Anthracnose leaf spot pathogens, *Colletotrichum fructicola* and *Colletotrichum cigarro,* associated with *Eucalyptus* seed produced in South Africa

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Abstract

The cost of *Eucalyptus* seed and adoption of strict phytosanitary regulations in seed trade makes it imperative to monitor the quality and ensure supply of pathogen-free seeds for both forestry regeneration and research. Based on seed health tests, two species in the *Colletotrichum gloeosporioides* species complex were found to be naturally associated with seeds of *Eucalyptus dunnii*, *E. nitens* and *E. macarthurii* produced in South Africa. Multiloci phylogenetic analyses based on the concatenated sequences of the ITS regions, *β*-tubulin, actin, glutamine synthetase and glyceraldehyde-3-phosphate dehydrogenase genes, identified representative isolates PPRI 24314 as *C. fructicola* and PPRI 24315 as *C. kahawae* subsp. *cigarro* (*C. cigarro*). Subsequent biochemical tests showed that isolate PPRI 24315 was able to utilise either ammonium tartrate or citric acid as a sole carbon source, confirming its identity as *C. cigarro*. Pathogenicity tests showed that both *C. fructicola* and *C. cigarro* caused anthracnose leaf spots on *E. camaldulensis*, *E. dunnii*, *E. nitens* and *E. viminalis* seedlings. Disease symptoms included irregular dark-brown leaf spots on seedlings six days after inoculation. The two fungi were exclusively re-isolated from disease spots, thereby fulfilling Koch's postulates. Sowing *Eucalyptus* spp. seed artificially inoculated with either of the two pathogens showed the seed-transmissibility of *C. fructicola* and *C. cigarro* in *E. camaldulensis*, *E. dunnii* and *E. nitens* seedlings. To our knowledge, this is the first report of *C. fructicola* and *C. cigarro* associated with *Eucalyptus*.

Key words: Anthracnose, *Colletotrichum* spp., *Eucalyptus* seed, seed-borne, seed-transmitted

Introduction

Anthracnose caused by *Colletotrichum* spp. is one of the most devastating plant diseases affecting commercial nursery seedlings and tree plantations globally (Shivanna 2005; Peres et al. 2008; Rodrigues et al. 2014). In South Africa, anthracnose leaf spot disease is commonly reported in *Eucalyptus* plantations, where above ground plant parts of susceptible hybrids and clones may be infected, resulting in partial or complete defoliation, or is later manifested as twig dieback of terminal leader shoots of trees (Viljoen et al. 1992; Smith et al. 1998). Despite recurring occurrences of anthracnose leaf spot in *Eucalyptus* nurseries and forest plantations, this disease is generally neglected and its overall impact underestimated.

Colletotrichum infections are most severe in nurseries with closely spaced seedlings under high temperature conditions and with prolonged exposure to excess moisture (Brown and Ferreira 2000). The disease symptoms appear as necrotic lesions on leaves and stems, as well as seedling blights (Viljoen et al. 1992). Necrotic lesions on leaves are light brown circular spots that are surrounded by a red-purple border in the early stages of symptom development. As the disease progress, the spots become more pronounced, with increased visibility of acervuli containing yellowish to pinkish conidial masses (Viljoen et al. 1992). As lesions coalesce, the photosynthetic leaf area is severely reduced.

Since the genus *Colletotrichum* consists of several cryptic species complexes (Damm et al. 2009; Weir et al. 2012), accurate identification is important not only to understand the epidemiology, but to improve biosecurity and for effective management of plant diseases (Cannon et al. 2012; De Silva et al. 2017b). Although previous surveys have reported *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. as an important pathogen in *Eucalyptus* plantations (Sharma et al. 1984; Smith et al. 1998), with the longstanding taxonomic confusion of *Colletotrichum* species (Hyde et al. 2009), there is need for

confirming the accuracy of names assigned to these pathogens. Prior to the molecular era, identification of *Colletotrichum* spp. was primarily based on highly variable methods that employed morphological and physiological characters such as conidial size and shape of appressoria, presence or absence of setae, growth rate and host range (Than et al. 2008). This is the reason several other researchers prior to 2009 tended to support the hypothesis that *C. gloeosporioides* was the sole pathogen associated with anthracnose disease occurring on a wide range of hosts around the world (Martínez-Culebras et al. 2000; Benyahia et al. 2003; Lubbe et al. 2004; Phoulivong et al. 2010).

Due to the paucity of useful morphological and physiological characters, DNA-sequence based identification methods were widely adopted to resolve taxonomy of *Colletotrichum* species complexes. Initially, multi-locus sequence datasets were not prevalent so single gene regions, mainly ITS or *β*-tubulin, were used (Cai et al. 2009). Thus, accuracy of species names in many of these previous studies remains doubtful, and authenticity of previously reported species are yet to be confirmed. Multigene phylogenetic analysis has successfully delineated cryptic species within species complexes such as *Colletotrichum acutatum* J.H. Simmonds and *C. gloeosporioides* (Guerber et al. 2003; Weir et al. 2012). Following this taxonomic correction, there has been an increase in reports of several *Colletotrichum* species other than *C. gloeosporioides* causing anthracnose on *Eucalyptus* and other hosts (Rodrigues et al. 2014; Diao et al. 2017; De Silva et al. 2017a; Guarnaccia et al. 2017; Rojaset al. 2018; Shi et al. 2018; Nodel et al. 2019).

Following reassessments on the systematics of *Colletotrichum* species complexes, this study was initiated to advance knowledge on *Colletotrichum* species associated with *Eucalyptus* seed produced in South Africa. A polyphasic approach that used both morphological characters and multi-gene sequence analysis was employed to confirm identify

of *Colletotrichum* species. Furthermore, effects of seed-borne *Colletotrichum* species on seed germination were determined, and their rates of transmission and pathogenicity were evaluated in greenhouse trials.

Materials and Methods

Screening *Eucalyptus* seed for the presence of *Colletotrichum* species

The presence of *Colletotrichum* species on commercially produced *Eucalyptus* seeds was determined by plating on agar. Screening tests were performed on locally produced seeds belonging to 15 species, namely: *Eucalyptus badjensis* Beuzev. & Welch, *E. benthamii* Maiden & Cambage, *E. camaldulensis* Dehnh., *E. dorrigoensis* (Blakely) L.A.S. Johnson & K.D. Hill, *E. dunnii* Maiden, *E. globulus* Labill., *E. grandis* W. Hill, *E. macarthurii* H. Deane & Maiden, *E. nitens* (H. Deane & Maiden) Maiden, *E. pellita* F. Muell., *E. saligna* Sm., *E. smithii* F. Muell. ex R.T. Baker, *E. tereticornis* Sm., *E. urophylla* S.T. Blake, and *E. viminalis* Labill. Seeds were surface disinfected in 1% sodium hypochlorite solution (NaOCl) for five minutes and rinsed three times in sterile distilled water. Seeds were dried under sterile conditions inside a laminar flow cabinet and plated on potato dextrose agar (PDA) (Biolabs, Midrand, South Africa), and incubated for 7 days at 25 ±1°C under alternating cycles of 12 h ultra violet (UV) (365 nm) light and 12 h darkness. Fungi growing from plated seeds were identified on the basis of their macroscopic and microscopic features (Cai et al. 2009), and incidence of *Colletotrichum* species was recorded. Selected fungal isolates were purified on PDA, stored at 4°C on PDA slants, and selected isolates were deposited in the National Collection of Fungi, ARC-Plant Health and Protection, Roodeplaat, Pretoria, South Africa.

Molecular identification

Genomic DNA was extracted from 7-day-old *Colletotrichum* cultures using a Zymo Fungal DNA MiniPrep extraction Kit™ (Zymo Research Corp., Irvine, USA) following the manufacturer's instructions. The DNA extracts were used as templates in polymerase chain reactions (PCR) with amplification of the partial glyceraldehyde-3-phosphate dehydrogenase (GPDH) gene region using primers GDF1 and GDR1 (Templeton et al. 1992); glutamine synthetase (GS) using primers GSF1 and GSR1 (Stephenson et al. 1997); chitin synthase (CHS-1) using primers CHS I-79F and CHS I-354R (Carbone and Kohn 1999); actin (ACT) using primers ACT-512F and ACT7-83R (Carbone and Kohn 1999; Glass and Donaldson 1995); *β*-tubulin (TUB2) using primers T1/Bt2b (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997) and the ribosomal internal transcribed spacer (ITS) region using primers ITS 1 and ITS 4 (White et al. 1990). PCR amplicons were submitted to Inqaba Biotechnical Industries (Pty) Ltd (Inqaba Biotec, Pretoria, South Africa) for Sanger sequencing. From forward and reverse sequences obtained, consensus sequences were compiled using BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and subjected to nBLAST searches in GenBank [National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/BLAST)]. Sequences derived in this study were deposited in GenBank at the National Centre for Biotechnology Information GenBank (NCBI, www.ncbi.nlm.nih.gov/genbank).

Colletotrichum species sequences from our study, together with reference sequences selected from Weir et al. (2012) were aligned for phylogenetic analyses using MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html). Maximum Likelihood analyses were performed using PhyML 3.0 (Guindon and Gascual 2003). Statistical selection of best-fit models of nucleotide substitution using the Akaike information criteria (AIC) was determined with jModeltest 2.1.7 (Darriba et al. 2012). Initial analyses showed that individual genes were broadly congruent, thus nucleotide alignments of all the genes were concatenated. A

TIM3+I+G model was used for the concatenated dataset, and 1000 bootstrap replicates were conducted. *Colletotrichum boninense* Moriwaki, Toy. Sato & Tsukib. (ICMP 17904) and *C. hippeastri* Yan L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai (ICMP 17920) were used as out groups in the analysis (Weir et al. 2012).

Substrate utilisation tests

Weir et al. (2012) classified *C. kahawae* into two subspecies: *C. kahawae* subsp. *kahawae* and *C. kahawae* subsp. *cigarro*. To determine the subspecies of *C. kahawae* isolates used in this study, a biochemical assay was conducted following a protocol used by Waller et al. (1993). In this test, agar plugs of 7-day-old cultures were inoculated on to a basal medium (Lynch et al. 1981), supplemented with either ammonium tartrate or citric acid (1% *w/v*). Visual assessments of growth of fungi were done by comparing with positive and negative controls that contained glucose or no additional carbon source, respectively.

Pathogenicity and host range tests

To evaluate the effects of *Colletotrichum* species identified in this study on seed germination, *Eucalyptus* spp. seeds were surface disinfected by immersion in 1% NaOCl solution for 5 min, rinsed in sterile distilled water and soaked for 10 min in a 1 x 10^6 conidia mL^{-1} *Colletotrichum* inoculum suspension. Inoculated seeds were air dried in laminar flow cabinet for 4 h. Surface disinfected seeds soaked in sterile distilled water served as controls. Thereafter, 25 seeds were plated per Petri dish (110 x 17 mm) for a total of 200 seeds for each *Eucalyptus* species. Fifteen *Eucalyptus* species seed samples were tested. Plated seeds were incubated in a growth chamber set at 25 ±1 ºC with alternating cycle of 16 h cool white light (Osram fluorescent tubes 58W/840 cool light; Russia) and 8 h dark period. Experimental units were laid in a completely randomised design, and repeated twice. Seedlings were

evaluated according to International Seed Testing Association (ISTA) rules, where final counts of seed germination and diseased seedlings were recorded after 21 days. Seedlings with discolorations on hypocotyls or seminal roots were scored as “diseased”.

Seed-transmission tests were performed in a greenhouse located at the Hatfield Experimental farm (University of Pretoria, Pretoria, South Africa). Inoculated seeds of *E. camaldulensis*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, and *E. viminalis* were sown singly in pasteurised loamy soil filled 5 cm diameter pots. Greenhouse conditions were adjusted and maintained at 25°C /17°C for day/night temperatures, respectively, and plants were watered every second day. Each experiment consisted of fifteen pots in a randomised complete block design and the experiment was repeated once. Assessments of transmission of anthracnose disease from seeds into seedlings were done after six months, where number of seedlings that developed anthracnose disease symptoms from inoculated seeds were counted in comparison with seedlings raised from controls, or non-inoculated, seeds. To confirm if *Colletotrichum* spp. were the cause of leaf spots, pathogens were re-isolated from symptomatic plants. Three leaves were randomly selected for each treatment, and leaf sections of 5 mm x 5 mm were excised, surface sterilised in 1% NaOCl solution for 5 min, rinsed in sterile distilled water and plated on PDA. After incubation at 25 °C for 5 d fungi were identified by microscopy.

Pathogenicity of the two *Colletotrichum* species was determined by detached leaf assays. Healthy leaves, three from six-month-old seedlings of *E. benthamii*, *E. camaldulensis*, *E. dorrigoensis*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. tereticornis* and *E. viminalis*, were surface sterilized in 70% ethanol, rinsed three times in sterile distilled water, and transferred to moistened sterilised blotters in glass Petri dishes. Inoculum of each of the two *Colletotrichum* species was sprayed on leaf surfaces at a concentration of 1 x 10^5 conidia

mL^{-1} until surface runoff. Leaves sprayed with sterilized distilled water served as controls. For each *Eucalyptus* species, two leaves were placed inside a glass Petri dish, and this was replicated five times. Experimental units were arranged in a completely randomised design in a growth chamber that was maintained at 25 °C with light/darkness cycles of 16 h and 8 h, respectively. Inoculated and non-inoculated leaves were monitored daily until appearance of disease symptoms, where results were recorded either as "having symptoms" or "symptom-free".

To investigate susceptibility of different *Eucalyptus* species seedlings to the two *Colletotrichum* species, greenhouse pot assays were performed on six-month-old seedlings of *E. camaldulensis*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens* and *E. viminalis*. Leaf surfaces of 10 healthy seedlings of each species were surface disinfected by spraying with 70% ethanol and washing with a continuous flow of sterile distilled water. Thereafter, leaf surfaces were sprayed with a 10^5 conidia mL^{-1} inoculum of each of the two *Colletotrichum* species until surface run-off, and seedlings covered with polythene bags for 72 h to maintain a high humidity. Controls also consisted of ten *Eucalyptus* species seedlings that were surface disinfected as above, but sprayed with sterile distilled water until run-off. To fulfil Koch's postulates, isolations were made from leaves of diseased and healthy plants and isolated fungi morphologically identified.

Assessments of disease development were done 14 d after inoculation and the experiment was repeated once. Data of lesions that developed on the leaves were recorded, where percentage disease incidence (DI) and disease severity (DS) were calculated using a formulae described by Awa et al. (2012). Severity of leaf spot disease was scored using a 1 to 5 point scale, where 1 = no visible symptoms, 2 = 1 to 3 lesions, 3 = 4 to 6 lesions, 4 = lesions coalesce to cover <30% of leaf surface area, and 5 = >30% leaf area covered with

lesions with severe to complete necrosis of the leaf. Percentage DI and DS were calculated as follows:

$\% DI = a/N*100$

$\% DS = \sum (a+b)/ (N*Z)* 100$

Where a = number of infected leaves; b = corresponding score of leaf infection; N= Total number of sampled leaves; Z= Highest score on the scale

The data was arcsine transformed and tested for normality by the Shapiro-Wilk test, from which data was above 0.05. This was interpreted as a positive confirmation of normality. Thereafter, data was analysed by parametric statistical methods using SAS v. 9.0 (Statistical Package, Cary, NC, USA), and comparisons between means used the Fisher's least significant difference (LSD) test at a threshold P value of 0.05. However, final results were presented as untransformed data.

Results

Morphological Identification

Two morphologically different *C. gloeosporioides*-like strains were isolated from seeds of *Eucalyptus* spp. examined. Mycelia in pure cultures of all isolates were light to dark grey. Cultures that produced a white to grey cottony upper surface and an orange to grey appearance on the reverse side, produced cylindrical, aseptate, hyaline conidia that had obtuse to slightly rounded ends, and were 10.5 to 22.5 μm long and 3 to 6.5 μm wide, consistently matched descriptions of morphological characteristics described for *Colletotrichum fructicola* Prihast., L. Cai and K.D. Hyde within the *C. gloeosporioides*

species complex (Prihastuti et al. 2009; Rojas et al. 2010). However, some dark grey cultures produced aerial mycelium with stromatic acervuli, and formed abundant sclerotia after two weeks. Conidia were hyaline, cylindrical tapering slightly from only one side, measuring 16 - 20 × 3.5 - 5.5 μm. These morphological descriptions matched those of *C. kahawae* subsp. *cigarro* (Weir et al. 2012). Purified *C. fructicola* and *C. kahawae* subsp. *cigarro* isolates were assigned South African National Collection of Fungi voucher numbers PPRI 24314 and PPRI 24315, respectively.

Colletotrichum spp. were isolated from three *Eucalyptus* species, with *C. fructicola* and *C. cigarro* on *E. dunnii* (3.5% and 0.5%, respectively), *E. nitens* (2.6% and 1.2%, respectively), and *E. macarthurii* (0.8% for only *C. fructicola*). However, *Colletotrichum* species were not isolated from the other *Eucalyptus* species tested.

Molecular identification

To confirm the identity of *Colletotrichum* spp. isolates, the ACT, CHS, GAPDH, GS, ITS and TUB2 gene regions were sequenced (GenBank Accession Nos for PPRI 24314: MK512735, MK512737, MK512733, MZ062209, MG641892 and MK512739; GenBank Accession Nos for PPRI 24315: MK512736, MK512738, MK512734, MZ062210, MG641893 and MK512740) and subjected to BLAST and Maximum likelihood analyses. The BLAST searches showed that both isolates belonged to the *C. gloeosporioides* species complex. Maximum likelihood analyses of the concatenated dataset of 2260 bp and 50 taxa, with representative reference isolates of all the accepted species in this species complex, gave strong support for PPRI 24314 belonging to *C. fructicola* and PPRI 24315 belonging to *C. kahawae* subsp. *cigarro* that is now classified as *C. cigarro comb. et stat. nov.* (Cabral et al. (2020) (Figure 1).

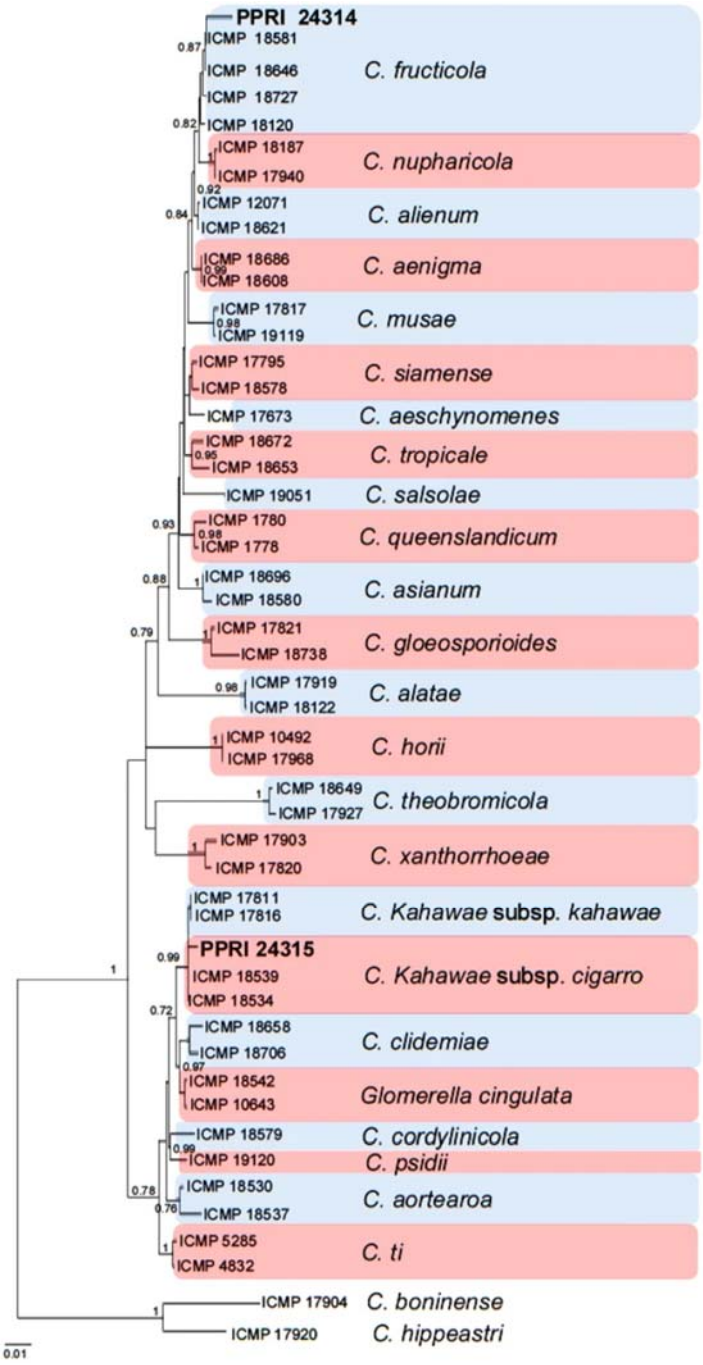

Figure 1. A Maximum likelihood phylogenetic tree of 50 isolates in the *Colletotrichum gloeosporioides* species complex. The tree was build using concatenated sequences of ACT, GAPDH, GS, CHS, ITS and *β*-tubulin genes. Maximum Likelihood bootstrap support values ≥ 0.7 are shown above nodes. The tree is rooted with *C. boninense* ICMP 17,904 and *C. hippeastri* ICMP 17,920. ICMP = International Collection of Microorganisms from Plants; PPRI = Plant Protection Research Institute (Pretoria, South Africa). Isolates from the current study are in bold

Substrate utilisation test

Biochemical tests showed that the *C. kahawae* isolate PPRI 24315 grew on basal medium containing either glucose or citric acid or ammonium titrate as a sole carbon source, confirming the identity of the isolate as *C. kahawae* subsp. *cigarro*.

Table 1 Effects of *Colletotrichum* species inoculation on seed germination of *Eucalyptus* species

***Eucalyptus* seeds**	**Non-inoculated control**	**Inoculated with *C. fructicola***	**Inoculated with *C. cigarro***
E. badjensis	78.3 *gh**x	42.0 ey	36 ez
E. benthamii	74.3 hx	29.8 gy	32.3 fy
E. camaldulensis	85.3 defx	46.3 dz	52.0 ay
E. dorrigoensis	88.3 bcdex	33.5 fz	41.8 dy
E. dunnii	94.3 ax	51.5 bcy	50.8 by
E. globulus	93.5 ax	47.5 dy	43.5 cdz
E. grandis	89.0 bcdx	56.8 ay	54.5 ay
E. macarthurii	88.8 bcdx	46 dy	45.8 cy
E. nitens	85.8 cdefx	39.5 ey	40.8 dy
E. pellita	90.5 abx	33.5 fy	33.3 efy
E. saligna	90.0 abcx	48.8 cdy	50.5 by
E. smithii	84.0 efx	47 dy	44.3 cdy
E. tereticornis	90.5 abx	53 by	45.5 cz
E. urophylla	91.3 abx	45.8 dz	54.8 ay
E. viminalis	81.5 fgx	47.0 dy	43.5 cdy
Cv%	2.0	2.0	2.0
LSD	4.4	3.1	3.7

*Means in the same column followed by the same letter do not differ significantly according to Fisher's LSD test at $p < 0.05$.

**Means in the same row for percentage germination not followed by the same letter are significantly different from each other ($p>0.05$).

Pathogenicity and host range tests

Germination capacity was significantly reduced on seed from all *Eucalyptus* species inoculated with either *C. fructicola* or *C. cigarro* (Table 1). For *Eucalyptus* seeds inoculated with *C. fructicola*, the highest percentage of seed germination was recorded from *E. grandis* (56.8%), and lowest seed germination observed on *E. benthamii* (29.8%). Seed germination

of *Eucalyptus* spp. seed inoculated with *C. cigarro* ranged from 32.3 to 54.8%, with the highest and lowest percentage seed germination recorded from *E. urophylla* (54.8%) and *E. benthamii* (32.3%), respectively. Seeds of *E. badjensis*, *E. globulus* and *E. tereticornis* were most susceptible to *C. cigarro*, whereas *E. camaldulensis*, *E. dorrigoensis*, *E. urophylla* were most susceptible to *C. fructicola*. There were similar responses to seed germination on *E. benthamii*, *E. dunnii*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. pellita*, *E. saligna*, *E. smithii* and *E. viminalis* seeds inoculated with either *C. fructicola* or *C. cigarro*.

Table 2 Effects of *Colletotrichum* species inoculation on percentage diseased *Eucalyptus* seedlings

Species	**Non-inoculated control**	**Inoculated with *C. fructicola***	**Inoculated with *C. cigarro***
E. badjensis	3.0 *ab**y	53.8 bx	55.3 ax
E. benthamii	4.3 ay	59.0 ax	58.3 ax
E. camaldulensis	2.0 bcdez	39.0 ex	35.5 gy
E. dorrigoensis	1.0 defz	44.3 cx	38 efgy
E. dunnii	0.8 efy	39.5 dex	38.0 efgx
E. globulus	0.8 efy	43.8 cdx	47.3 bx
E. grandis	1.5 cdefy	39.5 dex	38.3 efgx
E. macarthurii	2.3 bcdy	44.8 cx	41.8 cdex
E. nitens	2.0 bcdey	46.3 cx	46.3 bcx
E. pellita	1.0 defy	53.0 bx	53.8 ax
E. saligna	1.0 defz	44.0 cx	36.5 fgy
E. smithii	2.0 bcdez	47.5 cx	43.5 bcdy
E. tereticornis	0.5 fy	43.3 cdex	42.8 bcdex
E. urophylla	1.0 defz	44.0 cx	39.8 defgy
E. viminalis	2.8bcy	45.8 cx	41.5 cdefx
Cv%	2.0	2.0	2.0
LSD	1.5	4.5	5.2

Explanations: see Table 1

Inoculating *Eucalyptus* species seed with *Colletotrichum* species significantly increased the incidence of seedlings that developed disease symptoms ($P<0.05$) (Table 2). Disease symptoms initially appeared as slight discolorations with pale brown markings developing on green plant parts of seedlings, which rotted with spread of secondary infection. The number of diseased seedlings raised from different *Eucalyptus* species inoculated with *C.*

fructicola ranged from 39.0 to 59.0%. The highest and lowest number of diseased seedlings were raised from *E. benthamii* (59.0%) and *E. camaldulensis* (39.0%), respectively. For *Eucalyptus* species inoculated with *C. kahawae*, the number of seedlings that developed disease ranged from 35.5% to 53.8%, where highest and lowest number of diseased seedlings were raised from *E. pellita* (53.8%) and *E. camaldulensis* (35.5%), respectively. Seedlings raised from *E. camaldulensis*, *E. dorrigoensis*, *E. saligna*, *E. smithii* and *E. urophylla* seed inoculated with *C. fructicola* were most susceptible to diseases.

Detached leaf assays showed that both *C. fructicola* and *C. cigarro* were pathogenic on *Eucalyptus* species leaves (Figure 2). Six days after inoculation, all leaves showed irregular dark-brown leaf spots, whereas no symptoms were observed on controls. However, in greenhouse pot assays, anthracnose leaf spots appeared on all inoculated *Eucalyptus* seedlings after 11 days, except for *E. grandis, E. macarthurii* and the non-inoculated control seedlings (Figure 3). Incidence of anthracnose leaf spots caused by *C. fructicola* and *C. cigarro* was higher on *E. dunnii* and *E. nitens* (65.0% and 55.0%, respectively), and were most severe on *E. nitens* (67.0% and 63.0%, respectively). Both *C. fructicola* and *C. cigarro* were re-isolated from diseased seedlings, thereby fulfilling Koch's postulates and confirming them as causal agents of anthracnose leaf spots on *Eucalyptus*.

Sowing *E. camaldulensis*, *E. dunnii* and *E. nitens* seed artificially inoculated with *C. fructicola* resulted in transmission of the pathogen into the seedlings (25.5%, 38.3% and 64.0%, respectively), whereas seeds from the same *Eucalyptus* species inoculated with *C. cigarro* transmitted the pathogen in 27.3%, 30.5% and 56.3 of the seedlings, respectively. In contrast, *E. grandis* seed inoculated with either *C. fructicola* or *C. cigarro* did not result in transmission of either pathogen into the seedlings.

Control ***C. fructicola*** ***C. cigarro***

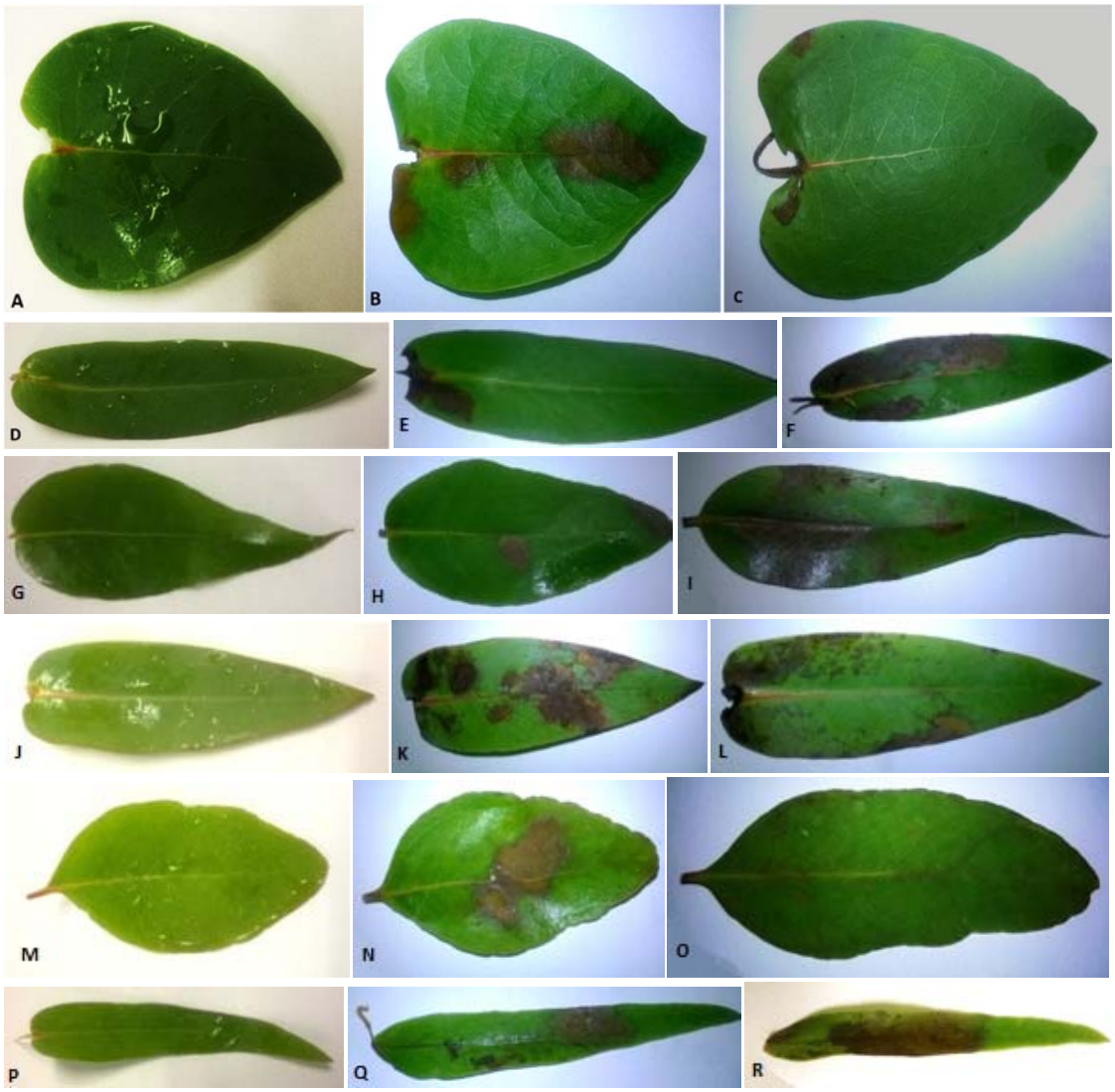

Figure 2 Example of symptoms of anthracnose leaf spots on *Eucalyptus* species: Left column are controls; middle column inoculated with *C. fructicola*; right column inoculated with *C. cigarro*. (A-C) *E. benthamii*, (D-F) *E. camaldulensis*, (G-I) *E. grandis*, (J-L) *E. nitens*, (M-O) *E. tereticornis*, (P-R) *E. viminalis*.

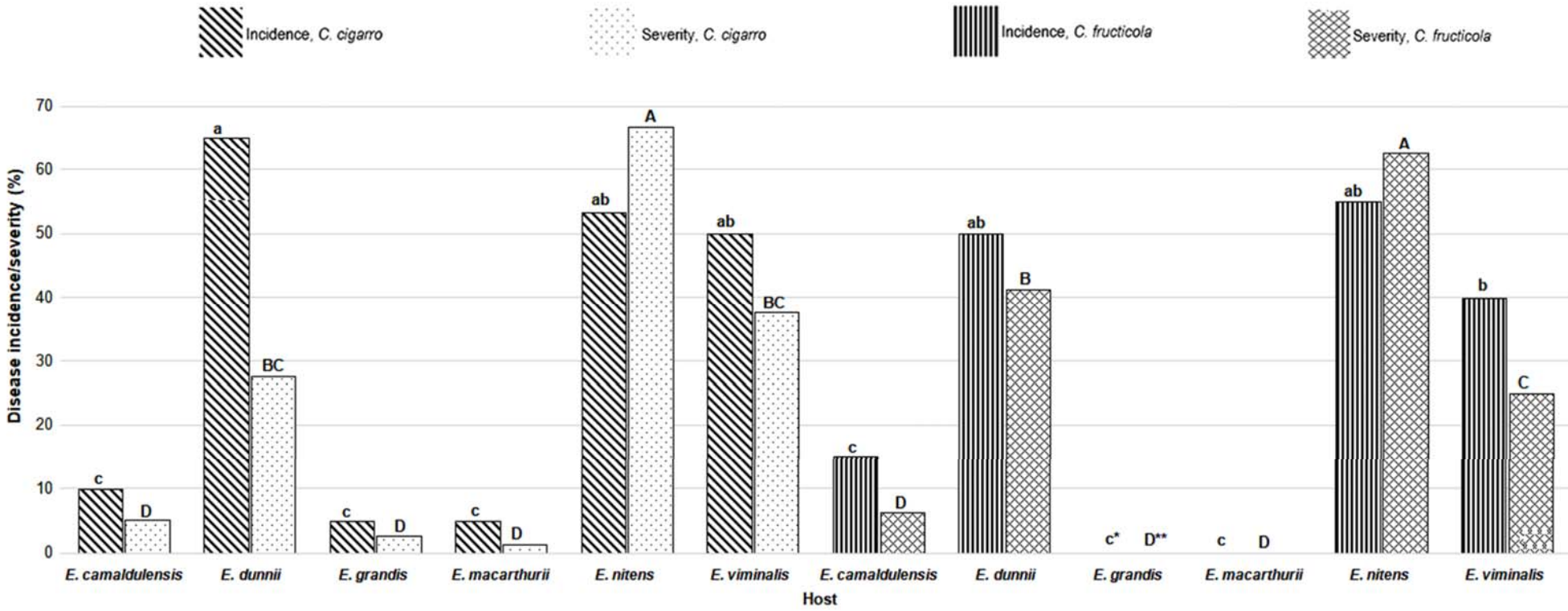

Figure 3 Mean disease incidence and severity of anthracnose leaf spot disease on *Eucalyptus* seedlings. Bars with the same letter (s) are not significantly different according to Fischer's LSD test ($p<0.05$).

Discussion

It is imperative to regularly monitor the health status of seed intended for seedling production. Apart from a study by Jimu et al. (2015), no work was previously done to explore seed-borne mycoflora associated with *Eucalyptus* seeds produced in South Africa. In this study, *C. fructicola* and *C. cigarro* are reported for the first time associated with seed of *Eucalyptus* species. Investigations were done to determine the pathogenicity of the two seed-borne fungi, their effects on seed germination and rates of transmission from seed to seedlings.

Screening tests showed that *Colletotrichum* species were naturally associated with seed from *E. dunnii*, *E. nitens* and *E. macarthurii*. The isolate PPRI 24314 was initially identified morphologically as *C. gloeosporioides* species complex. Morphological assessments are not always able to delineate species boundaries of cryptic species and reliance on these methods has resulted in taxonomic confusion of *Colletotrichum* species. For instance, conidia of *C. gloeosporioides* are described as aseptate, cylindrical with blunt ends, measuring from an average size of 10.4 to 16.7 μm × 4.2 to 5.8 μm (n=100) (Sutton, 1992) and may easily be confused with similar closely relating species in the *C. gloeosporioides* species complex such as *C. fructicola* that produces one celled conidia with obtuse to slightly rounded ends measuring 15.2 to 15.6 × 4.7 to 4.9 μm (n = 45) (Prihastuti et al. 2009).

Molecular methods have proven to be more objective in unravelling species richness of *Colletotrichum* species complexes (Chenet al. 2017; Ganet al. 2017; Hassan et al. 2018; Moreira et al. 2019). Based on multi-gene analyses of concatenated ATC, ITS, CHS, GAPDH and β-TUB2 sequences, PPRI 24314 isolate was identified as *C. fructicola* and PPRI 24315 as *C. kahawae* subsp. *cigarro.* Recently, *C. kahawae* subsp. *cigarro* has been classified as *C. cigarro* (Cabral et al. 2020). Although *Colletotrichum* spp. have been identified on

Eucalyptus spp. in South Africa (Viljoen et al. 1992; Smith et al. 1998), these isolates were no longer available for inclusion in this study. Few studies have successfully distinguished between the subspecies *C. kahawae* subsp. *kahawae* and *C. kahawae* subsp. *cigarro* on the basis of gene analyses of concatenated ATC, ITS, CHS, GAPDH, *β*-TUB2, calmodulin (CAL) genes (Batista et al. 2017). Failure to distinguish *C. kahawae* to the subspecies level has serious biosecurity implications considering that *C. kahawae* subsp. *kahawae* is listed as a quarantine pathogen outside the African continent (Batista et al. 2017). However, gene regions such as the glutamine synthetase (GS) (Weir et al., 2012), mating type1-2-1 (MAT1-2-1) and a fragment of DNA lyase Apn2 (Apn25L) (Silva et al., 2012) are more robust and can reliably distinguish species within *C. kahawae* clade. Additional biochemical assays confirmed the identity of isolate PPRI 24315 to be *C. kahawae* subsp. *cigarro* as it utilised either ammonium tartrate or citric acid as a sole carbon source. This is another distinct characteristic separating it from *C. kahawae* subsp. *kahawae,* which cannot metabolise any of the aforementioned substances as sole carbon sources (Waller et al. 1993).

Colletotrichum kahawae subsp. *cigarro* has been reported on various hosts including coffee (*Coffea arabica* L.), *Proteaceae* cut-flowers, tree tomato (*Solanum betaceum* Cav.), blackberry (*Rubus glaucus* Benth.), olives (*Olea europaea* L.), mango (*Mangifera indica* L.) and mandarin (*Citrus reticulate* Blanco) (Liu et al. 2013; Afanador-Kafuri et al. 2014; Mosca et al. 2014; Ismail et al. 2015; Perrone et al. 2016; Rojas et al. 2018). Previous studies have reported *C. fructicola* causing anthracnose lesions on other hosts including spotted laurel (*Aucuba japonica* Thunb.), chilli (*Capsicum* spp.), pear (*Pyrus bretschneideri* Rehder), apple (*Malus pumila* Miller), tea (*Camellia sinensis* (L.) Kuntze), cassava (*Manihot esculenta* Crantz) and fatsia (*Fatsia japonica* (Thunb.) Decne. and Planch.) (Sharma and Shenoy, 2014; Li et al. 2016; Bragança et al. 2016; Shi et al. 2017, 2018; Nodet et al. 2019).

This study demonstrated that the two seed-borne fungi, *C. fructicola* and *C. cigarro*, significantly reduced seed germination of all *Eucalyptus* species compared to non-inoculated controls. Seed inoculated with *Colletotrichum* spp. resulted in a significant increase in seedling disease in *E. badjensis*, *E. benthamii*, *E. dunnii*, *E. globulus*, *E. grandis*, *E. macarthurii*, *E. nitens*, *E. pellita*, *E. tereticornis* and *E. viminalis*.

Detached leaf assays and greenhouse pot assays provided evidence for the potential of both *C. fructicola* and *C. cigarro* to cause anthracnose leaf spots on *E. dunnii*, *E. nitens* and *E. viminalis* seedlings. Similarly, several *Eucalyptus* species and hybrids grown in plantations in Mpumalanga and KwaZulu-Natal provinces of South Africa are susceptible to anthracnose leaf spots (Smith et al. 1998). Although previously reported to be caused by *C. gloeosporioides*, anthracnose leaf spots were prominently reported on *Eucalyptus* cuttings and on *E. dives*, *E. grandis* and *E. globulus* seedlings (Viljoen et al. 1992). Pathogenicity investigations in this study showed that *E. grandis* and *E. macarthurii* were not susceptible to anthracnose disease caused by both *C. fructicola* and *C. cigarro*. It is possible that *Colletotrichum* species entered an extended quiescent phase or were endophytes as previously reported on other hosts (Cannon et al. 2012; O'Connell et al. 2012; Ranathunge et al. 2012; Manamgoda et al. 2013), which might have caused asymptomatic infection of *E. grandis* and *E. camaldulensis.* Smith et al. (1998) reported *C. gloeosporioides* causing leaf spots and dieback on trees exposed to environmental stress such as drought, frost and hot winds. Hence, it is possible that growth conditions in our study were not sufficiently stressful to allow similar development of disease symptoms.

In conclusion, this study demonstrated that *C. fructicola* and *C. cigarro* are both seed-borne and seed-transmitted but their overall impact on *Eucalyptus* seedling production in nurseries is not clear at this stage. Although pathogenicity tests showed high infection and

more severe anthracnose leaf spots on *Eucalyptus* leaves, this was derived with artificially inoculated seeds. Effects of seed-borne pathogens on plant growth and development depend on its transmissibility, but seed health tests showed that natural incidences of *Colletotrichum* spp. on *Eucalyptus* seed were low. Thus, local transmission via the seed pathway may not be as important as airborne dispersal. However, we illustrated the potential of *Eucalyptus* seed to transmit *Colletotrichum* spp. and subsequently cause disease in seedlings. Caution is needed to reduce the chance of long distance dispersal through infected seed in the trade of seed.

Conflict of Interest: The authors declare that they have no conflict of interest.

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