Tropical Plant Pathology, vol. 39(4):326-330, 2014

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SHORT COMMUNICATION

Colletotrichum theobromicola causes defoliation, stem girdling and death of mini-cuttings of eucalyptus in Brazil

Ana L. Rodrigues, Danilo B. Pinho, Daniela O. Lisboa, Robson J. Nascimento, Olinto L. Pereira, Acelino C. Alfenas & Gleiber Q. Furtado

Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil

Author for correspondence: Gleiber Q. Furtado, e-mail: gfurtado@ufv.br

ABSTRACT

Eucalyptus plantations cover approximately 5.1 million hectares of Brazil, an area that will likely increase given the demand for natural products from planted forests. In recent years, anthracnose diseases have been frequently found on eucalyptus in Brazilian nurseries. In 2012, rooted mini-cuttings of clones of *E. urophylla* x *E. grandis* ("urograndis") exhibiting leaf spot and stem girdling symptoms were collected from nurseries in the Brazilian states of Pará and Minas Gerais, and cultures of *Colletotrichum* were obtained from the lesions. The isolates were initially identified to species of the *C. gloeosporioides* species complex, according to searches of the Q-bank Fungi database with the DNA sequences of their internal transcribed spacer (ITS) regions. Subsequent phylogenetic analysis of the β-tubulin (TUB2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) regions, combined with morphological characterization, allowed us to conclude that the fungus belongs to *C. theobromicola.* Conidial suspension sprayed on "urograndis" clone plants induced similar symptoms as those found under natural conditions. Re-isolation of the fungus from symptomatic plants fulfilled the Koch´s postulate. To our knowledge, this is the first report of *C. theobromicola* in Brazil.
Key words: *Colletotrichum gloeosporioides*, anthracnose, forest pathology, tropical fungi.

Approximately 5.1 million hectares of Brazil are planted with eucalyptus. Given the increasing demand for forest products and the pressure to reduce the use of wood and wood products sourced from native forests, this acreage is likely to expand in the near future (ABRAF, 2013). The expansion of planted areas represents a risk for the appearance of new diseases. Among the diseases affecting eucalyptus plantations, anthracnose is especially harmful in nurseries due to high susceptibility of some eucalyptus genotypes and the environment favorable for the disease (Alfenas et al., 2009).

In nurseries, clonal hedges and plantations, anthracnose diseases occur at phenological stages A and B (Ferreira & Milani, 2002). The signs of the disease include the appearance of often-interconnected ellipsoidal lesions on shoots and cankers (Demuner & Ferreira, 1994). Leaf spots are circular, light brown to red in color and can lead to defoliation. Major damage has been observed in susceptible clones where dieback of mini-stumps is observed. During favorable environmental conditions, the lesions expand and can lead to stem girdling and plant death (Figure 1). Pink-cream spore masses are commonly observed on the lesions (Alfenas et al., 2009). The cause of the disease has been attributed to *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc (Ferreira & Milani, 2002; Alfenas et al., 2009; Mendes & Urben, 2013). However, the identification to species was based only on morphology, whereas an accurate identification of a *Colletotrichum* species should combine both morphological and molecular approaches (Hyde et al., 2010; Cai et al., 2011; Weir et al., 2012; Cannon et al., 2012).

Recently, several cryptic species of *C. gloeosporioides* sensu lato have been proposed within the *C. gloeosporioides* complex based on the analysis of DNA sequence data (Phoulivong et al., 2010; Cannon et al., 2012; Weir et al., 2012). The Q-bank Fungi database provides data of strains that are regarded as type material or reference strains of *Colletotrichum* spp. **(**Raak & Groenewald, 2013). The objective of this study was to determine the cause of a leaf spot disease leading to defoliation and death of eucalyptus mini-cuttings in nurseries based on DNA sequencing and morphological traits.

In 2012, plants of hybrid clones of *Eucalyptus urophylla* x *E. grandis* exhibiting leaf spots and stem girdling were collected from nurseries in the Brazilian states of Pará and Minas Gerais. Fungal structures were removed from symptomatic plants, mounted in drops of lactic acid on microscope slides and observed under a light microscope (FC Motic BA 210). The conidial sizes were determined based on 30 measurements.

Three isolates were obtained from single-spore suspensions and grown on potato-dextrose-agar (PDA) at 25ºC; the resulting cultures were then deposited at the Coleção de Culturas de Fungos Fitopatogênicos "Prof. Maria Menezes", Universidade Federal Rural de Pernambuco (UFRPE), Brazil (codes CMM 3559, CMM 3561 and

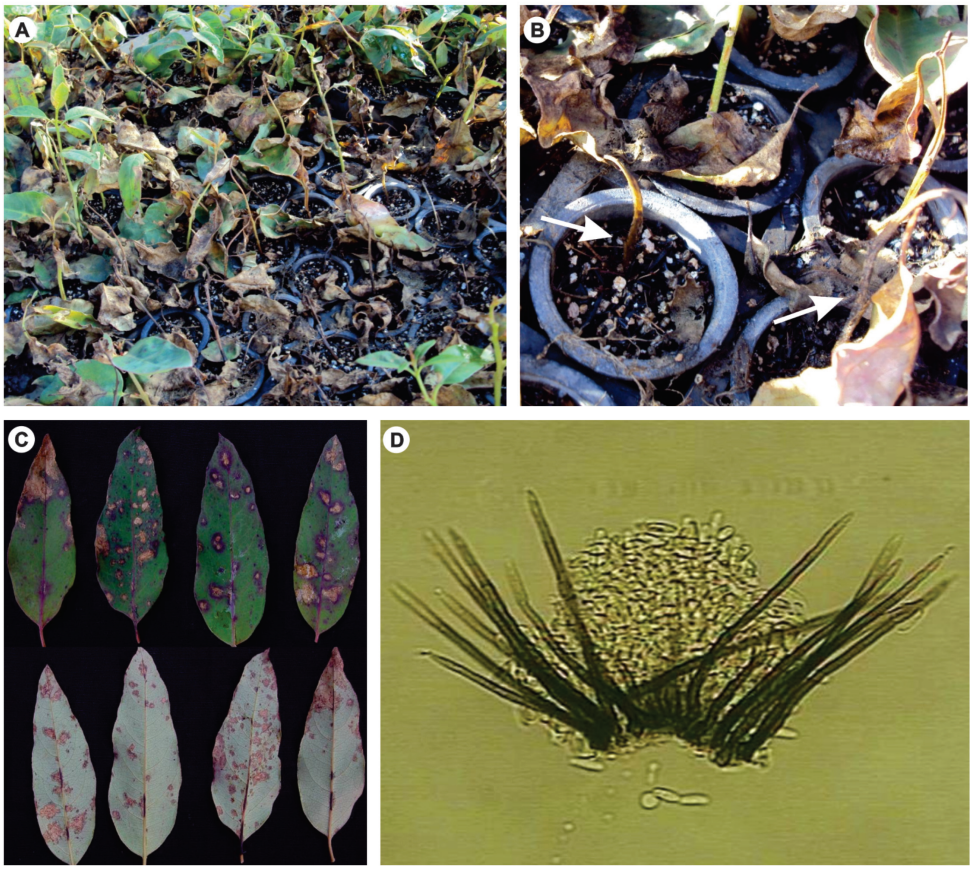

FIGURE 1 - Anthracnose in mini-cuttings of eucalyptus "urograndis" (*Eucalyptus grandis* x *Eucalyptus urophylla*) caused by *Colletotrichum theobromicola*. **A.** Mini-cuttings showing leaf spots and defoliation; **B.** stem girdling; **C.** spots on the abaxial and adaxial side of the leaf; **D.** acervulus of *C. theobromicola*.

CMM 3565). Total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) following the protocol described by Pinho et al. (2012).

Target regions of the internal transcribed spacer regions 1 and 2, including the 5.8S rRNA gene (ITS), β-tubulin (βt) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were amplified using primers ITS1 and ITS4 for ITS (White et al., 1990); Bt2a and Bt2b for partial βt (Glass & Donaldson, 1995) and GDF and GDR for partial GAPDH (Templeton et al., 1992).

PCR reactions included the following ingredients in each 25 µL reaction: 12.5 µL of Dream Taq TM PCR Master Mix 2X (MBI Fermentas); 1 µL of 10 µM of each forward and reverse primer, which were synthesized by Invitrogen; 1 µL of dimethyl sulfoxide (DMSO, Sigma–Aldrich); 5 µL of 100× (10 mg/mL) bovine serum albumin (BSA, Sigma–Aldrich); 2 µL of genomic DNA (25 ng/µL); and nuclease-free water to complete the total volume. PCR cycle parameters were the same as described by Weir et al. (2012). PCR products were purified and sequenced by Macrogen Inc., South Korea.

The nucleotide sequences were edited with BioEdit software (Hall, 2012). All sequences were manually checked, and nucleotides with ambiguous positions were clarified using the sequences from both primer directions. New sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov). Sequences for ITS, βt and GAPDH from the additional species were retrieved from GenBank (Table 1).

Consensus sequences of the ITS, βt and GAPDH regions were compared against the Q-bank Fungi database. In addition, DNA sequences were selected from Weir et al. (2012) to confirm the identity of *Colletotrichum*. The closest DNA sequence matches were aligned using MUSCLE (Edgar, 2004), built in MEGA v. 5 software (Tamura et al., 2011). The alignments were checked manually, and adjustments were made where needed. All the aligned regions within the dataset were included in the analysis. The resulting alignment and tree were deposited into TreeBASE (http://www.treebase.org; accession number: S14499).

Bayesian inference (BI) analysis employing a Markov Chain Monte Carlo method was performed with all sequences, first with each gene/locus separately and then with the concatenated sequences (ITS, βt and GAPDH). The SYM+I model of evolution was used for ITS, the HKY+G model for βt and the HKY+I model for GAPDH. The phylogenetic analysis of the concatenated alignment was performed using the CIPRES webportal (Miller et al., 2010) following the procedures described elsewhere (Pinho et al., 2012).

To confirm pathogenicity, a suspension of 10^5 conidia/mL was sprayed on healthy plants of a hybrid "urograndis" clone (CPC 011) of *Eucalyptus urophylla* x *E.*

grandis. Five 3-month old plants were inoculated with the conidial suspension, and five control plants were sprayed with autoclaved distilled water. The plants were incubated in a moist chamber for 24 h at 24ºC and then moved onto a 25ºC growth chamber where they were monitored daily until symptoms appearance.

Four days after inoculation, the plants showed irregular dark-brown leaf spots with dark brown coloration. The spots became necrotic, and leaves appeared dry. Fungal structures appeared in the center of the spot around one week later. The control plants showed no symptoms. *Colletotrichum* sp. was successfully re-isolated from the symptomatic plants.

TABLE 1 - GenBank accession numbers of the *Colletotrichum/Glomerella* DNA sequences used in the phylogenetic analysis.

Species	Isolate	ITS[1]	GAPDH[2]	TUB2[3]
C. aenigma	ICMP 18608	JX010244	JX010044	JX010389
C. aenigma	ICMP 18686	JX010243	JX009913	JX010390
C. aeschynomenes	ICMP 17673	JX010176	JX009930	JX010392
C. alatae	ICMP 17919	JX010190	JX009990	JX010383
C. alatae	ICMP 18122	JX010191	JX010011	JX010449
C. alienum	ICMP 12071	JX010251	JX010028	JX010411
C. aotearoa	ICMP 18537	JX010205	JX010005	JX010420
C. aotearoa	ICMP 18535	JX010201	JX009968	JX010423
C. asianum	ICMP 18696	JX010192	JX009915	JX010384
C. asianum	ICMP 18580	FJ972612	JX010053	JX010406
C. boninense	ICMP 17904	JX010292	JX009905	JQ005588
C. clidemiae	ICMP 18706	JX010274	JX009909	JX010439
C. clidemiae	ICMP 18658	JX010265	JX009989	JX010438
C. cordylinicola	ICMP 18579	JX010226	JX009975	JX010440
C. fructicola	ICMP 18581	JX010165	JX010033	JX010405
C. fructicola	ICMP17921	JX010181	JX009923	JX010400
C. gloeosporioides	ICMP 17821	JX010152	JX010056	JX010445
C. gloeosporioides	ICMP 19121	JX010148	JX010054	-
C. horii	ICMP 10492	GQ329690	GQ329681	JX010450
C. horii	ICMP 17968	JX010212	GQ329682	JX010378
C. kahawae	ICMP 17922	JX010238	JX010042	JX010432
C. kahawae	ICMP 17816	JX010231	JX010012	JX010444
C. musae	ICMP 19119	JX010146	JX010050	HQ596280
C. musae	ICMP 17817	JX010142	JX010015	JX010395
C. nupharicola	CMP 17938	JX010189	JX009936	JX010397
C. nupharicola	ICMP 18187	JX010187	JX009972	JX010398
C. psidii	ICMP 19120	JX010219	JX009967	JX010443
C. queenslandicum	ICMP 17921	JX010185	JX010036	JX010412
C. queenslandicum	ICMP 1778	JX010276	JX009934	JX010414
C. salsolae	ICMP 19051	JX010242	JX009916	JX010403
C. salsolae	ICMP 18693	JX010241	JX009917	-
C. siamense	ICMP 18578	JX010171	JX009924	JX010404
C. siamense	ICMP 18642	JX010278	JX010019	JX010410
C. theobromicola	ICMP 17957	JX010289	JX009962	JX010380
C. theobromicola	ICMP 17927	JX010286	JX010024	JX010373
C. theobromicola	ICMP 18649	JX010294	JX010006	JX010447
C. theobromicola	**CMM 3559**	**KF768556**	**KF768562**	**KF768559**
C. theobromicola	**CMM 3561**	**KF768555**	**KF768561**	**KF768558**
C. theobromicola	**CMM 3565**	**KF768554**	**KF768560**	**KF768557**
C. ti	ICMP 5285	JX010267	JX009910	JX010441
C. ti	ICMP 4832	JX010269	JX009952	JX010442
C. tropicale	ICMP 18653	JX010264	JX010007	JX010407
C. tropicale	ICMP 18672	JX010275	JX010020	JX010396
C. xanthorrhoeae	ICMP 17903	JX010261	JX009927	JX010448
C. xanthorrhoeae	ICMP 17820	JX010260	JX010008	-
G. cingulata f. sp. *camelliae*	ICMP 10643	JX010224	JX009908	JX010436
G. cingulata f. sp. *camelliae*	ICMP 18542	JX010223	JX009994	JX010429

[1]ITS: internal transcribed spacer regions; [2]GAPDH: glyceraldehyde-3-phosphate dehydrogenase; [3]TUB2: β-tubulin. The isolates used in this study are highlighted in bold. CMM stands for "Coleção de culturas de fungos fitopatogênicos Prof. Maria Menezes".

The isolates grown on PDA medium produced colonies with white to gray mycelial coloration that after 2 to 3 days became black, with orange spore masses. Conidia were hyaline, straight, subcylindrical to clavate, 10−17 × 4−6 µm in size and often had broadly rounded ends. These isolates were morphologically typical of the *C. gloeosporioides* species complex (Cannon et al., 2008; Rojas et al., 2010; Weir et al., 2012).

As differences between *Colletotrichum* spp. are slight and the ranges of conidial size overlap, morphology alone cannot discriminate species in the *C. gloesporioides* complex (Phoulivong et al., 2010; Weir et al., 2012). Weir et al. (2012) evaluated seven protein coding genes and recommend two genes (βt and GAPDH), in addition to ITS, for distinguishing members of the *C. gloeosporioides* species complex based on DNA sequence analysis. Searches on the Q-bank Fungi database using the ITS, βt and GAPDH gene sequences for our isolates retrieved *Colletotrichum theobromicola* Delacr. with 100, 100 and 99% identity, respectively. The resulting tree (Figure 2) supported this finding.

Colletotrichum theobromicola is one of 22 described species of the *C. gloeosporioides* species complex (Rojas et al., 2010; Weir et al., 2012). This fungus was first described in association with *Theobroma cacao* (Rojas et al., 2010),

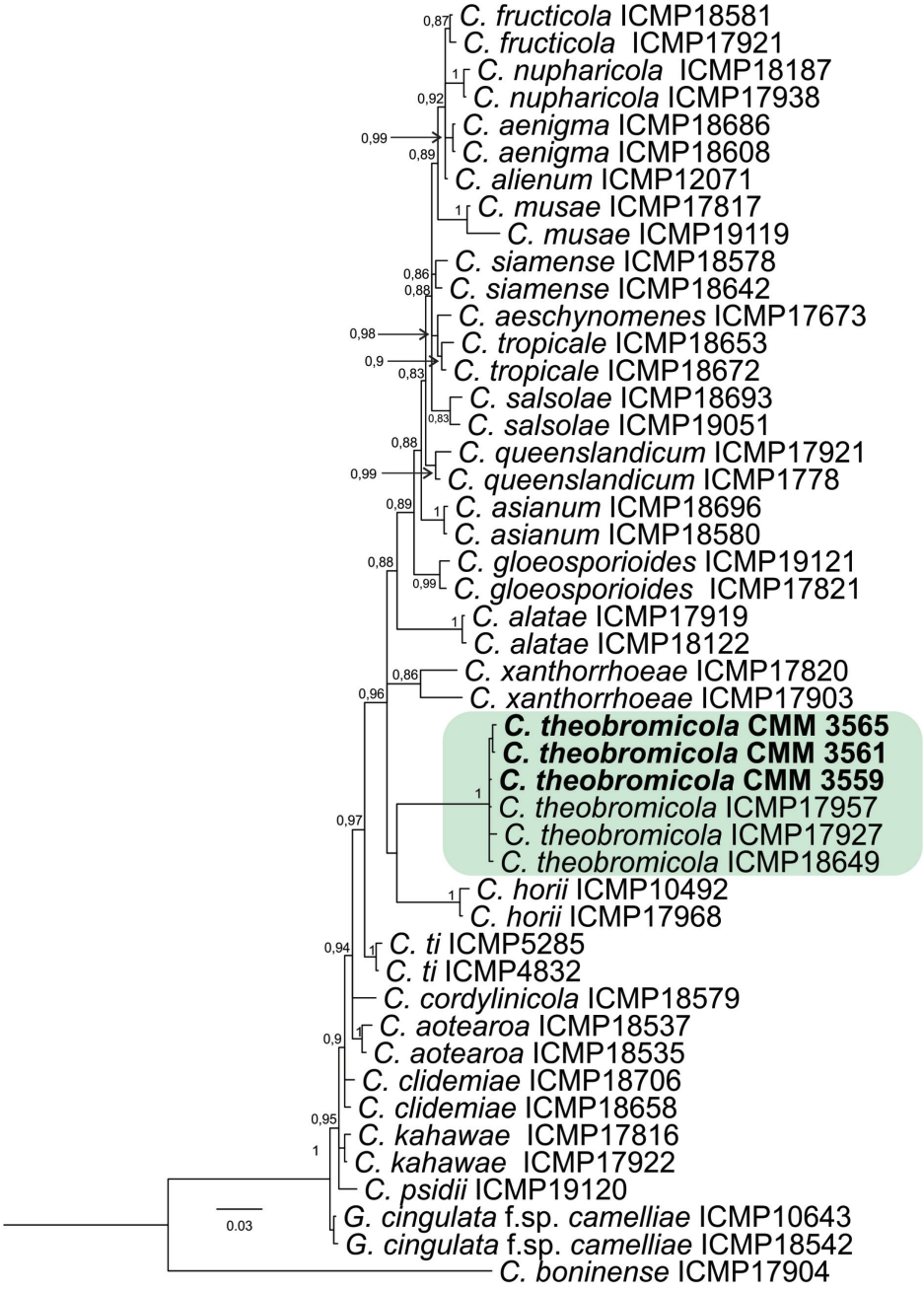

FIGURE 2 - Multi-locus phylogenetic tree inferred from Bayesian analysis using the ITS, β-tubulin and GAPDH regions. Bayesian posterior probability values >0.7 are indicated above the nodes. The isolates used in this study are highlighted in bold. The tree was rooted to *Colletotrichum boninense*.

and has been reported in several other plants including *Acca sellowiana*, *Annona diversifolia*, *Fragaria* × *ananassa*, *Fragaria vesca*, *Limonium* sp., *Olea europaea*, *Quercus* sp., *Stylosanthes guianensis* and *S. viscose*; the pathogen is only known to occur in Australia, Israel, Mexico, New Zealand, Panama and the United States of America (Weir et al., 2012). This is the first report of *C. theobromicola* causing leaf spot, stem lesions and defoliation on eucalyptus and the first record of the species in Brazil. The disease poses a threat to nurseries of eucalyptus mini-cuttings where spray irrigation facilitates inoculum dissemination and the environment is favorable for disease outbreaks.

ACKNOWLEDGEMENTS

We thank the Cenibra S/A for providing the eucalyptus clone used in this study. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES, and Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG.

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TPP-2013-0200
Submitted: 21 November 2013
Revisions requested: 29 December 2013
Accepted: 2 February 2014
Section Editor: Thomas Harrington