Ceratocystis neglecta sp. nov., infecting *Eucalyptus* trees in Colombia

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Commercial plantation forestry utilising species of non-native *Eucalyptus* trees forms an important industry in Colombia. These trees are, however, threatened by fungal diseases. In recent years a number of reports of *Ceratocystis fimbriata* s.l., causing wilt and death of *Eucalyptus* spp. have emerged from African and South American countries. In Colombia, the fungus is a serious pathogen of coffee, cacao and citrus where it enters wounds and causes a severe canker stain disease. *Ceratocystis fimbriata* has, however, not been found on *Eucalyptus* spp. in Colombia and the aim of this study was to consider whether it might infect wounds on these trees in the country. *Eucalyptus grandis* trees were artificially wounded in three different geographic zones of Colombia and a *Ceratocystis* sp. was commonly isolated from these wounds. Isolates of the fungus were identified based on morphology and through comparisons of sequences for the ITS regions of the rDNA operon. Morphological and DNA sequence comparisons showed that isolates from *E. grandis* in Colombia represent a new species of *Ceratocystis*, closely resembling *C. fimbriata* s.s. and for which the name *C. neglecta* sp. nov. is given. To determine the possible impact of *C. neglecta* on commercial forestry operations, two isolates were used in field pathogenicity trials on different clones of *E. grandis*. Isolates were shown to differ in their ability to cause lesions on *E. grandis*, with one isolate being highly pathogenic. The different clones of *E. grandis* also differed in their susceptibility to infection by the fungus.

Key words: fungal disease, plantation forestry, wounds

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Introduction

Colombia has a rapidly growing forestry industry supporting the production of solid wood and paper products. In the past, native trees have been exploited to produce these products, but recent trends are to grow trees for this purpose in intensively managed plantations. Non-native species of *Eucalyptus* and *Pinus* are the most commonly grown trees, and these currently make up approximately 150 000 hectares of plantations. In the case of *Eucalyptus*, large areas have been planted to clones of *E. grandis* Maiden and hybrids of this species with *E. urophylla* S.T. Blake, also known as *E. "urograndis*". Little is, however, known regarding diseases of these *Eucalyptus* trees in Colombia.

Ceratocystis fimbriata Ellis & Halst. s.l. includes some of the most important pathogens of woody plants, causing canker stain and vascular wilt diseases (Kile 1993). During the course of the last decade *C. fimbriata* s.l. has been reported from dying *Eucalyptus* trees with increasing frequency. *Ceratocystis fimbriata* s.l. was first reported as a pathogen of *Eucalyptus* trees in the 1990's after it was isolated from diseased and dying *Eucalyptus* trees in the Republic of Congo (Roux *et al*., 1999). At approximately the same time, it was found killing *Eucalyptus* trees in Brazil (Laia *et al*., 1999; Roux *et al*., 1999). It has since also been reported from dying *Eucalyptus* trees in

Uganda (Roux *et al*., 2001), Uruguay (Barnes *et al*., 2003a) and most recently from artificially wounded *Eucalyptus* trees in South Africa (Roux *et al*., 2004).

Ceratocystis fimbriata s.l. is a serious pathogen of coffee (*Coffea arabica* L.) (Pontis, 1951; Mourichon, 1994; Marin *et al*., 2003) and cacao (*Theobroma cacao* L.) (Garces, 1944; Marin *et al*., 2003) in Colombia. It has also been isolated from the Brazalian fire tree, *Schizolobium parahyba* (Vell.) S.F. Blake, in the country (Marin *et al*., 2003). Because of the importance of coffee to the Colombian economy, *C. fimbriata* s.l. is recognised as one of the most important agricultural pathogens in this country (Castro, 1998). The fungus infects trees via wounds made at the bases of coffee trees during farming operations (Marin *et al*., 2003). Its common occurrence in soil as chlamydospores (Kile, 1993) provides ample opportunity for infection of wounds that are generally at the bases of trees.

The taxonomy of *C. fimbriata* has been a source of considerable debate since its first description in the late 1800's. This confusion commenced with the misidentification of its fruiting bodies as pycnidia when the fungus was first found associated with sweet potato black rot (Halsted, 1890). Subsequent reports of *C. fimbriata* from a wide range of hosts and geographic areas led to further confusion because there is substantial variation in the morphology and pathogenicity of isolates. This led Webster and Butler (1967) to conclude that *C. fimbriata* "is a large and diverse species which consists of numerous strains." The advent of DNA sequencing and other less subjective techniques than morphological comparisons have shown that isolates of *C. fimbriata* s.l. represent phylogenetically distinct and cryptic species. In recent years several new species have thus been reported, all of which would earlier have been identified as *C. fimbriata* (Wingfield *et al*., 1996; Barnes *et al*., 2003b; Van Wyk *et al*., 2004; Baker Engelbrecht and Harrington, 2005; Johnson *et al*., 2005).

Ceratocystis fimbriata s.s. is now recognised to represent the sweet potato black rot pathogen (Baker Engelbrecht and Harrington, 2005) and not the many described and undescribed cryptic species in the *C. fimbriata* s.l. species complex. Many species in this group can be recognised in phylogenetic trees, representing large numbers of isolates, and these have yet to be described. In a recent study, Marin *et al*. (2003), for example showed that a large collection of *C. fimbriata* isolates from coffee, cocoa and citrus trees in Colombia reside in two distinct phylogenetic clades. These fungi are currently referred to as representing *C. fimbriata* s.l., but they apparently represent undescribed species.

There is growing evidence that *C. fimbriata* s.l. should be considered an important constraint to *Eucalyptus* plantation forestry. The wide spread occurrence of this pathogen on coffee and other croups in Colombia, often in areas in close proximity to *Eucalyptus* plantations, is a matter of concern. The aim of this study was, therefore, to determine whether *C. fimbriata* s.l. might occur on *Eucalyptus* spp. in this country. Furthermore, the potential threat of this fungus to *Eucalyptus* forestry in Colombia was considered in artificial inoculation experiments.

Materials and methods

Collection of isolates

Wounds were made on trees at eight farms in three different forestry zones of Colombia. These were at the San Jose and Vanessa farms in the Cauca zone, the Suiza, Cecilia and Samaria farms located in the Valle zone, and the Carolina, Cedral and Angela Maria farms in the Andina zone. At each of the farms in the three zones, twenty to forty trees were selected to be wounded. Wounds were made in June 2002, February 2004 and November 2005 by cutting a patch of bark (10 cm^2) from the stems of trees, to expose the cambium, similar to the method described by Barnes *et al*. (2003b).

Two to eight weeks after wounding wood and bark samples were collected from the wounds, placed in paper packets and transported to the laboratory for analyses. Isolations were made from discoloured wood using a combination of the carrot baiting technique (Moller and DeVay, 1968) and moisture chambering of material. For the carrot baiting technique pieces of wood (~ 2 cm^2) showing staining symptoms were wrapped

tightly between two slices (~ 1 cm thick) of carrot that had been surface disinfested with 70% ethanol. These carrot baits were incubated at 25ºC for up to two weeks and regularly inspected for the presence of *Ceratocystis* ascocarps. When present, the ascospore masses were removed from the apices of the ascocarps and transferred to 2% malt extract agar plates (MEA: 20g malt extract, 15g agar; Biolab Diagnostics Ltd, Midrand, South Africa), containing 100mg Streptomycin sulfate (SIGMA-ALDRICH CHEMIE Gmbh, STEINHEIM, Germany) and incubated at 25ºC. Half of the material collected was placed in plastic bags with moist tissue paper and incubated at 25ºC to induce fungal growth and sporulation. Ascospore drops and mycelium of a *Ceratocystis* sp. was transferred from the moisture chambers to MEA for purification. All isolates obtained were lodged in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Microscope slides bearing structures and dried down cultures have also been lodged in the National Collection of Fungi (PREM), Pretoria, South Africa and representative isolates deposited with the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands.

Morphological characteristics

The *Ceratocystis* isolates collected from wounds were grown on 2% MEA and identified based on morphological characteristics (Upadhyay, 1981). A representative of these isolates (CMW17808) was chosen and fifty measurements of all morphological structures were made after mounting the structures in lactophenol on microscope slides. Measurements are presented as (min-) (average - std. dev.) - (average + std. dev.) (-max). Rayner's (1970) colour charts were used to note the colour of the mycelium and other structures. To determine the optimum growth temperature of the isolates from Colombia, cultures (CMW18194, CMW11284) were grown on 2% MEA for seven days. Mycelial plugs (5 mm) were taken from the actively growing margins of these cultures and transferred to 2% MEA plates for incubation at temperatures ranging from five to 35ºC at 5ºC intervals. Five plates were prepared for each isolate tested at each temperature. Two measurements, perpendicular to each other, were made on the seventh day. The trial was repeated once.

DNA sequence comparisons

Four *Ceratocystis* isolates (CMW11285, CMW11284, CMW17808, CMW18194) from *Eucalyptus* in Colombia were used for DNA sequence comparisons. For DNA extraction, cultures were grown on 2% MEA for two weeks. Mycelial masses were scraped off, placed in an Eppendorf tube and freeze dried overnight. DNA extraction was performed as described by Van Wyk *et al*. (2006).

The Internal Transcribed Spacer regions (ITS1, 2), including the 5.8S rDNA operon were amplified using the polymerase chain reaction (PCR) and using primers ITS1 and ITS4 (White *et al*., 1990). Five ng of the DNA template was added to a 25 µl polymerase chain reaction (PCR) mixture containing 0.2 mM of each dNTP, 0.4 µM of each primer, 1 X FastStart buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme) and FastStart *Taq* enzyme (2 U) (Roche Diagnostics, Mannheim, Germany). The PCR amplification consisted of an initial denaturation step at 96°C for 4 min. This was followed by 10 cycles denaturation at 95°C for 40 s, annealing for 40 s at 55°C and an elongation step for 45 s at 70°C. Subsequently, 30 cycles consisting of 94ºC for 20 s, 55ºC for 40 s with a 5 s extension step, after each cycle and 70ºC for 45 s were performed with a final step of 10 min at 72ºC. PCR products were visualized using UV light after separation on a 2% agarose gel containing ethidium bromide. The products were then purified using 6% Sepha-dex G-50 columns (Steinheim, Germany).

PCR products were sequenced using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems, Foster City, CA) and the same primers as used in the PCR reactions. Sequencing reactions were run on an ABI Prism 3100 DNA sequencer (Applied Bio-Systems).

Sequences for the *Ceratocystis* sp. from *E. grandis* in Colombia were compared with

Table 1. *Ceratocystis* isolates used in DNA sequence comparisons and inoculation studies.

Species	Culture number[a]	Host	Country	GenBank accession number	Collectors
C. albifundus	CMW 4068[b]	*Acacia mearnsii*	South Africa	DQ520638	J. Roux
C. albifundus	CMW 5329	*A. mearnsii*	South Africa	AF388947	J. Roux
C. atrox	CMW 19383	*Eucalyptus* sp.	Australia	EF070414	M.J. Wingfield
C. atrox	CMW 19385	*Eucalyptus* sp.	Australia	EF070415	M.J. Wingfield
C cacaofunesta	CMW 15051	*Theobroma cacao*	Brazil	DQ520636	A.J. Hansen
C cacaofunesta	CMW 14809	*T. cacao*	Ecuador	DQ520637	C. Suarez
C. caryae	C 1829	*Carya* sp.	USA	AY907035	J.A. Johnson
C. caryae	C 1827	*Carya* sp.	USA	AY907034	J.A. Johnson
C. fimbriata s.s	CMW 15049	*Ipomoea batatas*	USA	DQ520629	C.F. Andrus
C. fimbriata s.s	CMW 1547	*I. batatas*	Papua New Guinea	AF264904	E.H.C. McKenzie
C. fimbriata	CMW 4844	*Coffea* sp.	Colombia	AF395691	B.L. Castro
C. fimbriata	CMW 4824	*Coffea* sp.	Colombia	AF395692	B.L. Castro
C. fimbriata	CMW 4829	*Citrus*	Colombia	AF395688	B.L. Castro
C. fimbriata	CMW 4835	*Coffea* sp.	Colombia	AF395689	B.L. Castro
C. fimbriata	CMW 8858	*Schizolobium parahyba*	Colombia	AY233865	B.L. Castro
C. fimbriata	C 1750	Cacao	Colombia	AY157955	N.A[c]
C. fimbriata	CMW 8860	Cacao	Colombia	AY233869	B.L. Castro
C. neglecta	CMW 11284[b]	*E. grandis*	Colombia	EF127988	C. Rodas
C. neglecta	CMW 11285[b]	*E. grandis*	Colombia	EF127989	C. Rodas
C. neglecta	CMW 17808[b]	*E. grandis*	Colombia	EF127990	C. Rodas
C. neglecta	CMW 18194[b]	*E. grandis*	Colombia	EF127991	C. Rodas
C. platani	CMW 1896	*Platanus* sp.	Switzerland	AF395681	O. Petrini
C. platani	CMW 2242	*Platanus* sp.	Italy	AF264903	A. Panconesi
C. pirilliformis	CMW 6569	*E. nitens*	Australia	AF427104	M.J. Wingfield
C. pirilliformis	CMW 6579	*E. nitens*	Australia	AF427105	M.J. Wingfield
C. polychroma	CMW 11424	*Syzygium aromaticum*	Indonesia	AY528970	M.J. Wingfield
C. polychroma	CMW 11436	*S. aromaticum*	Indonesia	AY528971	M.J. Wingfield
C. populicola	C 995	*Populus* sp.	Poland	AY907029	Gremmen
C. populicola	C 685	*Populus* sp.	Canada	AY907028	E. Smalley
C. smalleyi	C 1828	*Carya* sp.	USA	AY907032	J.A. Johnson
C. smalleyi	C 1410	*Carya* sp.	USA	AY907031	T.C. Harrington
C. variospora	C 1965	*Prunus* sp.	USA	AY907045	J.A. Johnson
C. variospora	C 1707	*Betula* sp.	Japan	AY907044	Matsuya
C. virescens	CMW 11164	*Fagus americana*	USA	N.A	D. Houston

[a] CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. ATCC = American Type Culture Collection (ATCC), Manassas, Virginia, USA. CBS = the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands. C = from the collection of T.C. Harrington.
[b] Isolates sequenced in this study.
Dried cultures representing CMW11285 (PREM57511) and CMW11284 (PREM57512) have also been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.
[c] N.A. = not available.

those of closely related *Ceratocystis* spp. obtained from the GenBank [NCBI (National Centre for Biotechnology Information)] nucleotide database (http://www.ncbi.nlm.nih.gov/) are published in previous studies (Barnes *et al*., 2003a; Marin et *al*., 2003; Van Wyk *et al*., 2004; Johnson *et al*., 2005) (Table 1). Sequences were manually aligned using the program Sequence Navigator version 1.0.1 (Applied Biosystems). The alignments were analysed using Phylogenetic Analysis Using Parsimony (PAUP) software, version 4.0b10 (Swofford, 2002). The heuristic search option based on parsimony with random stepwise addition and tree bisection reconnection (TBR) was used. Gaps were treated as fifth character and confidence intervals using 1000 bootstrap replicates were calculated. *Ceratocystis virescens* (R.W. Davidson) C. Moreau was included in the analyses as the out-group.

Pathogenicity tests

Three inoculation trials, using the *Ceratocystis* sp. collected from *Eucalyptus* in Colombia, were conducted in commercial *E.*

grandis plantations in the Valle zone of Colombia. The three plantations were situated on the Buenos Aires Farm, Trujillo, Valle (1994 masl, 1740 mm/y of precipitation, located at 76º21'24" W, 4º13'60" N), the Cedral farm in Darien, Valle (1692 masl, with an average precipitation of 1422 mm/y, located at 76º25'29" W, 4º1'05" N) and the La Suiza farm in Restrepo, Valle (precipitation 1279 mm/y, located at 1553 masl, 76º29'37" W, 3º51'35" N). At each of the three sites, 50 trees each of two clones (clone 301, clone 02) and one seed source (seed lot 211) of *E. grandis* were inoculated with two isolates (CMW 11284, CMW 11285) of the *Ceratocystis* sp. from *E. grandis*. At each site, 50 trees of each of the *E. grandis* clones or trees representing the seed source were also inoculated with sterile agar to serve as a negative control.

The two different clones and the trees representing the seed source were not present uniformly at the three different farms. At La Suiza and Buenos Aires, 50 trees of each of the *E. grandis* clones 301 and 02 were inoculated with the two *Ceratocystis* isolates and the control respectively. At Cedral, the same number of trees were inoculated but only clone 301 was available. Thus, instead of clone 02, 50 trees generated from seed belonging to the seed lot *E. grandis* 211 were used together with clone 301. In all cases, the trees were one-year-old and they were distributed in five blocks with 10 trees of each of the clones or the seed lot, selected for inoculation.

Inoculations were made on the stems of trees ~1 m above the ground using a six millimetre diameter cork borer. This instrument was used to remove a piece of bark from each stem to expose the cambium. A disc of the same size was taken from the edge of a rapidly growing 11-day-old *Ceratocystis* colony and placed into the exposed wound with the mycelium facing the cambium. In order to prevent desiccation, the inoculation sites were covered with tissue paper moistened with sterile water and secured with masking tape.

Internal lesion lengths were recorded in mm after 12 weeks. Data analysis was performed using SAS® (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, Version 8.2, running under VM/ESA on the University of Pretoria mainframe computer). The lack of normality of the data indicated that a non-parametric approach be followed. Kruskal/ Wallis determinations were thus performed with appropriate grouping variables and by specifying the "compare" option.

Results

Morphological characteristics

A *Ceratocystis* sp. (Fig. 1), resembling *C. fimbriata* s.l. in culture, was collected from wounds on *E. grandis* trees in La Suiza and Buenos Aires. Morphological characteristics of these isolates were typical for the genus *Ceratocystis* (Upadhyay, 1981), but some differences were observed. Compared to *C. fimbriata* s.s., the *Ceratocystis* sp. from *Eucalyptus* in Colombia had longer ascomatal necks and two morphological forms of phialides were observed, where isolates of *C. fimbriata* s.s. has only one phialide type. Furthermore, the Colombian isolates produced barrel-shaped conidia in chains, as well as cylindrical hyaline conidia. This is in contrast to isolates of *C. fimbriata* s.s. that do not produce barrel shaped conidia (Baker Engelbrecht and Harrington, 2005).

Cultures from *E. grandis* covered the plates in eight to 15 days and had a strong fruity aroma characteristic of many *Ceratocystis* spp. Growth studies in culture showed that the Colombian isolates have a growth optimum at 25°C, with colonies reaching 35 mm in seven days at this temperature. No growth was observed at 5, 10 or 35°C.

DNA sequence comparisons

The phylogenetic data set consisted of 33 in-group taxa, with the sequence of *C. virescens* defined as a monophyletic sister out-group taxon (Fig. 2). This data set consisted of 583 sequence characters of which 62 were parsimony-uninformative and 185 were parsimony-informative. Thirty trees were obtained from the heuristic search and one was selected for presentation (Fig. 2). The tree had a length of 439 steps, with a Consistency Index (CI) of 0.7312, a Retention Index (RI) of 0.8590 and Re-scaled Consistency Index (RC) of 0.6281 (TreeBase SN3474).

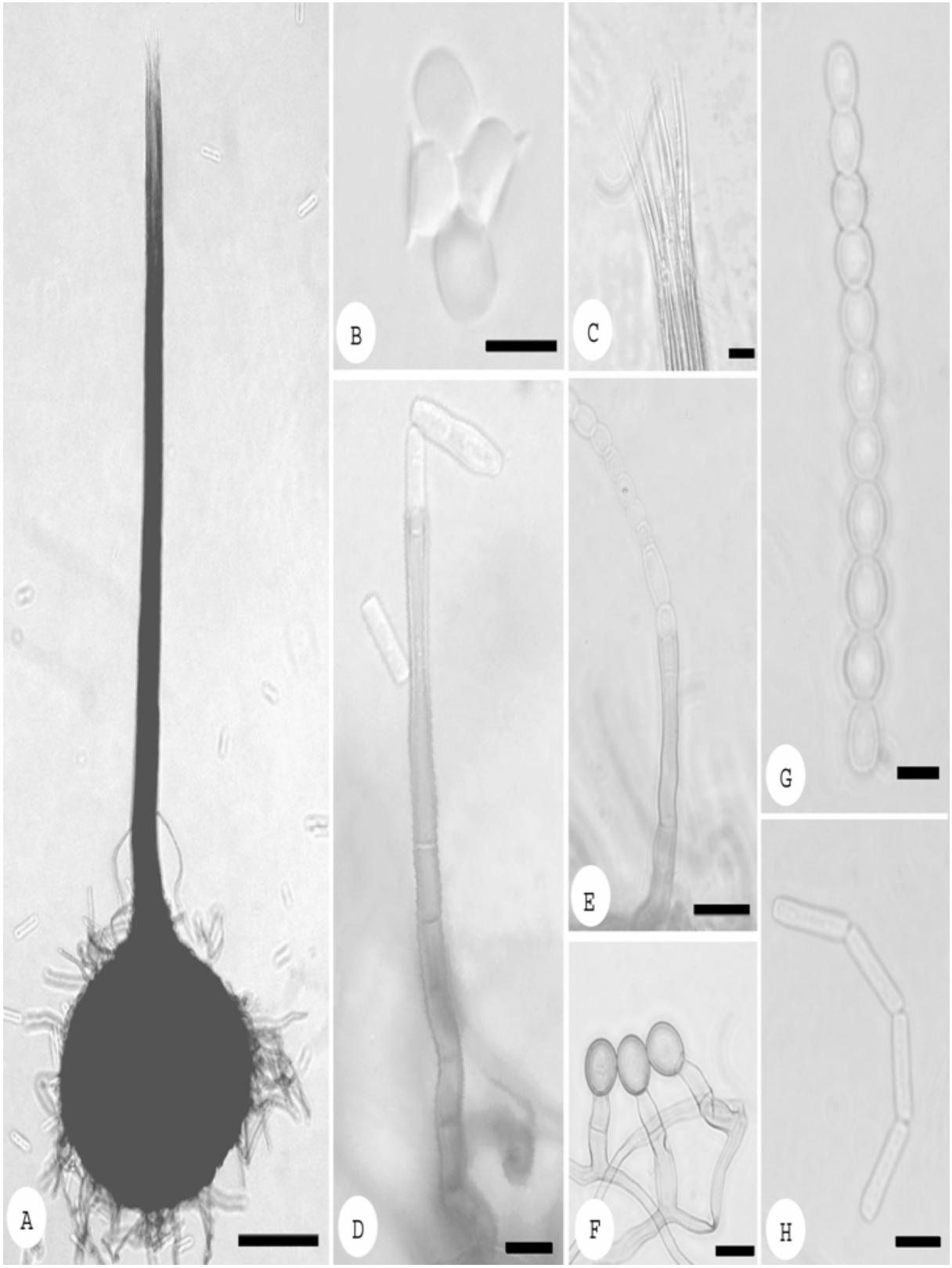

Fig. 1. Morphological features of *Ceratocystis neglecta* from *Eucalyptus grandis* in Colombia. **A.** Ascomata with globose bases. **B.** Hat-shaped ascospores. **C.** Divergent ostiolar hyphae. **D.** Primary phialide. **E.** Secondary phialide. **F.** Chlamydospores. **G.** Barrel-shaped conidia. **H.** Cylindrical conidia. Bars: A = 100 µm, C-H = 10 µm; B = 5 µm.

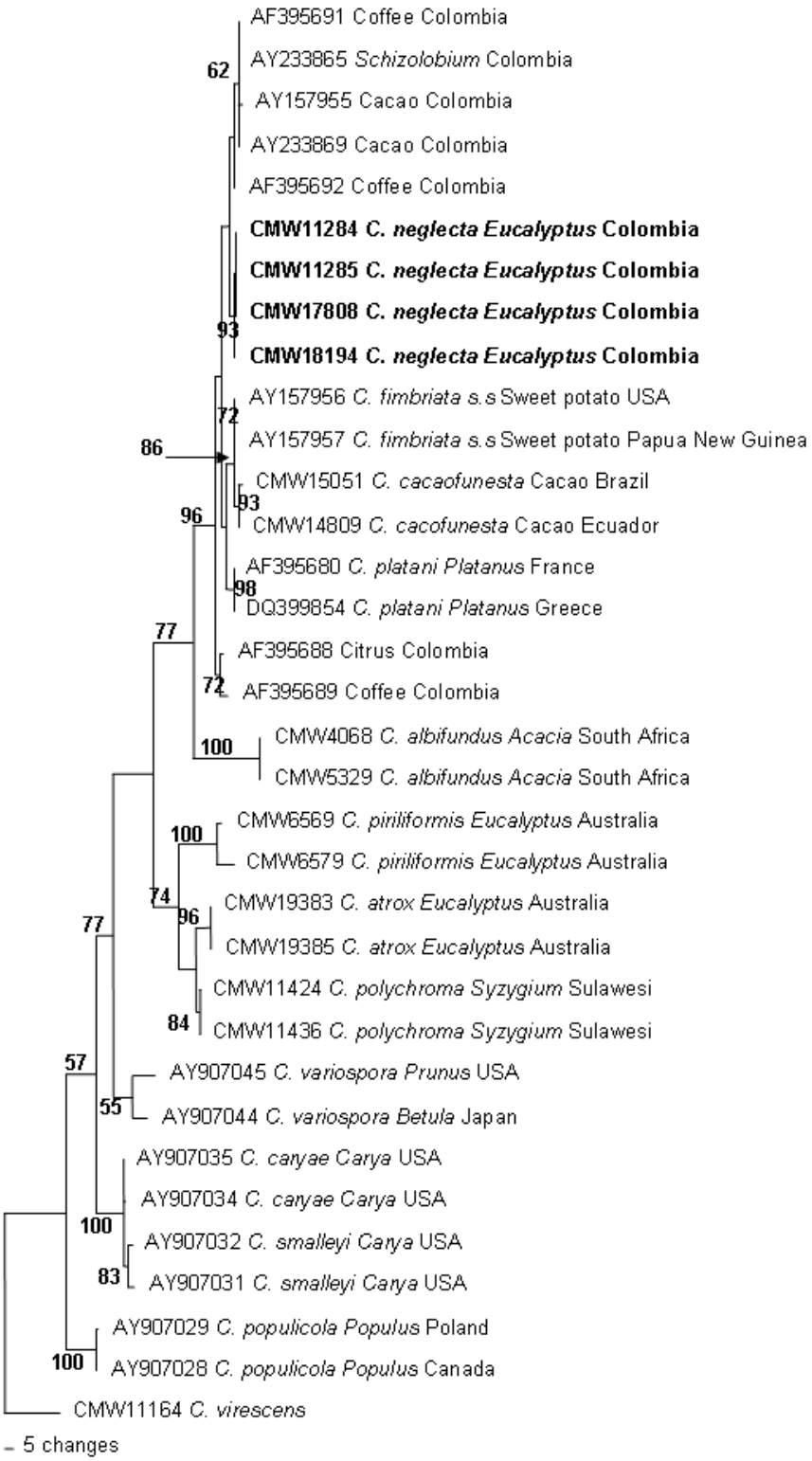

Fig. 2. A phylogenetic tree (tree length = 439 steps, CI =0.7312, RI =0.8590, HI = 0.2688, RC = 0.6281) generated from DNA sequences of the ITS1/2 regions of the rDNA for various *Ceratocystis* species. Bootstrap values (1000 replicates) are indicated in bold. An isolate of *C. virescens* was used as the out-group taxon.

The *Ceratocystis* isolates from *E. grandis* in Colombia grouped alone in a well-resolved clade, separate from all other lineages, including those published previously for other Colombian isolates (Marin *et al*., 2003) (Fig. 2; Bootstrap support 93%). *Ceratocystis fimbriata* s.s. from sweet potato (type) grouped separately from all other isolates considered in this study (Bootstrap support 72%). The other lineages represent *C. platani* (J.M. Walter) Engelbr. & T.C. Harr. (Bootstrap support 98%), *C. albifundus* M.J. Wingf., De Beer & M.J. Morris (Bootstrap support 100%), *C. pirilliformis* I. Barnes & M.J. Wingf. (Bootstrap support 100%), *C. polychroma* M. van Wyk, M.J. Wingf. & E.C.Y. Liew (Bootstrap support 84%), *C. variospora* (R.W. Davidson) C. Moreau (Bootstrap support 55%), *C. smalleyi* J.A. Johnson & T.C. Harr. (Bootstrap support 83%), *C. caryae* J.A. Johnson & T.C. Harr. (Bootstrap support 100%), *C. cacaofunesta* Engelbr. & T.C. Harr. (Bootstrap support 93%), *C. populicola* J.A. Johnson & T.C. Harr. (Bootstrap support 100%) and *C. atrox* M. van Wyk & M.J. Wingf. (Bootstrap support 96%).

Taxonomy

Comparison of the DNA sequence data for the ITS regions, including the 5.8S rDNA, confirmed morphological observations that the *Ceratocystis* isolates from *Eucalyptus* trees in Colombia are related to, but different from *C. fimbriata* s.s. The fungus thus represents a new and previously undescribed species of *Ceratocystis* and it is described as follows:

Ceratocystis neglecta M. van Wyk, Jol. Roux & C. Rodas, **sp. nov.** (Fig. 1)
MycoBank: 10947.

Anamorph: *Thielaviopsis* sp.

Etymology: The name neglecta refers to the fact that this fungus has been overlooked in the past.

Coloniae glaucovirides. *Hyphae* laeves, non segmentatae, (2-) 4-6 µm latae. *Bases ascomatum* atrobrunneae vel nigrae, globosae, (173-)202-244(-281) µm latae, (153-)178-228(-250) µm longae, (163-)190-236(-266) µm diametro. *Colla ascomatum* basi atrobrunneae, apicem versus pallescentia, basi (27-)31-39(-46) µm, apice (14-)16-20 (-22) µm lata, (691-)745-840(-889) µm longa. *Hyphae ostiolares* divergentes, hyalinae, (35-)41-49(-54) µm longa. *Asci* non visi. *Ascosporae* in massa alba vel flavobubalina in apicibus collorum crescunt; lateraliter visa cucullata vel pileata, non septata, hyalina, vaginata, 3-6 µm longa, 4-7 µm lata.

Anamorpha Thielaviopsis: Conidiophorae biformes, in mycelio singulae, primaria hyalina, (75-)80-114(-152) µm longae, basi (4-)5-7(-8) µm, apice (3-)4-6(-7) µm lata; secondaria hyalina phialida primaria brevior, (38-) 48-76(-89) µm longa, basi apiceque (3-)5-7(-8) µm lata. Evolutio *conidiorum* phialidica per formatione parietum annularium, *conidia* biformia, singula vel concatenata, primaria cylindrica (11-)15-27(-30) µm longa, (3-)5-6 µm lata; secondaria orculiformia (6-)10-11 µm longa, (4-)5-7(-9) µm lata. Chlamydosporae adsunt, (8-)10-12(-13) µm lata, (9-)10-14(-16) µm longa.

Colony color greenish glaucous (33'''f). *Hyphae* smooth, non-segmented, (2-)4-6 µm wide. *Ascomatal bases* dark brown to black, globose, (173-)202-244(-281) µm wide, (153-)178-228 (-250) µm in length, (163-)190-236(-266) µm in diameter. *Ascomatal necks* dark brown at base becoming lighter towards apex, (27-)31-39(-46) µm wide at base of neck, (14-)16-20(-22) µm wide at tip of neck, (691-)745-840(-889) µm in length. *Ostiolar hyphae* divergent, hyaline, (35-)41-49(-54) µm in length. *Asci* not observed. *Ascospores* accumulate in a round, white to yellow (yellow-buff 19d) mass at the apices of the ascomatal necks, hat-shaped in side view, aseptate, hyaline, invested in sheath, 3-6 µm in length and 4-7 µm in width.

Thielaviopsis anamorph: Conidiophores of two types occurring singly on mycelium, primary conidiophores hyaline, long, (75-)80-114(-152) µm in length, (4-)5-7(-8) µm wide at base, (3-)4-6(-7) µm wide at tip, secondary conidiophores hyaline, shorter than primary phialides, (38-)48-76(-89) µm in length, (3-)5-7(-8) µm wide at base, (3-)5-7(-8) µm wide at tip. *Conidium* development phialidic through ring wall building, *conidia* of two types formed singly or in chains; primary conidia, bacilliform cylindrical, (11-)-15-27(-30) µm in length, (3-)5-6 µm wide, secondary conidia, doliiform (barrel-shaped) (6-)10-11 µm in length, (4-)5-7(-9) µm wide. *Chlamydospores* present, (8-)10-12(-13) µm wide and (9-)10-14(-16) µm in length.

Habitat: On inner bark and cambium of wounded living *Eucalyptus* spp.

Known distribution: Colombia

Material examined: COLOMBIA, from artificial wounds on *Eucalyptus grandis* trees, 2004, C. Rodas & J. Roux (**holotype** Herb. PREM 59616); culture ex-type CMW 17808. Colombia, artificial wounds on *E. grandis*

trees, 2004, C. Rodas & J. Roux, **paratype** PREM 59617; culture ex-paratype CMW 18194. Colombia, from artificial wounds on *E. grandis* trees, 2002, C. Rodas & J. Roux, **paratype** PREM 59618; culture ex-paratype CMW 11284 = CBS 121349. Colombia, from artificial wounds on *E. grandis* trees, 2002, C. Rodas & J. Roux, culture ex-paratype CMW 11285.

Pathogenicity tests

Ceratocystis neglecta gave rise to lesions of varying length on inoculated *E. grandis* trees (Figs 3-5). Isolate (CMW11285) from wounds on *E. grandis* at La Suiza, was highly pathogenic and produced extensive lesions significantly different ($P = 0.0001$) to that of the control and other isolate used (Figs 3-5).

Eucalyptus grandis clone 301 planted at all three sites was most susceptible with lesions extending up to 350 mm in length (Fig. 3-5). Clone 02 was clearly more tolerant to *C. neglecta* ($P < 0.05$). Trees representing the seed lot *E. grandis* 211 collectively had a level of susceptibility to *C. neglecta* intermediate between that of clones 301 and 02 (Fig. 5).

Some differences were observed in the results between the different farms. For CMW11284, Clone 301 did not differ significantly in susceptibility at the Cedral and La Suiza farms. However, at the Buenos Aires farm significantly larger lesions were produced when Clone 301 was inoculated with CMW 11284 ($P < 0.05$). For isolate CMW11284, Clone 02 differed significantly in susceptibility at the Buenos Aires and La Suiza Farms ($P < 0.05$). In all cases the control lesions were significantly less than those of the *C. neglecta* isolates.

For isolate CMW11285, Clone 301 did not differ significantly at the Buenos Aires and Cedral farms, but at the La Suiza farm it was significantly more tolerant to infection that at the former two farms ($P < 0.05$). For isolate CMW11285 and Clone 02, there were no statistically significant lesions between Buenos Aires Farm and La Suiza Farm ($P > 0.05$). In all cases the control lesions were significantly less than those of the *C. neglecta* isolates.

Discussion

Results of this study gave rise to the discovery of a new *Ceratocystis* sp. now known as *C. neglecta*, which is closely related to, but distinct from *C. fimbriata* s.s. This fungus was isolated from artificially inflicted wounds on *E. grandis* trees in Colombia. These infections, however, appear not to be common and occurred on only a few trees occurring in the three different climatic zones considered in this study. Although wounds became infected with *C. neglecta*, no indication was found of trees dying due to these infections. This may be due to the fact that trees were inspected only once after wounding, which might not have been sufficiently long for symptoms to develop. Another explanation could be that trees wounded in this study were not highly susceptible to infection by *C. neglecta*.

Morphologically, *C. neglecta* is distinct from *C. fimbriata* s.s. and all other closely related species previously considered as *C. fimbriata*. When *C. neglecta* is compared with *C. fimbriata* s.s., differences can be observed in the length of the ascomatal necks, with those of *C. neglecta* being longer than those of the latter species. Furthermore, the ostiolar hyphae of *C. neglecta* are much shorter than those of *C. fimbriata* s.s., *C. platani* and *C. cacaofunesta*. The conidiophores of *C. neglecta* are also shorter than those of *C. fimbriata*, while they are longer than those of *C. platani* and *C. cacaofunesta*. The ascomatal necks of *C. cacaofunesta* are much longer than those of *C. neglecta* and other species in this group, except for *C. polychroma*, which also has long necks. Similar to other species in the *C. fimbriata* s.l. complex, *C. neglecta* has divergent ostiolar hyphae, which distinguishes it from *C. pirilliformis* which has convergent ostiolar hyphae.

In phylogentic studies, *C. neglecta* grouped separately from all other described species of *Ceratocystis* in the *C. fimbriata* s.l. species complex. Comparison with isolates from Colombia that have previously also been studied using DNA sequence comparisons (Barnes *et al*., 2003a; Marin *et al*., 2003) also showed that *C. neglecta* represents a different species from these lineages. A study of *C. fimbriata* s.l. isolates from coffee growing regions in Colombia previously showed that at least two distinct phylogenetic lineages exist for those isolates (Marin *et al*., 2003). This

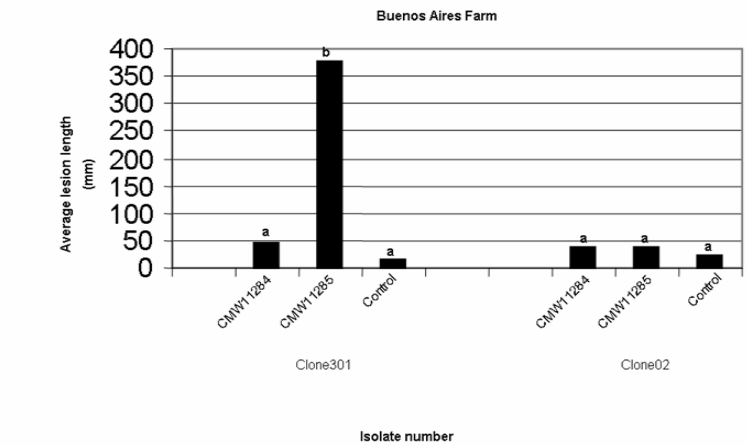

Fig. 3. Results of an inoculation trial with isolates of *Ceratocystis neglecta* from *Eucalyptus grandis* (CMW11285 and CMW11284) from Colombia and a negative control. Inoculations were done on *E. grandis* clones 301 and 02 at Buenos Aires farm, Trujillo Valle. Different letters above bars indicates statistical differences between isolates and clones at Buenos Aires Farm ($P < 0.05$).

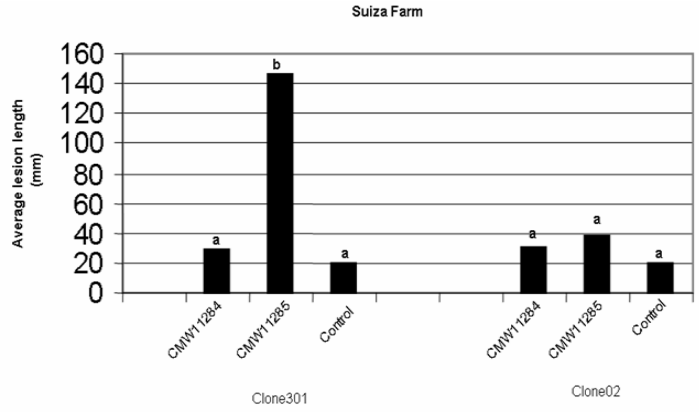

Fig. 4. Results of an inoculation trial with isolates of *Ceratocystis neglecta* from *Eucalyptus grandis* (CMW11285 and CMW11284) from Colombia and a negative control. Inoculations were done on *E. grandis* clones 301 and 02 at La Suiza farm, Restrepo Valle. Different letters above bars indicates statistical differences between isolates and clones at La Suiza Farm ($P < 0.05$).

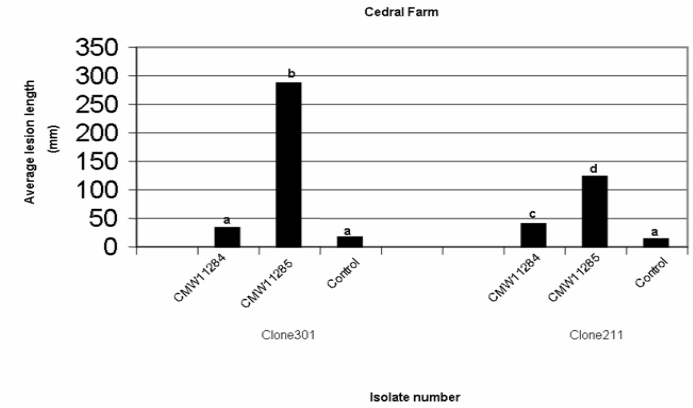

Fig. 5. Results of an inoculation trial with isolates of *Ceratocystis neglecta* from *Eucalyptus grandis* (CMW11285 and CMW11284) and a negative control. Inoculations were done on clone 301 and seed lot 211 at Cedral farm, Darien, Valle. Different letters above bars indicates statistical differences between isolates and clones at Cedral Farm ($P < 0.05$).

suggests that additional, currently undescribed species in *C. fimbriata* s.l. exist in Colombia.

Although *C. neglecta* was not found associated with naturally infected and dying *Eucalyptus* trees in this study, we were able to show that isolates of this fungus can give rise to distinct lesions when inoculated onto susceptible *Eucalyptus* trees. The fungus is evidently a potentially important pathogen of these trees. Furthermore, pathogenicity tests showed clearly that one *E. grandis* clone deployed in Colombian plantations is highly susceptible to infection by *C. neglecta*. Previously unexplained deaths of trees in plantations could well have been due to this fungus, which can also be difficult to isolate.

An important and interesting outcome of this study was the fact that different clones of *E. grandis* differ substantially in their susceptibility to infection by *C. neglecta*. Thus, Clone 301 was highly susceptible to infection by the most pathogenic isolate of *C. neglecta*, at all three sites where this clone was tested. This is in contrast to Clone 02 that was considerably less susceptible to the isolates tested. The fact that the trees generated from seed were more susceptible to infection by the most pathogenic isolate than one of the clones, is typical of results found in other studies (Zauza *et al*., 2004). Thus, seedling material harbours a wide range of susceptibility to pathogens and it displays a wide variability in response to infection.

In this study, artificially inflicted wounds were made on trees to determine whether these might become infected by isolates of *C. fimbriata* s.l. Similar wounding studies have previously been used on *Eucalyptus* trees in Australia (Barnes *et al*., 2003b; Kile *et al*., 1996) and South Africa (Roux *et al*., 2004) and these have led to the discovery of new species of *Ceratocystis* spp. as well as the detection of *C. fimbriata* s.l. where it was previously not known to occur. *Ceratocystis* spp. are well-known to infect wounds on trees and these infections probably originated from infected sap-feeding insects visiting wounds (Hinds, 1972; Juzwik and French, 1983; Teviotdale and Harper, 1991). We believe that *C. neglecta* infection of the wounds made on *Eucalyptus* in this study originated from insects visiting these wounds, although further studies are needed to confirm this.

Inoculations in this study showed that one isolate of *C. neglecta* from wounds on *Eucalyptus*, was significantly more pathogenic than the other isolate chosen for inoculation trials. Variability in virulence of individuals of a pathogen is a well-recognised phenomenon and emphasises the importance of choosing appropriate isolates when screening planting stock for resistance. If this isolate had not been included in the trials, *C. neglecta* would not have been recognised as a potentially important pathogen of *Eucalyptus* in Colombia.

Results of this study have shown that *C. neglecta* is a potentially important pathogen of *Eucalyptus* in Colombia. Where trees die due to wilt and where vascular discoloration is noted, this fungus should be included amongst the possible causes of death. In these cases, isolation techniques suitable for recognising *Ceratocystis* infections should be included. Results have also shown that clones differ markedly in their susceptibility to infection. If *C. neglecta* becomes an important pathogen in the future, there will be excellent opportunities to reduce losses through the selection of disease tolerant planting stock.

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