Plant Pathology (2004) **53**, 414–421 Doi: 10.1046/j.1365-3059.2004.01014.x

Ceratocystis species infecting stem wounds on *Eucalyptus grandis* in South Africa

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Reports of *Ceratocystis* spp. causing disease of exotic plantation hardwood species have increased in recent years. *Ceratocystis fimbriata* causes wilt and canker on *Eucalyptus* spp. in Africa and South America, and *C. albofundus* results in wilt and death of *Acacia mearnsii* in Africa. *Ceratocystis* spp. generally infect wounds on trees, and artificial stem wounding can thus be used to determine the presence of these fungi. The aim of this study was to identify *Ceratocystis* spp. infecting wounds on *Eucalyptus grandis* in South Africa. Isolated *Ceratocystis* spp. were identified using morphological characteristics and comparisons of DNA sequence data for the ITS and 5·8S regions of the rRNA operon. Pathogenicity trials were conducted in the greenhouse to determine the possible role that these *Ceratocystis* spp. could have in disease development. These trials were also conducted under field conditions. Three *Ceratocystis* spp. were collected: *C. fimbriata*, *C. moniliformis* and *C. pirilliformis*. This is the first report of *C. fimbriata* and *C. pirilliformis* from *Eucalyptus* spp. in South Africa, and the first report of the latter fungus outside Australia. Both *C. fimbriata* and *C. pirilliformis* caused significant lesions on inoculated *E. grandis* trees. This is the first evidence that *C. pirilliformis* is a pathogen of *Eucalyptus* spp. From the results of both greenhouse and field trials, it has the potential to cause serious disease problems in *Eucalyptus* plantations.

Keywords: canker, forestry, pathogens, phylogeny, vascular wilt, wounds

Introduction

Ceratocystis species and their *Thielaviopsis* anamorphs are well known pathogens of agricultural and forestry crops worldwide (Kile, 1993; Paulin & Harrington, 2000). They cause a wide range of disease symptoms including stem cankers, root and fruit rots, as well as vascular wilts. Important tree diseases caused by *Ceratocystis* spp. and their anamorphs include oak wilt in the USA (Henry *et al*., 1944; Sinclair *et al*., 1987); canker and death of *Platanus* spp. in Europe (Walter, 1946; Walter *et al*., 1952); stone fruit tree diseases (De Vay *et al*., 1963); wilt of *Nothofagus cunninghamii* in Tasmania (Kile & Walker, 1987); and canker of coffee and *Gmelina arborea* (Pontis, 1951; Muchovej *et al*., 1978).

Ceratocystis spp. were first reported occurring on exotic hardwood plantation tree species in the late 1980s, when *C. fimbriata* was reported to cause canker and death of *Acacia decurrens* trees in Brazil (Ribeiro *et al*., 1988). Shortly thereafter, this fungus was reported as a pathogen of *A. mearnsii* trees in South Africa (Morris *et al*., 1993), but the fungus was later recognized as a distinct species now known as *C. albofundus* (Wingfield *et al*., 1996). The first report of a *Ceratocystis* sp. causing disease on *Eucalyptus* spp. was in 1999, when *C. fimbriata* was described as the cause of rapid wilt and death of *Eucalyptus* spp. in the Republic of Congo and Brazil (Roux *et al*., 1999). This was followed by a report of *C. fimbriata* causing wilt of *Eucalyptus grandis* in Uganda (Roux *et al*., 2001) and, more recently, in Uruguay (Barnes *et al*., 2003a).

Three *Ceratocystis* ssp. other than *C. fimbriata* have been reported from *Eucalyptus* spp. They are *C. eucalypti* (Kile *et al*., 1996), *C. moniliformopsis* (Yuan & Mohammed, 2002) and *C. pirilliformis* (Barnes *et al*., 2003b), all of which occur in Australia. *Ceratocystis eucalypti* and *C. moniliformopsis* were described as nonpathogenic wound colonists that may cause vascular stain in the case of *C. eucalypti*. Nothing is known regarding the infection biology or importance of *C. pirilliformis*, although it is phylogenetically closely related to the pathogenic *C. fimbriata* (Barnes *et al*., 2003b).

Ceratocystis albofundus is the only *Ceratocystis* sp. recorded as a pathogen of plantation forestry trees in South Africa. It is considered to be the most important pathogen of exotic *A. mearnsii* trees, resulting in rapid

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Accepted 21 February 2004

wilt and death after wounding (Morris *et al*., 1993; Roux & Wingfield, 1997). Although *Eucalyptus* spp. are widely planted in South Africa, and *C. fimbriata* is recognized as a pathogen of these trees elsewhere, including other African countries, the pathogen has not been reported in this country. The only other reports of *Ceratocystis* spp. from South Africa are from agronomic crops (Crous *et al*., 2000).

Ceratocystis spp. are known to infect freshly made wounds on trees, and species such as *C. eucalypti* and *C. pirilliformis* have been described from artificially inflicted wounds on *Eucalyptus* spp. (Kile *et al*., 1996; Barnes *et al*., 2003b). To determine the presence and identity of *Ceratocystis* spp. on *Eucalyptus* trees in South Africa, artificial stem wounds were made on *E. grandis* trees. More specifically, the aim was to assess the potential threat of *Ceratocystis* spp. to intensively propagated *Eucalyptus* spp. in South Africa. Isolates were identified using morphological characters and comparisons of DNA sequence data, and their pathogenicity was determined in inoculation experiments.

Materials and methods

Collection of isolates

Stem-wounding trials similar to those described by Barnes *et al*. (2003b) were established on ≈ 5-year-old *E. grandis* trees at four sites in South Africa. These were in the Mpumalanga Province near the towns of Sabie (S25°03·242′–E030°51·680) and Bushbuckridge (S24°56·829′–E030°55·213′), and in the Kwazulu-Natal Province near the towns of Paulpietersburg (S27°31·843′–E030°48·123′) and Richardsbay (S28°42·747′–E032°07·753′). Twenty trees were randomly selected in a single compartment at each site and wounds made on the stems 1·5 m above ground. These wounds consisted of 10 cm^2 sections of bark removed from the stems of trees to expose the cambium. For each wound a horizontal slit into the wood (5 mm deep) was also made in the centre of the wound. Samples were collected from the wounds at monthly intervals starting 8 weeks after wounding. This was done by removing ≈ 5 mm thick pieces of wood from the wound sites. Sections of wood and bark from below and above the wounds, displaying blue/brown discoloration, were collected from each tree. Samples from each tree were placed in separate paper bags and transported to the laboratory.

Two isolation techniques were used to determine the presence of *Ceratocystis* spp. Sections of the wood samples were first inspected microscopically for *Ceratocystis* spp. on their surfaces. Samples were then placed in moist chambers to induce further sporulation. Sections of stained wood (2 × 2 cm) were also wrapped between two slices of carrot (5 mm thick) and placed in plastic bags that were incubated at room temperature (20–25°C) for 10–14 days to bait for *Ceratocystis* spp. (Moller & DeVay, 1968). Carrot slices were surface disinfected before use in sterile water containing streptomycin, to inhibit bacterial growth.

When ascomata developed on the surfaces of the wood samples or carrot slices, single-ascospore masses were transferred to 2% malt extract agar (MEA; Biolab, Midrand, South Africa) containing streptomycin sulphate (0·001 g vol^{-1}, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and incubated at 25°C under natural day/night conditions. All isolates collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Identification of isolates

Morphology and comparisons of DNA sequence data were used to identify the isolates collected. Isolates were grown on MEA until sporulation commenced. Ascomata were mounted in lactophenol on microscope slides and examined using a Zeiss Axiocam light microscope. The shape of ascospores and the shape and ornamentation of ascomatal bases were the primary morphological characteristics used to distinguish between isolates of *Ceratocystis* spp. (Hunt, 1956; Upadhyay, 1981; Wingfield *et al*., 1996; Barnes *et al*., 2003b).

Cultures (CMW9043, 9590, 10136, 10314, 11722, 11723, 12280), selected after identification based on morphology (Table 1), were grown in 1000 μL malt extract broth in Eppendorf tubes for 1–2 weeks to obtain mycelium for DNA extraction. Mycelial mats were separated from the broth by centrifugation at 13 000 ***g*** and transferred to new Eppendorf tubes to which 500 μL extraction buffer was added (200 mM Tris-HCL, 250 mM NaCl, 25 mM EDTA, 0·5% SDS). The mycelium was ground to a fine powder in liquid nitrogen using a pestle, and DNA was isolated using a modified version of the method described by Raeder & Broda (1985). The DNA was resuspended in 100 μL sterile water and stored at –20°C until use.

For DNA sequencing the internal transcribed spacer regions (ITS) and 5·8S gene of the ribosomal RNA operon were amplified using primers ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). The polymerase chain reaction (PCR) mixtures included 0·04 U μL^{-1} Expand (High Fidelity PCR system, Roche Diagnostics GmbH, Mannheim, Germany), 0·2 mM dNTPs, 10× buffer, 1 mM $MgCl_2$ supplied by the manufacturer, 0·75 mM of each primer and ≈ 2 ng μL^{-1} DNA. The PCR reaction conditions included the following steps: an initial denaturation at 96°C for 1 min, followed by 35 cycles of primer annealing at 55°C for 30 s, chain elongation at 72°C for 1 min and denaturation at 92°C for 1 min. A final chain-elongation step was included at 72°C for 5 min. PCR products were visualized in agarose gels stained with ethidium bromide under UV illumination. Products of successful amplifications were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and stored at –20°C until further use.

PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit with Amplitaq DNA

Table 1 *Ceratocystis* species used in DNA sequence analysis and pathogenicity trials

Species	Host	Geographic origin	Genebank no.	CMW no.[a]
Ceratocystis albofundus	*Acacia mearnsii*	South Africa	AF264910	2148
	A. mearnsii	South Africa	AF043605	2475
Ceratocystis fimbriata	–	Papua New Guinea	AF264904	1547
	Platanus sp.	Switzerland	AF395681	1896
	Platanus sp.	France	AF395679	2219
	Platanus sp.	France	AF043604	2220
	Platanus sp.	Italy	AF264903	2242
	Populus sp.	Canada	AF395696	2901
	Populus sp.	USA	AF395694	2913
	Populus sp.	USA	AF395695	3205
	A. mearnsii	South Africa	–	4102
	Citrus sp.	Colombia	AF395688	4829
	Soil from coffee plantation	Colombia	AY177235	5768
	Coffea sp.	Colombia	AY177238	10844
	Soil from coffee plantation	Colombia	AY177236	10875
	Eucalyptus grandis	Brazil	AF395683	4903
	E. grandis	Uganda	AF395687	5312
	E. grandis	Republic of Congo	AF264905	4769
	E. grandis	Republic of Congo	AF395684	4793
	E. grandis	Uruguay	AF453438	7383
	E. grandis	South Africa	–	10312[bc]
	E. grandis	South Africa	–	10313[bc]
	E. grandis	South Africa	AY428863	10314[bc]
	E. grandis	South Africa	AY428862	12280[b]
Ceratocystis moniliformis	*E. grandis*	South Africa	AY431101	9590
	E. grandis	South Africa	AY428867	10136[b]
	Erythrina sp.	South Africa	AF043597	3782
Ceratocystis pirilliformis	*E. grandis*	South Africa	AY428866	9043[c]
	E. grandis	South Africa	AY428864	11722[bc]
	E. grandis	South Africa	AY428865	11723[bc]
	E. nitens	Australia	AF427104	6569
	E. nitens	Australia	AF427106	6574
Petriella setifera	Rock hyrax dung	Kenya	AF043596	–

[a]CMW numbers refer to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
[b]Sequenced in this study.
[c]Used in pathogenicity trials.

polymerase, FS (Perkin-Elmer, Warrington, UK), according to the manufacturer's protocol, on an ABI PRISM 377 DNA Autosequencer (Perkin Elmer). For sequencing the ITS regions, primers ITS1 and ITS4 were used. All sequences were analysed with SEQUENCE NAVIGATOR ver. 1·0·1 (Perkin Elmer Applied Biosystems, Foster City, CA, USA). DNA sequences were compared with those published by Barnes *et al*. (2003a, 2003b), Marin *et al*. (2003), and with sequences obtained from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov).

DNA sequences were manually aligned by the insertion of gaps and analysed using PAUP 4·0 (Swofford, 1998). Gaps were treated as a 'fifth character' (newstate). Analysis was done using parsimony with dendrograms generated by heuristic searches with simple addition and tree bisection reconnection (TBR) branch swapping and MULPAR effective. Bootstrap values of the branch points were generated using the heuristic search option and 1000 replicates (Felsenstein, 1988). *Petriella setifera* (AF043596) was used as an outgroup for the analysis. This taxon was treated as a monophyletic sister group to the ingroup.

Pathogenicity tests

Glasshouse inoculation studies were conducted on an *E. grandis* clone (ZG14) known to be susceptible to a number of pathogens. Three South African isolates of *C. fimbriata* (CMW10312, 10313, 10314) and one isolate of *C. pirilliformis* (CMW9043) were used in the first trial. Twenty trees were inoculated for each test strain, and an equal number of trees were inoculated with sterile agar plugs to serve as controls. Isolates were grown on 2% MEA for 14 days and then inoculated onto the stems of 1-year-old trees (≈ 1 cm diameter). This was accomplished by removing a disc of bark (6 mm diameter) to expose the cambium on the stems of each tree using a cork borer. A plug of mycelium of equal size, overgrown with the test fungus, was placed into each wound with the mycelium facing the

cambium. The wounds and plugs were sealed with Parafilm to protect them against desiccation. The greenhouse trial was repeated once including additional *C. pirilliformis* isolates (CMW11722, CMW11723).

Pathogenicity trials were also conducted once under field conditions in South Africa, on trees of a 1-year-old *E. grandis* clone. Three *C. fimbriata* (CMW10312, CMW10313, CMW10314) and three *C. pirilliformis* (CMW9043, CMW11722, CMW11723) isolates were tested using the same technique as described for the greenhouse inoculations. Stem diameters of inoculated trees at inoculation height were ≈ 10 cm at the time of inoculation. The trial was situated in a commercial *Eucalyptus*-growing area near the town of Kwambonambi in the Kwazulu-Natal Province. This area is classified as subtropical with annual rainfall exceeding 1000 mm per annum, and high temperatures and relative humidity. The field trial was not repeated.

Lesion lengths were assessed 6 weeks after inoculation. Both bark lesions and cambial lesions were recorded at the time of scoring. Reisolations were made from lesions to meet the requirements of Koch's postulates. Data were analysed using programmes in SAS (SAS Institute Inc., 1999). All data were tested for agreement with the normal distribution and analysed according to the general linear model (GLM). Confidence limits were determined at 95%.

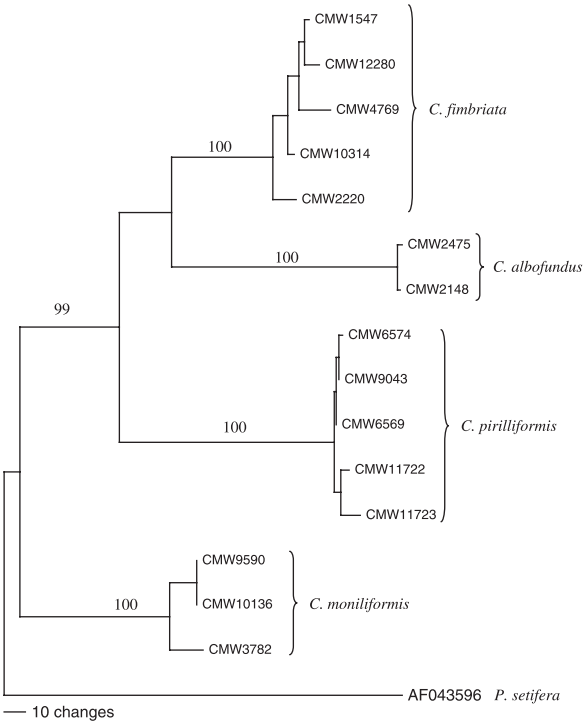

Figure 1 Phylogram obtained from sequence data of the ITS region of various *Ceratocystis* spp. Bootstrap values are indicated above the branches. *Petriella setifera* was treated as outgroup in the analysis.

Results

Collection of isolates

Ceratocystis spp. were commonly isolated from wood samples at all four sites. Ascocarps were found sporulating on the carrot slices as well as on the wood samples placed in moist chambers. The majority of *Ceratocystis* spp. were obtained from the cambium, just below the bark, and most were from the Sabie and Paulpietersburg sites.

Identification of isolates

The *Ceratocystis* spp. isolated from *E. grandis* all had hat-shaped ascospores and *Thielaviopsis* anamorphs. Based on the morphology of the ascomatal bases, they could easily be divided into those with spines on the bases; those with round to oval bases; and those with pear-shaped bases. Using morphology the isolates were identified as *C. pirilliformis*, *C. moniliformis* and *C. fimbriata*. The latter was the most commonly isolated species, followed by *C. pirilliformis* and *C. moniliformis*. At the Bushbuckridge site, however, *C. pirilliformis* was the most common species obtained.

Manual alignment of the ITS and 5·8S sequences obtained for the *Ceratocystis* isolates sequenced, resulted in a total of 585 characters. Four most parsimonious phylograms, differing only in their internal arrangements but with similar topologies, with tree lengths of 705, were obtained from the heuristic search. One of these was chosen for representation (Fig. 1). Of the characters analysed, 302 were parsimony informative, 83 parsimony uninformative, and 200 constant. The consistency index (CI), retention index (RI) and rescaled consistency index (RC) values were 0·8170, 0·8995 and 0·7349, respectively.

Isolates CMW9043, CMW11722 and CMW11723 grouped with *C. pirilliformis* isolates from Australia with a 100% bootstrap value. The *C. fimbriata* (CMW-10314, 12280) and *C. moniliformis* (CMW9590, 10136) isolates, grouped within their respective clades with bootstrap values of 100%, confirming morphological identification.

A separate analysis with only *C. fimbriata* isolates produced six most parsimonious phylograms that differed only in internal arrangement. One such, with CI = 0·7834, RI = 0·8914, RC = 0·7230, was selected for representation (Fig. 2). Alignment resulted in 520 characters of which 104 were parsimony informative, 23 variable characters parsimony uninformative and 393 constant. Midpoint rooting was used for the analysis. *Ceratocystis fimbriata* isolates from North America grouped separately from all other *C. fimbriata* isolates with a bootstrap value of 100%. The second major clade consisted of isolates from Colombia (76% bootstrap), while the third clade was made up of a mixture of isolates from different continents and hosts. Within this clade, *C. fimbriata* isolates from Europe grouped together with 100% bootstrap support. South African *C. fimbriata* isolates grouped within two separate subclades including *C. fimbriata* isolates from *Eucalyptus* spp. in Africa and South America.

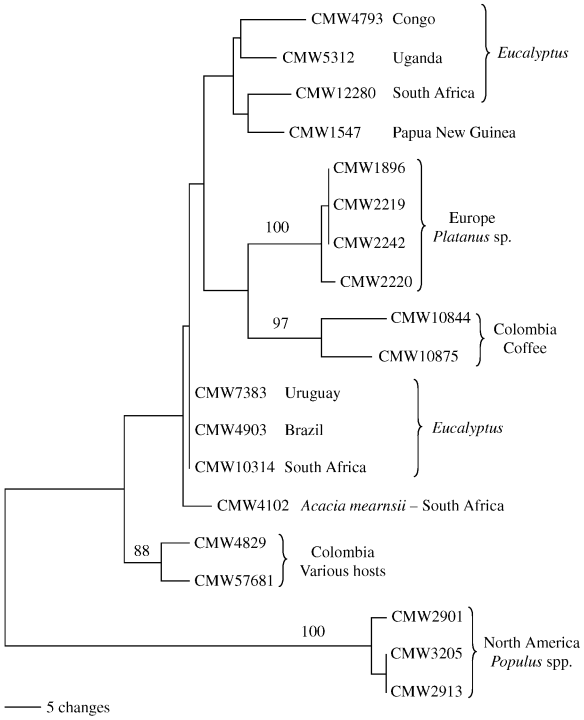

Figure 2 Phylogram obtained from sequence data of the ITS region of various *Ceratocystis fimbriata* isolates. Bootstrap values are indicated above the branches.

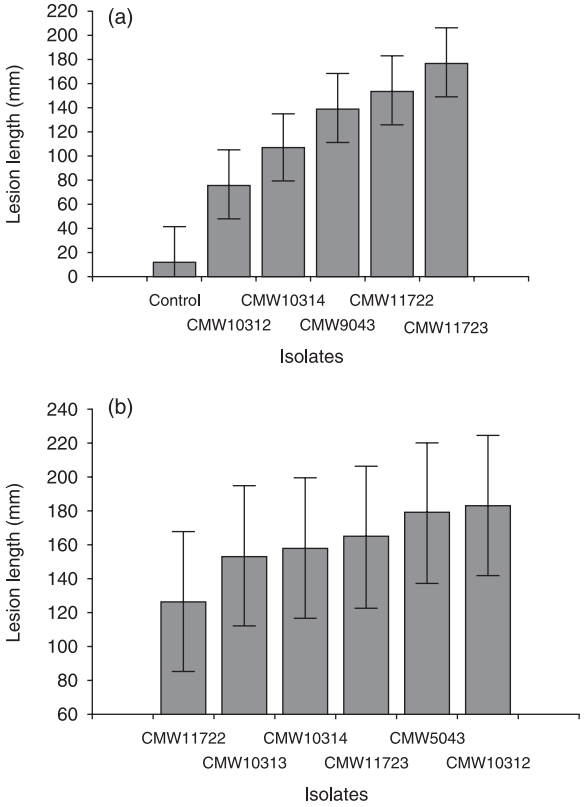

Figure 4 Histogram showing results of artificial inoculation trials with *Ceratocystis fimbriata* (CMW10312, 10313, 10314) and *Ceratocystis pirilliformis* (CMW9043, 11722, 11723) on *Eucalyptus grandis* clones in the field and greenhouse. Results of (a) second greenhouse inoculation trial, $R = 0{\cdot}44$, CV = 56·33, $P < 0{\cdot}0001$; (b) field inoculation trial, $R = 0{\cdot}04$, CV = 57·9, $P < 0{\cdot}0001$, confidence limit = 95%.

Pathogenicity studies

Six weeks after inoculation both *C. fimbriata* and *C. pirilliformis* gave rise to distinct lesions on the stems of inoculated *E. grandis* trees, under both greenhouse and field conditions (Fig. 3). In the greenhouse some trees were already completely girdled and producing epicormic shoots. Significant differences ($P < 0{\cdot}0001$) in lesion lengths were found between the *C. fimbriata* and *C. pirilliformis* isolates when compared to the control inoculations (Fig. 4). The differences between the *Ceratocystis* isolates and the control inoculations were supported by the confidence limits, with confidence bars showing no overlap

Figure 3 Disease symptoms on a *Eucalyptus grandis* clone after inoculation with *Ceratocystis fimbriata* and *Ceratocystis pirilliformis* under field conditions. (a) Bark discoloration caused by *C. fimbriata* isolate; (b) extensive discoloration of the cambium after inoculation with *C. pirilliformis*.

between the controls and the fungal isolates. In greenhouse experiments *C. pirilliformis* isolates produced extensive lesions in the bark of the inoculated trees, while the *C. fimbriata* isolates produced only small lesions in the bark and extensive lesions in the sapwood. The control inoculations produced no lesions in the outer bark or in the xylem, and all had started producing callus tissue around the wounds to heal them. Reisolations from the lesions consistently yielded the inoculated fungi.

Under field conditions, symptoms associated with inoculation were similar for *C. fimbriata* and *C. pirilliformis*, with both producing small or no lesions in the bark and large lesions in the cambium and xylem (Fig. 3). Reisolations from the lesions consistently yielded the inoculated fungi.

Discussion

Wounds artificially inflicted on *E. grandis* stems were used in this study to show that *Ceratocystis* spp. commonly infect freshly made wounds on these trees in South Africa. Of greater significance is the fact that the important *Eucalyptus* pathogen *C. fimbriata* appears to be common in plantations of this tree in South Africa. *Ceratocystis fimbriata* is generally not easily isolated from diseased tissue unless specific techniques are used, and it is possible that this fungus, as well as *C. pirilliformis*, has been the cause of many unexplained deaths of *Eucalyptus* in the country. Results of this study represent the first report of *C. fimbriata* and *C. pirilliformis* from *Eucalyptus* spp. in South Africa. This is also the first report of *C. pirilliformis* from a country outside Australia.

The results showed that both *C. fimbriata* and *C. pirilliformis* are present in all four geographic areas considered, and that they are widespread in South Africa. Pathogenicity tests have not previously been conducted with *C. pirilliformis*. Interestingly, the trials have shown that this fungus is pathogenic and that levels of pathogenicity on *Eucalyptus* appear to be equal to *C. fimbriata*, which is an important *Eucalyptus* pathogen. *Ceratocystis pirilliformis* was first isolated from Australia where it was discovered on artificially made wounds on *E. nitens* (Barnes *et al*., 2003b). Its occurrence in South Africa raises the question as to the origin of this fungus. It is most likely that *C. pirilliformis* is native to Australia, where it would have coevolved with *Eucalyptus* spp., explaining why no disease symptoms have been associated with the fungus in that country. Assuming that it was introduced from Australia, the fact that it is already widely spread in South Africa suggests that it has probably been in the country for an extended period. Detailed population genetic studies are planned to consider the origin of *C. pirilliformis*.

Although it has been known from wounds on *Eucalyptus* trees for a number of years (M.J.W., unpublished data), this study represents the first published report of *C. moniliformis* from *Eucalyptus* spp. Compared with *C. fimbriata* and *C. pirilliformis*, only a relatively small number of *C. moniliformis* isolates were obtained in this study. This fungus was most common on fresh wounds, and it was difficult to isolate from older wounds. It is known, however, to occur on a wide range of plants including woody angiosperms, fruits, vegetables and other crops (Grylls & Seifert, 1993), but not as a pathogen.

Ceratocystis fimbriata was the most common of the three *Ceratocystis* spp. isolated in this study, and it was found at all sites where collections were made. A limited number of reports of *C. fimbriata* exist for the African continent. In South Africa *C. fimbriata* was first mentioned as a pathogen of *Protea* spp. (Gorter, 1977), but this is now recognized to be a record of *C. albofundus* (Wingfield *et al*., 1996). The only other reports of *C. fimbriata* from the African continent are from *Crotalaria* sp. in the Ivory Coast (Davet, 1962), and from *Hevea* sp. in Uganda and the Democratic Republic of Congo (Ringoet, 1923; Snowden, 1926; Anonymous, 1948). More recent records are those of *C. fimbriata* causing disease of *Eucalyptus* spp. in the Republic of Congo (Roux *et al*., 1999) and Uganda (Roux *et al*., 2001). A single isolate of the fungus was also found on the stump of an *A. mearnsii* tree in South Africa in 1996 (Roux, 1998) and 2003 (J.R., unpublished data). Results of the present study show clearly that *C. fimbriata* is much more common in Africa, especially in South Africa, than previously recognized.

The *Ceratocystis* spp. isolated from *Eucalyptus* wounds in this study are all characterized by hat-shaped ascospores. There are only six *Ceratocystis* spp. with hat-shaped ascospores described to date. They are *C. acericola*, *C. albofundus*, *C. fimbriata*, *C. moniliformis*, *C. moniliformopsis* and *C. pirilliformis* (Upadhyay, 1981; Wingfield *et al*., 1996; Yuan & Mohammed, 2002; Barnes *et al*., 2003b). These species can, however, be distinguished from each other, especially based on ascomatal shape, size and ornamentation. The easiest of these species to distinguish from others are *C. albofundus*, which is the only species with light to cream coloured ascomatal bases (Wingfield *et al*., 1996), and *C. acericola* which has no ostiolar hyphae or known anamorph (Upadhyay, 1981). *Ceratocystis fimbriata* is characterized by round to oval-shaped ascomatal bases without basal spines and with convergent ostiolar hyphae (Upadhyay, 1981). *Ceratocystis pirilliformis* has distinctly pear-shaped ascomatal bases without basal spines and with convergent ostiolar hyphae (Barnes *et al*., 2003b). Both *C. moniliformis* and *C. moniliformopsis* have pigmented spines ornamenting the ascomatal bases, and the bases of the necks are disciform (Hunt, 1956; Upadhyay, 1981; Yuan & Mohammed, 2002). The anamorph of *C. moniliformopis* is, however, distinct from *C. moniliformis* in that it has phialides with percurrent proliferation in addition to those with cylindrical collarettes (Yuan & Mohammed, 2002). Morphological characteristics can thus be relatively easily used to distinguish between the three *Ceratocystis* spp. isolated from wounds on *Eucalyptus* spp. in South Africa.

Inoculations in this study provide the first evidence that *C. pirilliformis* is a pathogen of *Eucalyptus* spp. Under greenhouse conditions it produced lesions similarly sized or larger than those associated with the known *Eucalyptus* pathogen *C. fimbriata*. Clear differences were also observed

in the symptoms produced by these two fungi. *Ceratocystis fimbriata* is known to cause root rot, stem cankers and vascular wilt diseases of numerous plant hosts (Kile, 1993). On *Eucalyptus* spp. in Africa it causes a vascular wilt. This symptom was confirmed in the greenhouse experiments, where distinct vascular staining was observed. In contrast, *C. pirilliformis* produced no vascular streaking in either of the greenhouse trials. Rather, it produced rapidly developing discoloration of the bark and sapwood/phloem of trees. Under field conditions, symptoms associated with the two fungi were more similar, with little bark discoloration. However, *C. pirilliformis* produced more discoloration associated with superficial tissues than *C. fimbriata* with its vascular staining.

DNA sequence data generated in this study confirmed the identification of the three *Ceratocystis* spp. based initially on morphological characteristics. These data also provided confirmation of previous phylogenetic evidence (Barnes *et al*., 2003b) showing that *C. pirilliformis* resides in a subclade with *C. fimbriata* and *C. albofundus*, which is distant from other *Ceratocystis* spp. Species within this clade are all important plant pathogens. It has been suggested that *C. fimbriata* represents a species complex (Webster & Butler, 1967; Harrington & Baker, 2002; Marin *et al*., 2003). Recent work shows a clear phylogenetic distinction between isolates of *C. fimbriata* originating from North America, South America and Asia (Harrington, 2000; Harrington & Baker, 2002), while previous studies using biological, DNA sequence and simple sequence repeat (SSR) data (Webster & Butler, 1967; Barnes *et al*., 2001; Harrington & Baker, 2002) also show that *C. fimbriata* isolates have some degree of host specificity.

The South African isolates of *C. fimbriata* resulting from the current study grouped in two clades distinct from isolates from North America. They were rather more closely related to those from South America, Africa and the rest of the world. They also grouped in a clade made up mostly of isolates from *Eucalyptus* spp., which is consistent with the view that the fungus in some situations might represent host-specialized groups (Webster & Butler, 1967; Harrington, 2000; Barnes *et al*., 2001).

The consistent association of *C. fimbriata* with *E. grandis* recognized in this study is of economic relevance. This fungus is a well known pathogen of forest plantation trees and of agricultural crops. *Ceratocystis* spp. probably play a more important role in diseases of eucalypts than has previously been recognized. Although these fungi have not been isolated from trees showing disease symptoms, wilt and rapid death of *Eucalyptus* spp. is relatively common in South African plantations. Because these fungi are difficult to isolate, it is suspected that they have been overlooked in disease surveys. Further studies are planned to gain a more comprehensive understanding of their importance to forestry in South Africa.

Acknowledgements

We are grateful to the South African Forestry Company Ltd (SAFCOL), SiyaQhubeka Forests (SQF) and Mondi Ltd for providing trial sites that made this study possible. We also thank the members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry, South Africa and the University of Pretoria for financial assistance. Dr Benjamin Eisenberg is thanked for his assistance with the statistical analyses of data from the inoculation studies.

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