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Southern Forests: a Journal of Forest Science

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tsfs20

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To cite this article: Myriam Solís, Michael J Wingfield, Izette Greyling & Nam Q Pham (2022): A serious shoot and leaf disease caused by *Colletotrichum theobromicola* discovered on eucalypts in South Africa, Southern Forests: a Journal of Forest Science, DOI: 10.2989/20702620.2021.2005454

To link to this article: https://doi.org/10.2989/20702620.2021.2005454

Published online: 22 Mar 2022.

Southern Forests 2022, 84(1): 01–13
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This is the final version of the article that is published ahead of the print and online issue

SOUTHERN FORESTS
ISSN 2070-2620 EISSN 2070-2639
https://doi.org/10.2989/20702620.2021.2005454

A serious shoot and leaf disease caused by *Colletotrichum theobromicola* discovered on eucalypts in South Africa

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Eucalypt plantations in South Africa make up an important part of the local forestry industry. Recently, one-year-old nursery plants of a *Eucalyptus grandis* × *Eucalyptus urophylla* variety displayed symptoms of leaf and shoot anthracnose disease. Samples were collected from these plants and isolations were made from the disease symptoms. Isolates were identified based on their morphological characteristics and DNA sequence data for eight gene regions. Phylogenetic analyses led to the isolates being identified as *Colletotrichum theobromicola* and the reduction of *Colletotrichum pseudotheobromicola* to synonymy with the former species. Pathogenicity trials with isolates of *C. theobromicola* were conducted on clones of *E. grandis* and hybrids of *E. grandis* × *E. urophylla* and *E. grandis* × *Eucalyptus camaldulensis.* Resulting symptoms were similar to those found on naturally infected plants and the fungus was re-isolated from the infections. *Colletotrichum theobromicola* is known to cause anthracnose on various plants including eucalypts in Brazil, but this is the first record of the pathogen in South Africa.

Keywords: anthracnose disease, *Eucalyptus* hybrids, fungal pathogens, nursery diseases

Introduction

The genus *Eucalyptus* (Myrtaceae) includes approximately 700 species that are native to Australia, Indonesia, the Philippines and New Guinea (Potts and Pederick 2000). Plantations of these trees, mostly as non-natives, are amongst the most important sources of wood and pulp products in the southern hemisphere, covering an area of more than 20 million hectares worldwide (Turnbull 2000; Booth 2013).

South Africa has approximately 1.2 million ha of commercial forest plantation, with eucalypts making up a substantial component of this resource (https://www.forestry.co.za/statistical-data/). As is true for many other regions where plantation forestry is based on non-native species, the accidental introduction of non-native pathogens has resulted in serious losses for the industry (Wingfield et al. 2015; Burgess and Wingfield 2017). Likewise, host shifts where native pathogens have adapted to infect non-native eucalypts have added to these disease problems (Slippers et al. 2005; Burgess and Wingfield 2017).

Apart from the many diseases that affect *Eucalyptus* spp. in plantations, diseases of seedlings and cutting plants in nurseries also result in considerable economic losses (Sharma et al. 1984; Old et al. 2003). In this regard, diseases in nurseries can result in failure to produce sufficient planting material, loss of important germplasm and down-stream losses in biomass from plantations (Keane 2000; Old et al. 2003).

Most eucalypt nursery pathogens are known to be either soil-, seed- or water-borne and they can arise from the time of sowing through to out-planting in the field (Sharma et al. 1984; Mohanan 2014). Among the more commonly encoun-tered pathogens that can affect eucalypts in nurseries are species of *Ralstonia* (Alfenas et al. 2006), *Phytophthora* (Simamora et al. 2017), *Botrytis* (Viljoen et al. 1992), *Calonectria* (Lombard et al. 2010) and *Colletotrichum* (Rodrigues et al. 2014).

In 2021, during routine observations of eucalypt plants in a Pretoria nursery, symptoms of a serious leaf and shoot disease were observed on one-year-old *E. grandis* × *E. urophylla* hybrid plants. The aim of this study was to identify the causal agent of this disease. This was achieved using routine isolation procedures, identification of the associated fungus based on morphological characteristics, as well as DNA sequence analyses, after which pathogenicity of the putative pathogen was tested on several different eucalypt varieties.

Materials and methods

Disease symptoms, sampling and fungal isolation

Symptoms of a leaf and shoot disease were observed on an *E. grandis* × *E. urophylla* variety in a nursery where the conditions included a range of temperatures from 20 °C to 32 °C and high levels of relative humidity (> 80%). The disease was typified by anthracnose symptoms, including circular to irregular,

red-brown to black necrotic leaf spots on young and newly expanded leaves (Figure 1). In more advanced stages of the disease, necrotic spots were found on the stems, resulting in rotting of the young shoots (Figure 1).

Fresh *E. grandis* × *E. urophylla* leaves showing typical anthracnose symptoms were collected and placed in paper bags and transferred to the laboratory for isolations. Direct isolations of spores were made from fungal structures using a sterile needle and placed on to the surface of Malt Extract Agar (MEA; 20 g malt extract, 20 g Difco agar, 1 L ionised water) in Petri dishes and incubated for 3–5 days at 25 °C. Single hyphal tips from primary isolations were transferred to MEA plates and incubated at 25 °C for seven days to obtain pure cultures. Resulting isolates were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction, polymerase chain reaction (PCR) sequencing and phylogenetic analyses

Single hyphal-tip isolates grown on MEA for seven days at 25 °C were used for DNA extraction. Mycelium was scraped from the surface of the cultures using a sterile needle and transferred to 1.5 ml Eppendorf tubes. DNA was extracted using Prepman® Ultra Sample Preparation Reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocols. Eight gene regions, namely *ACT*, *CAL*, *CHS1*, *GAPDH*, *GS*, ITS, *ApMat* and *TUB2*, were used for polymerase chain reaction (PCR) amplification and subsequent sequencing (Table 1). Initially, the ITS region was amplified for all isolates. Based on these preliminary results, representative isolates were chosen for further sequencing of the remaining seven gene regions for phylogenetic analyses.

The PCR reactions were conducted using an Applied Biosystems ProFlex PCR System (Thermo Fisher Scientific,

Figure 1: Symptoms of anthracnose on *E. grandis* × *E. urophylla*: (a) Leaf spots in shoots and young leaves in the upper leaf surface; (b) Leaf spots present in the abaxial leaf surface; (c) Stem and shoot rot; (d) Leaf spots in new shoots and stem; (e) Necrotic spots on the adaxial leaf surface with sporulation of the pathogen

Waltham MA, USA) following conditions suggested by Gan et al. (2013) and Khodadadi et al. (2020). Amplified fragments were purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Amplicons were sequenced in both directions using an ABI PRISM™ 3100 DNA sequencer (Applied Biosystems, USA) at the Sequencing Facility of the Faculty of Natural and Agricultural Sciences, University of Pretoria, South Africa. Raw sequences were assembled and edited using Geneious Prime 2021.1.1 (https://www.geneious.com). All sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) with details provided in (Table 2).

Sequences of species related to those emerging from this study were sourced from the GenBank database (http://www.ncbi.nlm.nih.gov/) (Table 2). Alignments of all sequences were assembled using MAFFT v. 7 (http://mafft.cbrc.jp/alignment/server) (Katoh and Standley 2013), then confirmed manually in MEGA v. 7 (Kumar et al. 2016). A concatenated data set was generated comprising of *ACT*, *CAL*, *CHS1*, *GAPDH*, *GS*, ITS and *TUB2* sequences. Alignment of *ApMat* sequences was analysed individually because sequence data were not equally available for the gene regions and species considered. Maximum likelihood (ML) analyses were conducted using RaxML v. 8.2.4 on the CIPRES Science Gateway v. 3.3 (Stamatakis 2014) with default GTR substitution matrix and 1 000 rapid bootstraps. Final consensus trees were viewed using MEGA v. 7 (Kumar et al. 2016).

Morphological observations

Morphological characteristics of the isolates obtained from diseased tissues were observed using three representative cultures (CMW 56826, CMW 56827 and CMW 56828). The cultures were grown on MEA for 7 days and incubated at 25 °C in the dark to induce sporulation. Fungal structures that emerged were initially mounted in water and this was then replaced with 85% lactic acid. Morphological observations were made using an Eclipse Ni, SMZ 18 (Nikon, Tokyo, Japan) stereoscope and an Axioskop 2 plus (Zeiss, Oberkochen, Germany) microscope.

Pathogenicity tests

To confirm pathogenicity of the putative causal agent of the disease, plants of *E. grandis* × *E. urophylla*, *E. grandis* and *E. grandis* × *E. camaldulensis* varieties were used. Plants were grown in pots in a greenhouse at 25 °C with 16h light/8h dark cycles. Two representative isolates (CMW 56826 and CMW 56827) were selected for the inoculations, and these were grown on MEA at 25 °C for 7 days.

A conidial suspension was prepared by adding sterile distilled water to the cultures and gently scraping their surface with a sterilised scalpel. The suspension was filtered through a layer of cheesecloth to remove mycelial fragments and the concentration of the conidia was adjusted to 10^6 spores ml^{-1} using a haemocytometer. Six plants for each eucalypt variety were inoculated with conidia from the two isolates by spraying these on to the apical parts of the plants until runoff. Six additional plants for each variety were inoculated with sterile water and maintained as controls.

Foliage of the inoculated plants and the controls were enclosed in plastic bags to which balls of cotton wool, soaked in sterile distilled water, had been added to ensure the leaf

Table 1: Primers used in this study, with sequences and sources

Region	Primer	Direction	Sequence	References
Actin (*ACT*)	ACT-512F	Forward	ATGTGCAAGGCCGGTTTCGC	Carbone and Kohn (1999)
	ACT-783R	Reverse	TACGAGTCCTTCTGGCCCAT	Carbone and Kohn (1999)
Calmodulin (*CAL*)	CL1	Forward	GARTWCAAGGAGGACGCTTCTC	O'Donnell et al. (2000)
	CL2a	Reverse	TTTTTGCATCATGAGTTGGAC	O'Donnell et al. (2000)
Chitin synthase (*CHS1*)	CHS-79F	Forward	TGGGGCAAGGATGCTTGGAAGAAG	Carbone and Kohn (1999)
	CHS-345R	Reverse	TGGAAGAACCATCTGTGAGAGTTG	Carbone and Kohn (1999)
Glutamine synthetase (*GS*)	GSF	Forward	ATGGCCGAGTACATCTGG	Stephenson et al. (1997)
	GSR	Reverse	GAACCGTCGAAGTTCCAC	Stephenson et al. (1997)
Glyceraldehyde-3-phosphate (*GAPDH*)	GDP1	Forward	CAACGGCTTCGGTCGCATTG	Berbee et al. (1999)
	GDP2	Reverse	GCCAAGCAGTTGGTTGTGC	Berbee et al. (1999)
Intergenic spacer and partial MAT1-2 gene (*ApMat*)	CgDL-F6	Forward	AGTGGAGGTGCGGGACGTT	Rojas et al. (2010)
	CgMAT1F2	Reverse	TGATGTATCCCGACTACCG	Rojas et al. (2010)
Internal transcribed spacer (ITS)	ITS-1F	Forward	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
	ITS-4	Reverse	TCCTCCGCTTATTGATATGC	White et al. (1990)
β-Tubulin 2 (*TUB2*)	T1	Forward	AACATGCGTGAGATTGTAAGT	O'Donnell and Cigelnik (1997)
	Bt2b	Reverse	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson (1995)

Table 2: Collection details and GenBank accessions of isolates included in the phylogenetic analyses

Species	Isolate number	Host/Substrate	Country	GenBank accession number								Reference
				ACT	*ApMat*	*CAL*	*GAPDH*	*GS*	ITS	*TUB2*	*CHS*	
C. aeschynomenes	ICMP 17673, ATCC 201874T	*Aeschynomene virginica*	USA	JX009483	KM360145	JX009721	JX009930	JX010081	JX010176	JX010392	JX009799	Vieira et al. (2017)
C. alatae	CBS 304.67, ICMP 17919T	*Dioscorea alata*	India	JX009471	KC888932	JX009738	JX009990	JX010065	JX010190	JX010383	JX009837	Liu et al. (2015)
C. alatae	ICMP 18122	*Dioscorea alata*	Nigeria	JX009470	N/A	JX009739	JX010011	JX010136	JX010191	JX010449	JX009846	Liu et al. (2015)
C. asianum	GM595, MTCC 11680	*Mangifera indica*	India	JQ894545	JQ894554	KC790789	JQ894623	N/A	JQ894679	JQ894601	JQ894616	Liu et al. (2015)
C. asianum	ICMP 18580, CBS 130418T	*Coffea arabica*	Thailand	JX009584	FR718814	FJ917506	JX010053	JX010096	FJ972612	JX010406	JX009867	Liu et al. (2015)
C. boniense	MAFF 305972, CBS 123755T	*Crinum asiaticum* var. *sinicum*	Japan	JQ005501	N/A	JQ005674	JQ005240	N/A	JQ005153	JQ005588	JQ005327	Liu et al. (2015)
C. changpingense	MFLUCC 15-0022, SA0016	Rhizome of *Fragaria* × *ananassa*	China	KP683093	N/A	N/A	KP852469	N/A	KP683152	KP852490	KP852449	Jayawardena et al. (2016)
C. changpingense	SA0050	Rhizome of *Fragaria* × *ananassa*	China	KY214470	N/A	N/A	KY214472	N/A	KY214473	KY214474	KY214471	Jayawardena et al. (2016)
C. endophytica	MFLUCC 130417, LC1216	*Pennisetum purpureum*	Thailand	KC692467	N/A	KC810017	KC832853	N/A	KC633853	N/A	N/A	Vieira et al. (2017)
C. endophytica	MFLUCC 130418, LC0324T	*Pennisetum purpureum*	Thailand	KF306258	N/A	KC810018	KC832854	N/A	KC633854	N/A	N/A	Vieira et al. (2017)
C. gloesporioides	LC3686, LF916	*Camellia sinensis*	China	KJ954493	KJ954629	KJ954777	KJ954927	KJ955076	KJ955226	KJ955371	N/A	Liu et al. (2015)
C. gloesporioides	IMI 356878T, ICMP 17821, CBS 112999	*Citrus sinensis*	Italy	JX009531	JQ807843	JX009731	JX010056	JX010085	JX010152	JX010445	JX009818	Vieira et al. (2017)
C. grevileae	CBS 132879T, CPC 15481	*Grevillea* sp.	Italy	KC296941	N/A	KC296963	KC297010	KC297033	KC297078	KC297102	KC296987	Vieira et al. (2017)
C. grossum	CAUG7T	*Capsicum* sp.	China	KP890141	N/A	KP890147	KP890159	N/A	KP890165	KP890171	KP890153	Diao et al. (2017)
C. grossum	CAU31	*Capsicum* sp.	China	KP890142	N/A	KP890148	KP890160	N/A	KP890166	KP890172	KP890154	Diao et al. (2017)
C. grossum	CAUG32	*Capsicum* sp.	China	KP890143	N/A	KP890149	KP890161	N/A	KP890167	KP890173	KP890155	Diao et al. (2017)
C. grossum	INIFAT-4144	*Magifera indica*	Cuba	MG826117	MG826119	MG826121	MG826116	MG826120	MG812307	MG826118	N/A	Manzano León et al. (2018)
C. horii	ICMP 17968	*Diospyros kaki*	China	JX009547	N/A	JX009605	GQ329682	JX010068	JX010212	JX010378	JX009811	Liu et al. (2015)
C. horii	ICMP 10492T	*Diospyros kaki*	Japan	JX009438	JQ807840	JX009604	GQ329681	JX010137	GQ329690	JX010450	JX009752	Vieira et al. (2017)
C. makassarense	CPC 28556	*Capsicum annuum*	Indonesia	MH781478	MH728833	N/A	MH728821	MH748262	MH728815	MH846561	N/A	de Silva et al. (2019)
C. makassarense	CBS 143664, CPC 28612T	*Capsicum annuum*	Indonesia	MH781480	MH728831	N/A	MH728820	MH748264	MH728812	MH846563	MH805850	de Silva et al. (2019)
C. proteae	CBS 132882, CPC 14859T	*Protea* sp.	South Africa	KC296940	N/A	KC296960	KC297009	KC297032	KC297079	KC297101	KC296986	Liu et al. (2015)
C. proteae	CBS 134301, CPC 14860	*Protea* sp.	South Africa	KC842373	N/A	KC842375	KC842379	KC842381	KC842385	KC842387	KC842377	Liu et al. (2015)
C. pseudotheobromicola	MFLUCC 18–1602T			MH853681	N/A	N/A	MH853675	N/A	MH817395	MH853684	MH853678	Chethana et al. (2019)
C. queenslandicum	ICMP 18705	*Coffea* sp.	Fiji	JX009490	N/A	JX009694	JX010036	JX010102	JX010185	JX010412	JX009890	Liu et al. (2015)
C. queenslandicum	ICMP 1778T	*Carica papaya*	Australia	JX009447	KC888928	JX009691	JX009934	JX010104	JX010276	JX010414	JX009899	Vieira et al. (2017)
C. salsolae	ICMP 19051T	*Salsola tragus*	Hungary	JX009562	KC888925	JX009696	JX009916	JX010093	JX010242	JX010403	JX009863	Vieira et al. (2017)
C. salsolae	CBS 119296, ICMP 18693	*Glycine max* (inoculated)	Hungary	JX009559	N/A	JX009695	JX009917	N/A	JX010241	N/A	JX009791	Weir et al. (2012)
C. siamense	LC2940, LF148	*Camellia* sp.	China	KJ954370	KJ954504	KJ954641	KJ954789	KJ954939	KJ955088	KJ955237	N/A	Liu et al. (2015)
C. siamense	ICMP 18578T, CBS 130417	*Coffea arabica*	Thailand	FJ907423	JQ899289	FJ917505	JX009924	JX010094	JX010171	JX010404	JX009865	Vieira et al. (2017)
C. tainanense	CBS 143666, CPC 30245T	*Capsicum annuum*	Taiwan	MH781475	MH728836	N/A	MH728823	MH748259	MH728818	MH846558	MH805845	de Silva et al. (2019)
C. tainanense	UOM1120, Coll1298	*Capsicum annuum*	Taiwan	MH781487	MH728824	N/A	MH728819	MH748271	MH728805	MH846570	MH805857	de Silva et al. (2019)
C. theobromicola	GM592 = MTCC 11673	*Mangifera indica*	India	JQ894544	JQ894578	KC790788	JQ894631	N/A	JQ894678	JQ894593	JQ894615	Sharma et al. (2013)
C. theobromicola	CBS 124945T, ICMP 18649	*Theobroma cacao*	Panama	JX009444	KC790726	JX009591	JX010006	JX010139	JX010294	JX010447	JX009869	Vieira et al. (2017)
C. theobromicola	GJS B1160843	*Theobroma cacao*	Panama	N/A	GU994447	N/A	N/A	N/A	GU994356	GU994476	N/A	Vieira et al. (2017)
C. theobromicola	GA002	Persea americana	Israel	KX620137	KX620170	KX620198	KX620234	KX620267	KX620300	KX620333	N/A	Sharma et al. (2017)
C. theobromicola	GA006	Persea americana	Israel	KX620138	KX620171	KX620199	KX620235	KX620268	KX620301	KX620334	N/A	Sharma et al. (2017)

Table 2 *continued*

Species	Isolate number	Host/Substrate	Country	GenBank accession number								Reference
				ACT	*ApMat*	*CAL*	*GAPDH*	*GS*	ITS	*TUB2*	*CHS*	
C. theobromicola	ICMP 18566	*Olea europaea*	Australia	JX009496	N/A	JX009593	JX009953	JX010071	JX010282	JX010376	JX009801	Weir et al. (2012)
C. theobromicola	ICMP 18565	*Olea europaea*	Australia	JX009449	N/A	JX009594	JX010029	JX010070	JX010283	JX010374	JX009802	Weir et al. (2012)
C. theobromicola	ICMP 18567	*Olea europaea*	Australia	JX009457	N/A	JX009599	JX009985	JX010069	JX010287	JX010377	JX009803	Weir et al. (2012)
C. theobromicola	MUCL 42295, ICMP 17958, CBS 124250	*Stylosanthes guianensis*	Australia	JX009498	N/A	JX009598	JX009948	JX010067	JX010291	JX010381	JX009822	Weir et al. (2012)
C. theobromicola	ICMP 18576	*Limonium* sp.	Israel	JX009532	N/A	JX009595	JX010022	N/A	JX010279	N/A	JX009771	Weir et al. (2012)
C. theobromicola	ICMP 17895	*Annona diversifolia*	Mexico	JX009568	N/A	JX009600	JX010057	JX010066	JX010284	JX010382	JX009828	Weir et al. (2012)
C. theobromicola	ICMP 15445	*Acca sellowiana*	New Zealand	JX009509	N/A	JX009601	JX010027	N/A	JX010290	N/A	JX009893	Weir et al. (2012)
C. theobromicola	CBS 125393, ICMP 18650	*Theobroma cacao*	Panama	JX009503	N/A	JX009590	JX009982	N/A	JX010280	N/A	JX009872	Weir et al. (2012)
C. theobromicola	ICMP 17099	*Fragaria* × *ananassa*	USA	JX009493	N/A	JX009588	JX009957	N/A	JX010285	N/A	JX009778	Weir et al. (2012)
C. theobromicola	ICMP 17100	*Quercus* sp.	USA	JX009507	N/A	JX009596	JX009947	N/A	JX010281	N/A	JX009781	Weir et al. (2012)
C. theobromicola	IMI 348152, ICMP 17814	*Fragaria vesca*	USA	JX009448	N/A	JX009589	JX010003	JX010062	JX010288	JX010379	JX009819	Weir et al. (2012)
C. theobromicola	CBS 142.31, ICMP 17927	*Fragaria* × *ananassa*	USA	JX009516	N/A	JX009592	JX010024	JX010064	JX010286	JX010373	JX009830	Weir et al. (2012)
C. theobromicola	MUCL 42294, ICMP 17957, CBS 124251	*Stylosanthes viscosa*	Australia	JX009575	N/A	JX009597	JX009962	JX010063	JX010289	JX010380	JX009821	Weir et al. (2012)
C. theobromicola	CMM 3559	*Eucalyptus* sp.	Brazil	N/A	N/A	N/A	KF768562	N/A	KF768556	KF768559	N/A	Rodrigues et al. (2014)
C. theobromicola	CMM 3561	*Eucalyptus* sp.	Brazil	N/A	N/A	N/A	KF768561	N/A	KF768555	KF768558	N/A	Rodrigues et al. (2014)
C. theobromicola	**CMW 56824**	*Eucalyptus* sp.	South Africa	OK584040	OK584046	OK584034	OK584058	OK584052	OK485037	OK584022	OK584028	This study
C. theobromicola	**CMW 56825**	*Eucalyptus* sp.	South Africa	OK584041	OK584047	OK584035	OK584059	OK584053	OK485038	OK584023	OK584029	This study
C. theobromicola	**CMW 56826**	*Eucalyptus* sp.	South Africa	OK584042	OK584048	OK584036	OK584060	OK584054	OK485039	OK584024	OK584030	This study
C. theobromicola	**CMW 56827**	*Eucalyptus* sp.	South Africa	OK584043	OK584049	OK584037	OK584061	OK584055	OK485040	OK584025	OK584031	This study
C. theobromicola	**CMW 56828**	*Eucalyptus* sp.	South Africa	OK584044	OK584050	OK584038	OK584062	OK584056	OK485041	OK584026	OK584032	This study
C. theobromicola	**CMW 56829**	*Eucalyptus* sp.	South Africa	OK584045	OK584051	OK584039	OK584063	OK584057	OK485042	OK584027	OK584033	This study
C. tropicale	CBS 124949T, ICMP 18653	*Theobroma cacao*	Panama	JX009489	GU994425	JX009719	JX010007	JX010097	JX010264	GU994454	JX009870	Vieira et al. (2017)
C. tropicale	CMM 4243	*Musa* sp.	Brazil	KU213596	KU213597	KU213599	KU213601	KU213602	KU213603	KU213604	KU213600	Vieira et al. (2017)
C. xanthorrhoeae	IMI 350817a, ICMP 17820	*Xanthorrhoea* sp.	Australia	JX009479	N/A	JX009652	JX010008	N/A	JX010260	N/A	JX009814	Liu et al. (2015)
C. xanthorrhoeae	BRIP 45094, ICMP 17903, CBS 127831T	*Xanthorrhoea preissii*	Australia	JX009478	KC790689	JX009653	JX009927	JX010138	JX010261	JX010448	JX009823	Liu et al. (2015)

N/A = information not available

ACT = actin

ApMat = Intergenic spacer and partial MAT1-2 gene

ATCC = American Type Culture Collection, Virginia, USA

BRIP = Queensland Plant Pathology Herbarium, Australia

CAL = calmodulin

CBS = The culture collection of Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands

CHS1 = Chitin synthase

CMM = Culture Collection of Phyto-pathogenic Fungi Prof. Maria Menezes, Federal Rural University of Pernambuco, Brazil

CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), South Africa

CPC = Pedro Crous working collection housed at Westerdijk Fungal Biodiversity Institute

GAPDH = Glyceraldehyde-3-phosphate

GS = Glutamine synthetase

Bold type = isolates obtained in this study

ICMP = International Collection of Microorganisms from Plants

IMI = CABI Genetic Resource Collection, UK

ITS = Internal transcribed spacer

LC = Working collection of Lei Cai, housed at CAS, China

LF = Working collection of Fang Liu, housed at CAS, China

MAFF = Ministry of Agriculture, Forestry and Fisheries, Japan

MFLUCC = Mae Fah Luang University Culture Collection, Thailand

MUCL = Belgian Co-ordinated Collections of Micro-organisms, (agro)industrial fungi and yeasts, Belgium

T = ex-type strain

TUB2 = β-tubulin 2

UOM = University of Melbourne culture collection, Victoria, Australia

wetness and to retain high levels of humidity. Plants were maintained in a growth chamber at 25 °C and subjected to a 16h light/8h dark cycle. Inoculated plants were monitored daily until symptoms appeared. To comply with Koch's postulates, re-isolations were made from symptomatic leaves on the inoculated plants. The resulting isolates were identified based on morphological characteristics and DNA sequence data for the ITS region.

Results

Fungal isolations

A total of 20 isolates, morphologically resembling a *Colletotrichum* species, were obtained from the disease symptoms. All of these isolates were either from leaf samples or from infected stems. All the colonies had a similar morphology on MEA and grew rapidly to cover the surface of the agar in the Petri dishes within four days.

Phylogenetic analyses

Based on the preliminary sequencing results for the ITS region, all *Collectotrichum* isolates resided in the *Colletotrichum gloeosporioides* complex. Six representative isolates were chosen for further study. Amplicons of approximately 260 bp were generated for the *ACT* gene region, 730 bp for the *CAL,* 240 bp for the *CHS1,* 620 bp for the *GAPDH,* 910 bp for the *GS,* 590 bp for the *ITS,* 800 bp for the *ApMat* and 730 bp for the *TUB2*.

The 7-locus combined sequence data set used in the phylogenetic analyses included 61 ingroup taxa, with *C. boninense* (CBS 123755) as the out-group. The data set contained 4 750 characters including the alignment gaps. Phylogenetic analysis of the aligned sequences for the *ApMat* locus included 30 taxa and 892 characters with alignment gaps. The tree was rooted with *C. alatae* (CBS 304.67). The phylogenetic trees for the seven-locus combined data set and the *ApMat* locus with bootstrap support values are presented in Figures 2 and 3 respectively. The two trees were found to be congruent, with similar topologies overall.

The phylogenetic tree presented in Figure 2 showed that all South African isolates considered in this study were all identical and clustered in a clade (ML = 100), residing together with representative isolates of *C. theobromicola sensu stricto* (Weir et al. 2012). This was further supported by the *ApMat* tree where these six isolates from eucalypts also formed a monophyletic clade with sequences for isolate GJS B1160843 and the ex-type isolate CBS 124945, representing *C. theobromicola* (Figure 3).

Isolate MFLUCC 18–1602, previously recognised as *C. pseudotheobromicola* based on a multilocus analysis (Chethana et al. 2019), clustered with *C. theobromicola* in our seven-gene multilocus analysis (Figure 2). Sequences for this isolate formed a clade together with isolates ICMP 18565 and ICMP 18576, previously identified as *C. theobromicola* (Weir et al. 2012).

Sequences for three isolates, GM529 (from *Mangifera indica*), GA002 and GA006 (from *Persea americana*), were previously identified as *C. theobromicola* (Sharma et al. 2013; Sharma et al. 2017). However, in our analyses, sequences for these isolates fell outside of the main *C. theobromicola* clade and grouped more closely with those representing *C. grossum* (Figure 2). The same pattern was observed in the *ApMat* analysis, where sequences for these three isolates clustered with isolate INIFAT-4144 of *C. grossum* and were clearly distinct from the clade accommodating the ex-type isolate of *C. theobromicola* (Figure 3). Hence, isolates GM529, GA002 and GA006 were considered as representing *C. grossum*.

Taxonomy

Collectotrichum pseudotheobromicola, identified from diseased leaves of *Prunus avium* in China (Chethana et al. 2019), was shown not to be phylogenetically distant from *C. theobromicola* based on phylogenetic analyses of sequence data generated in this study. *Colletotrichum pseudotheobromicola* was primarily distinguished from the ex-type of *C. theobromicola* based on ITS, *GAPDH*, *CHS1*, *ACT* and *TUB2* sequences data (Chethana et al. 2019). Sequences for this isolate failed to form a genetically distinct lineage when a greater number of *C. theobromicola* isolates were included in the analysis compared to one conducted with a limited number of reference sequences in the original description of the species. Based on this result, *C. pseudotheobromicola* is reduced to synonymy with *C. theobromicola*, as follows:

Colletotrichum theobromicola Delacr. *Bulletin de la Société Mycologique de France* 21: 191 (1905)

= *Colletotrichum pseudotheobromicola* Chethana, J.Y. Yan, X.H. Li & K.D. Hyde. *Mycosphere* 10: 518 (2019)

Morphology

The isolates grown on MEA medium produced a white to gray mycelium and the underside of the colony was uniformly black (Figure 4). After five to seven days, the aerial mycelium became darker with orange spore masses obvious (Figure 4). Setae were produced on black acervuli on cultures after 10 days, and these were simple, light brown, with 6–10 septa (Figure 4). Conidia were hyaline, straight to cylindrical, aseptate and 7–8 × 2–3 µm in size (Figure 4).

Pathogenicity

The two isolates used in the pathogenicity tests produced circular anthracnose-like leaf spots two to three days after inoculation. These symptoms were similar to those found on naturally infected plants in the nursery (Figure 5). Symptoms included brown-red leaf spots on both leaf surfaces, and these became black and necrotic at 10 days post inoculation, with the presence of fungal structures at the site of infection (Figure 5). These symptoms developed only on the *E. grandis* × *E. urophylla* hybrid plants. No symptoms developed on the inoculated *E. grandis* or *E. grandis* × *E. camaldulensis* plants (Figure 5) or those used as controls.

Colletotrichum theobromicola was successfully re-isolated on MEA from the lesions on all the *E. grandis* × *E. urophylla* plants inoculated. The isolates were morphologically identical to those used for the pathogenicity test and this identification was confirmed based on sequence data for the ITS region.

Discussion

The results of this study showed that a potentially serious leaf and shoot disease that has emerged in a South African eucalypt nursery was caused by *C. theobromicola*. This was

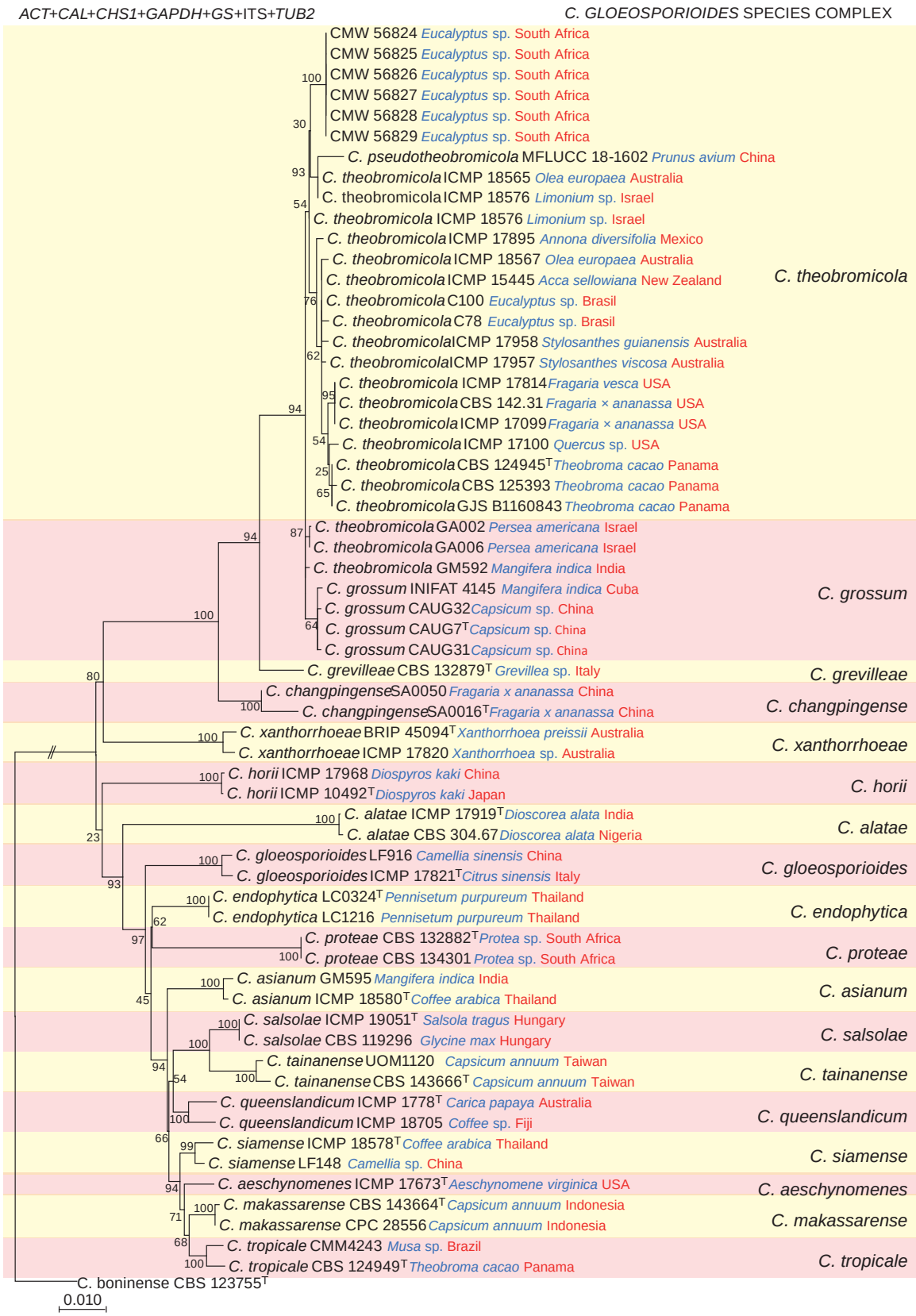

Figure 2: Phylogenetic tree based on maximum likelihood (ML) analysis of a combined DNA data set of *ACT*, *CAL*, *CHS1*, *GAPDH*, *GS*, ITS and *TUB2* sequences for *Colletotrichum* spp. Isolates sequenced in this study are presented in bold face. Host species are highlighted in blue and the countries of origin of the isolates are in red. Bootstrap values for ML analyses are indicated at the nodes. Isolates representing ex-type material are marked with a 'T'. *Colletotrichum boninense* (isolate CBS 123755) represents the outgroup taxon.

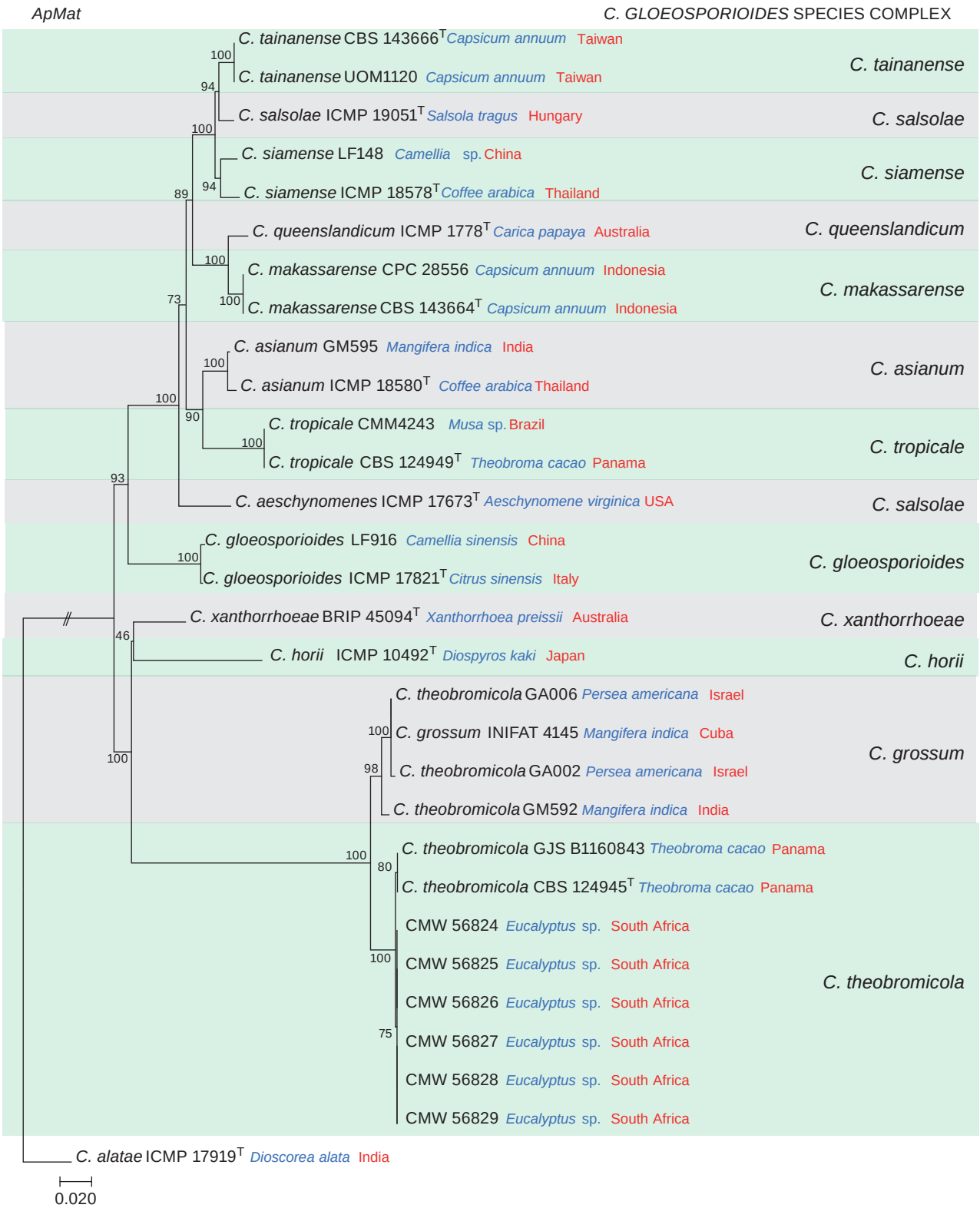

Figure 3: Phylogenetic tree based on maximum likelihood (ML) analysis of *ApMat* locus sequences for *Colletotrichum* sp. Isolates used in this study are presented in bold face. Host species are highlighted in blue and the countries of origin of the isolates are given in red. Bootstrap values for ML analyses are indicated at the nodes. Sequences representing the ex-type isolates are marked with 'T'. The tree was rooted to *C. alatae* (CBS 304.67)

based on isolations from symptomatic tissues, identification of the resulting cultures using analyses of DNA sequences for eight gene regions and pathogenicity tests. This is the first record of *C. theobromicola* from South Africa.

The morphological characteristics of the isolates obtained in this study are typical of species in the *C. gloeosporioides* species complex (von Arx 1970; Sutton 1992; Weir et al. 2012). It is well known that differentiating among species within this species complex is challenging due to a lack of distinctive morphological features and a plasticity of their phenotypic characters (Cai et al. 2009; Weir et al. 2012; Jayawardena et al. 2016). For this reason, identification of *Colletotrichum* spp. is carried out using a polyphasic approach that combines morphological and cultural characteristics with multilocus phylogenetic analyses of DNA sequences, which has transformed the taxonomy of the genus (Cai et al. 2009; Weir et al. 2012; Liu et al. 2016). We used the combination of eight gene regions to effectively resolve the phylogenetic placement of the isolates associated with a newly observed disease of eucalypts in South Africa. In addition, the resulting data provided justification to reduce *C. pseudotheobromicola* to synonymy with *C. theobromicola*.

Colletotrichum theobromicola occurs mostly in tropical, subtropical and temperate regions and it has a relatively wide host range, including economically important crops (Bragança et al. 2014; Araújo et al. 2018; Hawk et al. 2018; Lima et al. 2019). It is also known to cause anthracnose in Brazilian eucalypt nurseries causing symptoms similar to those observed in this study (Rodrigues et al. 2014). The disease described from Brazil also appeared to be specific to shoots and stems of a hybrid clone of *E. grandis* × *E. urophylla*. Importantly, this pathogen is able to cause severe defoliation and death of plants, and this can occur rapidly when conditions are conducive to disease development.

Some differences in morphology and DNA sequence data were observed in this study for *C. theobromicola* isolates from South Africa, compared to those reported from Brazil.

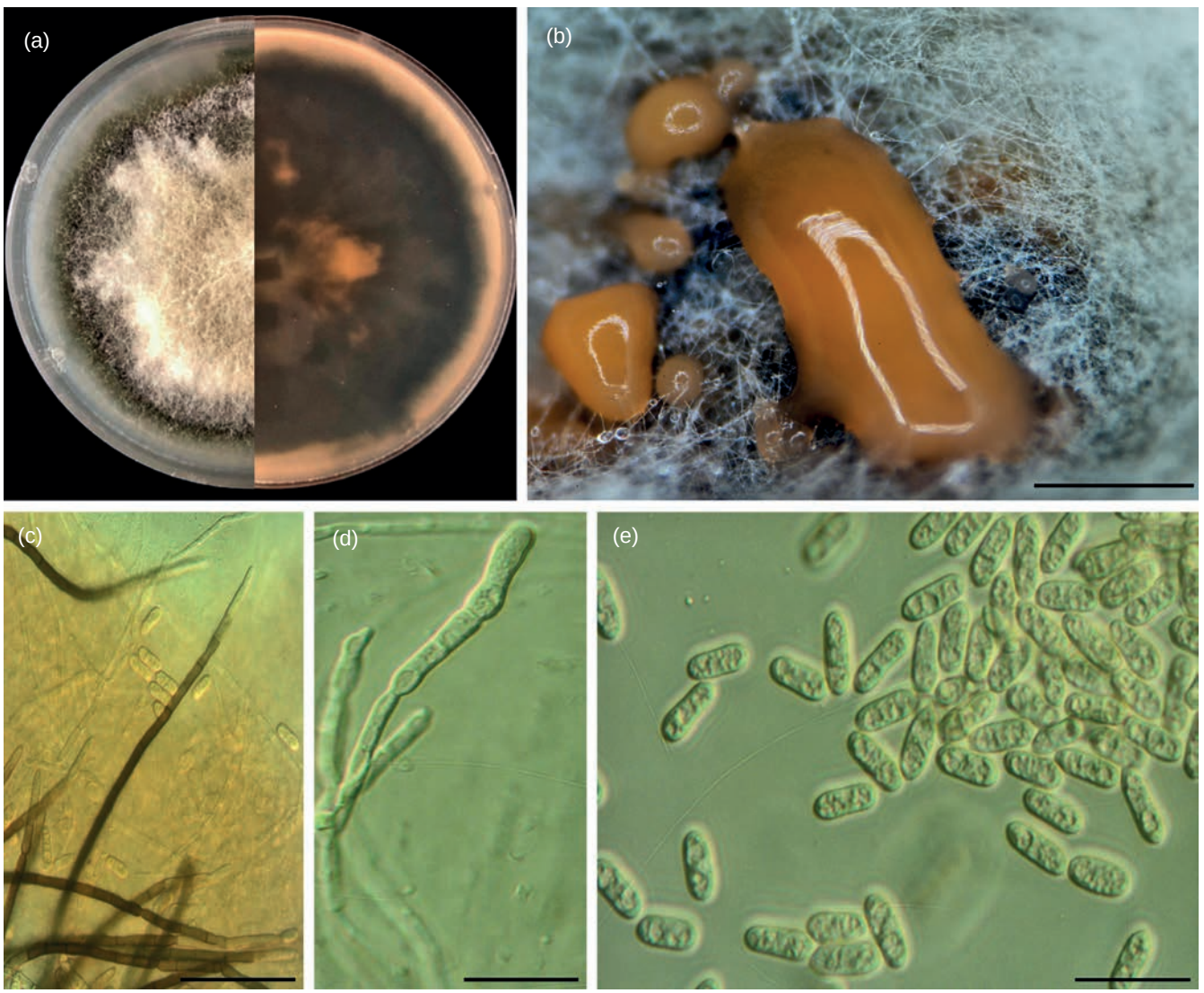

Figure 4: Morphology of *C. theobromicola*: (a) Seven-day-old colony from above and from below; (b) Spore masses exuding from conidiomata on MEA; (c) Setae on 10-day-old culture; (d) Conidiophores; (e) Conidia; Scale bars: b = 500 **µm**; c–e = 10 **µm**

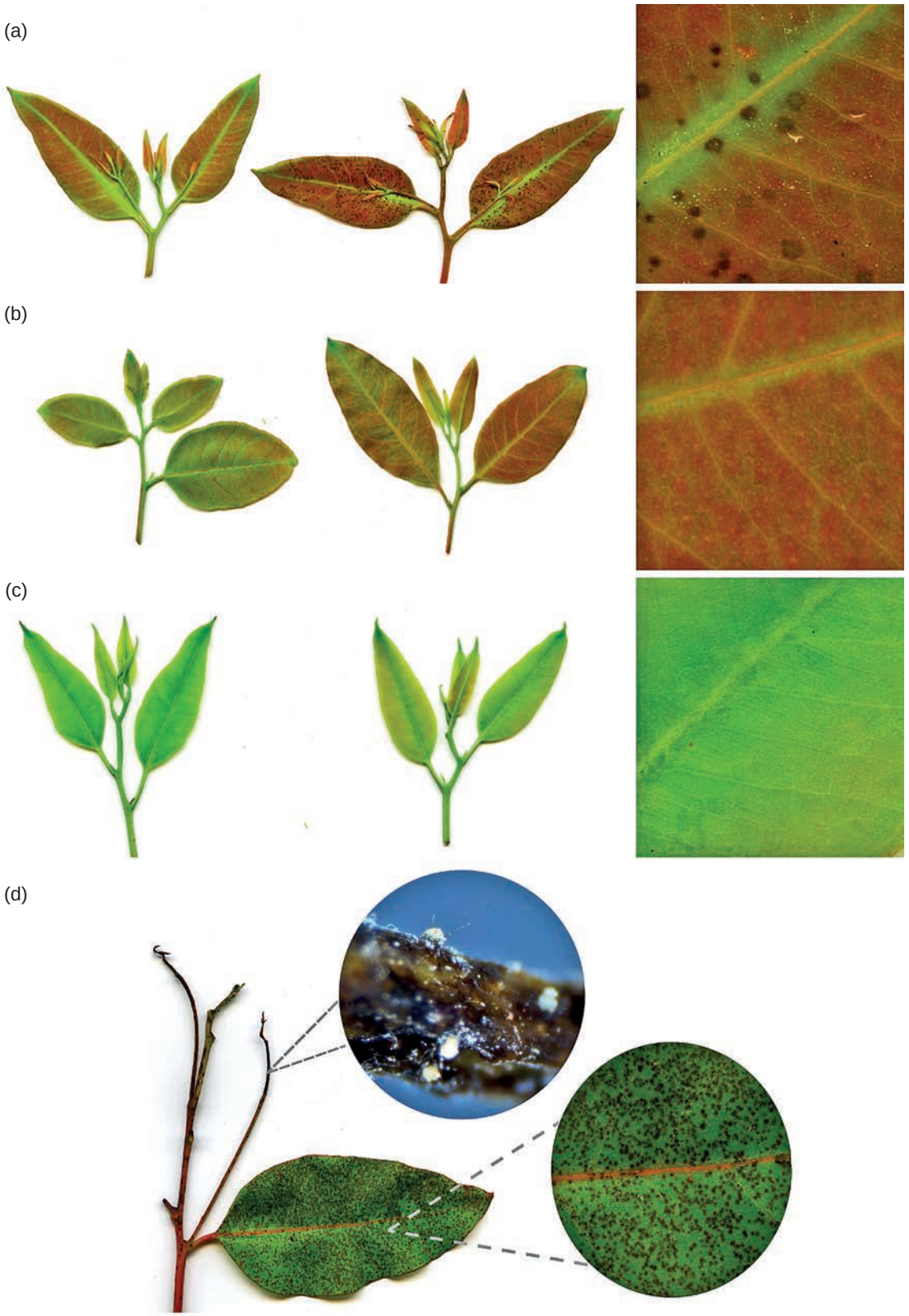

Figure 5: Results of the pathogenicity test with *C. theobromicola* on different eucalypt varieties: (a) *E. grandis* × *E. urophylla*, control (left) and leaf spots three days after inoculation (right); (b) *E. grandis*, control (left) and after three days (right); (c) *E. grandis* × *E. camaldulensis*, control (left) and after three days (right); (d) *E. grandis* × *E. urophylla*, 10 days after inoculation, black necrotic spots in the abaxial leaf surface, sporulation in stems and defoliation

Conidia produced on culture in this study (avg. 7–8 × 2–3 µm) were smaller than those reported on eucalypts in Brazil (avg. 10–17 × 4–6 µm) (Rodrigues et al. 2014). While phylogenetic analyses showed that the South African isolates clustered together as a well-supported clade with representative isolates of *C. theobromicola*, they clustered separately from two Brazilian isolates. This might suggest that *C. theobromicola* in South Africa does not share a similar origin to those causing diseases on eucalypts in Brazil.

Inoculation tests in this study showed that *C. theobromicola* was specifically pathogenic on a single variety of an *E. grandis* × *E. urophylla* hybrid. This is an intriguing result given that this fungus has a wide host range, including for example olive (*Olea europaea)*, onion (*Allium fistulosum*) (Matos et al. 2017) and sapote (*Manilkara zapota*) (Martins et al. 2018). While fungal pathogens of eucalypts are known to display differential pathogenicity on different varieties of these (van Heerden et al. 2005; Chen et al. 2013), it seems likely that *C. theobromicola* has a broader host range than has emerged from this study. Consequently, eucalypt nursery surveys will need to be conducted to clarify this important question, which has a direct impact on the management programme for this emerging disease.

The origin of *C. theobromicola* causing disease on a eucalypt variety in this study is unknown. However, it is well known that *Colletotrichum* spp. can be seed borne (Meon and Nik 1988; Begum et al. 2007; Pecchia et al. 2019). And consistent with this fact, two other *Colletotrichum* spp., *C. ciggaro* and *C. fructicola*, were recently recorded for the first time in South Africa after being isolated from the seed of various eucalypt species (Mangwende et al. 2021). It is thus plausible to hypothesise that *C. theobromicola* was accidently introduced into this country with seed imported from elsewhere. One possibility would be Brazil where the fungus is known to occur on eucalypts (Rodrigues et al. 2014).

Various important tree pathogens are thought to have been introduced into South Africa via seed used for plantation establishment. Notable examples are the pine pitch canker pathogen *Fusarium circinatum* (Coutinho et al. 2007) and the eucalypt canker pathogen *Teratosphaeria zuluense* (Jimu et al. 2016). The appearance of *C. theobromicola* in this study as well as two other *Colletotricum* spp. in the recent study reported by Mangwende et al. (2021) suggests a need for increased attention to be paid to seed pathogens that might accidentally be introduced into South Africa in the future.

Colletotrichum theobromicola appears to be a pathogen of increasing prevalence in countries such as Argentina (Lima et al. 2019), Australia (Wang et al. 2021), Israel (Sharma et al. 2016), United States (Hawk et al. 2018) and Brazil (Rodrigues et al. 2014), where it is found in both agriculture and forestry settings. Generally, little is known regarding its impact and given its recent discovery, this is also true for South Africa. Surveys should now be undertaken in South African forestry nurseries to determine where the pathogen occurs and how it might be managed.

Conclusions

Infections by *C. theobromicola* found in this study were associated with plants in a greenhouse and where plants were exposed to high levels of humidity and regular periods of leaf wetness. This might imply that anthracnose caused by the pathogen was facilitated by these conditions, which could be managed in commercial forestry nurseries. Furthermore, there was clear evidence of tolerance to infection in some eucalypt varieties, reducing the concern that the pathogen will emerge as an important constraint to large-scale propagation of these plants. It is also important to recognise that *Colletotrichum* spp. are known as opportunistic pathogens, which would also allow for effective management of *C. theobromicola* in the future.

Acknowledgements — We thank Prof. Jolanda Roux for her valuable advice regarding this study. We acknowledge the financial support from members of the Tree Protection Co-operative Programme (TPCP) and the Department of Science and Technology–National Research Foundation Centre of Excellence in Plant Health Biotechnology (CPHB), South Africa.

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