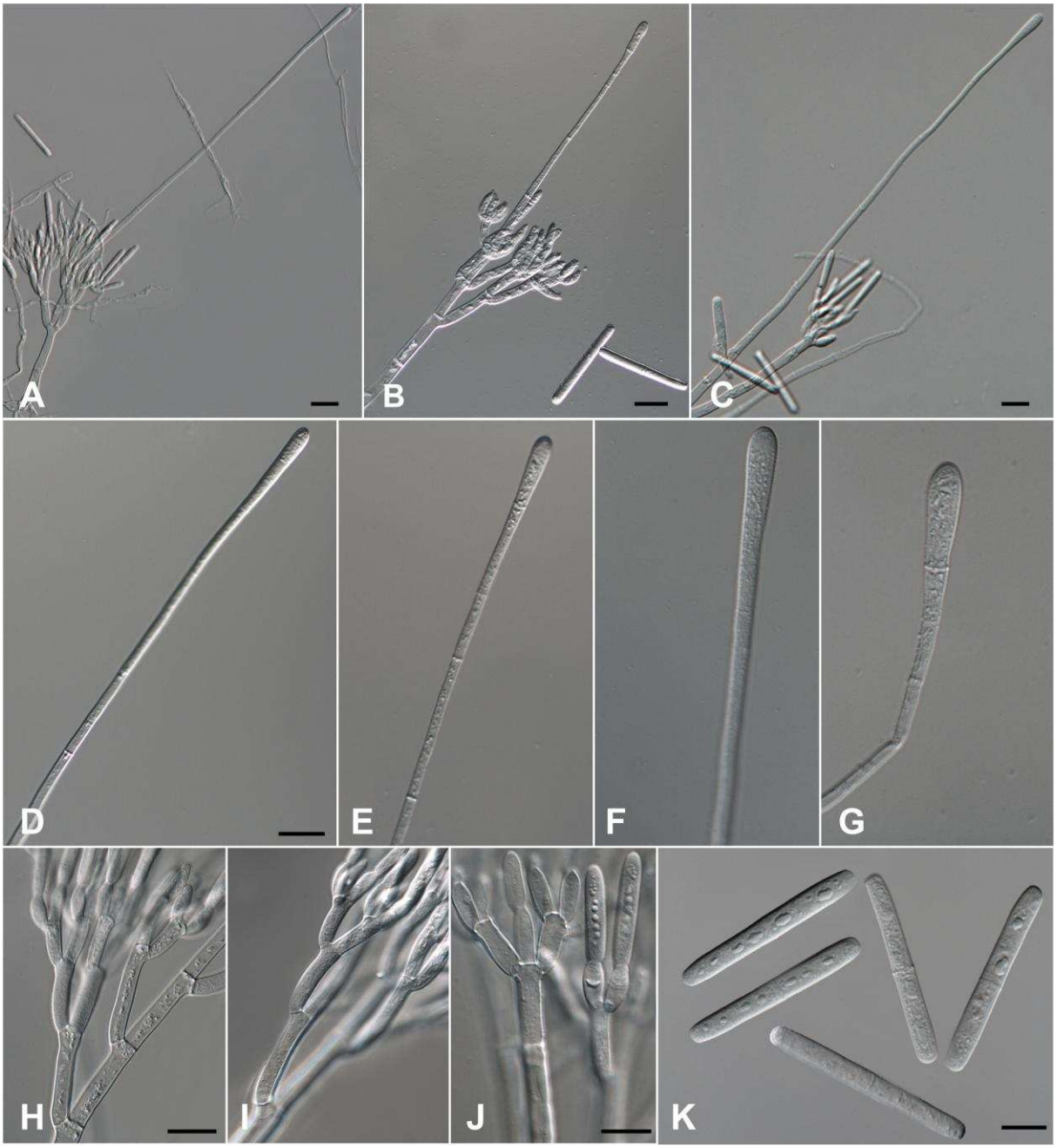

Fig 6: Morphological characteristics of Calonectria sp. 2. A–C: Macroconidiophores containing clavate vesicles; D–G: Clavate vesicles; H–I: Macroconidiophores; J: Phialide doliiform to reniform; K: Uniseptate macroconidia. Scale bars: A–C = 20 µm, D–K = 10 µm

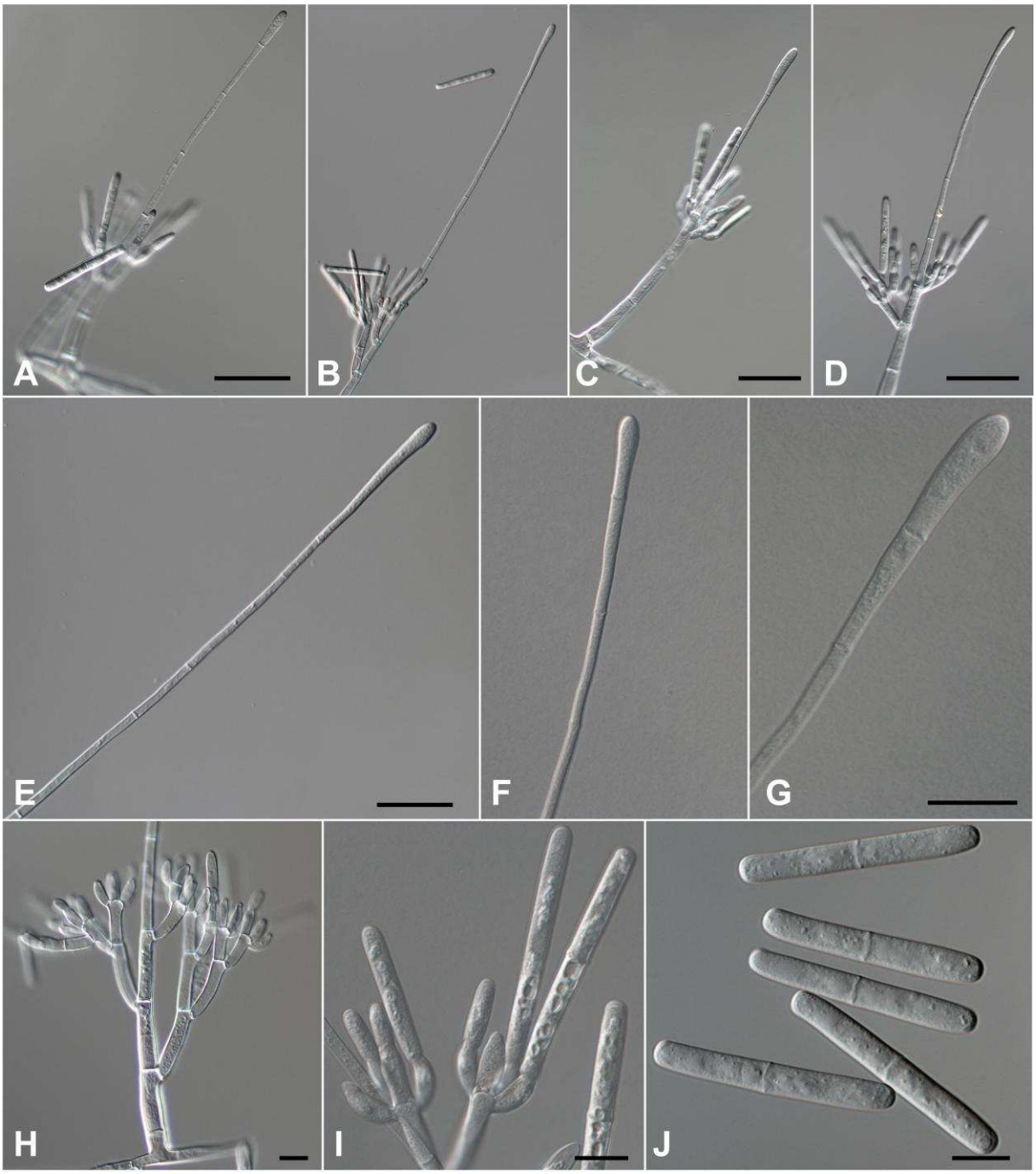

Fig 7: Morphological characteristics of Calonectria sp. 3. A–D: Macroconidiophores containing clavate vesicles; E–G: Clavate vesicles; H: Macroconidiophores; I: Phialide doliiform to reniform; J: Uniseptate macroconidia. Scale bars: A–D = 50 µm, E–J = 10 µm

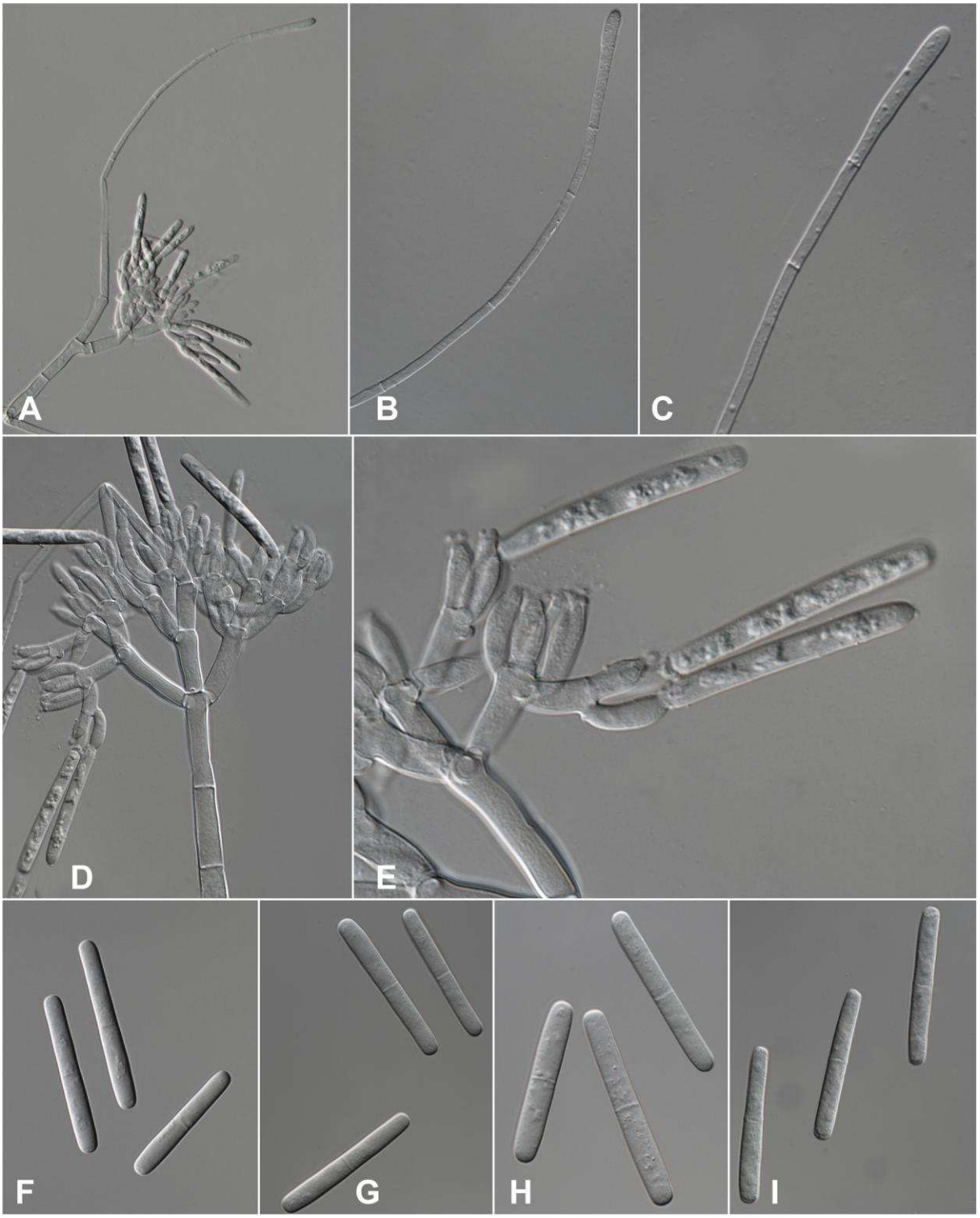

Fig 8: Morphological characteristics of Calonectria sp. 4 A: Macroconidiophores containing clavate vesicle; B–C: Clavate vesicles; D: Macroconidiophores; E: Phialide doliiform to reniform; F–I: Uniseptate macroconidia.

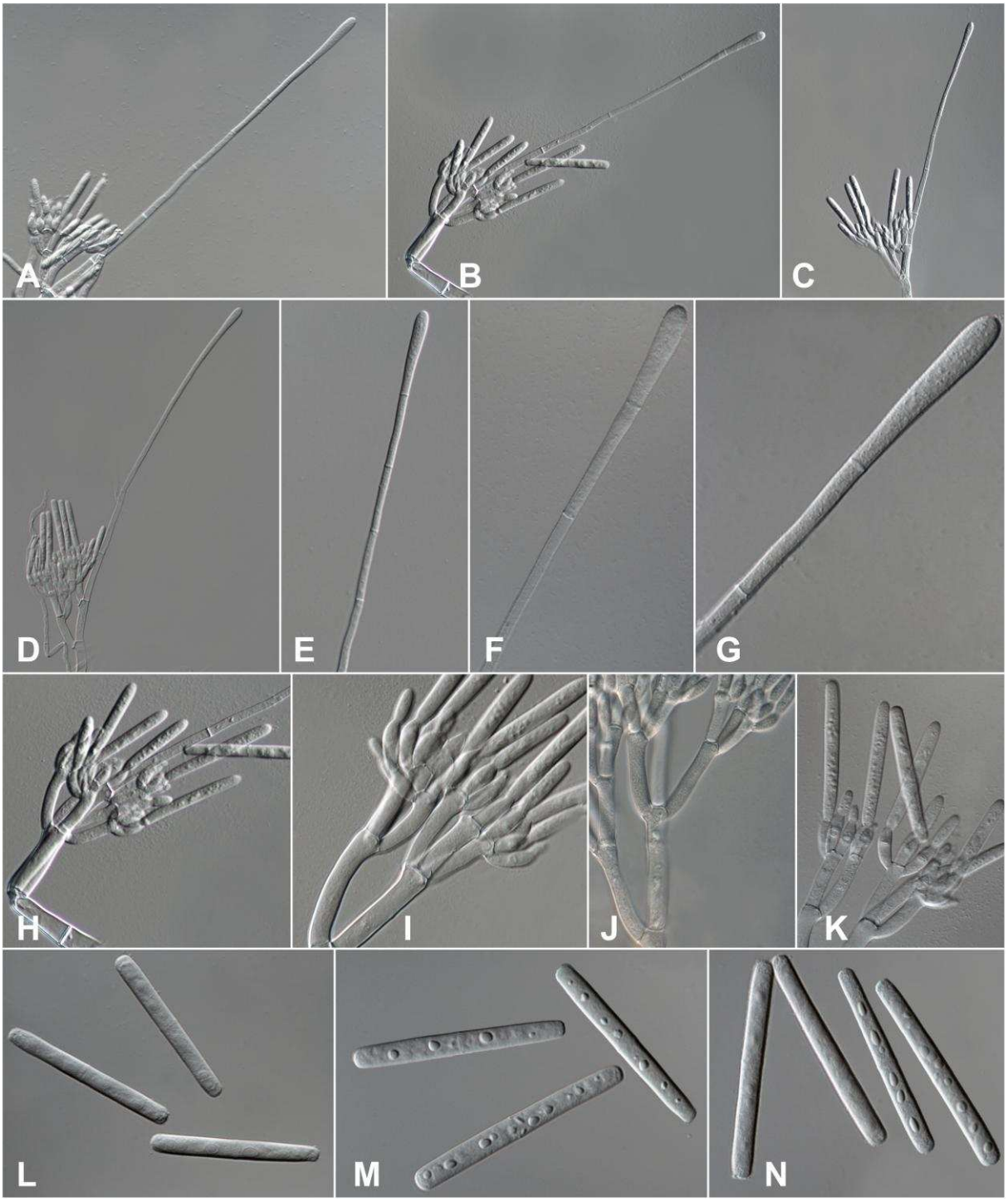

Fig 9: Morphological characteristics of Calonectria sp. 5 A–D: Macroconidiophores containing clavate vesicle; E–G: Clavate vesicles; H–J: Macroconidiophores; K: Phialide doliiform to reniform; L–N: Uniseptate macroconidia.

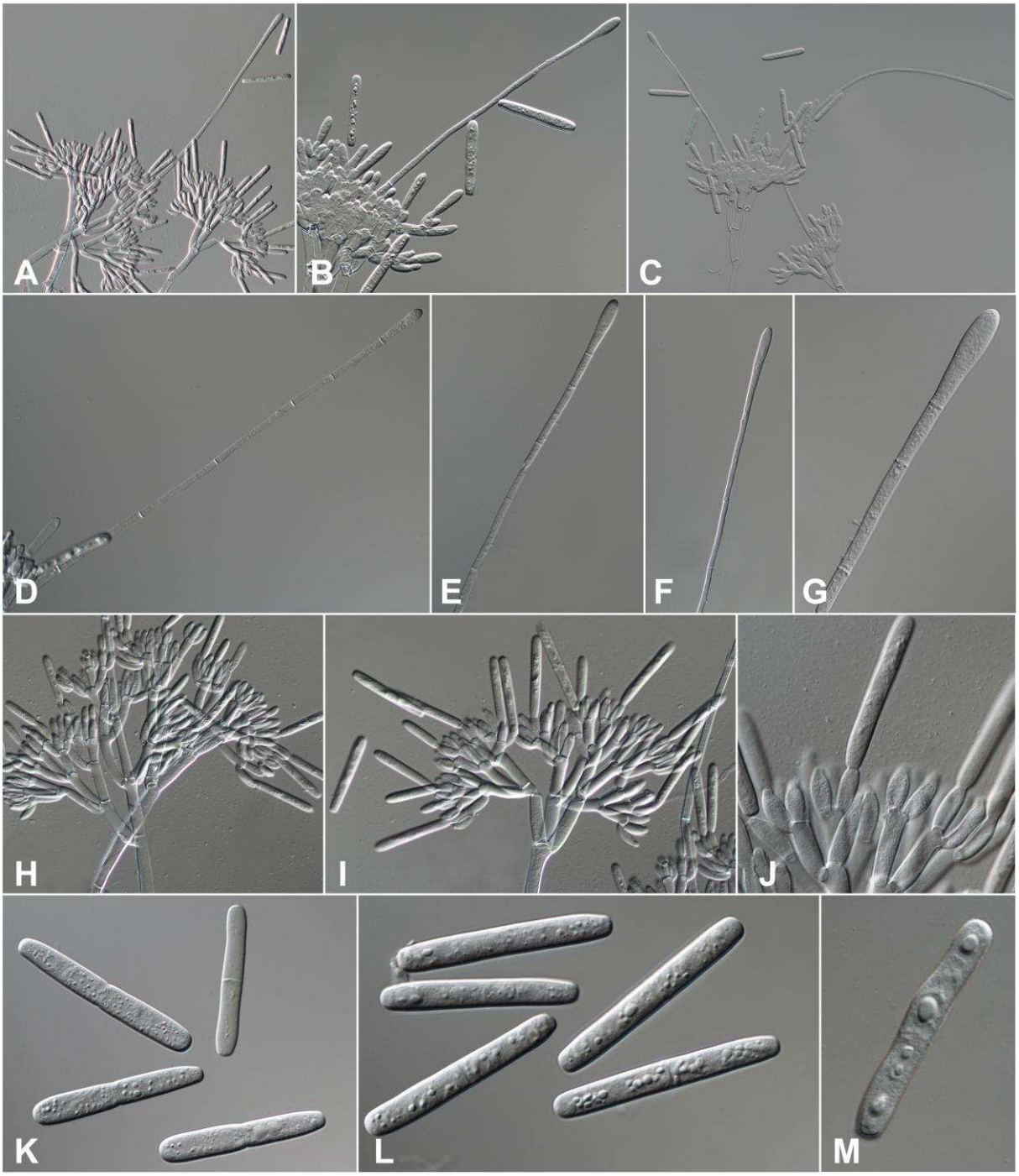

Fig 10: Morphological characteristics of Calonectria sp. 6. A–D: Macroconidiophores containing clavate vesicles; E–G: Clavate vesicles; H–I: Macroconidiophores; J: Phialide doliiform to reniform; K–M: Uniseptate macroconidia.

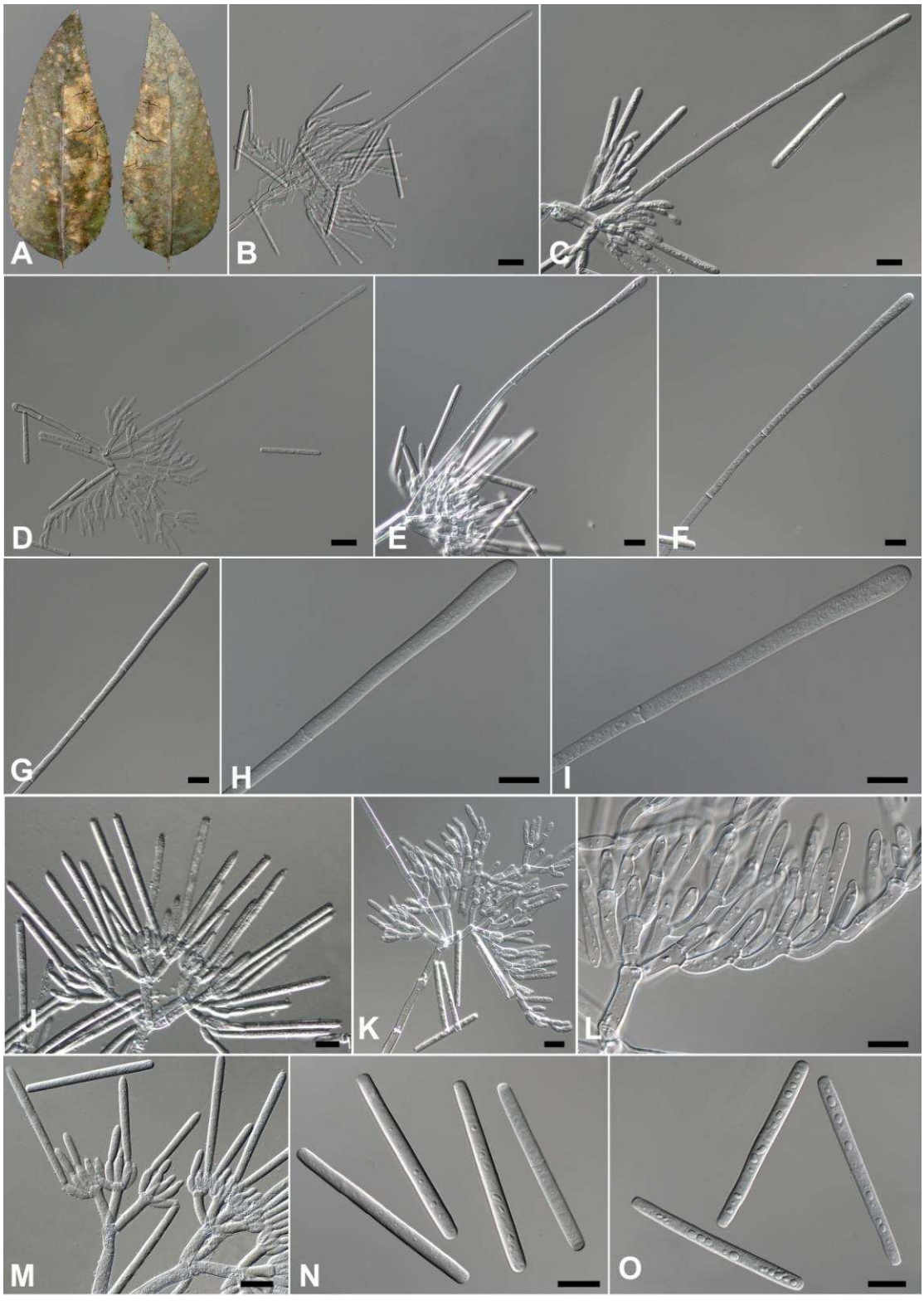

Fig 11: Leaf blight and morphological characteristics of Calonectria sp. 7. A: Small and rounded lesions of Calonectria sp 7 in Eucalyptus sp; B–E: Macroconidiophores containing clavate vesicles; F–I: Clavate vesicles; J–M: Macroconidiophores containing phialide doliiform to reniform; N–O: Uniseptate macroconidia. Scale bars: B–D = 20 µm; E–O = 10 µm.

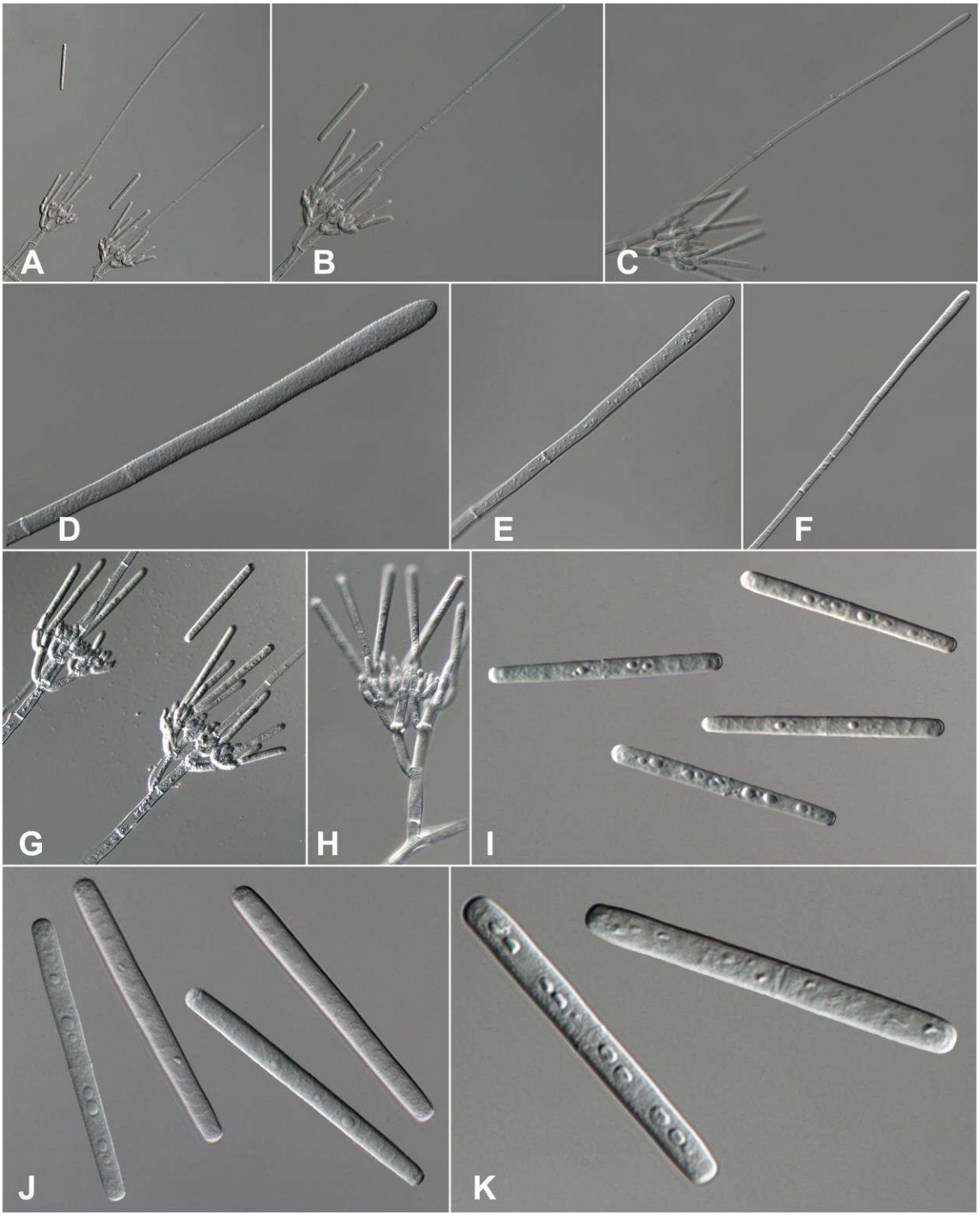

Fig 12: Morphological characteristics of Calonectria sp. 8. A–C: Macroconidiophores containing clavate vesicles; D–F: Clavate vesicles; G–H: Macroconidiophores; I–K: Uniseptate macroconidia.

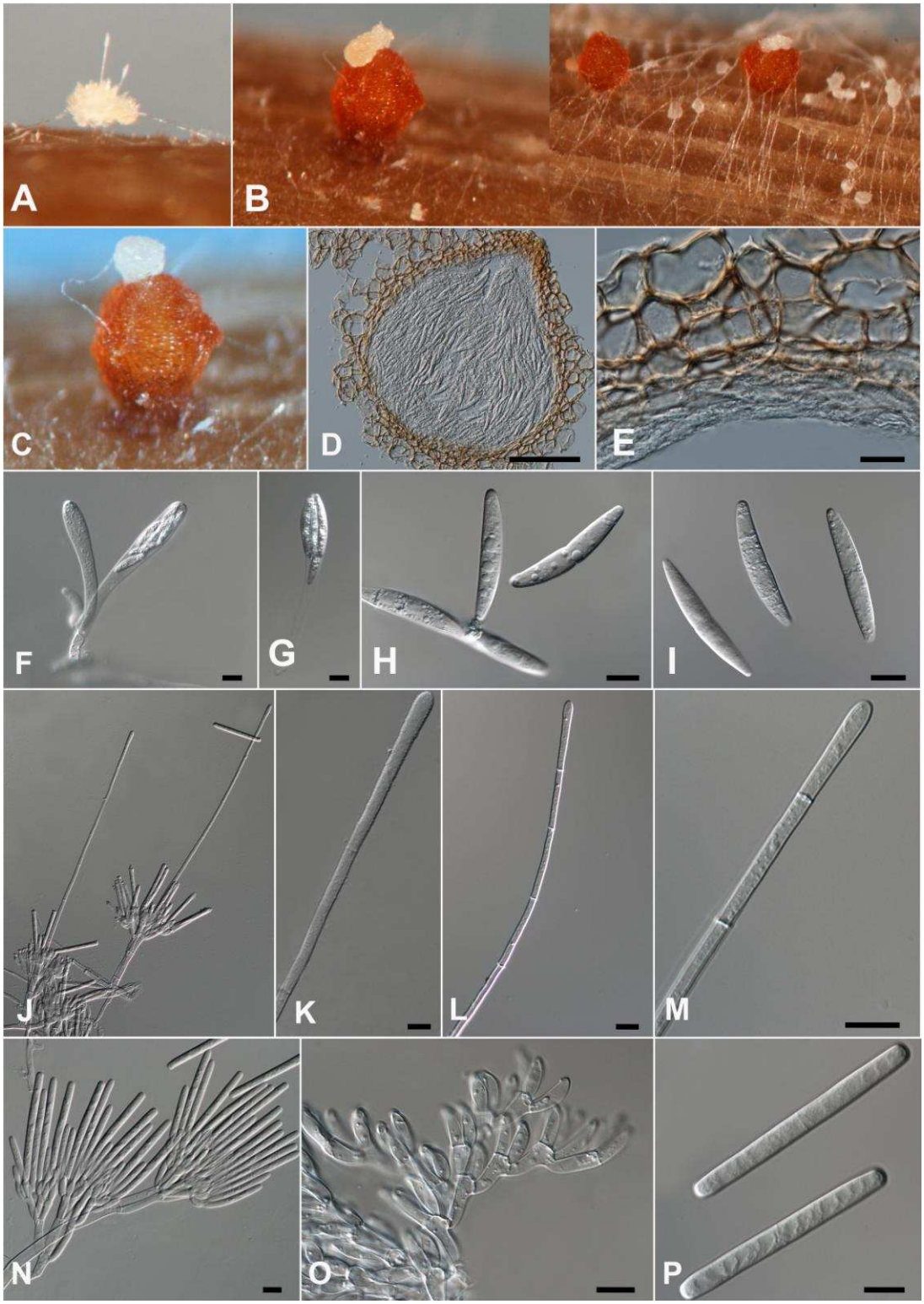

Fig 13: Morphological characteristics of Calonectria sp. 9. A: Typical sporulation of Calonectria sp. 9; B–C: Perithecium; D: Vertical section through a perithecium; E: Section through laterial perithecial wall; F–I: Asci and ascospores; J: Macroconidiophores containing clavate vesicle; K–M: Clavate vesicles; N–O: Macroconidiophores and P: Uniseptate macroconidia. Scale bars: D = 100 µm and others = 10 µm.

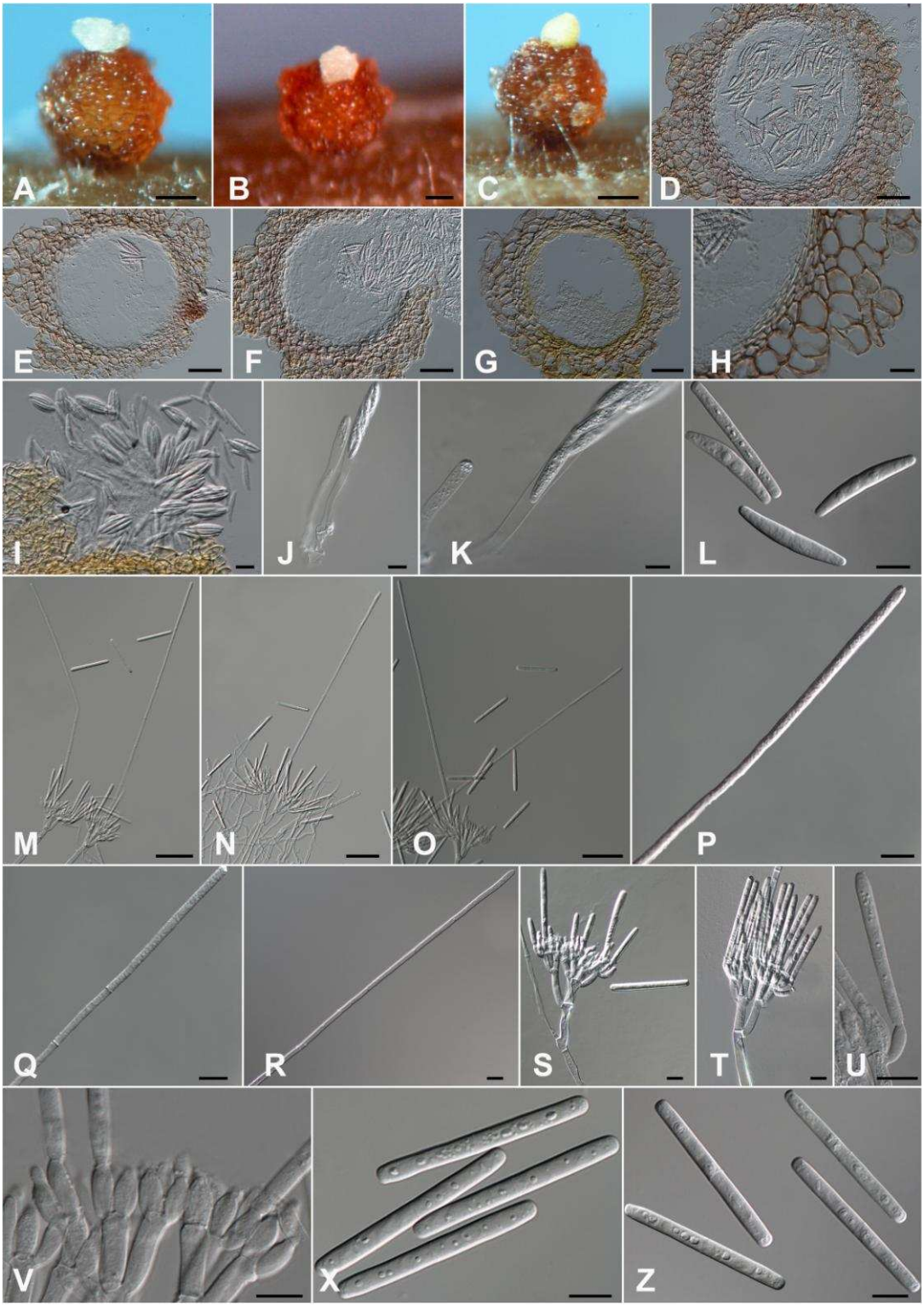

Fig 14: Morphological characteristics of Calonectria sp. A–C: Perithecium; D–G: Vertical section through a perithecium; H: Section through laterial perithecial wall; I–L: Asci and ascospores; M–O: Macroconidiophores containing clavate vesicle; P–R: Clavate vesicles; S–T: Macroconidiophores, U–V: Phialide and X–Z: Uniseptate macroconidia. Scale bars: A–C = 100 µm, D–G and M–O = 50 µm, H–J = 20 µm and others = 10 µm.

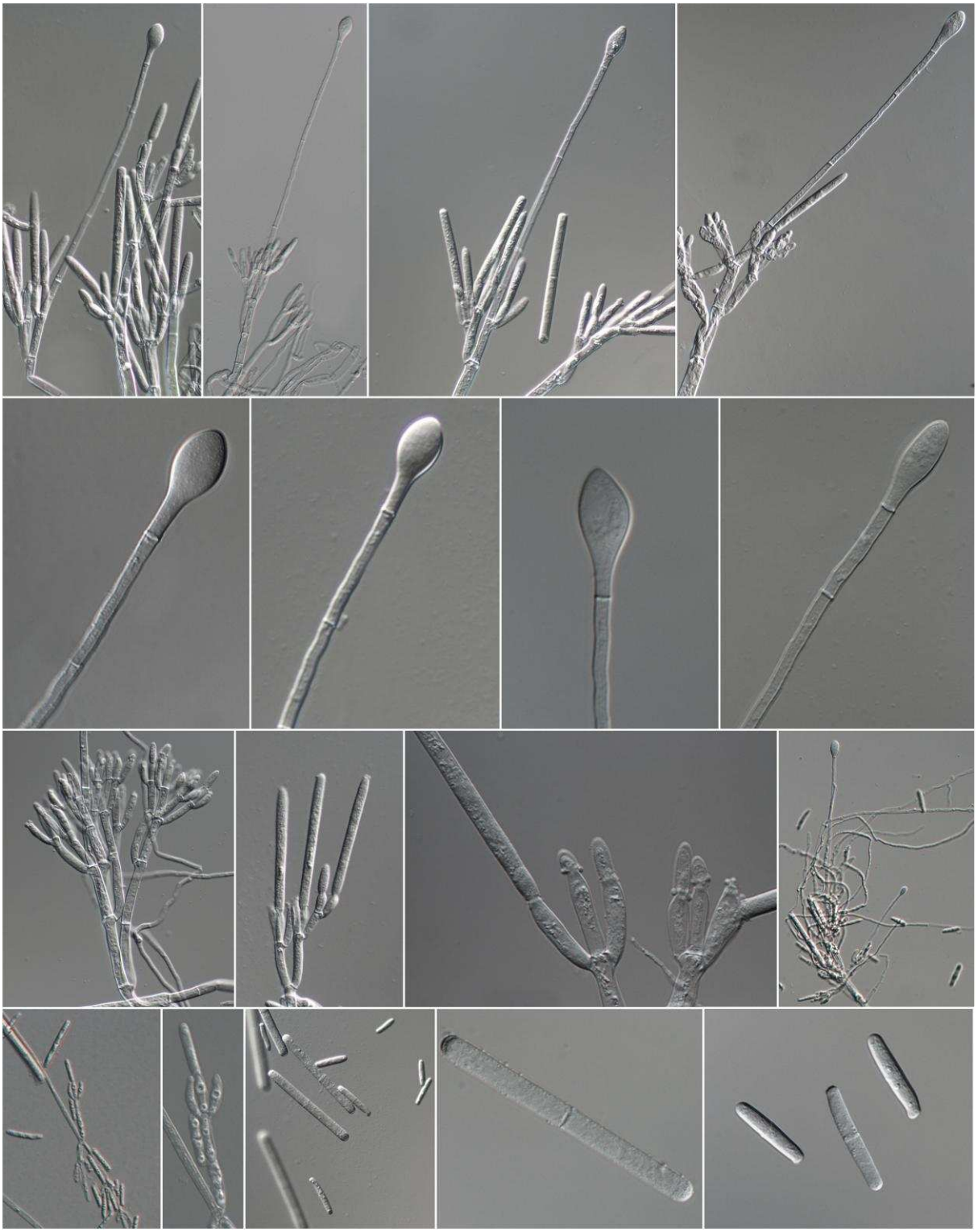

Fig 15: Morphological characteristics of Calonectria sp nov 10.

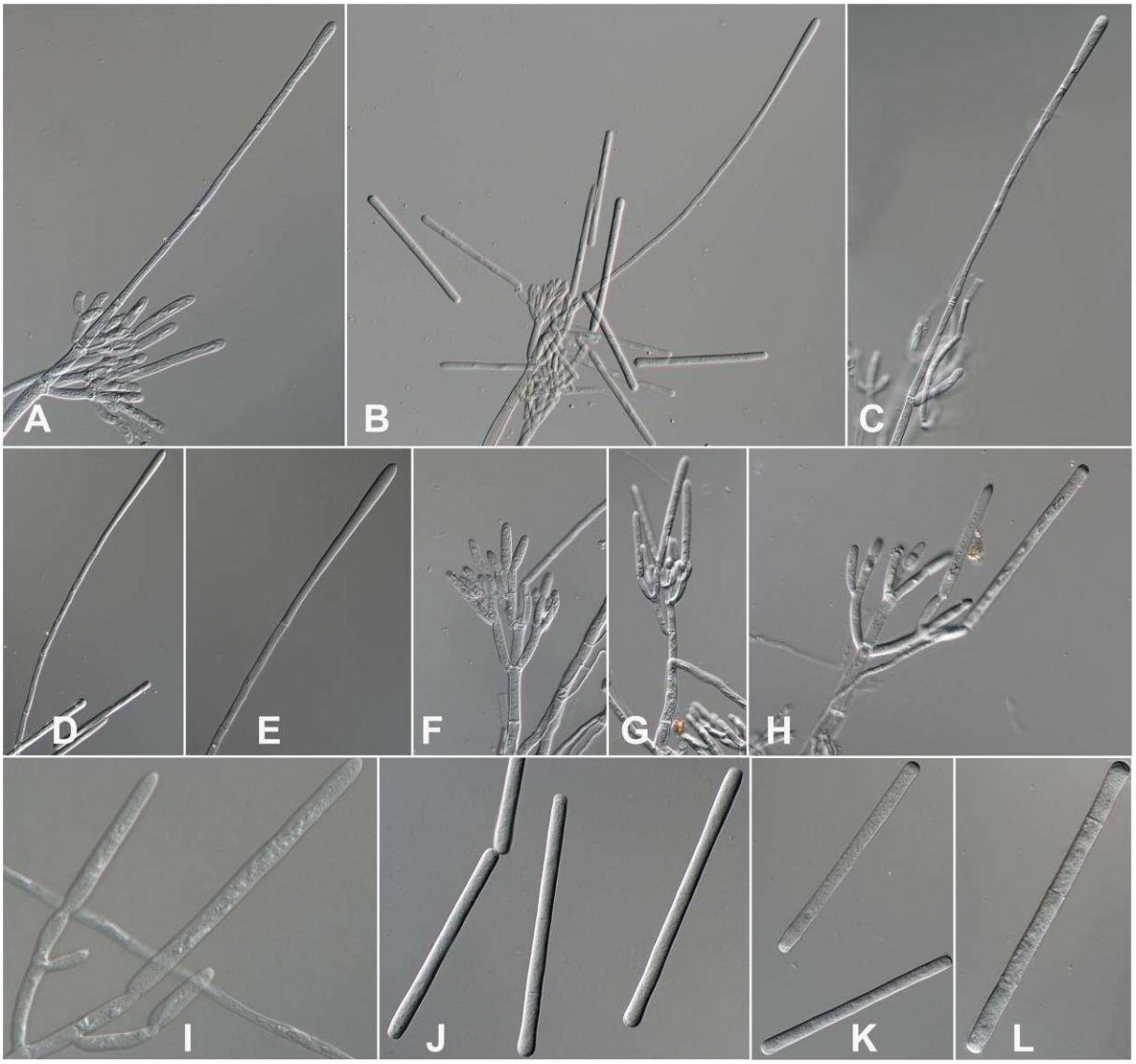

Fig 16: Morphological characteristics of Calonectria sp nov 11. A–C: Macroconidiophores containing clavate vesicle; D–E: Clavate vesicles; F–H: Macroconidiophores, I: Phialide elongate doliiform to reniform and J–K: 1(–3)-septate macroconidia.

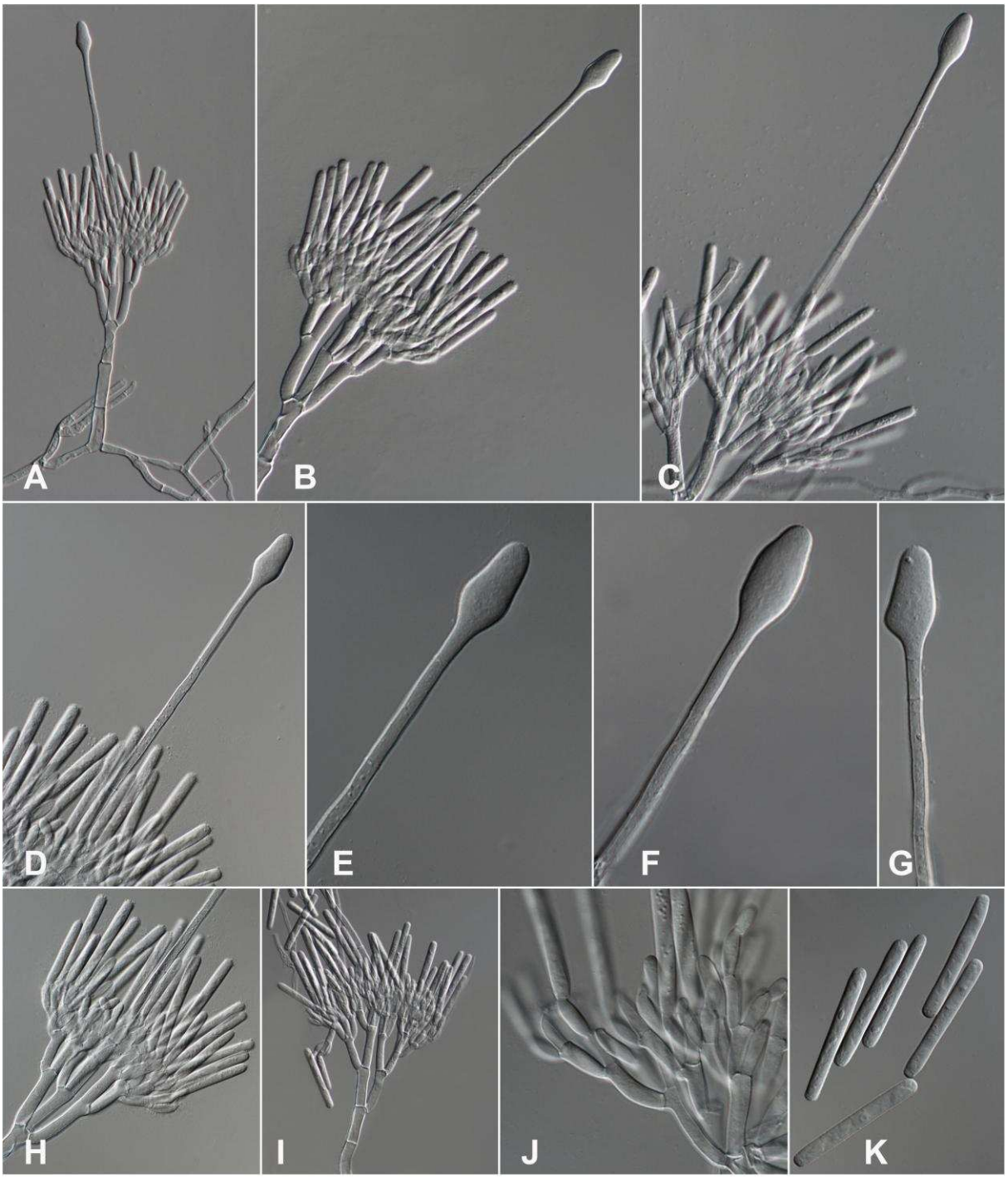

Fig 17: Morphological characteristics of Calonectria sp 22 (LPF081). A–C: Macroconidiophores containing obpyriform vesicles; D–G: Variation in vesicle shape;Clavate vesicles; H–I: Macroconidiophores; J: Phialide doliiform to reniform and K: Uniseptate macroconidia.

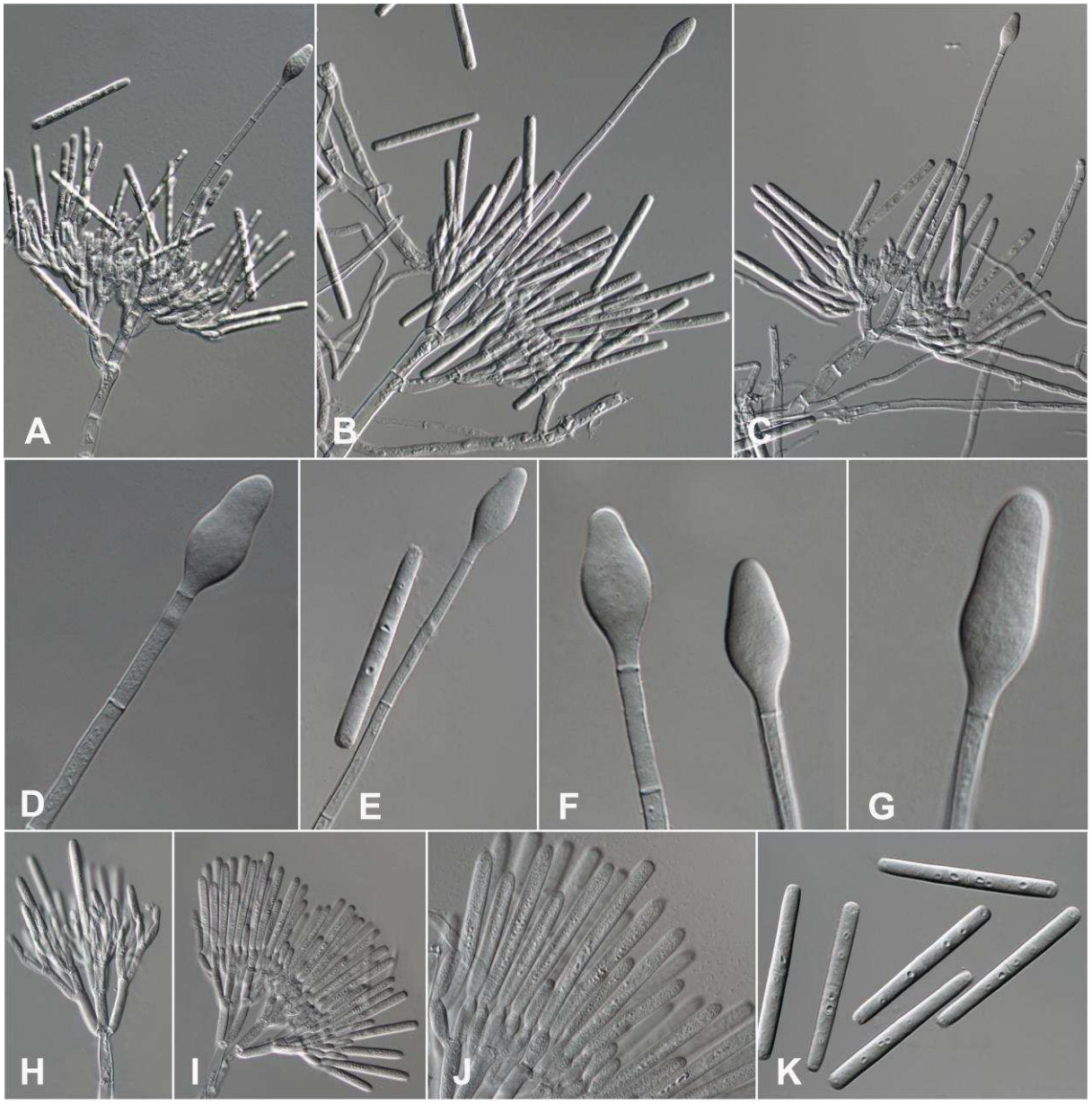

Fig 18: Morphological characteristics of Calonectria sp 24. A–C: Macroconidiophores containing obpyriform vesicles; D–G: Variation in vesicle shape;Clavate vesicles; H–I: Macroconidiophores; J: Phialide doliiform to reniform and K: Uniseptate macroconidia.

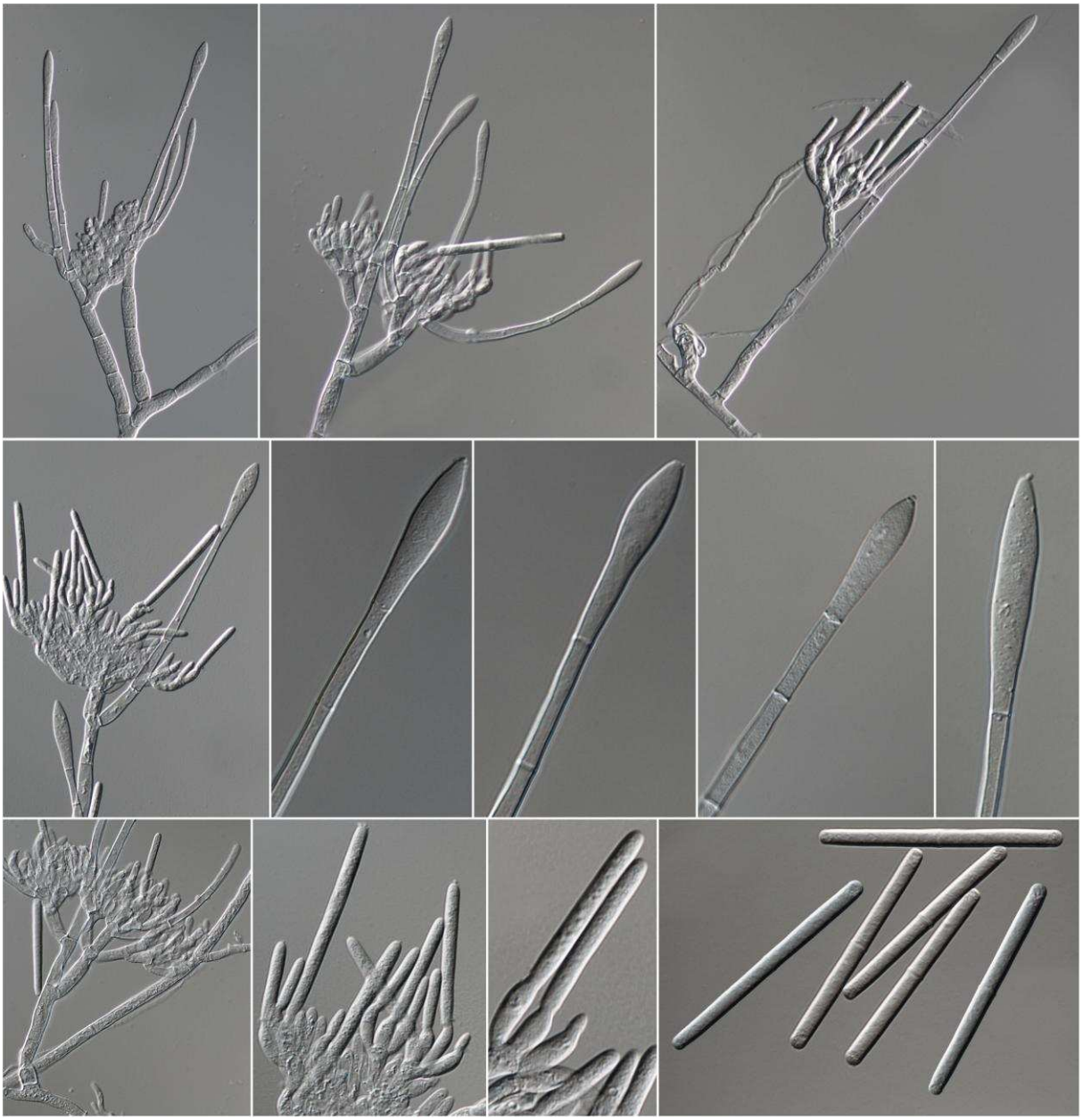

Fig 19: Morphological characteristics of Calonectria sp 25.

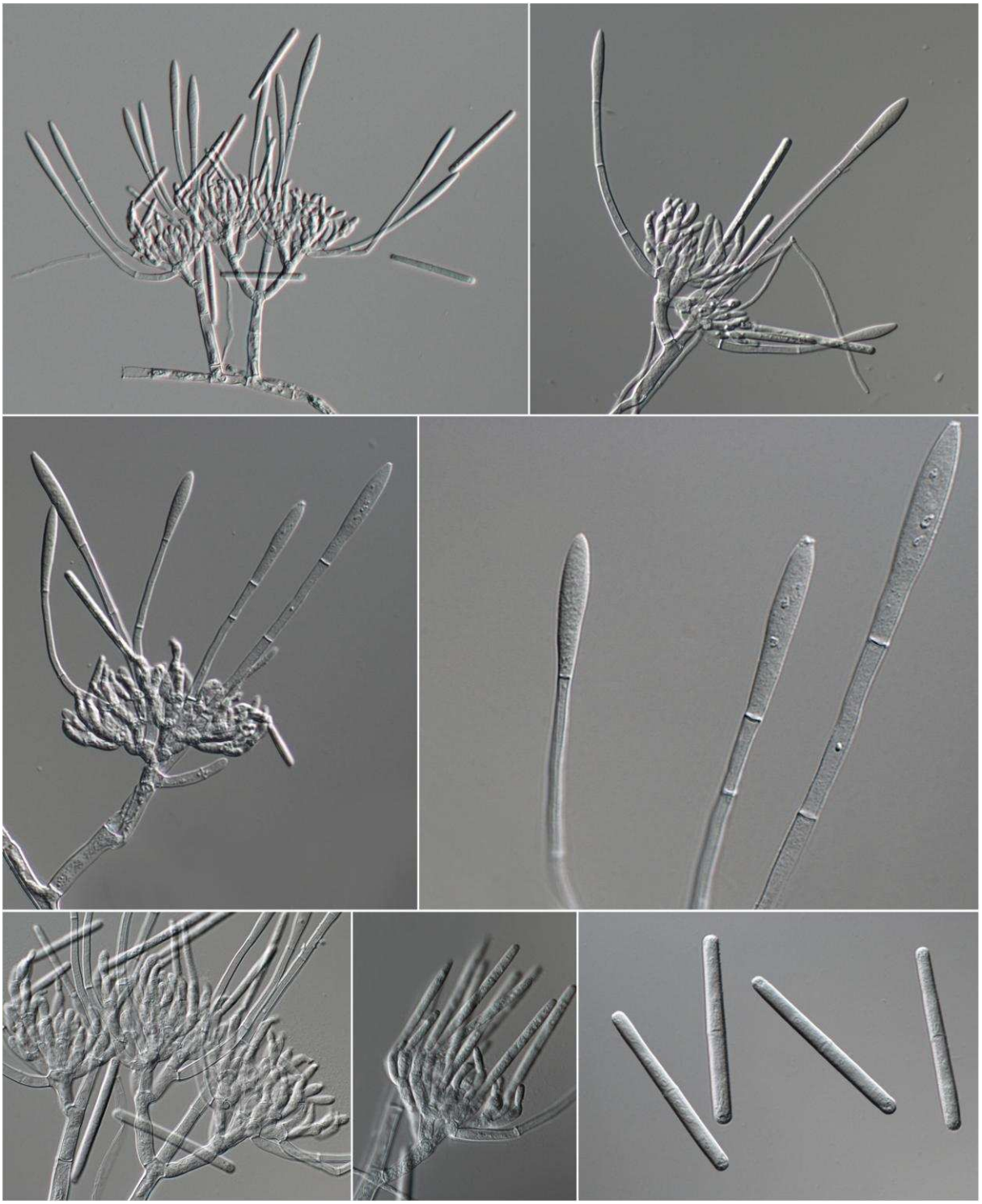

Fig 20: Morphological characteristics of Calonectria sp 26.

CHAPTER 4

Resistance of Eucalyptus species to Calonectria pteridis leaf blight

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Summary

Calonectria-leaf-blight (CLB), caused by Calonectria pteridis, is a major foliar disease of eucalyptus plantations in the warm and high rainfall regions of Brazil. The use of resistant genotypes is the best method for disease control in the field; therefore, the identification of new sources of resistance is highly strategic for the long-term genetic breeding programs of eucalyptus. In this study, the resistance of 17 species of Eucalyptus to CLB was evaluated by the spray inoculation of a spore suspension ($1x10^4$ conidia/mL) of the pathogen under controlled conditions. Eucalyptus brassiana, E. saligna, E. scias and E. aglomerata were the most resistant; E. urophylla, E. camaldulensis, E. cloeziana, E. longirostrata, E. pellita, E. robusta and C. torelliana were susceptible, and E. tereticornis, E. pilularis, C. maculata, E. grandis, E. dunnii and C. citriodora were the highly susceptible species. The broad inter- and intra-specific variability of the tested species demonstrate the potential for the introgression of resistance genes into valuable genotypes as a strategy for eucalyptus breeding programs. Superior resistant plants that have been derived from crosses can be multiplied for clonal trials, silvicultural and wood property evaluations and the subsequent establishment of seed orchards containing the best resistant genotypes.

Keywords: Corymbia, Cylindrocladium, defoliation, breeding for resistance

INTRODUCTION

In the 1970's, the total area that was planted with eucalyptus in Brazil was concentrated in the states of São Paulo and Minas Gerais. The seedling plantations had a relatively low annual mean increment (AMI) of approximately 25 m^3 ha^{-1} y^{-1} (Guimarães et al., 2010, Alfenas et al., 2009). With the advent and progress of clonal propagation techniques by cuttings, along with the employment of modern silvicultural and management practices, there has been a significant increase in the productivity of eucalyptus plantations in Brazil, reaching an average 40 m^3 ha^{-1} y^{-1} (ABRAF, 2012). The increasing demand for wood products combined with the awareness of the public's opinion on the preservation of the native forests has stimulated the worldwide expansion of eucalyptus plantations, which in Brazil alone constitute approximately 4.8 million hectares (ABRAF, 2012). However, the extension of the plantations to warmer and more humid regions, the use of more productive genetic materials without prior knowledge of their disease resistance, the implementation of new management techniques and the successive cycles of culture in the same planting area have favored the emergence of diseases whose pathogens were endemic or accidentally introduced to these regions (Alfenas et al., 2011). Calonectria-leaf-blight (CLB), caused by Calonectria pteridis Crous, MJ Wingf. & Alfenas, is one of the major foliar diseases of Eucalyptus spp., causing leaf lesions and intense defoliation in susceptible genotypes, especially in the warm and high rainfall regions in Brazil, which are favorable for pathogen infection (Ferreira et al., 1995). The disease was first reported in the mid 1990's in southeastern Bahia, where it caused severe defoliation in plantations of Eucalyptus grandis (Ferreira et al., 1995). Since then, C. pteridis has been the most common species found in commercial plantations, mainly in the provenances of E. camaldulensis Dehnh., E. cloeziana F. Muell., E. grandis, E. saligna Smith, E. tereticornis Smith, E. urophylla S.T. Blake and a hybrid of E. grandis x E. urophylla ("urograndis"), among others (Alfenas et al., 2009). In

most Eucalyptus species, the disease is characterized by several small rounded or elongated leaf spots (Ferreira & Milani, 2002, Ferreira et al., 1995) that are generally surrounded by a callus as a consequence of a rapid host reaction. With the progress of the disease, the lesions change in color from light gray to light brown and may occupy a large proportion of the leaf blade, inducing severe defoliation (Figure 1) (Ferreira et al., 1995).

Losses from defoliation in eucalyptus by CLB have not been measured, but levels of artificial pruning equal to or greater than 75 % of the crown in 1-year-old E. grandis plants reduces the volume increase by 45 % at seven years of age (Pulrolnik et al., 2005, Pires 2000). Based on this information, as well as the fact that levels of defoliation equal to or higher than 75 % are observed in susceptible clones in the field, it is believed that growth reduction is equal to or greater than 45 % of the volume as a consequence of toxic metabolites that are most likely produced by the fungus (Von Wallbrunn et al., 2001, Takayama et al., 1984, Hirota et al., 1973).

The use of resistant genotypes is the best method for CLB control (Alfenas et al., 2009). Observations in the field under natural infection and the results of inoculations under controlled conditions indicate the existence of inter and intra-specific variability for resistance in eucalyptus (Fonseca et al., 2010, Zarpelon et al. 2011). Although there are approximately 700 described species of Eucalyptus, only a limited number of species are planted commercially in Brazil, mainly E. grandis, E. urophylla and their hybrids "urograndis". Therefore, to minimize the impact of CLB, it is important to amplify the genetic basis of breeding populations by the introgression of resistance genes from other species of Eucalyptus and Corymbia, which can provide disease resistance (Grattapaglia et al., 2012, Fonseca et al., 2010). Thus, the objective of this study was to identify and select sources of resistance to CLB in the following Corymbia and Eucalyptus species: C. citriodora, C. maculata, C. torelliana, E. aglomerata, E. brassiana, E. camaldulensis, E. cloeziana, E.

dunnii, E. grandis, E. longirostrata, E. pellita, E. pilularis, E. robusta, E. saligna, E. scias, E. tereticornis and E. urophylla.

MATERIALS AND METHOD

Plant material

A total of 17 seed lots of the species that are most used in Brazilian breeding programs (C. citriodora, C. maculata, C. torelliana, E. agglomerate, E. brassiana, E. camaldulensis, E. cloeziana, E. dunnii, E. grandis, E. longirostrata, E. pellita, E. pilularis, E. robusta, E. saligna, E. scias, E. tereticornis and E. urophylla) were selected to evaluate and identify sources of resistance to CLB. The seeds of these species were seeded in tubes of 50 cm^3 capacity containing Mecplant substrate (Telêmaco Borba, Paraná, Brazil) that was enriched with Simples Superphosphate (6.0 kg m^{-3}) and Osmocote® (19:06:10 at 1.5 kg m^{-3}). At 90 days age, the seedlings were transplanted to 5–L plastic bags containing the same mixture of potting medium previously described. The plants were kept in greenhouse and fertilized biweekly with 100 mL NPK solution (05:10:30 at 6 g L^{-1}) per plant until reaching the stage suitable for inoculation as previously described (Graça et al., 2009). Thirty plants of each species in a completely randomized design were used. Five replicates of the hybrid (E. grandis x E. urophylla) clones CLR-221 and CLR-236 were used as resistant controls, and CLR-158 was used as a susceptible control.

Mass inoculum production and inoculation

The mass inoculum production of a single spore culture (LPF059) of C. pteridis (Graça et al., 2009), used routinely in our laboratory, was performed on Malt-Yeast-Extract-Agar (MYEA) as previously described (Alfenas et al., 2013). The plants were homogeneously

mist cooling inoculated by mist-spraying 200 mL/plant of an inoculum suspension at $1x10^4$ conidia mL^{-1}. After inoculation, the plants were incubated at 25 °C in a mist chamber for 48 h with an intermittent mist every 30 min for 10 sec and subsequently transferred to the greenhouse. The plants were mist irrigated every hour for 2 min until they were scored for disease severity.

Disease evaluation and statistical analysis of the data

The assessment of disease severity was performed 50 days after inoculation by quantifying the percentage of defoliation in four branches of the basal lower portion of the crown of each plant (Graça et al., 2009). A scale with four levels of defoliation was used to quantify the frequency of plants at each level of resistance (0 – 30 % = resistant; 30 – 50 % = moderately susceptible; 50 – 80 % = susceptible and 80 – 100 % = highly susceptible). The frequency of plants in each class of disease severity was determined according to the level of defoliation.

The data were also submitted to ANOVA, and the means were compared by Dunnett's test (P = 0.05) to compare each treatment with the resistant (CLR-221 and CLR-236) and susceptible (CLR-158) controls in SAS (SAS Institute, Cary, NC). Furthermore, the statistical software STATISTICA (StatSoft, Inc, Tulsa, OK, USA) was also used to perform Tukey's t-test (P = 0.05) to compare the means of each treatment.

RESULTS

As found in the field, under conditions of natural infection (Ferreira et al., 1995; Alfenas et al., 2009), the inoculated plants in the present study showed small, circular or elongated leaf lesions that were light gray to light brown (Figure 2). On the light gray lesions, sparse sporulation was observed. As the disease progressed, the lesions became light brown,

surrounded by a callus-like structure, and generally no sporulation was observed. Intense defoliation was found in highly susceptible genotypes. As observed in other species of Eucalyptus under natural infection in the field (Alfenas & Ferreira, 1979, Alfenas et al., 1979), the CLB caused by different species of Calonectria was more severe in the expanded leaves, and the highest percentage of defoliation occurred in the branches of the basal third of the canopy as has been previously found (Figure 3) (Guimarães et al., 2010, Graça et al., 2009). The average percentage of defoliation in all of the species assessed in our work ranged from 13 % to 91 %, indicating the existence of inter-specific variability for resistance (Table 1). Among the species tested, E. brassiana, E. saligna, E. scias, E. aglomerata C. citriodora, E. dunnii, E. grandis, C. maculata, E. pilularis and E. tereticornis differ from the resistant standard clones (CLR-221 and CLR-236) that were used for comparison, and they were therefore classified as susceptible (Table 2). E. aglomerata, E. brassiana, E. saligna and E. scias did not differ from the resistant standard clone that was used for comparison and they were therefore classified as resistant (Table 1). Nevertheless, intra-specific variability for resistance to CLB was found even in the most susceptible species (Table 2). Eucalyptus brassiana is the best source of resistance to CLB because more than 85 % of the plants had less than 30 % of defoliation (Table 2). However, C. citriodora, E. dunnii, E. grandis, C. maculata, E. pilularis and E. tereticornis were highly susceptible (Table 1). Highly resistant genotypes displaying 0 – 30 % defoliation can be cloned by rooted cuttings and potentially used for commercial plantations or as a source of resistance for breeding.

DISCUSSION

The inter- and intra-specific variability for the resistance of Eucalyptus spp. that were found in the present study make possible the selection of resistant genotypes for commercial plantations or for tree improvement, preferably under controlled pollinated crosses.

Eucalyptus brassiana was the best source of resistance, followed by E. saligna, E. scias and E. aglomerata. Although a limited number of seed sources were tested, E. brassiana, E. saligna, E. scias and E. aglomerata may not be significantly affected by C. pteridis in the field. However, these former four species contained some genotypes with more than 50 % defoliation, and they may be affected by CLB in the field. However, Corymbia citriodora, E. dunnii, E. grandis, C. maculata and E. pilularis had a greater than 60 % frequency of plants that were classified as susceptible and highly susceptible. Nevertheless, is possible to find resistant genotypes within highly susceptible species by testing other Australian provenances. Because the species of Eucalyptus generally do not breed with those of Corymbia, the crosses should be performed between species of the same genus (Dickinson et al., 2013, Fonseca et al., 2010).

Based on the effects of artificial pruning on the tree growth of Eucalyptus grandis (Pires, 2000), in this work we considered as highly resistant those plants displaying up to 30 % defoliation. Although the effects of CLB have not yet been quantified on a physiological basis, foliar pathogens play a major negative effect on the photosynthetic processes (Berger et al., 2007, Domiciano et al., 2009) and, consequently, on plant growth. Depending on the level of CLB, there might be significant negative effects on tree growth in the field, as was found by Pires (2000) by artificially pruning E. grandis and by Alves et al. (2011) on the leaf gas exchanges in Eucalyptus urophylla clones that were infected with Puccinia psidii Winter. If C. pteridis produces phytotoxic metabolites during the infection process, it is possible that the negative effects of CLB-induced defoliation greater than 30 % on Eucalyptus spp. is equal to or greater than those observed by the artificial pruning of Eucalyptus grandis.

Our results differ from those obtained by Blum et al. (1992), where E. robusta, E. urophylla, C. citriodora, E. pellita, E. grandis and C. maculata were resistant to Calonectria brassicae (Panwar & Borha) L. Lombard, MJ Wingf. & Crous (syn. Cylindrocladium

clavatum Hodges & L. C. May) and Calonectria morgani Crous, Alfenas M.J. Wingf. (syn. Cylindrocladium scoparium Morgan). However, these differences may be attributed to the seed source, inoculation method, age and architecture of the plants.

Currently, most commercial clones of Eucalyptus that are planted in Brazil are "urograndis" (E. grandis x E. urophylla) (Fonseca et al., 2010). Inoculations under controlled conditions in our laboratory have shown that over 65% of commercial clones that are evaluated are susceptible to CLB (Santos et al., 2008). Therefore, the introgression of resistant genes from different Eucalyptus species with complementary silvicultural and technological characteristics is needed to broaden the genetic base for the resistance of breeding populations as well as of commercial clones (Alfenas et al., 2009). The resistant genotypes of the different species found in this work are being cloned by rooted cuttings and the replicates will be tested in the field to evaluate their performance and confirm their resistance under conditions of natural infection. The superior resistant plants can be potentially used for commercial plantation and/or breeding.

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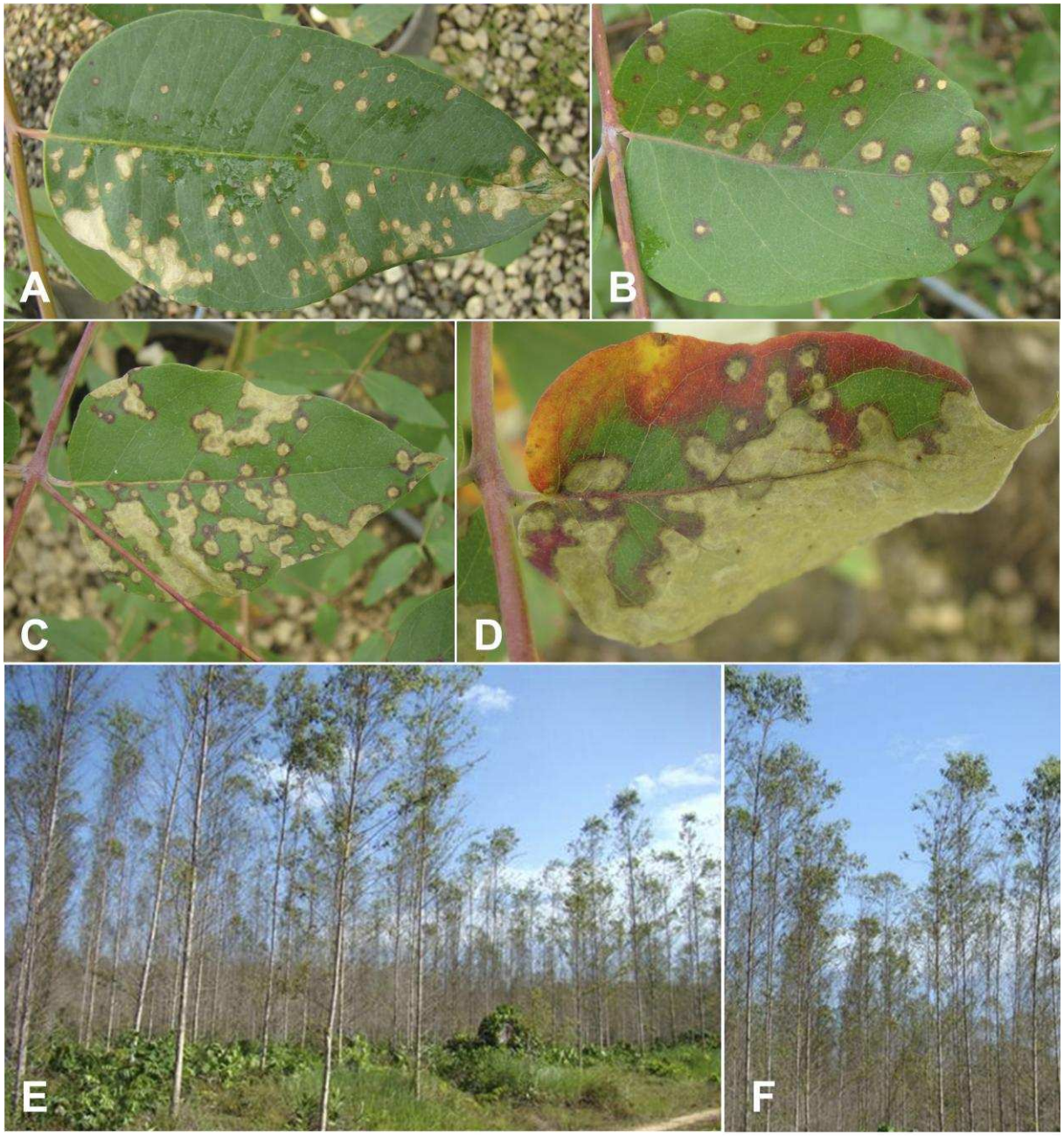

Figure 1: Leaf blight and defoliation of Eucalyptus spp. caused by Calonectria pteridis. **A-B**: Typical small and rounded lesions caused by C. pteridis. **C-D**: With disease progression, the lesions change in color and may occupy a large proportion of the leaf. **E-F**: Intense defoliation.

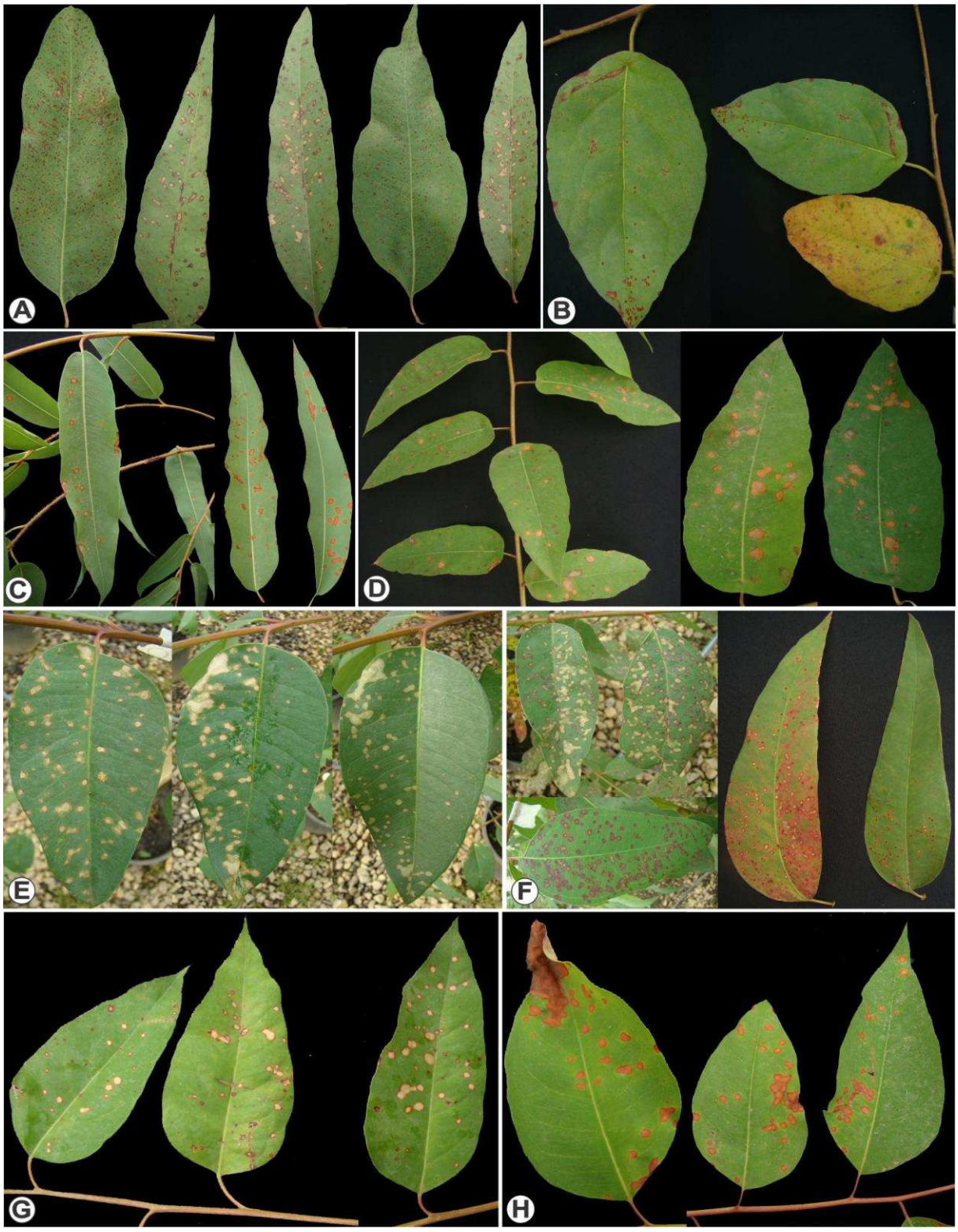

Figure 2: Variation of symptoms in Eucalyptus spp. inoculated with Calonectria pteridis. A- Eucalyptus brassiana, B- Corymbia torelliana, C- Corymbia citriodora, D- Corymbia maculata, E- Eucalyptus robusta, F- Eucalyptus tereticornis, G- Eucalyptus urophylla and H- Eucalyptus cloeziana.

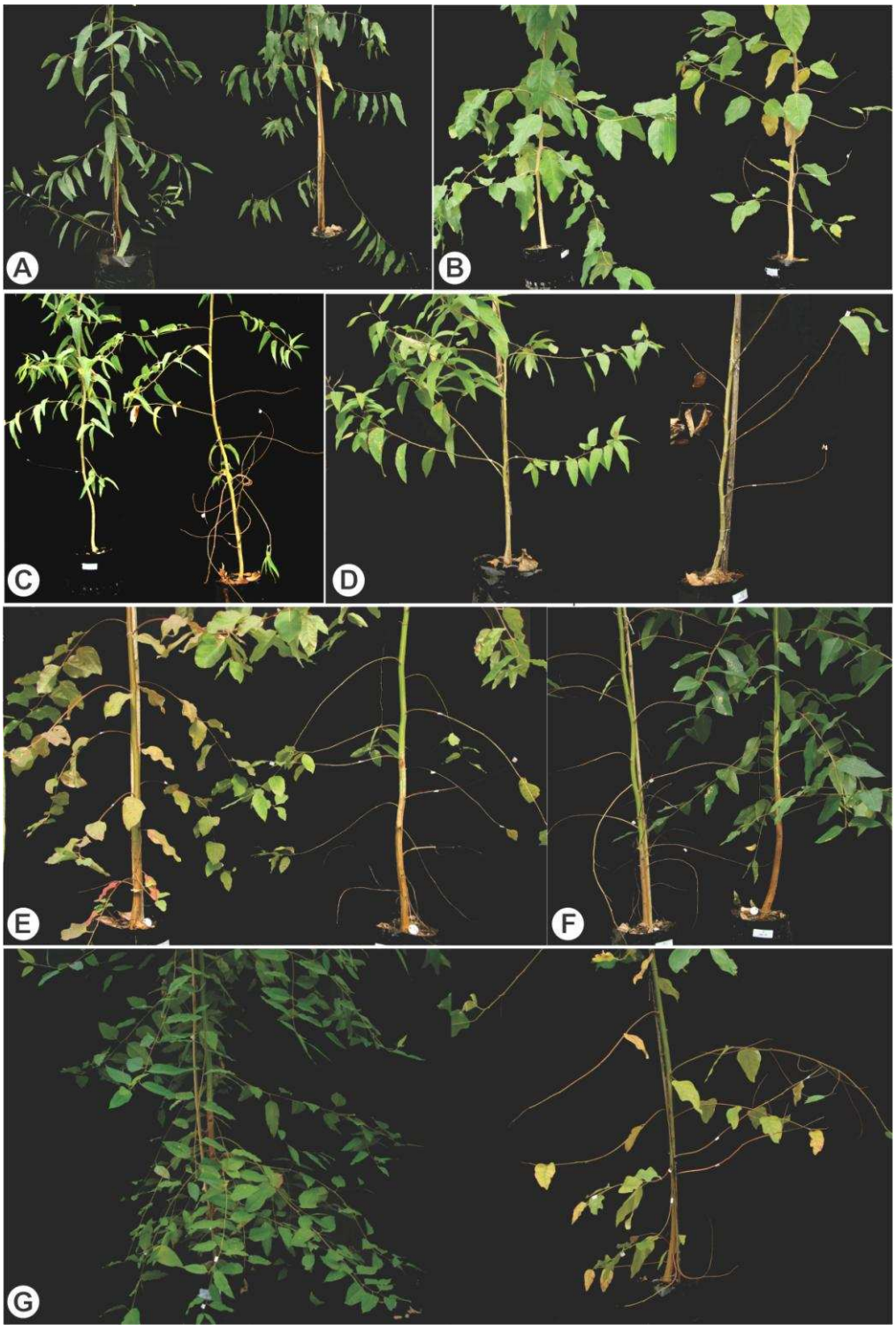

Figure 3: Variation in the phenotype of Eucalyptus species inoculated with Calonectria pteridis, showing intraspecific variability for resistance to Calonectria-leaf-blight. Resistant genotypes are next to susceptible genotypes. A - Eucalyptus brassiana, B- Corymbia torelliana, C- Corymbia citriodora, D- Corymbia maculata, E- Eucalyptus pellita, F- Eucalyptus robusta and G- Eucalyptus urophylla.

Table 1: Average percentage of defoliation in seventeen Eucalyptus species, three species of Corymbia and the resistant (CLR-221 and CLR-236) and susceptible (CLR-158) controls 50 days after inoculation with Calonectria pteridis Statistically significant differences between the species studied are represented by Tukey's t-test at 5%.

Phynotype	Species	Av. Defoliation (%)	a	b	c	d	e	f	g	h
R	CLR-236 (R)	12.97	**	**						
	E. brassiana	16.95	**							
	CLR-221 (R)	22.66	**	**	**					
	E. saligna	26.72	**	**	**					
	E. scias	28.82	**	**	**					
	E. agglomerata	29.07	**	**	**					
S	E. urophylla	32.94	**	**	**	**				
	E. longirostrata	33.19	**	**	**	**				
	C. torelliana	35.64	**	**	**	**	**			
	E. camaldulensis	36.07	**	**	**	**	**			
	E. robusta	38.42	**	**	**	**	**	**		
	E. pellita	44.77		**	**	**	**	**		
	E. cloeziana	47.11			**	**	**	**	**	
	CLR-158 (S)	47.42	**	**	**	**	**	**	**	
HS	E. tereticornis	52.20				**	**	**	**	
	E. pilulares	57.46					**	**	**	
	C. maculata	60.38					**	**	**	
	E. grandis	61.55						**	**	
	E. dunnii	68.76							**	**
	C. citriodora	90.60								**

334 **Table 2:** The average percentage of defoliation in seventeen Eucalyptus species, three
335 species of Corymbia and the resistant (CLR-221 and CLR-236) and susceptible (CLR-
336 158) controls 50 days after inoculation with Calonectria pteridis. Comparisons that
337 were significant at the 0.05 level by Dunnett's test are indicated by ***.

Species	**Av. defoliation (%)**	**Resistant controls**		**Susceptible control**
		CLR-221	**CLR-236**	**CLR-158**
CLR-221	22.7	Control		
CLR-236	13.0		Control	***
CLR-158	47.4		***	Control
E. agglomerata	29.1			
E. brassiana	17.0			***
E. camaldulensis	36.1			
C. citriodora	90.6	***	***	***
E. cloeziana	45.8		***	
E. dunnii	68.8	***	***	
E. grandis	61.6	***	***	
E. longirostrata	33.2			
C. maculata	60.4	***	***	
E. pellita	44.8		***	
E. pilulares	57.5	***	***	
E. robusta	38.4			
E. saligna	26.7			
E. scias	28.8			
E. tereticornis	52.2	***	***	
C. torelliana	35.6			
E. urophylla	32.9			

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339 **Table 3:** Frequency of plants distributed across four levels of defoliation.

Species	Defoliation levels			
	0 – 30 %	**30 – 50 %**	**50 – 80 %**	**80 – 100 %**
E. agglomerata	62.1	20.7	13.8	3.4
E. brassiana	87.0	8.7	0.0	4.3
E. camaldulensis	46.7	30.0	23.3	0.0
C. citriodora	0.0	0.0	21.4	78.6
E. cloeziana	33.3	23.8	33.3	9.5
E. dunnii	3.3	23.3	30.0	43.3
E. grandis	25.0	3.6	25.0	46.4
E. longirostrata	46.7	26.7	26.7	0.0
C. maculata	33.3	5.6	5.6	55.6
E. pellita	35.7	21.4	21.4	21.4
E. pilulares	11.5	26.9	53.8	7.7
E. robusta	47.4	42.1	0.0	10.5
E. saligna	64.3	21.4	7.1	7.1
E. scias	65.5	13.8	20.7	0.0
E. tereticornis	20.0	32.0	28.0	20.0
C. torelliana	44.4	29.6	22.2	3.7
E. urophylla	50	50	0	0

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