GABRIELLE AVELAR SILVA

SPECIES OF *Chrysoporthe* PATHOGENIC TO MELASTOMATACEAE IN BRAZIL

LAVRAS-MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Doutora.

Prof(a). Dr(a). Maria Alves Ferreira
Orientadora

Prof(a). Dr(a). Mara Elisa Soares de Oliveira
Coorientadora

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A Deus por nunca me desamparar e me segurar pelas mãos nos momentos difíceis.

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RESUMO

Chrysoporthe spp. são conhecidas por causar cancro e mortalidade em espécies lenhosas das famílias Myrtaceae e Melastomataceae, sendo a última uma das maiores famílias de angiospermas em todo o mundo, abrigando espécies amplamente utilizadas na arborização urbana e ambientalmente importantes para a recuperação de áreas degradadas, atuando como espécies pioneiras durante o processo de sucessão secundária. Desse modo, o presente trabalho teve por objetivo realizar a caracterização filogenética, morfológica e patogênica de isolados fúngicos obtidos de árvores pertencentes à família Melastomataceae com sintomas de cancro, seca de galhos e mortalidade no sudeste do Brasil. O primeiro artigo trata da descrição de uma nova espécie denominada *Chrysoporthe brasiliensis* encontrada no sudeste do Brasil causando cancro e mortalidade em *Miconia* spp. e *Rhynchanthera grandiflora*. O segundo artigo trata da ocorrência de *Chrysoporthe doradensis* em *R. grandiflora* e d*e Chrysoporthe puriensis* em *R. grandiflora* e *Miconia theaezans* no sul estado de Minas Gerais.

Palavras-chave: Filogenia. Patogenicidade. Cryphonectriaceae. *Miconia* spp. *Rhynchanthera grandiflora.*

ABSTRACT

Chrysoporthe species are well known to cause canker and mortality in woody species of the Myrtaceae and Melastomataceae families. The later family is one of the largest families of angiosperms worldwide, with species widely used in urban afforestation and environmentally important for the recovery of degraded areas, acting as pioneer species during the process of secondary succession. Thus, the present study aimed to conduct the phylogenetic, morphological and pathogenic characterization of the of fungal isolates obtained from trees belonging to the Melastomataceae family with symptoms of canker, branch dryness and mortality in southeastern Brazil. The first article treats the description of a new species of Chrysoporthe found in southeastern Brazil causing canker and mortality in *Miconia* spp. and *Rhynchanthera grandiflora*. The second article is related the occurrence of *Chrysoporthe doradensis* on *R. grandiflora* and of *Chrysoporthe puriensis* on *R. grandiflora* and *Miconia theaezans* in the south of Minas Gerais state.

Keywords: Phylogeny. Pathogenicity. Cryphonectriaceae. *Miconia* spp. *Rhynchanthera grandiflora*.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

O gênero *Chrysoporthe* Gryzenhout & M.J. Wingf. pertence à família Cryphonectriaceae, ordem Diaporthales e filo Ascomycota. As espécies de *Chrysoporthe* são conhecidas por causarem cancro principalmente em árvores de *Eucalyptus* spp. L'Hér e membros da família Melastomataceae A. Juss. (WINGFIELD et al., 2001; GRYZENHOUT et al., 2004; RODAS et al., 2005).

Atualmente, o gênero abriga nove espécies: *Chrysoporthe cubensis*, *Chrysoporthe Chrysoporthe deuterocubensis*, *Chrysoporthe hodgesiana*, *Chrysoporthe zambiensis*, *Chrysoporthe syzygiicola*, *Chrysoporthe doradensis*, *Chrysoporthe inopina*, *Chrysoporthe austroafricana* e *Chrysoporthe puriensis*, descrita recentemente, (HODGES et al., 1976; GRYZENHOUT et al., 2005; CHUNGU et al., 2010; SOARES et al., 2018; OLIVEIRA et al., 2021). As espécies de *Chrysoporthe* são muito similares morfologicamente, sendo diferenciadas de modo sutil pelo tamanho dos esporos e temperatura ótima de cultivo entre 25 a 30°C. (GRYZENHOUT et al., 2009). A identificação conclusiva só é possível com a comparação das sequências de fragmentos de DNA, sendo mais utilizados para essa diferenciação o gene β-tubulina e a região ITS do rDNA (GRYZENHOUT et al., 2004; GRYZENHOUT et al., 2009; van der MERWE et al., 2013; SOARES et al., 2018; OLIVEIRA et al; 2021).

A primeira espécie do gênero a ser descrita foi *C. cubensis* (Bruner) Gryzenhout & M.J. Wingf. em 1917 em Cuba, como *Diaporthe cubensis*, causando cancro em *Eucalyptus* spp. (BRUNER, 1917). Posteriormente, a espécie foi recolocada dentro do gênero *Cryphonectria* devido ao fato de suas características culturais e morfológicas se assemelharem às das espécies desse gênero. Porém, em 2004, estudos das sequências de ITS e β-tubulina apontaram diferenças filogenéticas entre *C. cubensis* e as demais espécies do gênero *Cryphonectria*. Sugeriu-se a inclusão de dois novos os gêneros *Chrysoporthe* e *Chrysoporthella*, abrigando as espécies *Chrysoporthe cubensis, Chrysoporthe austroafricana* Gryzenh. & M.J. Wingf. *(*originária da África do Sul) e *Chrysoporthe hodgesiana* Gryzenh. & M.J. Wingf. (sin. = *Chrysoporthella hodgesiana*) (originária da Colômbia).

Baseando-se em diferenças morfológicas e filogenéticas, trabalhos posteriores descreveram ainda as espécies *Chrysoporthe doradensis* Gryzenh. & M.J. Wingf., *Chrysoporthe inopina* Gryzenh. & M.J. Wingf. e *Chrysoporthe deuterocubensis* Gryzenh. &

M.J. Wingf. (GRYZENHOUT et al., 2005; GRYZENHOUT et al., 2006; van der MERWE et al., 2010).

As espécies *Crhysoporthe zambiensis* Chungu, Gryzenh. & Jol. Roux e *Crysoporthe syzygiicola* Chungu, Gryzenh. & Jol. Roux foram descritas simultaneamente em 2010 por Chungu et al. (2010) na Zâmbia, no continente africano causando sintomas de cancro em *Eucalyptus grandis* W. Hill e *Syzygium guineense* Wall., respectivamente. *Chrysoporthe puriensis* M.E.S. Oliv., T.P.F. Soar. & M.A. Ferr. é a espécie do gênero descrita mais recentemente, em 2021, no Brasil provocando cancro em *Pleroma* spp. D. Don. (sin. = *Tibouchina* spp. Aubl.) (OLIVEIRA et al., 2021).

Dessas espécies, apenas *C. cubensis*, *C. doradensis* e *C. puriensis* já foram relatadas no Brasil (HODGES, 1980; SEIXAS et al., 2004; BARRETO et al., 2006; ALFENAS et al., 2009; SOARES et al., 2018; OLIVEIRA et al., 2021).

Chrysoporthe cubensis foi relatada no Brasil pela primeira vez em 1973, atacando plantios de *Eucalyptus* spp. (HODGES et al., 1973), sendo nessa década a doença biótica mais importante da eucaliptocultura brasileira e impulsionando o desenvolvimento da patologia florestal, silvicultura e melhoramento genético na busca de resistência à doença (FERREIRA, 1989). *C. cubensis* já foi identificada no Brasil em diversas espécies de Melastomataceae e Myrtaceae, sendo elas *Eucalyptus* spp.,*Syzygium aromaticum* (L.) Merr. & L.M.Perry, *Psidium cattleianum* Sabine, *Marlierea edulis* Nied., *Pleroma* spp. e *Corymbia citriodora* (Hook.) K.D.Hill & L.A.S.Johnson (HODGES, 1980; SEIXAS et al., 2004; BARRETO et al., 2006; ALFENAS et al., 2009; SOARES et al., 2018).

Chrysoporthe doradensis foi descrita no Equador causando cancro em plantios de *E. grandis* e *Eucalyptus deglupta* Blume, com sintomas semelhantes aos causados por *C. cubensis* (GRYZENHOUT et al., 2005). Sua ocorrência no Brasil foi relatada em 2018 em espécies de *Eucalyptus* nos estados do Maranhão e Minas Gerais e em *Pleroma granulosum* (Desr.) D. Don. em Minas Gerais (SOARES et al., 2018). A espécie *C. puriensis* foi descrita causando cancro e até mesmo morte em árvores de *Pleroma* spp. nos estados de Minas Gerais, Bahia e na região do Parque Nacional do Itatiaia no Rio de Janeiro (OLIVEIRA et al., 2021).

As espécies da família Melastomataceae estão entre os principais hospedeiros de *Chrysoporthe* spp. Esta família classifica-se na ordem Myrtales e é representada por aproximadamente 4570 espécies e 150 gêneros distribuídos mundialmente em regiões tropicais e subtropicais. No Brasil as espécies de Melastomataceae são encontradas em todos os estados e corresponde à sexta família mais importante no cerrado brasileiro (CLAUSING e

RENNER, 2001; ALBUQUERQUE et al., 2013). As espécies de Melastomataceae possuem hábito arbóreo, arbustivo ou herbáceo e podem ser facilmente reconhecida por suas folhas com nervuras acródomas e abriga várias espécies cultivadas como árvores ornamentais devido à sua beleza, sendo conhecidas como quaresmeiras, manacá, orelha-de-onça, dentre outros nomes (FREITAS et al., 2016).

As espécies de Melastomataceae são consideradas pioneiras de grande potencial para recuperação de áreas degradadas. Apresentam características que permitem rápido desenvolvimento em ecossistema que sofreu perturbação antrópica: são heliófilas, de crescimento rápido e pouco exigentes quanto à fertilidade do solo. Também produzem frutos carnosos, pequenos e arredondados com grande quantidade de sementes e possuem ampla gama de polinizadores e dispersores de sementes, principalmente aves (JANSEN et al., 2002; ARZOLLA et al., 2010; HÜLLER et al., 2011; ALBUQUERQUE et al., 2013; JESUS et al., 2016).

Muitas espécies de Melastomataceae, dentre elas *Miconia theaezans* (Bonpl.) Cogn., *Miconia ibaguensis* (Bonpl.) Triana e *Rhynchanthera grandiflora* (Aubl.) DC. atuam como nucleadoras, aumentando a conectividade entre fragmentos florestais e as áreas em processos de restauração ecológica (ALBUQUERQUE et al., 2013).

A importância ambiental das espécies de Melastomataceae no processo de recuperação de áreas degradadas aliada aos relatos recentes de *Chrysoporthe* spp. parasitando-as no Brasil reforçam a necessidade de investigações mais abrangentes de novos patossistemas em território nacional, ressaltando a gravidade dos sintomas provocados no hospedeiro e as condições climáticas do País, ideais ao desenvolvimento do patógeno.

Portanto o presente trabalho teve como objetivos realizar a caracterização filogenética, morfológica e patogênica de isolados de *Chrysoporthe* spp. encontrados em espécies da família Melastomataceae no Brasil.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 - *Chrysoporthe brasiliensis* sp. nov. associated with *Miconia* spp. and *Rhynchanthera grandiflora* in southeast Brazil

Artigo preparado em concordância com as normas do periódico "Journal of fungi".

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ABSTRACT

Species of Melastomataceae family are among the main hosts of fungi of the species of genus *Chrysoporthe*. These fungi cause canker symptoms, death of branches and even death of the host. During field research conducted in Southeast Brazil, in the states of Minas Gerais and Rio de Janeiro, *Chrysoporthe*-like fungi were observed in *Miconia* spp. and *Rhynchanthera grandiflora*. The aims of this study were to isolate and identify the fungus through morphological and phylogenetic characteristics. In addition, artificial inoculations were conducted to assess its pathogenicity on *R. grandiflora* and *M. theaezans*. The results showed that fungus associated with canker in Melastomataceae in Southeast Brazil is a new species of *Chrysoporthe* which is described in this study and named *Chrysoporthe brasiliensis* sp. nov. Further that this species causes disease on *R. grandiflora* and *M. theaezans*.

Keywords: Cryphonectriaceae; Melastomataceae; Phylogeny; Canker; Pathogenicity.

1. Introduction

The family Melastomataceae belongs to the order Myrtales and is represented by approximately 150 genera which include 4570 species, these species occur in the tropics and sub tropics worldwide [1]. Members of this family are pioneer species and are thus important for habitat to recovery of degraded areas. They are also heliophiles, grow quickly and are undemanding in terms of soil fertility. Furthermore, they have small and fleshy fruits with an abundance of seeds, which favours establishment [2-6].

Many species in fungal family Cryphonectriaceae family are pathogens of the Melastomataceae. The family Cryphonectriaceae was established in 2006, and includes pathogenic, saprophytic, and endophytic species [7,8]. The pathogens cause canker, dead branches, and mortality of tree species. The fungi of this family differ from others in the same order by presenting orange stromal tissue at some stage of their life cycle, turn purple colour when reacting with KOH, and yellow when in contact with lactic acid [9,10].

The genus *Chrysoporthe* is one of the most studied genera in the Cryphonectriaceae family. Species in this genus cause typical canker symptoms, especially on species of the families Melastomataceae and Myrtaceae in tropical and subtropical regions [9,11]. The different species of the genus are morphologically very similar, and have subtle differences in spore size and optimal growth temperatures. Thus, DNA sequences and phylogenetic analyses are necessary for accurate identification [12-14].

Nine species of *Chrysoporthe* have been described, namely, *C. cubensis*, *C. deuterocubensis*, *C. hodgesiana*, *C zambiensis*, *C. syzygiicola*, *C. doradensis*, *C. inopina*, *C. austroafricana*, and, most recently, *C. puriensis*. Among them, only *C. cubensis*, *C. doradensis*, and *C. puriensis* have been reported to occur in Myrtaceae and Melastomataceae in Brazil [13-17].

The first species of the genus *Chrysoporthe* reported in Brazil was *C. cubensis*, causing canker in commercial plantations of *Eucalyptus* spp., as well native plants [18,19]. The fungus was initially reported in Cuba in *Eucalyptus* spp. [20]. and has been identified in several tropical and subtropical countries [21-24]. It was first reported in Brazil in the 1970s [15] and has become the most important fungal pathogen in Brazilian eucalyptus plantations. The importance of this pathogen has warranted the development of forest pathology and breeding programs for genotypes resistant to disease [25,26].

The species *C. doradensis* was described in Ecuador, causing canker in *E. grandis* and *E. deglupta* [16]. In Brazil, this species was reported in 2015 attacking *Eucalyptus* spp. in the states of Maranhão and Minas Gerais and *Pleoroma granulosum* (= *Tibouchina granulosa*) in Minas Gerais (Soares et al., 2018), the symptoms caused by the fungus are typical of canker, like those caused by *C. cubensis* [16,17].

Recently in Brazil was described *C. puriensis*, causing canker lesions in *Pleroma* spp. in the states of Minas Gerais, Bahia, and Rio de Janeiro. The symptoms caused by *C. puriensis* often lead to the death of host trees, and pathogenicity tests have proven its ability to cause canker in *Eucalyptus* hybrids [14].

Recent reports of new *Chrysoporthe* species in Brazil, and other genera in the Cryphonectriaceae causing canker in *Caryocar brasilieinse* trees [27] demonstrate that more comprehensive studies of new pathosystems throughout the country are necessary. This is especially given Brazil's tropical and subtropical climatic conditions are favourable to the development of fungi of the Cryphonectriaceae family [9].

During field research conducted in southeastern Brazil, symptoms, and signs typical of those caused by Cryphonectriaceae were observed in *Miconia* spp. and *Rhynchanthera grandiflora* plants, which belongs to the Melastomataceae family. The aim of the present

study was to isolate and identify this fungus through phylogenetic and morphological analyses, as well to assess its pathogenicity on *R. grandiflora*.

2. Materials and methods

2.1. Cultures

Samples were collected from *Miconia ibaguensis*, *Miconia theaezans* and *Rhynchantera grandiflora* plants in Rio de Janeiro and Minas Gerais states. These trees showed symptoms and typical signs of pathogens belonging to the Cryphonectriaceae family. The samples were stored in paper bags, labelled, and sent to the Laboratório de Patologia Florestal - Universidade Federal de Lavras, Brazil.

Single spores were isolated to obtain monosporic cultures. For this purpose, a single fruiting body was removed from each sample and suspended in water, and the suspensions were transferred to Petri dishes containing potato dextrose agar (PDA) medium supplemented with rifamycin (100 mg/L). The Petri dishes were incubated at 28 °C for 24 hours until germination. After germination, a single germinated spore was transferred to a new Petri dish containing PDA medium and supplemented with rifamycin (100 mg/L), then incubated at 28 °C for 7 days to store a pure culture of the fungus.

The isolate TILU 103 was deposited in the Coleção de Culturas de Microorganismos – Departamento de Ciências dos Alimentos (CCDCA) at the Universidade Federal de Lavras and other isolates were stored in tubes containing sterilized water [28] and kept at room temperature in the Laboratório de Patologia Florestal. In addition, the samples of branches from which the TILU 103 isolate were deposited in the Coleção Micológica do Herbário UB – Universidade de Brasília.

2.2. DNA extraction, amplification, and sequencing

The mycelium used for DNA extraction was obtained according to Myburg et al. (1999) [29]. Mycelial discs 5 mm in diameter were transferred to Erlenmeyer flasks containing liquid culture medium composed of malt extract (20 g/L), yeast extract (2 g/L), and dextrose (5 g/L). The flasks were incubated for 7 days at 28°C for the fungus to grow. The mycelia were removed, filtered, and dried on filter paper and then ground in liquid nitrogen.

DNA was extracted following the cationic hexadecyl trimethyl ammonium bromide (CTAB) protocol with some modifications [30]. For cell lysis, an extraction buffer containing Tris-Cl, EDTA, NaCl, and CTAB was used. The quality and concentration of the extracted fungal DNA was evaluated using agarose gel electrophoresis stains visualized in ultraviolet-light image capture system (DNR Bio Imaging System, MiniBis Pro) and Nanodrop Thermo Scientific 2000c equipment®, respectively.

Polymerase chain reactions (PCRs) were performed as described by Glass and Donaldson (1995) [31]. Each PCR had a final volume of 25 µL and was run in the Techne Prime G thermal cycler. The amplified genomic regions were the ITS region of the rDNA gene (ITS1-5.8S-ITS2), targeted by the primer pair ITS1 and ITS4 [32] and the BT1 and BT2 fragments of the β-tubulin gene, targeted by the primer pairs BT1a/BT1b and BT2a/BT2b, respectively [31]. PCR conditions were adjusted for each gene. For the ITS region and BT1 fragment of the β-tubulin gene, the cycling conditions included denaturation at 94 °C for 1 minute, followed by 35 cycles at 94 °C for 30 seconds, 55 °C for 55 seconds, and 72 °C for 2 minutes, with a final extension of 72 °C for 5 minutes. For the BT2 fragment, the cycling

conditions were denaturation at 94 °C for 1 minute; 35 cycles at 94 °C for 30 seconds, 62 °C for 45 seconds and 72 °C for 1 minute; and a final extension at 72 °C for 5 minutes.

The amplification products were evaluated by electrophoresis in 1% agarose gel with the addition of GelRed®. The bands were visualized using an ultraviolet-light image capture system (DNR Bio Imaging System, MiniBis Pro). Purification of the PCR product and sequencing were performed by Macrogen Company (Korea).

2.3. Phylogenetic analyses

The electropherograms generated were analysed, and the sequences were manually edited, when necessary, using SeqAssem software [33]. After editing, the sequences were compared with other *Chrysoporthe* sequences from the GenBank database. The sequences generated in this study were also deposited in NCBI/GenBank (Table 1).

Multiple alignments of the sequences were performed using the online interface of MAFFT software [34]. To assess the possibility of doing a combined analysis of ITS and β-tubulin sequences by means of gene concatenation, the partition homogeneity test was applied [35] using the software PAUP* 4.0 [36], with 1000 replicates. Phylogenetic analyses were performed using the maximum likelihood, maximum parsimony, and Bayesian inference methods. The maximum likelihood analysis was done in MEGA software version 6.0 [37]. For the combined tree of the ITS, BT1, and BT2 regions, the three-parameter Tamura evolutionary model was applied, as determined by MEGA [38], in addition to the discrete gamma distribution to model the evolutionary differences between sites. The confidence level of the branches was established using 1000 bootstrap replicates.

Table 1 *Chrysoporthe* spp. and *Amphilogia gyrosa* (outgroup) reference sequences used in the present study.

Species	Isolate number	Host/substrate	Location	GenBank accession number		
				ITS	BT1	BT2
Chrysoporthe brasiliensis	**MIL06**	*Miconia theaezans*	Minas Gerais - Brazil	MT886851	OP714218*	
	MIL104B	*Miconia theaezans*	Minas Gerais - Brazil	MT886850	OP714219*	
	MILS01E	*Miconia theaezans*	Minas Gerais - Brazil	ON000431	OP714220*	
	MIPNI01	*Miconia ibaguensis*	Rio de Janeiro - Brazil	MT886852	OP714221*	
	TILU103	*Rhynchanthera grandiflora*	Minas Gerais - Brazil	MT886849	OP714222*	
	TILU104	*Rhynchanthera grandiflora*	Minas Gerais - Brazil	MT886848	OP714223*	
	TILU104D	*Rhynchanthera grandiflora*	Minas Gerais - Brazil		OP714224*	
	TILU308	*Rhynchanthera grandiflora*	Minas Gerais - Brazil	ON000430	OP714225*	
Chrysoporthe doradensis	CMW11286	*Eucalyptus grandis*	Ecuador	AY214290	AY214218	AY214254
	CMW11287	*Eucalyptus grandis*	Ecuador	GQ290156	GQ290179	GQ290190
Chrysoporthe inopina	CMW12729	*Pleroma lepidotum*	Colombia	DQ368778	AH015656*	
	CMW12727	*Pleroma lepidotum*	Colombia	DQ368777	AH015657*	
Chrysoporthe hodgesiana	CMW10625	*Miconia theaezans*	Colombia	AY956970	AH014900*	
	CMW10641	*Pleroma semidecandrum*	Colombia	AY692322	AY692326	AY692325
Chrysoporthe deuterocubensis	CMW12745	*Pleroma urvilleanum*	Singapur	DQ368764	GQ290183	DQ368781
	CMW18515	*Melastoma malabathricum*	Indonesia	JN942341		

Chrysoporthe cubensis	CMW14394	*Eucalyptus grandis*	Cuba	DQ368773	AH015642*	
	CMW10639	*Eucalyptus grandis*	Colombia	AY263421	AY263419	AY263420
Chrysoporthe puriensis	CT13	*Pleroma granulosum*	Brazil	MN590029	MN590041*	
	TCL01	*Pleroma candolleanim*	Brazil	MN590030	MN590042*	
Chrysoporthe syzygiicola	CMW29940	*Syzygium guineense*	Zambia	FJ655005	FJ805230	FJ805236
	CMW29941	*Syzygium guineense*	Zambia	FJ655006	FJ805231	FJ805237
Chrysoporthe zambiensis	CMW29930	*Eucalyptus grandis*	Zambia	FJ655004	FJ858711	FJ805235
	CMW29928	*Eucalyptus grandis*	Zambia	FJ655002	FJ858709	FJ805233
Chrysoporthe austroafricana	CMW2113	*Eucalyptus grandis*	South Africa	AF046892	AF273067	AF273462
	CMW9327	*Pleroma granulosum*	South Africa	GQ290158	GQ290185	AF273455
Amphilogia gyrosa	CMW10469	*Ealeocarpus dentatus*	New Zealend	AF452111	AF525707	AF525714
	CMW10470	*Ealeocarpus dentatus*	New Zealend	AF452112	AF525708	AF525715

Isolates in bold were sequenced in this study.

CMW= Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

*Sections of BT1/BT2 combined of the gene β-tubulin.

The analysis of maximum parsimony was performed in the software PAUP* 4.0 [36], using only parsimony-informative characters. The analysis consisted of 100 replicates, using the random addition of sequences and subsequent tree bisection and reconnection branch swapping. The degree of branch consistency was calculated using 1000 bootstrap replicates. The number of parsimony-informative characters, consistency index, retention index, and rescaled consistency index were also calculated [39,40].

Bayesian inference was performed using MrBayes software, version 3.2.1 [41]. The branches were supported by the posterior probability values [42]. The Markov chain Monte Carlo algorithm [43] was generated from a random tree and executed with 10^7 generations. A total of 25% of the initial samples collected were discarded (burn-in), and the remaining samples were used to calculate the posterior probability values.

Sequences of *Amphilogia gyrosa* were used as outgroups in all analyses, and the trees were analysed and edited in FigTree version 1.3.1 (http://tree.bio.ac.uk/software).

2.4. Morphological characterization

For the morphological characterization of the species, longitudinal anatomical sections of the fungal structures were made, and then slides were made with 50% glycerol mounting medium. The size, shape, and colour of the pycnidia, loculi, conidia, and conidiophores were evaluated. Measurements were performed by visualizing 50 replicates of each structure under a Nikon Eclipse E200 microscope equipped with an Infinity Analyze image capture system and Infinity 1 software. Fungal structures formed in culture medium were also observed under a Nikon SMZ 1500 stereoscope microscope.

For better observation of conidia and conidiophores, scanning electron microscopy (SEM) was used. For this purpose, the fruiting bodies and spore mass of the pathogen were collected from the samples and fixed in Karnovsky solution (2.5% glutaraldehyde, 2.5% paraformaldehyde in cacodylate buffer pH 7.2) in microtubes for 24 hours at 4 °C. The suspension was centrifuged, the excess supernatant was reduced, and the remaining suspension was transferred to coverslips treated with poly-L-lysine for adhesion of the material.

After 10 minutes, the material was post fixed in osmium tetroxide (OsO_4) and washed in 0.05 M cacodylate buffer three times for 10 minutes. The samples were then dehydrated in graded acetone solutions at concentrations of 25, 50, 75, 90, and 100%. After dehydration of the samples to the critical point using the CPD 030 Bal-Tec critical point dryer, the coverslips containing the structures were adhered to the stub and gold-coated in an SCD 050 Bal-Tec sputter coater (sputtering).

After the treatments, the samples were evaluated in an LEO EVO 40XVP scanning electron microscope at the Laboratório de Microscopia Eletrônica - Universidade Federal de Lavras.

2.5. Pathogenicity tests

For the pathogenicity tests, *R. grandiflora* six seedlings and ten stems of *M. theaezans* with approximately 10mm in diameter were artificially inoculated with the fungus. Disks from the bark are removed until the wood exposition, with cylindrical cutter of 5mm diameter. Subsequently, mycelium discs (seven days old on PDA medium) of the same diameter were placed in contact with the cambium and protected with plastic film against desiccation. Disks containing PDA medium only were used as control in others six seedlings and ten stems. The experiment was repeated two times.

The plastic film was kept on the seedlings for 30 days after inoculation (d.a.i.) and the evaluation was performed at 60 d.a.i assessing the length of stem lesion with a tape measure. In stems the plastic film was kept and evaluation was performed at 21 d.a.i. To compare the mean lesion lengths of the control and inoculated plants a t-test was used and values where $P \leq 0.05$ were considered as significant.

After evaluation an indirect re-isolation was performed for *M. theaezans* and a direct re-isolation from the fungal structures formed on the lesion was performed on *R. grandiflora*, to fulfil the Koch's postulates.

3. Results

3.1. Obtaining isolates and symptomatology

A total of eight isolates (Table 1) were obtained in the present study, of which one was from *M. ibaguensis* tree in Parque Nacional do Itatiaia - Rio de Janeiro. Three isolates were from trees of *M. theaezans* in the Parque Ecológico Quedas do Rio Bonito -Lavras - Minas Gerais (Figure 1B) and three were from samples of *R. grandiflora* trees from the city of Luminárias, Minas Gerais (Figure 1A).

The isolates were taken from different symptomatic trees located near water courses. The symptoms observed were different for *Miconia* spp. and *R. grandiflora*. In *Miconia*, there were slits in the bark of trunk (Figure 1C), many dry branches (Figure 1D) and intense defoliation, causing partial death of the plant. In *R. grandiflora*, the branches and trunk were all dry, indicating death of the individuals.

On both hosts blackened bark caused by the presence of pycnidia were observed on the surface of the trunks and branches (Figure 1E, F and G).

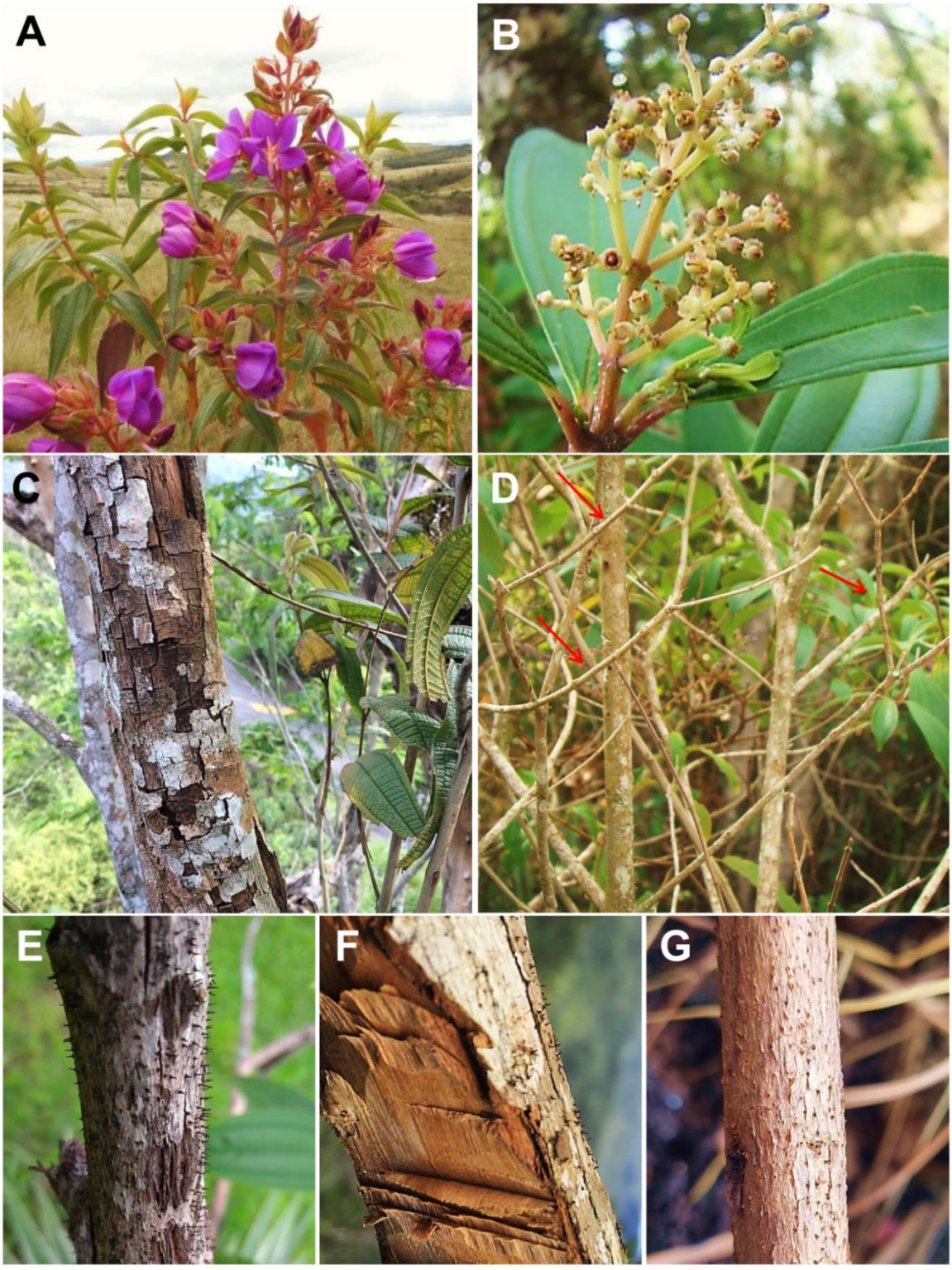

Figure 1 *Chrysoporthe brasiliensis* hosts and symptomatology. (A) *R. grandiflora*. (B) *Miconia theaezans* of Minas Gerais State. (C) *Miconia ibaguensis* from Rio de Janeiro State. Trunk with slits in the bark and typical symptoms of canker. (D) Drying and death of branches with the presence of pathogen typical structures (indicated by red arrows) in

Miconia theaezans (E) Signs of *C. brasiliensis* on *Miconia theaezans*. (F) Signs on *Miconia ibaguensis*. (G) Signs of *C. brasiliensis* on *R. grandiflora.*

3.2. Phylogenetic analyses

The sequences obtained by PCR were approximately 437 bp for the ITS region and 685 bp for BT1 and BT2. The partition homogeneity test returned P = 0.01, indicating congruence between the sequences of the ITS region and the β-tubulin gene, and thus enabling concatenated analysis of the sequences. The trees generated by the maximum likelihood, maximum parsimony, and Bayesian inference analyses showed the same topology and well-supported branches, with high bootstrap and posterior probability values.

The alignment of the combined BT2, BT1 and ITS sequences had a total of 1,122 characters, of which 1000 were constant, ten were non informative, and 112 were informative. The values obtained were 0.78 for the consistency index, 0.21 for homoplasy index, 0.87 for the retention index, and 0.68 for the rescaled consistency index.

By all analyses performed, the *Chrysoporthe* spp. sequences were grouped into nine distinct and well-supported clades (more than 70% bootstrap and 0.9 posterior probability). The eight isolates of this study were grouped into a distinct clade, supported by high bootstrap values for maximum likelihood (99%) and maximum parsimony (100%) and 1.0 of posterior probability for Bayesian inference (Figure 2), indicating it was a *Chrysoporthe* species not yet described.

The alignment of the β-tubulin and ITS sequences of all *Chrysoporthe* species showed that the difference between them was due to the presence of 53 polymorphic nucleotides, of which nine were exclusive to the isolates that represented a new taxon (Table 2).

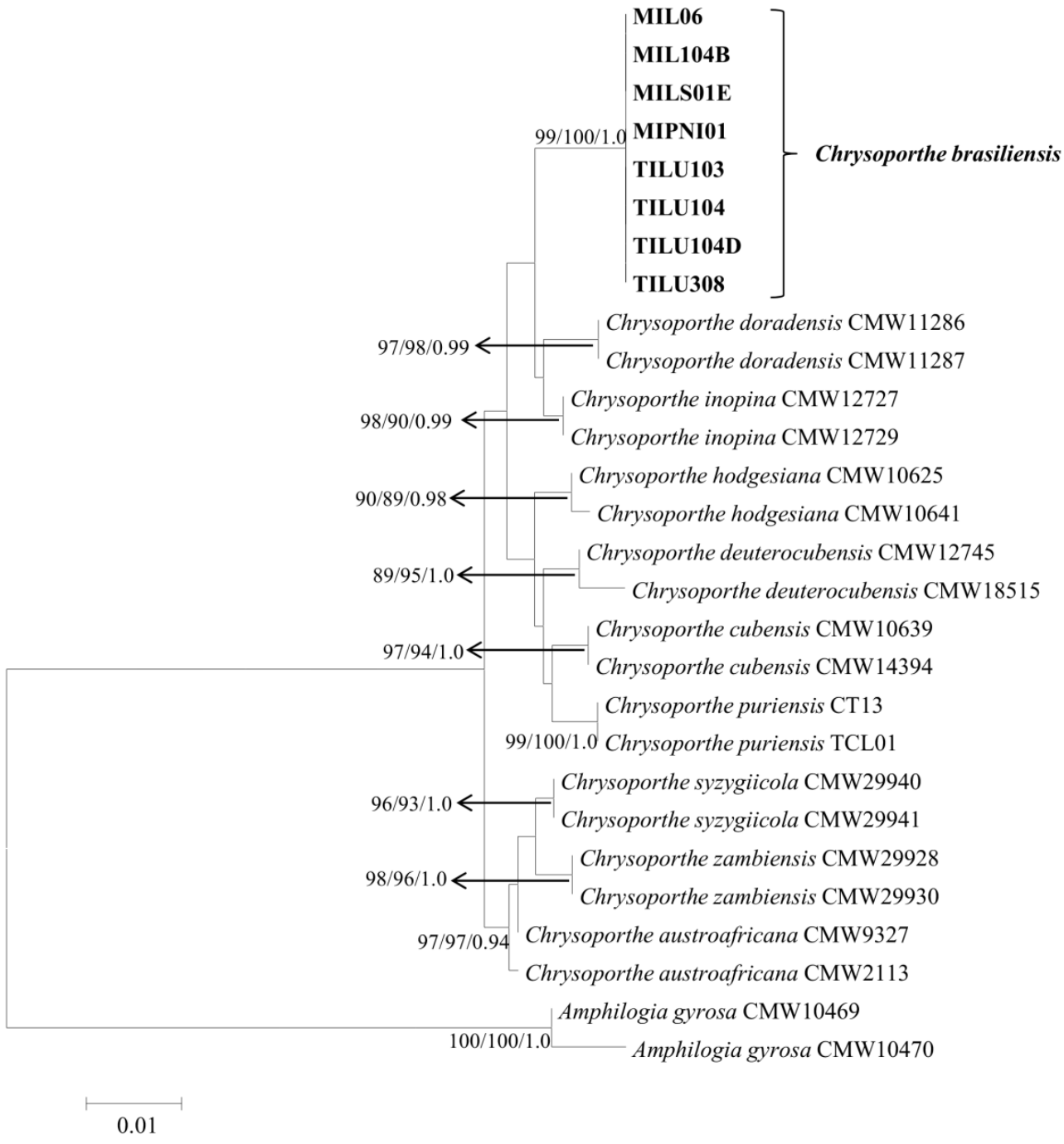

Figure 2 Phylogenetic tree of the combined BT1, BT2, and ITS regions obtained by the likelihood method. The bootstrap/posterior probability values are shown in the branches according to the maximum likelihood, maximum parsimony, and Bayesian inference

analyses. *Amphilogia gyrosa* was defined as the outgroup. The isolates of *Chrysoporthe brasiliensis* sp. nov. are highlighted in bold.

Table 2 Summary of polymorphic sites found in the IT region and β-tubulin gene sequences for the different *Chrysoporthe* species. Variations unique to *Chrysoporthe brasiliensis* are highlighted.

Species	Beta tubulin 2										Beta tubulin 1																ITS				
	27	66	85	95	97	145	201	289	298	319	411	413	424	425	431	432	446	467	471	511	574	577	601	628	727	749	1032	1069	1070
Chrysoporthe brasiliensis	C	g	**t**	C	g	c	**T**	t	c	**c**	C	c	c	**c**	g	g	a	**c**	c	**t**	c	g	c	t	**t**	**t**	a	c	**a**
Chrysoporthe doradensis	C	g	c	c	g	c	G	t	c	t	C	c	t	g	a	g	a	-	c	c	c	g	a	t	c	c	a	c	g
Chrysoporthe inopina	C	g	c	c	a	c	A	t	c	t	C	c	c	g	g	g	a	t	c	c	c	g	c	t	c	c	a	c	g
Chrysoporthe hodgesiana	C	a	c	c	g	c	A	c	c	t	c	c	c	g	g	g	g	-	c	c	c	g	c	t	c	c	a	c	g
Chrysoporthe deuterocubensis	C	g	c	c	g	c	A	t	t	t	c	c	c	g	g	g	a	-	c	c	c	g	c	t	c	c	g	c	g
Chrysoporthe cubensis	C	g	c	c	g	c	A	t	c	t	c	c	c	g	g	a	a	-	c	c	c	g	c	t	c	c	a	c	g
Chrysoporthe puriensis	T	g	c	t	g	c	A	t	c	t	t	t	c	g	g	g	a	-	t	c	c	g	c	t	c	c	a	c	g
Chrysoporthe syzygiicola	C	g	c	c	g	c	A	t	c	t	c	c	c	g	g	g	a	-	c	c	a	t	c	t	c	c	a	c	g
Chrysoporthe zambiensis	C	g	c	c	g	a	A	t	c	t	c	c	c	g	g	g	a	-	c	c	c	g	c	a	c	c	a	a	g
Chrysoporthe austroafricana	C	g	c	C	g	c	A	t	c	t	c	c	c	g	g	g	a	-	c	c	c	g	c	t	c	c	a	c	g

3.3. Taxonomy and morphology

Analysis of the DNA sequences showed that the isolates of the present study were of an unknown *Chrysoporthe* species. Morphologically, the characteristics found were similar to those of *Chrysoporthe* spp. (Table 3), with subtle differences, such as smaller conidiomata, conidiophores, and conidia. The isolates analyzed in the present study are described as a novel taxon.

Chrysoporthe brasiliensis, **G.A. Silva, M.E.S. Oliv., & M.A. Ferr., sp. nov.**

Etymology: The name "*brasiliensis*" is because the species in question was found in Southeast Brazil.

Ascostromata: not observed.

Conidiomata: The pycnidia are superficial to semi-immersed in the bark of branches and trunks of the host. They have a matte black colour, are mostly pulvinate, and may be conical or pyriform, with a base size of 57-(206.5)-372 µm. They have a single ostiolate neck measuring 75.2-(205.0)-354.1 µm. Most of them have a single locule, or rarely two, convoluted (Figure 3 A), measuring 27.5-(104.0)-212.5 µm in length by 23.5-(87.5)-170.5 µm in width. Its stromal tissue has a golden-brown colour and a globular texture closer to the base and an epidermoid texture close to the conidiogenous cells. The conidiophores are hyaline, with globular to rectangular basal cells and a total length of 6.0-(9.5)-12.0 µm. Occasionally, paraphyses are found between the conidiophores (Figure 3 B). The conidiogenous cells are hyaline, with phialidic conidiogenesis and a cylindrical shape with attenuated apices, flask-shaped, measuring 3.5-(5.5)-7.5 µm (Figure 3 C). The conidia are aseptate, hyaline, and oblong, with a wide variety of shapes—cylindrical, fusoid, oval, or ellipsoid (Figure 3 D), measuring 0.5-(1.0)-1.5 µm in width and 2.0-(2.5)-4.5 µm in length.

Culture characteristics: Colonies obtained in PDA medium have a mycelium with a fluffy appearance of white colour that becomes brownish orange as the colony ages. They grow best at 25 to 30 °C. When incubated in this temperature range for approximately 30 days, they sprout fruiting bodies with golden-yellow to orange sporulation, varying according to the isolate (Figure 3 E, F).

Substrate: Bark of branches and trunks of *Miconia ibaguensis*, Miconia *theaezans* and *Rhynchanthera grandiflora*

Distribution: Southeast Brazil: Minas Gerais and Rio de Janeiro States.

Material examined: Brazil, Minas Gerais State, Luminárias, *Rhynchanthera grandiflora*, collected in 2018, MA Ferreira, Holotype TILU 103 = UB24348 (branches with mature pycnidia), Ex-type culture TILU 103 = CCDCA11647

Others specimens examined: MIPNI01, Brazil, Rio de Janeiro State, Itatiaia National Park (22°29′45″S and 44°39′39″W), *Miconia ibaguensis*, MES Oliveira and MA Ferreira. MIL06, MIL 104B and MILS01E, Brazil, Minas Gerais State, Lavras, Quedas do Rio Bonito Ecological Park (21°19′44″S and 44°58′6″W), *Miconia theaezans*, collected in 2018 by GA Silva, MES Oliveira and MA Ferreira. TILU104, TILU 104D and TILU 308, Brazil, Minas Gerais State, Luminárias (21°19′44″S and 44°58′6″W), *Rhynchanthera grandiflora*, MA Ferreira, and GA Silva.

Table 3 Morphological characteristics found for species of the genus *Chrysoporthe* in different studies.

Species	Conidiomata		Conidial locules	Conidium			Conidiogenous cells length(µm)	Paraphyses	References
	Shape	Base width (µm)		Shape	Length(µm)	Width(µm)			
Chrysoporthe "brasiliensis"	Pulvinate to conic, pyriform	57-372	unilocular or multilocular	oblong, fusoid, cylindrical, oval	2.0-4.5	0.5-1.5	6.0-12.0	Present	This study
Chrysoporthe doradensis	Pyriform to pulvinate	100-290	multilocular	oblong, oval, cylindrical, alantoid	3-6.5	1.5-2.5	9.5-21.5	Absent	Gryzenhout et al., 2005; Soares et al., 2018;
Chrysoporthe inopina	Pyriform to pulvinate	70-710	unilocular or multilocular	Oblong	3.0-4	1.5-2.5	11-29.5	Absent	Gryzenhout et al., 2006
Chrysoporthe hodgesiana	Pulvinate to pyriform	145-635	unilocular or multilocular	Oblong	3-5.5	1.5-2.5	12-33.0	Absent	Gryzenhout et al., 2004
Chrysoporthe deuterocubensis	Pyriform, clavate to pulvinate	100-950	unilocular or multilocular	Oblong	3-5.0	1.5-2.5	12-24.5	Absent	Van der Merwe et al., 2010
Chrysoporthe cubensis	Pyriform to clavate	100-950	unilocular or multilocular	Oblong	2.5-5	1.5-3	10-26.5	Absent	Gryzenhout et al., 2004; Chen et al., 2010; Soares et al., 2018;
Chrysoporthe puriensis	Pyriform to pulvinate	95-470	unilocular or multilocular	oblong, fusoid to oval	3-6.5	1.5-2.5	9.0-17	Present	Oliveira et al., 2021
Chrysoporthe syzygiicola	Globose	250-500	unilocular	Oblong to oval	2.0-4	1-2.0	11-13.5	Absent	Chungu et al., 2010
Chrysoporthe zambiensis	Rostrate to globose	208-310	unilocular	Oblong	2.5-4	1-2.0	14.0-17.5	Absent	Chungu et al., 2010
Chrysoporthe austroafricana	Pyriform to clavate	80-120	unilocular or multilocular	Oblong to oval	3-3.5	1.5-2	7.5-28	Absent	Gryzenhout et al., 2004; Heath et al., 2006

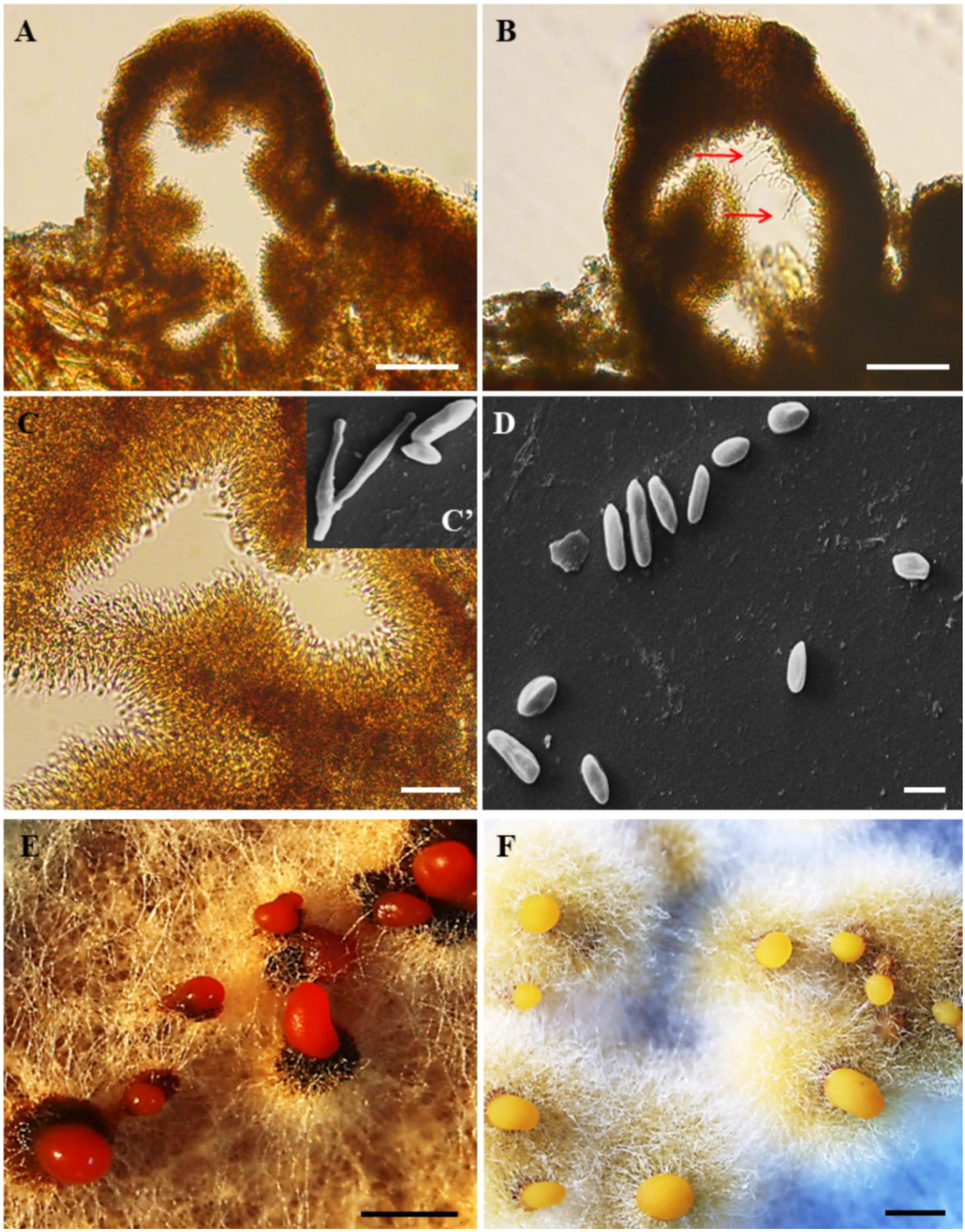

Figure 3 Pycnidia, conidiogenous cells, conidia, and culture of *Chrysoporthe brasiliensis.* (A) Presence of a single convoluted locule. (B) Locule with presence of paraphyses (indicated by red arrows). (C) Locule and conidigenous cells. (D)Variety of *C. brasiliensis* conidia shapes

seen by SEM. (E-F) Sporulation of colonies of isolates TILU104 and MIL06, respectively, in PDA medium after approximately 30 days of incubation. Scale bar: A and B= 50 µm; C= 20 µm; D= 2 µm; E and F= 500µm.

3.4. Pathogenicity

Lesions with discoloration in the xylem were observed in all six seedlings and in the all ten stems inoculated with the isolate fungus. The same was not observed in the control treatments. Analysis of variance revealed significant differences among treatments ($P \leq 0.05$). the experiment was repeated two times.

The Koch's postulates were fulfilled with the re-isolation of tissues from *M. theaezans* stem lesions and pycnidia formed in the lesion 90 days after inoculation on *R. grandiflora* seedlings.

4. Discussion

The present study shows that the fungal isolates obtained from *Miconia* spp. and *R. grandiflora* in the Southeast region of Brazil are a new species, not yet described, belonging to the genus *Chrysoporthe*.

Chrysoporthe brasiliensis isolates were grouped into a separate clade with high bootstrap and posterior probability support. Like other species of the genus, it has a superficial to semi-immersed conidioma with a matte-black colour. As in *C. puriensis*, its conidiomata occasionally have paraphyses [14], unlike those of other *Chrysoporthe* species.

The phylogenetic differences that separate *C. brasiliensis* as a new species may be

reflected by the smaller sizes of its conidiophores and conidiomata and by the marked variety of its conidia shapes, which may be cylindrical, fusoid, oval, or ellipsoid [9,12,13,14,16,44].

The symptoms and structures found in the field are characteristic of those caused by *Chrysoporthe*: desiccation and death of branches and trunk, cankers and pycnidia causing a blackening of the branch surface. Was demonstrate that *C. brasiliensis* causes mortality on *R. grandiflora* a species from which this pathogen was initially isolated. The potential of this pathogen to cause disease on plant in the family Melastomataceae is especially of concern as species in this family play a prominent role in floristic restoration of degraded areas. Species of Melastomataceae are considered pioneer species of great potential because they are heliophilous, with low soil fertility requirements and fast growth. They tolerate acidic and aluminum-rich soils, in which few plant species can grow. They produce many seeds with efficient zoochorous dispersal, characteristics that make them the first species to restore a degraded ecosystem, enabling the establishment of climax species [2,3,5,6].

Another important aspect arising from the report of a new species of *Chrysoporthe* in Brazilian territory is the fact that the genus includes pathogens that impact eucalyptus plantations, which can decrease the productivity of plantations which then result in financial losses to the Forestry industry [25,9,45]. Brazil is one of the largest producers of *Eucalyptus* spp. in the world, with a planted area of 9.3 million hectares [46].

Chrysoporthe species can host-jump between native and planted species of the order Myrtales [8,11,14]. The host jump process is related to the phylogenetic proximity between hosts and can be facilitated by ecological factors, such as physical proximity between species [47]. Host jump capacity is observed in *C. austroafricana*, which initially occurred only in native species but was later reported to be causing canker in exotic species of *Eucalyptus* planted in South Africa. It was also observed in *C. cubensis* and which is probably a pathogen

of native species of Melastomataceae in South America but later began to infect exotic *Eucalyptus* plantations [11,48].

Genetic studies of *C. cubensis* populations from different locations in Brazil indicate high genetic variability between these populations from different locations, showing that the pathogen is well established, occurring in hosts of the family Melastomataceae, in areas of native vegetation, and in hosts of the family Myrtaceae in forest plantations [49]. *Chrysoporthe puriensis*, despite being found in Brazil only in *Pleoroma* spp., caused canker in *Eucalyptus* hybrids in the pathogenicity tests conducted by Oliveira (2021) [14], also demonstrating its potential to cause disease in other tree species.

Future studies on genetic variability of *Chrysoporthe* spp. in Brazil may help in the understanding of the evolutionary relationship between pathogens and hosts, helping to protect native Melastomataceae stands and commercial *Eucalyptus* spp. plantations. Through morphological and molecular analyses, this study identified the pathogen found in Melastomataceae in Southeast Brazil as a new species which has been designated as *Chrysoporthe brasiliensis*.

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Abbreviations: ITS= internal transcribed spacer BT1= β-tubulin gene region 1. BT2= β-tubulin gene region 2. PDA= potato dextrose agar medium. d.a.i.= days after inoculation.

ARTIGO 2 - New reports of *Chrysoporthe doradensis* and *C. puriensis* on Melastomateceae in Brazil

Artigo preparado em concordância com as normas do periódico "Forests".

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ABSTRACT

The Melastomataceae family is one of the most abundant worldwide and in the Brazilian cerrado, with great environmental importance in the process of regeneration of degraded areas, especially in those previously occupied by pasture. In recent years, *Chrysoporthe doradensis* and two new species of *Chrysoporthe* were reported in Brazil causing canker, branch dieback and mortality in native Melastomataceae, which leads to the demand for further investigations and understanding of these pathossystems. During field surveys, typical *Chrysoporthe* signs and symptoms were found on *Rhynchanthera grandiflora* and *Miconia theaezans* in southern Minas Gerais. Through phylogenetic analysis of the BT1 and BT2 fragments of β-tubulin gene, morphological characterization of the isolates obtained and the pathogenicity tests, was possible to identify *C. doradensis* occurring and pathogenic to *R. grandiflora* and *C. puriensis* occurring and pathogenic to *R. grandiflora* and *M. theaezans*.

Keywords: Canker; *Rhynchanthera grandiflora*; *Miconia theaezans*; Phylogeny; Pathogenicity.

1. Introduction

Chrysoporthe spp. are known to causing canker in species of the families Myrtaceae, especially in *Eucalyptus* spp., and Melastomataceae. *Chrysoporthe* species have brown to black-fuscous fruiting bodies which are semi-immersed in the bark of the host causing lesions, branch dryness, typical cankers and even plant death [1-8].

Ten species of *Chrysoporthe* were described: *C. cubensis* (the first specie described and most common), *C. deuterocubensis*, *C. doradensis*, *C. inopina*, *C. hodgesiana C. zambiensis*, *C. syzygiicola*, *C. austroafricana* and more recently *C. puriensis* and *C. brasiliensis*. These species are very similar morphologically, being separated by subtle differences in spore size and optimal growth temperature [4]. The accurate identification is possible with molecular analysis using the ITS region and especially the β-tubulin gene [4,8,9].

Until 2018 only *C. cubensis* has reported in Brazil causing canker especially in *Eucalyptus* spp. and in other native Myrtaceae and Melastomataceae species. However, in 2018, Soares et al. [7] reported the occurrence of *C. doradensis* in *Eucalyptus* species and *Pleroma granulosum* in the states of Maranhão and Minas Gerais. In 2021, Oliveira et al. [8] described *C. puriensis* causing canker and death on *Pleroma* spp. in the Bahia, Minas Gerais, and Rio de Janeiro states. Recently, a new species was described causing canker, drying of branches and trunks and death of *Miconia* spp. and *Rhynchanthera grandiflora* (unpublished data).

The incidence of *Chrysoporthe* in species of the Melastomataceae becomes a concern, because this is one of the largest families of angiosperms in the world and one of most present in the Brazilian cerrado, having great importance in the secondary regeneration process in regions degraded especially by pasture [10].

Due to recent reports of new *Chrysoporthe* species occurring in native Melastomataceae and the high environmental importance of this family, the present study aimed to identify possible new occurrences of *Chrysoporthe* spp. in species of this family in southern Minas Gerais, Brazil.

2. Materials and methods

2.1. Disease symptoms, samples, and fungal isolates

Samples were obtained from stems and trunks of symptomatic plants with typical *Chrysoporthe* spp. signs. Thus, samples of *Rhynchanthera grandiflora* and *Miconia theaezans* were collected in the city of Luminárias - MG and in the Parque Ecológico Quedas do Rio Bonito in the city of Lavras, MG, respectively.

The samples were stored in paper bags, labelled, and sent to the Laboratório de Patologia Florestal – Universidade Federal de Lavras, to perform fungal isolations and subsequent molecular and morphological identification and proof of pathogenicity.

Fungal isolations were performed by monosporic culture. For this purpose, a single fruiting body was removed from each sample and suspended in water. The suspensions were transferred to Petri dishes containing potato dextrose agar (PDA) medium supplemented with rifamycin (100 mg/L). The Petri dishes were incubated at 28 °C for 24 hours until germination and then a single germinated spore was transferred to a new Petri dish containing PDA medium and supplemented with rifamycin, then incubated at 28 °C for seven days to store a pure culture of the fungus.

2.2. DNA extraction, PCR and sequencing

After obtaining the pure culture on Petri dishes, mycelial discs 5 mm in diameter were transferred to Erlenmeyer flasks containing 200 mL of liquid culture medium, composed of malt extract (20 g/L), yeast extract (2 g/L), and dextrose (5 g/L). The flasks were incubated at 28°C for 7 days for the fungus to grow. The mycelia were removed, filtered, and dried on filter paper and then ground in liquid nitrogen.

DNA extractions were performed using the Wizard® Genomic DNA purification Kit (Promega, Madson, WI, USA), following the manufacturer's recommendations The quality and concentration of the extracted fungal DNA was evaluated using agarose gel electrophoresis and Nanodrop® Thermo Scientific 2000c equipment, respectively.

Polymerase chain reactions (PCRs) were performed as described by Glass and Donaldson (1995) [11]. Each PCR had a final volume of 25 µL and was run in the Techne Prime G thermal cycler. The regions BT1 and BT2 of the β-tubulin gene were amplified, targeted by the primer pairs BT1a/BT1b and BT2a/BT2b, respectively [11]. PCR conditions were adjusted for each region.

The amplification products were evaluated by electrophoresis in 1% agarose gel with the addition of GelRed®. The bands were visualized using a transilluminator (Loccus®, L-Pix EX). Purification of the PCR product and sequencing were performed by Macrogen Company (Korea).

2.3. Phylogeny

The electropherograms generated were analysed, and, when necessary, the sequences were manually edited using SeqAssem software [12]. The sequences were compared with

other *Chrysoporthe* spp. sequences from the GenBank database. Sequences generated in this study were also deposited in NCBI/GenBank (Table 1). Multiple alignments of the sequences were performed using the online interface of MAFFT software [13].

Table 1 Species, acession number of cultures, host/substrate, location, and Genbank accession number of *Chrysoporthe* species and outgroup sequences included in this study.

Species	Culture accession number	Host/substrate	Location	GenBank accession number	
				BT1	BT2
Amphilogia gyrosa	CMW10469	*Ealeocarpus dentatus*	New Zealend	AF525707	AF525714
	CMW10470	*Ealeocarpus dentatus*	New Zealend	AF525708	AF525715
Chrysoporthe austroafricana	CMW2113	*Eucalyptus grandis*	South Africa	AF273067	AF273462
	CMW9327	*Pleroma granulosum*	South Africa	GQ290185	AF273455
Chrysoporthe brasiliensis	CCDCA 11647	*Rhynchanthera grandiflora*	Minas Gerais Brazil		
	MIL06	*Miconia theaezans*	Minas Gerais Brazil		
Chrysoporthe cubensis	CMW14394	*Eucalyptus grandis*	Cuba	AH015642*	
	CMW10639	*Eucalyptus grandis*	Colombia	AY263419	AY263420
Chrysoporthe deuterocubensis	CMW12745	*Pleroma urvilleanum*	Singapur	GQ290183	DQ368781
	CMW18515	*Melastoma malabathricum*	Indonesia	AH15467*	
Chrysoporthe doradensis	CMW11286	*Eucalyptus grandis*	Ecuador	AY214218	AY214254
	CE3	*Eucalyptus sp.*	Maranhão Brazil	KX639087	KX639104
	TILU502A1	***Rhynchanthera grandiflora***	**Minas Gerais Brazil**		
	TILU503A1	***Rhynchanthera grandiflora***	**Minas Gerais Brazil**		
Chrysoporthe hodgesiana	CMW10625	*Miconia theaezans*	Colombia	AH014900*	
	CMW10641	*Pleroma semidecandrum*	Colombia	AY692326	AY692325
Chrysoporthe inopina	CMW12729	*Pleroma lepidotum*	Colombia	AH015656*	
	CMW12727	*Pleroma*	Colombia	AH015657*	

		lepidotum			
Chrysoporthe puriensis	CML3738	*Pleroma granulosum*	Brazil	MN590041*	
	TCL01	*Pleroma candolleanum*	Brazil	MN590042*	
	MIL102D	***Miconia theaezans***	**Minas Gerais Brazil**		
	MIL103PA	***Miconia theaezans***	**Minas Gerais Brazil**		
	TILU101	***Rhynchanthera grandiflora***	**Minas Gerais Brazil**		
	TILU102	***Rhynchanthera grandiflora***	**Minas Gerais Brazil**		
Chrysoporthe syzygiicola	CMW29940	*Syzygium guineense*	Zambia	FJ805230	FJ805236
	CMW29941	*Syzygium guineense*	Zambia	FJ805231	FJ805237
Chrysoporthe zambiensis	CMW29930	*Eucalyptus grandis*	Zambia	FJ858711	FJ805235
	CMW29928	*Eucalyptus grandis*	Zambia	FJ858709	FJ805233

Isolates in bold are obtained in this study.

*Sections of BT1/BT2 combined of the gene beta-tubulin.

Phylogenetic analyses were performed using the maximum likelihood, maximum parsimony, and Bayesian inference methods.

The maximum likelihood analysis was performed in MEGA software version 6.0 [14]. For the combined tree of the BT1, and BT2 regions of the beta- tubulin gene the three-parameter Tamura evolutionary model was applied, as determined by MEGA [15], in addition to the discrete gamma distribution to model the evolutionary differences between sites. The confidence level of the branches was established using 1000 bootstrap replicates.

The maximum parsimony analysis was performed in the software PAUP* 4.0 [16], using only parsimony-informative characters, the analysis consisted of 100 replicates, using the random addition of sequences and subsequent tree bisection and reconnection branch swapping. The degree of branch consistency was calculated using 1000 bootstrap replicates.

The number of parsimony-informative characters, consistency index, retention index, and rescaled consistency index were also calculated [17,18].

Bayesian inference was performed using MrBayes software [19] with branches supported by the posterior probability values [20]. The Markov chain Monte Carlo algorithm [21] was generated from a random tree and executed with 10^7 generations. A total of 25% of the initial samples collected were discarded (burn-in), and the remaining samples were used to calculate the posterior probability values.

As the outgroup, in all analyses, sequences of *Amphilogia gyrosa* were used. The trees were analysed and edited in FigTree version 1.3.1 (http://tree.bio.ac.uk/software).

2.4. Morphology

For morphology, longitudinal anatomical sections of the fungal structures were made, and then slides were made with lactophenol as mounting medium. The size, shape, and colour of the pycnidia, conidia, and conidiophores were evaluated. Measurements were performed by visualizing 30 replicates of each structure and image capture was performed with the aid of a Nikon Eclipse E200 microscope equipped with an Infinity Analyze image capture system and Infinity 1 software. Fungal structures formed in culture medium were also observed under a Nikon SMZ 1500 stereoscope microscope.

2.5. Pathogenicity tests

To assess the pathogenicity of the isolates obtained, *R. grandiflora* seedlings and *M. theaezans* stems were artificially inoculated. Five *R. grandiflora* seedlings, approximately 12 months old, were inoculated with *C. doradensis*, five seedlings with *C. puriensis* and five

seedlings used as control. For pathogenicity tests on *M. theaezans*, ten stems with, approximately, 10 mm diameter and 100 mm long were inoculated with *C. puriensis* and ten were used as control. The design was entirely random. The experiments were repeated two times.

For the artificial inoculations, a 5mm diameter cylindrical cutter was used to remove the bark and expose the cambium. Subsequently, mycelium disks (seven days old on PDA medium) of the same diameter were placed in contact with the cambium. Disks containing only PDA medium were used as a control. The inoculated area was protected with plastic film for 15 days after inoculation (d.a.i.) to prevent desiccation. The seedlings of *R. grandiflora* were kept in greenhouse and the stems were kept in a humid chamber at 25º. Evaluation was performed after six weeks for seedlings and three six for stems; assessing the length of lesion with a tape measure. To compare the mean lesion lengths of the control and inoculated plants a T-test was used and values where $P \leq 0.05$ were considered as significant. After evaluation a re-isolation of lesion was performed to fulfil the Koch's postulates.

3. Results

3.1. Symptomatology and isolates obtained

A total of 37 isolates were obtained in the present study (Table 2), of which 28 was from *R. grandiflora* trees (Figure 1 A) in Luminárias City, Minas Gerais and nine isolates were from trees of *M. theaezans* (Figure 1 B) in the Quedas do Rio Bonito Ecological Park in the city of Lavras, Minas Gerais. All isolates were obtained from symptomatic trees located close to waterways.

In both hosts it was possible to observe the presence of cankers on the woody tissue (Figure 1 C and D) and the presence of dark pycnidia on the bark, similar to the typical structures of *Chrysoporthe* spp. (Figure 1 E). In *M. theaezans* was observed, in addition to canker, many dry branches. In *R. grandiflora*, in most of the times, the branches and trunks were all dry with defoliation, and death of individuals.

Table 2 Detailed information for isolates obtained in this study

Samples	City/State	Geographic coordinates	Species	Host	Number of isolates
TILU105, TILU502A1*, TILU502A2, TILU502B1, TILU502B2, TILU502C3, TILU502C4, TILU503A1*, TILU503A3, TILU503B1, TILU503B4, TILU503C3, TILU503C4, TILU504A2, TILU504A3, TILU504B2, TILU504C2, TILU504C3	Luminárias/Minas Gerais	21°32’14”S 44°49’34”W	*C. doradensis*	*Rhynchanthera grandiflora*	18
TILU101*, TILU102*, TILU106, TILU107, TILU111, TILU304, TILU306, TILU307, TILU309, TILU310	Luminárias/Minas Gerais	21º19’45”S 44º58’18”	*C. puriensis*	*Rhynchanthera grandiflora*	10
MIL102D*, MIL102E, MIL102F, MIL102G, MIL102H, MIL103PA*, MIL103PB, MIL103PB, MIL103PC, MIL103PD	Parque Ecológico Quedas do Rio Bonito – Lavras/Minas Gerais	21° 31' 34" 44° 52' 53"	*C. puriensis*	*Miconia theaezans*	9

* Isolates selected for Phylogenetic tree

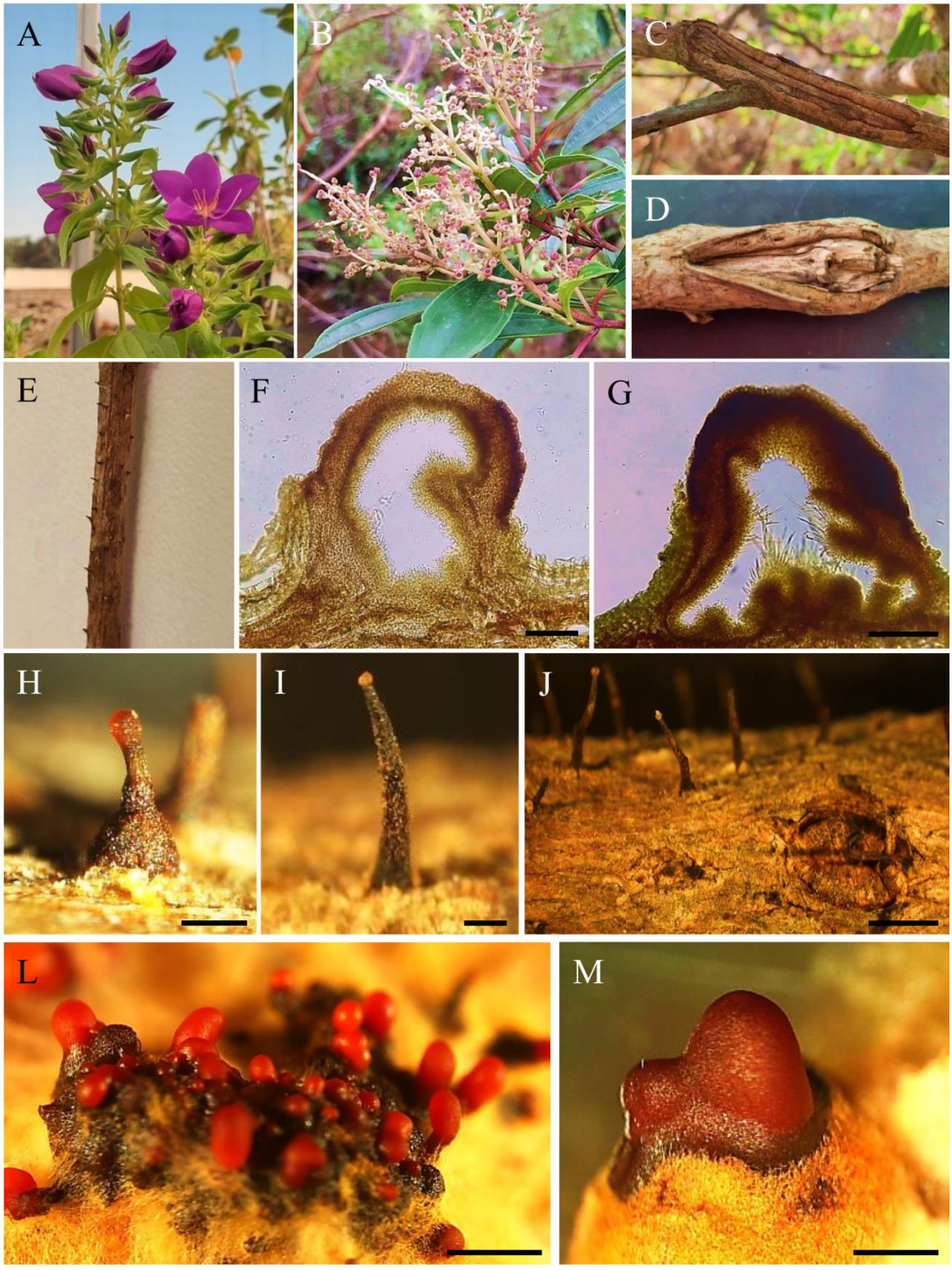

Figure 1 A) *Rhyncathera. grandiflora*. B) *Miconia. theaezans*. C) Canker symptoms in branches of *M. theaezans*. D)Canker symptoms in *R. grandiflora.* E) Signs of *Chrysoporthe* in *R. grandiflora.* F) Conidiomata of *Chrysoporthe doradensis* in transversal section G) Conidiomata of *C. doradensis* in transversal section. Red arrows indicate paraphyses. H and

Pycnidia of *C. doradensis* and *C. puriensis*, respectly, in *R. grandiflora*. J) Pycnidia of *C puriensis* in *M. theaezans*. L) Anamorfs of *C. puriensis* produced on PDA medium. M) Anamorfs of *C. doradensis* produced on PDA. Scale bar: F and G= 100 µm; H, I and J= 200 µm; L and M= 500 µm.

3.2. Phylogeny

PCR amplification result in fragments of 345bp for BT1 (BT1a/BT1b primers) and 337bp for BT2 (BT2a/BT2b primers) before alignment. Aligned sequences of the combined BT2 and BT1 regions of beta- tubulin gene result in 682 characters, of which 592 were constant, four were non informative, and 86 were informative. Values of 0.85 for the consistency index (CI), 0.15 for homoplasy index (HI), 0.92 for the retention index (RI) and 0.79 for the rescaled consistency index (CI) were obtained.

The maximum likelihood, maximum parsimony and Bayesian inference trees presented the same topology, with well-supported branches, with high values of bootstrap and posterior probability. In all analyses performed, *Chrysoporthe* species separated from each other in distinct clades with high bootstrap and posterior probability values (more than 80% bootstrap and more than 0.8 posterior probability) (Figure 2).

Among the 28 isolates obtained from *R. grandiflora*, 18 clustered with the *C. doradensis* clade and ten grouped with *C. puriensis*. The nine isolates of *M. theaezans* also clustered with the *C. puriensis* clade. The clustering of all isolates was well supported, with more than 90% bootstrap and 0.9 for posterior probability.

Two isolates from each host and each clade were selected for the phylogenetic tree, because they had similar sequences (Table 2; Figure 2).

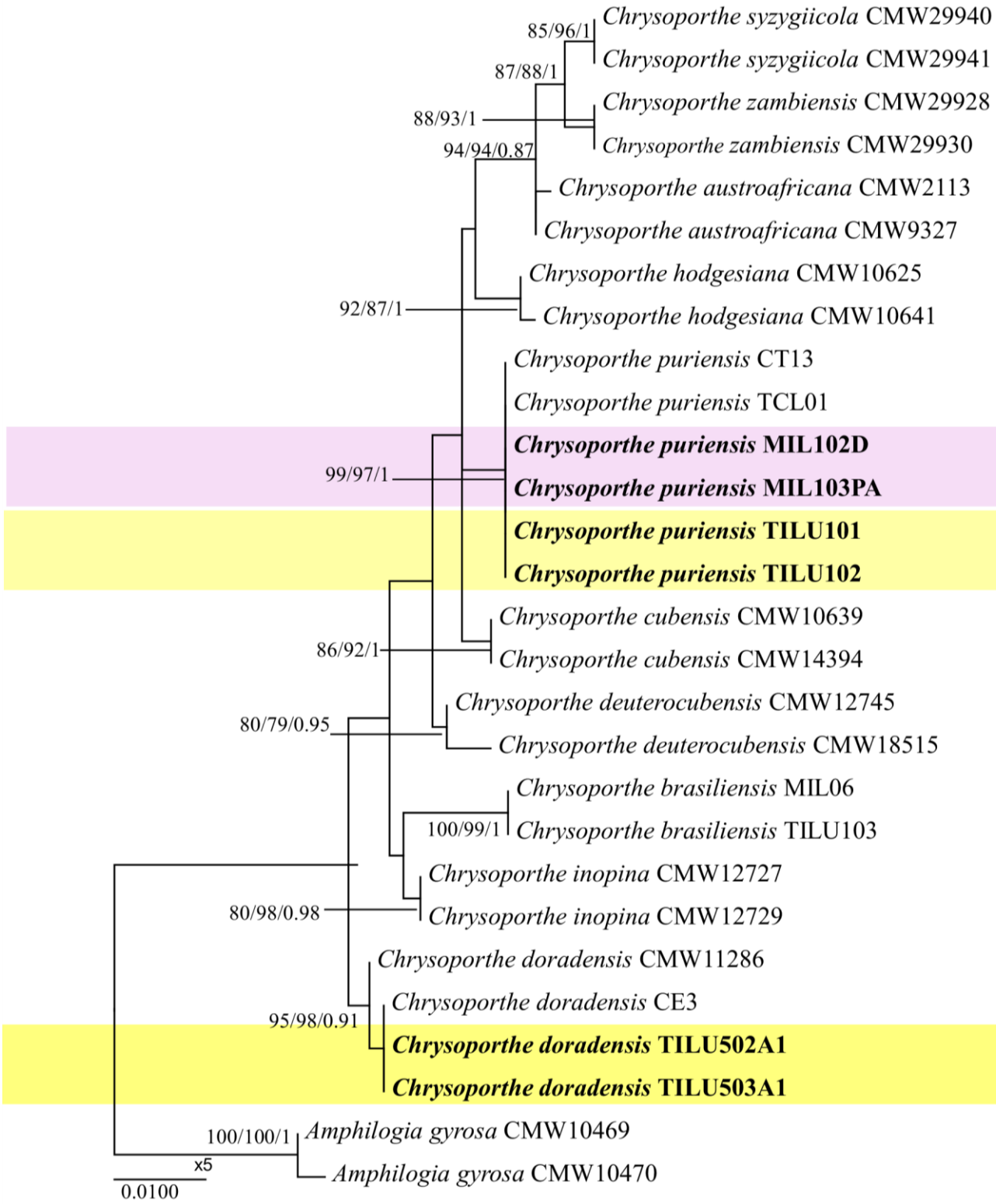

Figure 2 Phylogenetic tree of the combined BT1 and BT2 of β-tubulin gene, obtained by the likelihood method. The bootstrap/posterior probability values are shown in the branches

according to the maximum likelihood, maximum parsimony, and Bayesian inference analyses. *Amphilogia gyrosa* was defined as the outgroup. The isolates in bold are obtained in this study, highlighted in violet are from *Miconia theaezans*, and in yellow from *Rhyncanthera grandiflora*.

3.3. Morphology

According to the results obtained in the phylogenetic analyses, the isolates TILU502A1, TILU101 and MIL103PA were selected for morphological characterization. In both host, only the anamorph was found. The isolate TILU502A1 showed typical features of *C. doradensis*: conidiomata superficial to slightly immersed, pyriform to pulvinate exuding conidia in luteal drops [3] with yellowish-brown color (Figure 1 H) according to Soares et al., 2018 [7]. Conidiomata measuring about 135- 490 μm long x 247-386,5 μm wide (Figure 1 F), conidiogenous cells cylindrical to flask-shaped with attenuated apices meausuring 1.5-4.5 μm wide and conidia 3.5–4.0-μm long x 1.5–2.0-μm wide, non-septate, oblong, ovoid, cylindrical and ocassionally alantoid. Cultures sporulate on PDA medium and produce few anamorphs (Fig. 1 M) and no teleomorphs.

Isolates TILU101 and MIL103PA had the typical morphology of *C. puriensis*: conidiomata pyriform to pulvinate, usually with a single neck with conidial masses exuding as bright luteous droplets (Figure 1 I, J) [8]. In some conidiomas are found paraphyses (Figure 1 G) occurring between conidiophores. Conidiogenous cells cylindrical to flask-shaped with attenuated apices. Conidia hyaline, aseptate, oblong, fusoid to oval. The measurements obtained for the TILU 101 isolate were 175-523 μm long x 190-390 μm wide for conidiomata, 5-12 μm long for conidiogenous cells and 2.5-5 μm long x 1.5-2.5 μm wide for conidia. To MIL103PA isolate, the measurements were 350-450 μm long x 145-335 μm wide for

conidiomata, 3.5-6.5 μm long for conidiogenous cells and 4.5-6 μm long x 1.5-2 μm wide for conidia. *C. puriensis* from both hosts produced anamorphs on PDA medium (Figure 1 L).

3.4. Pathogenicity tests

In the evaluation of pathogenicity tests, vertical necrosis on the outside of the wood were observed all *M. theaezans* stems artificially inoculated with *C. puriensis* (Figure 3 B) and in all *R. grandiflora* seedlings inoculated with *C. puriensis* and *C. doradensis* (Figure 3 D, E), which was not observed in the control (Figure 3 A, C). Analysis of variance revealed significant differences among treatments ($P \leq 0.05$). At the site of the lesion caused by *C. puriensis* on *R. grandiflora* it was possible to observe the presence of pycnidia (Figure 3 F). The Koch's postulates were fulfilled with the re-isolation of the lesion tissues or pycnidia.

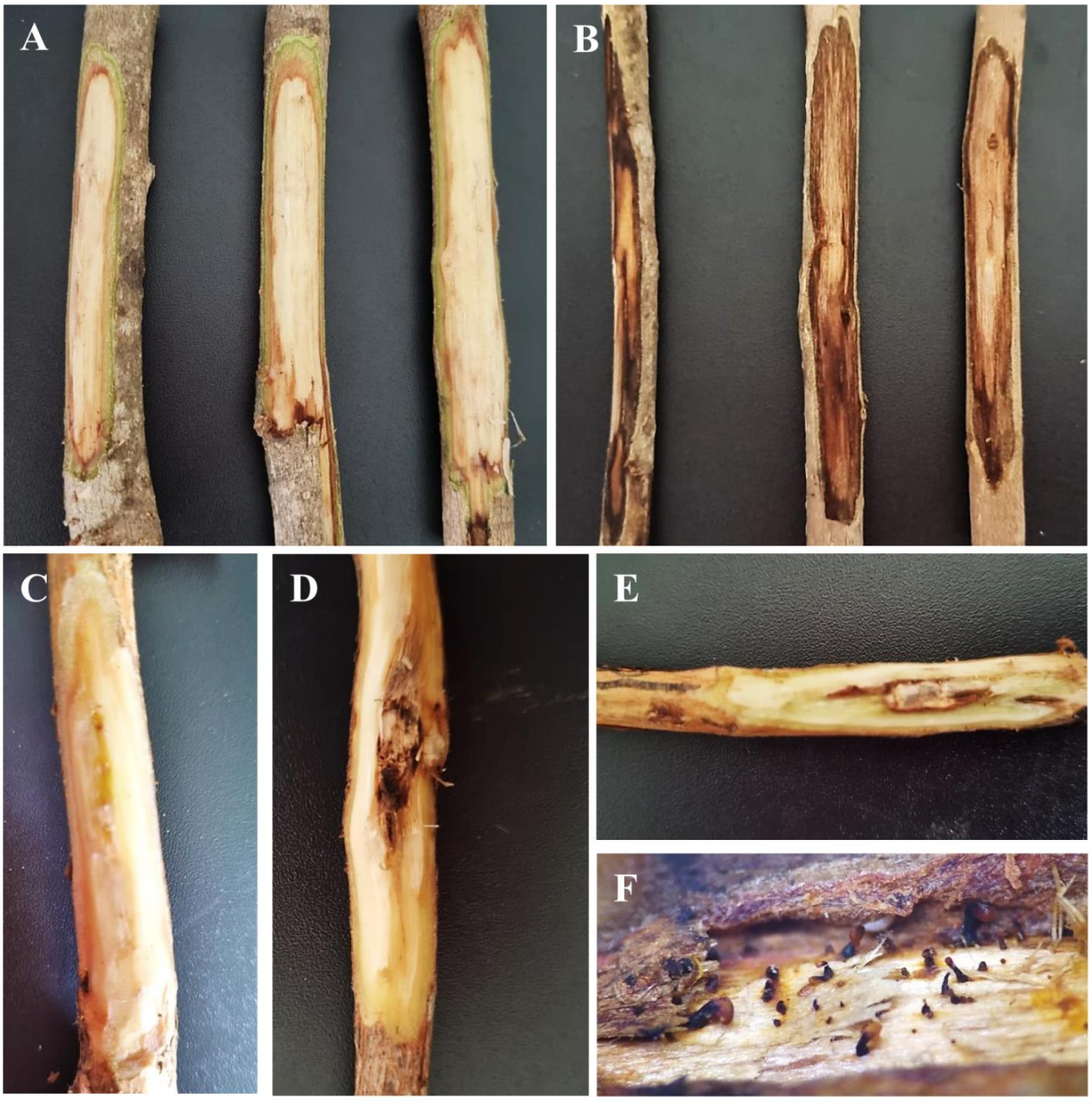

Figure 3 Pathogenicity test. A) Stems control of *Miconia theaezans*. B) Stems of *M. theaezans* inoculated with *Chrysoporthe puriensis* C) Control seedling of *Rhincathera grandiflora*. D and E) Seedlings of R. grandiflora inoculated with *C. doradensis* and *C. puriensis*, respectly. F) Pycnidia on lesions caused by *C. puriensis* on *R. grandiflora.*

4. Discussion

The isolates obtained from *R. grandiflora* in the city of Luminárias-MG were identified as *C. doradensis and C. cubensis*. In *M. theaezans*, *C. puriensis* was identified. Phylogenetic characterization of the isolates was well supported in the analyses of maximum likelihood, maximum parsimony and Bayesian inference with high bootstrap and posterior probability values. Also, we noted that the Brazilian isolates of *C. doradensis* grouped in a subclade distinct from the Ecuador isolate, evidencing the need for future studies with more isolates from distinct locations to understand the genetic variability and possible origin of the pathogen.

The morphology results obtained for *C. doradensis* were compatible with those described in previous papers in terms of the variety of conidia shapes (ranging from oblong to cylindrical and rarely allantoids), shape of conidiogenous cells, shape and form of sporulation of conidiomata [3,7]. The coloration of the conidia mass was yellowish-brown, the same observed by Soares et al. (2018) [7], similarly to other *Chrysoporthe* species. Was observed that *C. doradensis* isolated from *R. grandiflora* had larger conidiogenous cell size (1.5-4.5 μm) and conidiomata size (135- 490 μm long x 247-386.5 μm wide).

The morphology of *C. puriensis* was the same as in the description of the species [8] for both hosts. Only an increase in conidiogenous cell length was observed: 5-12 μm in *R. grandiflora* and 3.5-6.5 μm in *M. theaezans*. It possible to observe paraphyses in some conidiomata.

Miconia theaezans was previously reported as a host species for *C. cubensis* in Colombia, and symptoms described were like those found in the present study: branch die-back and cankers on branches, trunks or on base of the tree, resulting in the death of tree parts or all trees [22].

The severity of the symptoms caused by *C. doradensis* and *C.puriensis* in Melastomataceae species in Brazil is concern because the family is, environmentally, important. Melastomataceae species can survive and reproduce in hostile environments, with low soil fertility and high soil degradation, serving as pioneer species during the process of secondary succession, improving environmental conditions and supporting the establishment of other species [10,22,23].

The present study is the first report of both *C. doradensis* and *C. puriensis* on *M. thaezans* and *R. grandiflora*, two native Melastomataceae in Brazil, which is pathogenic and may lead to the death hosts.

Environmental conditions of high temperature and humidity are prevalent in Brazil and favorable to the development of *Chrysoporthe* species [4]. This associated with the great diversity of Melastomataceae species encourage more studies of these pathosystems.

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