CHAPTER 4

Cylindrocladium blight of *Eucalyptus grandis* in Colombia

ABSTRACT

Species of *Cylindrocladium* represent one of the most important groups of pathogens that affect *Eucalyptus grandis* plantations in Colombia. Disease symptoms include both leaf blotch and shoot blight and these can lead to severe defoliation. This affects the productivity of *E. grandis* in forestry zones with high humidity. The objective of this study was to identify the *Cylindrocladium* spp. associated with defoliation of *E. grandis* plantations in three important forestry regions of Colombia. Isolates were obtained from samples collected from these areas and the morphology and DNA sequence data was used for identification. Results of both morphological comparisons and analysis of β-tubulin gene sequences showed that only *C. spathulatum* was present in the evaluated areas. This is in contrast to previous reports of a number of other *Cylindrocladium* spp. of *Eucalyptus* in the country. Evaluation of a *Eucalyptus* clonal trial showed that clones differ greatly in their susceptibility to infection by *C. spathulatum*. This presents excellent opportunities for disease avoidance in the future.

INTRODUCTION

Colombia has a large and growing forestry industry with 145 759 hectares (SITEP 1999) planted mainly to various species of *Pinus* and *Eucalyptus*. These trees are used to produce structural timber, pulpwood and paper. Approximately 47 700 ha (33 %) of the forestry areas are planted to *Eucalyptus* species. Propagation of these trees is rapidly becoming a major component of the forestry industry in Colombia (Osorio, Wright & White 1995).

Eucalyptus planted as exotics in commercial plantations have many advantages. They have valuable wood and pulp characteristics, as well as many useful sylvicultural properties such as rapid growth rates and adaptability to a wide range of soils and climates (Turnbull 2000). As exotics, *Eucalyptus* spp. have been separated from their natural enemies and this has enhanced the productivity of plantations (Burgess & Wingfield 2003, Turnbull 2000, Wingfield *et al.* 2001, Wingfield 2003). However, new pests and pathogens are appearing in exotic plantations at an increasing rate and this threatens the sustainability of exotic *Eucalyptus* forestry (Turnbull 2000, Wingfield *et al.* 2001, Wingfield 2003).

In Colombia, leaf and shoot blight associated with *Cylindrocladium* spp. is recognised as one of the most common threats to *Eucalyptus grandis* W. Hill & Maiden. A possible reason for the common occurrence of these fungi, is the high humidity areas where plantations have been established. During the course of the last six years, leaf spots and defoliation symptoms caused by *Cylindrocladium* spp. have been observed in young (one to two years old) plantations at Smurfit Carton de Colombia company located in the Caldas, Quindio, Risaralda and Valle provinces in Colombia.

Cylindrocladium represents an important group of pathogens associated with diverse hosts in tropical and subtropical regions of the world (Crous 2002). These fungi are associated with a wide range of disease symptoms including damping-off, root rot, crown canker, leaf spot, seedling and shoot blight, needle blight, wilt, fruit rot, tuber rot, cutting rot, die-back and stem lesions (Schoch 1999, Crous 2002). In Colombia the most common symptom on *Eucalyptus* is leaf and shoot blight on young trees that develops from the base of trees upwards.

The main symptoms of *Cylindrocladium* diseases in commercial *E. grandis* plantations in Colombia include leaf spots initially on the mature leaves on the lower branches of young trees. Defoliation ascends upwards from the base and centers of trees and in severe cases can affect 100 % of the tree canopies. Depending on the severity of the disease and the extent of defoliation, tree death can also occur.

Cylindrocladium spp. have *Calonectria* teleomorphs (Crous 2002, Rossman 1979). The species are distinguished based on the morphological features of the anamorph, such as conidium shape and size, vesicle shape and phialide morphology, as well as cultural characteristics. Morphological features of the teleomorph tend to be more conserved and species identification based on these characters alone is generally not possible (Crous & Wingfield 1994, Crous 2002). The *Cylindrocladium* anamorphs represent the state most frequently encountered in the field and nearly all species can be distinguished based solely on their asexual characters (Schoch 1999, Crous 2002).

Preliminary disease surveys between 1993 and 1995 led to the identification of a number of *Cylindrocladium* spp. in *Eucalyptus* in Colombia. These include *C. candelabrum* (Bugn.) Boesew., *C. gracile* (Bugn.) Boesew., *C. parasiticum* Crous, M.J. Wingfield & Alfenas and *C. reteaudii* (Bugn.) Boesew. These originated on a wide range of hosts and also from soil samples in *Eucalyptus* plantations. Collection data pertaining to these species are presented in the monograph of Crous (2002). Although the presence of these species was of interest in previous studies, the work was largely of a taxonomic nature and the relative importance of these species was not determined.

The objective of this study was to identify *Cylindrocladium* spp. associated with outbreaks of severe leaf blight, specifically in *E. grandis* plantations in three different geographic areas of Colombia. Identification of the fungi resulting from field surveys were based on β-tubulin sequence comparisons, as well as cultural and morphological characteristics.

MATERIALS AND METHODS

Isolates

Isolates were obtained from leaf spots on *E. grandis* in plantations displaying leaf blight symptoms (Fig. 1). Samples were collected from 14 farms located in three different geographic areas in Colombia (Fig. 2). Twenty diseased leaves from each of ten randomly selected trees were collected at each of 14 farms sampled. These covered most of the areas affected by leaf blight in *E. grandis* plantations belonging to the forestry company Smurfit Carton de Colombia. Samples were packed into brown paper bags and transported to the laboratory for further examination. Selected diseased tissue was placed in moisture chambers and incubated at 25 °C for approximately ten days to promote sporulation. Conidia produced on typical *Cylindrocladium* conidiophores were then transferred onto 2% malt extract agar (MEA; Biolab, Midrand, South Africa) in Petri dishes. Dishes were incubated for eight days at 25 °C under continuous near-ultraviolet light. The cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1). Dried cultures of representative isolates have been lodged with the National Collection of Fungi in Pretoria, South Africa (PREM) (Table 2).

Morphological characteristics

Isolates were plated onto carnation leaf agar (CLA) (Crous 1992) to induce production of both anamorph and teleomorph structures. These plates were incubated at 25 °C under near-ultraviolet light and examined after 6-7 days. Cultural and morphological characteristics were determined as described by Crous (2002). Conidiophores on the surface of carnation leaves were mounted onto microscope slides in lactophenol and twenty measurements of vesicles, stipes and conidia were made using a light microscope with an Axiocam digital camera and Axiovision 3.1 software (Carl Zeiss, Mannheim, Germany). Measurements are presented as (min-)(average – std. dev.) – (average + std. dev.)(-max).

DNA sequence

Five isolates (Table 2) utilized in the DNA sequencing and subsequent phylogenetic analyses, were selected from those collected from different farms in the three different geographic areas in Colombia. They were as follows: CMW 10369 and CMW 10357 from Valle, samples CMW 10363 and CMW 10367 from Cauca, and CMW 10374 from the Andina zone. The single conidial isolates were grown on MEA plates from which mycelium was collected and freeze–dried. The freeze-dried mycelium was ground to a fine powder in liquid nitrogen with a mortar and pestle. DNA was extracted using the technique described by Crous *et al.* (1993).

A 473 bp fragment of the β-tubulin gene was amplified using primers T1 (5' AACATGCGTGAGATTGTAAGT 3') (O'Donnell & Cigelnik 1997) and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC 3") (Glass & Donaldson 1995). The PCR reactions of 25 µl comprised of 2.5 units of Taq (Roche Molecular Biochemicals, Almeda, California, USA), 10x buffer, 1 mM $MgCl_2$ (as supplied by the manufacturer), 0.25 mM deoxynucleoside triphosphates, 0.5 µM primers and approximately 30 ng of fungal genomic DNA as target. The β-tubulin gene was sequenced as more informative for this species. PCR reactions were performed on a Mastercycler (Eppendorf, Hamburg, Germany) using the same reaction conditions as those described by Schoch *et al.* (2001). The PCR amplified fragments were purified using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Almeda, California, USA).

Each DNA strand of the PCR products was sequenced in both directions with the primers used for the PCR amplifications. Sequencing reactions were done using the ABI PRISM ™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California, USA). The reactions were run with capillary electrophoresis on an ABI PRISM ™ 310 DNA Autosequencer (Applied BioSystems). Sequence data were processed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California, USA). The nucleotide sequences were aligned manually by inserting gaps where necessary and phylogenetic relationships were determined using PAUP version 4.0b10 (Swofford 2002). Gaps were treated as fifth characters and "Ns" missing and confidence intervals were determined using 1000 bootstrap replications. To establish the

phylogenetic relationships and identities of the *Cylindrocladium* isolates from Colombia, 17 sequences of known *Cylindrocladium* species (Table 2) obtained by Schoch *et al.* (2001) and Crous (2002) were taken from GenBank and included in the alignment. *Fusarium circinatum* Nirenberg & O'Donnell was used as the outgroup taxon in the analyses.

Susceptibility of Eucalyptus *clones*

A natural outbreak of Cylindrocladium leaf blight occurred in an *E. grandis* clonal trial in Colombia during 1998. This trial was of two-year-old trees planted at Angela Maria farm in Santa Rosa, Risaralda at 1864 masl, with an average of 2437 mm/year precipitation and located at 75° 11' 14" W, 6° 8' 46" N. A total of 420 *E. grandis* trees, representing 42 clones distributed in five blocks with two trees per clone, were evaluated for the percentage of leaves infected. Two branches, one from the lower half and the other from the upper half of each tree, were cut from opposite positions on the stems in order to evaluate incidence of the disease. All leaves were collected from the branches and the total number of diseased leaves based on the presence of any *Cylindrocladium* symptoms was enumerated. The presence of *Cylindrocladium* was confirmed using a dissection microscope and isolates were made from a random sample of leaves for identifications. Statistical analysis of the infection data was carried out using SAS analytical programs (1990). Analysis of variance tables were produced, as well as tables of means with the 95% confidence limits for each mean.

RESULTS

Morphological characteristics

White conidiophores typical of *Cylindrocladium* spp. (Fig. 3a) were common on the surface of the *E. grandis* leaves showing symptoms of infection. Cultures on MEA resulting from isolations from these structures were similar for all 24 isolates collected from the 14 farms (Figs 3b-d). Perithecia were common on the carnation leaves (Fig. 3e) and in culture (Fig. 3f), and these contained typical *Calonectria* ascospores (Figs 3g-h).

Two isolates from each of the three geographical locations were randomly selected for further study. Morphological characters including macroconidiophores, the shape and diameter of the terminal vesicles extending from the conidiophore stipes, and the conidial shape and size, showed that all isolates were those of *C. spathulatum* El-Gholl, Kimbr., E. L. Barnard, Alfieri & Schoult, as described by Crous (2002). The stipe and extensions were septate, straight, hyaline, (210-)269-307 µm in length and terminated in ellipsoid to obpyriform vesicles, (3-)5-7(-9) µm in diam (Figs 3i-j). Each terminal branch of the fertile branches produced approximately five phialides (Figs 3i-j). Phialides were cylindrical, straight, doliiform to reniform, hyaline and aseptate (Fig. 3j). The conidia were cylindrical, rounded at the ends, straight, 3–septate (Fig. 3k). The size computed for 90 conidia was: (48-)53-73(-90) x (3-)4-6(-8) µm (average = 63 x 5.5 µm).

DNA sequence comparisons

A dataset of 21 ingroup taxa and one outgroup taxon, *F. circinatum*, was analysed. The alignment of the β-tubulin gene fragments gave rise to a data set of 473 characters of which 278 were constant, 99 were parsimony-uninformative and 96 parsimony-informative (Fig. 4). One tree from 54 most parsimonious trees was chosen for presentation (Fig. 5). The trees had a length of 294 steps, consistency index = 0.844, retention index = 0.832 and rescaled consistency index = 0.156. The phylogenetic tree (Fig. 5) clearly showed that all five randomly selected *Cylindrocladium* isolates from Colombia grouped in the clade representing *C. spathulatum* (94% bootstrap support).

Susceptibility of* Eucalyptus *clones

All samples taken from the clonal field trial at Andina had *Cylindrocladium* infections caused by *C. spathulatum*. Evaluation of the 42 *E. grandis* clones for percentage infection by *C. spathulatum* showed that clones differed distinctly in their susceptibility to infection (Fig. 6). There was a clear continuum of levels of susceptibility of clones. However, at the upper and lower limits, clones could be classified as highly susceptible and highly tolerant to *C. spathulatum*. Differences in susceptibility of clones were highly significant (P = 0.0001) showing that under natural conditions these differences are

quantifiable. Clones 25, 29 and 36 were the least affected by *C. spathulatum* and clones 14, 17 and 18 were most susceptible, with infection percentages of 90% and above.

Infection was not linked to the relative position of the leaves in the tree, or the placement of the trees. Analysis of variance (Table 3) showed no differences in susceptibility based on position of the branches either lower and higher in the canopy (P = 0.2181). Likewise, there were no statistical differences based on the position of the trees in the trial (P = 1).

DISCUSSION

In this study, we have shown that *C. spathulatum* is the major leaf blight pathogen on *E. grandis* in four different sites in Colombia. *Cylindrocladium spathulatum* was also the only *Cylindrocladium* sp. found amongst a large collection of isolates. This result is interesting as previous preliminary surveys (Crous 2002, Wingfield unpublished) identified four other species of *Cylindrocladium* in Colombian plantations. There are two possible explanations for this disparity. One is that the previous surveys were more random, included soil samples and were not necessarily linked to major outbreaks of leaf blight. It is also known that *Cylindrocladium* spp. responsible for leaf blight in *Eucalyptus* plantations can change over time (Crous 2002), although it is unusual that the major species presently responsible for leaf blight was not collected in earlier preliminary surveys.

Cylindrocladium spathulatum is a well known pathogen of *Eucalyptus* in South America. The fungus was first described as a leaf spot pathogen of *Eucalyptus* spp. from Brazil (Crous & Wingfield 1994, El-Gholl *et al.* 1986). In subsequent studies comparing numerous isolates associated with leaf spotting on *Eucalyptus* from various countries in South America, this pathogen was found in Brazil, Argentina, Colombia and Ecuador (Crous & Kang 2001). Although various other species of *Cylindrocladium* are found on *Eucalyptus* leaves in South America, we believe that *C. spathulatum* has become the dominant species associated with *Eucalyptus* leaf blight in the area.

Results of this study suggest that climate does not affect the species of *Cylindrocladium* responsible for leaf blight of *Eucalyptus* in Colombia. We have shown that the single species, *C. spathulatum* was the only species present in three different planting zones that

differ markedly in climate. However, all four sites are typified by humid conditions that clearly facilitate infection. Our observations also showed clearly that trees between 12 and 32 months old, are most susceptible and thereafter, they appear to recover. This is typical of Cylindrocladium leaf blight of *Eucalyptus* where young trees with closed canopies and thus high humidity levels within and between trees, are most susceptible to blight (Park *et al.* 2000).

Evaluation of a clonal field trial made up of 42 different clones, showed that clones differ markedly in their susceptibility to infection by *C. spathulatum* in Colombia. This result is consistent with observations pertaining to Cylindrocladium leaf blight elsewhere in the world (Henry & Chase 1986). Our results are encouraging from a management perspective as it should be possible to select planting stock with high levels of resistance to Cylindrocladium leaf blight in Colombia. The use of trees with such resistance in a breeding program is likely to reduce the impact of Cylindrocladium leaf blight in Colombia in the longer term.

REFERENCES

Burgess, T. & Wingfield, M. J. (2003) Impact of fungal pathogens in natural forest ecosystems: a focus on *Eucalyptus*. In *Microorganims in Plant conservation and Biodiversity*. (K. Sivasithamparam & K. W. Dixon, eds.). Kluwer Academic Press. (In press).

Crous, P. W. (1992) *Cylindrocladium* and *Cylindrocladiela* with special reference to species occurring in South Africa. PhD dissertation, University of the Orange Free State, Bloemfontein, South Africa.

Crous, P. W., Janse, B. J. H., Victor, D., Marais, G. F. & Alfenas, A. C. (1993) Characterization of some *Cylindrocladium* species with three septate conidia using morphology, isozyme banding patterns and DNA polymorphisms. *Systematic & Applied Microbiology* **16**: 266-273.

Crous, P. W. & Wingfield, M. J. (1994) A monograph of *Cylindrocladium*, including anamorphs of *Calonectria*. *Mycotaxon* **51**: 341-345.

Crous, P. W. & Kang, J. C. (2001) Phylogenetic confirmation of *Calonectria spathulata* and *Cylindrocladium leucothoes* based on morphology, and sequence data of the ß-tubulin and ITS rRNA genes. *Mycoscience* **42**: 51-57.

Crous, P. W. (2002) *Taxonomy and pathology of* Cylindrocladium *(*Calonectria*) and allied genera*. The American Phytopathological Society, St. Paul, Minnesota.

El-Gholl, N. E., Kimbrough, J. W., Barnard, E. L., Alfieri, S. A. & Schoulties, C. L. (1986) *Calonectria spathulata* sp. nov. *Mycotaxon* **16**: 115-164.

Glass, N. L & Donaldson, G. (1995) Development of primer sets for use with PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* **61**: 1323-1330.

Henry, R. J. & Chase, A. R. (1986) Screening *Spathiphyllum* species and cultivars for resistance to *Cylindrocladium spathiphylli. Hortscience* **21**: 515-516.

O'Donnell, K. & Cigelnik, E. (1997) Two divergent intragenomic rDNA ITS types with a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics & Evolution* **7**: 103-116.

Osorio, L. F., Wright, J. W. & White, T. (1995) Breeding strategy for *Eucalyptus grandis* at Smurfit Carton de Colombia. In Eucalyptus *plantations, improving fibre yield and quality*. (B. M. Pitts, N. M. G. Borralho, J. B. Reid, R. N. Cormer, W. N. Tibbits & C. A. Raymond, eds.): 264-266. CRC/ IUFRO Conference, February 1995, Hobart, Australia.

Park, R. F., Keane, P. J., Wingfield, M. J. & Crous, P. W. (2000) Fungal diseases of eucalypt foliage. In *Diseases and pathogens of eucalypts*. (P. J. Keane, G. A. Kile, F. D. Podger & B. N. Brown, eds.): 153-239. CSIRO publishing, Australia.

Rossman A. Y. (1979) A preliminary account of the taxa described in *Calonectria. Mycotaxon* **8**: 485-558.

SAS Statistical Software (1990). *SAS / STAT User Guide*, version 6, Fourth Edition, Vols 1 & 2. SAS Institute, Cary, NC, USA.

Schoch, C. L. (1999) Phylogenetic relationships and population dynamics of *Calonectria*. PhD disseration, University of Stellenbosch, Stellenbosch, South Africa.

Schoch, C. L., Crous, P. W., Wingfield, B. D. & Wingfield, M. J. (2001) Phylogenetic of *Calonectria* based on comparison of β-tubulin DNA sequences *Mycological Research* **105**: 1045-1052.

Swofford, D. L. (2002) *PAUP. Phylogenetic Analysis Using Parsimony*. Version 4.0b10. Sinauer Associates Inc. Publishers, Sunderland, Massachusets.

Turnbull, J. W. (2000) Economic and social importance of eucalypts. In *Diseases and pathogens of eucalypts*. (P. J. Keane, G. A. Kile, F. D. Podger & B. N. Brown eds.). p. 1-9. CSIRO Publishing, Australia.

Wingfield, M. J., Roux, J., Coutinho, T. A., Govender, P. & Wingfield, B. D. (2001) Plantation disease and pest management in the next century. *Southern African Forestry Journal* **190**: 67-71.

Wingfield, M. J. (2003) Increasing threat of diseases to exotic plantation forestry in the Southern Hemisphere. Lessons from Cryphonectria canker. *Australasian Plant Pathology* (in press).

Table 1. *Cylindrocladium* isolates from *Eucalyptus* in Colombia used in this study.

Isolate number (CMW) [a]	Locality / Zone [b]	Altitude (masl)	Collector
10356	Samaria/ Valle	1825	C. A. Rodas
10357	Samaria / Valle	1825	C. A. Rodas
10358	Samaria/ Valle	1825	C. A. Rodas
10359	Suiza / Valle	1469	C. A. Rodas
10360	A. Maria/ Andina	1864	C. A. Rodas
10361	Ignacia/ Cauca	2000	C. A. Rodas
10362	Ignacia/ Cauca	2000	C. A. Rodas
10363	D Miguel/ Cauca	1750	C. A. Rodas
10364	Calichares/ Cauca	2000	C. A. Rodas
10365	Claridad/ Cauca	1750	C. A. Rodas
10366	La Paz / Cauca	1850	C. A. Rodas
10367	Sta Maria/ Cauca	1850	C. A. Rodas
10368	Hato Frio/ Cauca	2000	C. A. Rodas
10369	Suiza/ Valle	1469	C. A. Rodas
10370	Samaria/ Valle	1825	C. A. Rodas
10371	Tesorito/ Valle	1800	C. A. Rodas
10372	Alpes/ Valle	1613	C. A. Rodas
10373	Libano/ Andina	2102	C. A. Rodas
10374	A. Maria/ Andina	1864	C. A. Rodas

[a] Isolate numbers are those of the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] Locality refers to a farm belonging to Smurfit Carton de Colombia and Zone is an area defined by climate and indicated in Fig. 2.

Table 2. Species of *Cylindrocladium* used in the phylogenetic analyses.

Isolate number[a]	Species identity	GenBank numbers
CMW 10359 [b]	*Cylindrocaldium spathulatum*	n.a.
CMW 10374 [b]	*C. spathulatum*	n.a.
CMW 10367 [b]	*C. spathulatum*	n.a.
CMW 10363 [b]	*C. spathulatum*	n.a.
CMW 10370 [b]	*C. spathulatum*	n.a.
STE U 2712	*C. spathulatum*	AF 308463
STE U 599	*C. spathulatum*	AF 308464
STE U 925	*C. pauciramosum* C. L. Schoch & Crous	AF 210470
STE U 416	*C. pauciramosum*	AF 210869
STE U 1677	*C. candelabrum* Viégas	AF 210858
STE U 1674	*C. candelabrum*	AF 210857
ATCC 46300	*C. scoparium* Morgan	AF 210873
ATCC 38227	*C. scoparium*	AF 210872
STE U 616	*C. insulare* C. L. Schoch & Crous	AF 210860
STE U 768	*C. insulare*	AF 210853
STE U 1237	*C colhounii* Peerally	AF 231953
STE U 1339	*C. colhounii*	AF 232851
STE U 516	*C. reteaudii* (Bugn.) Boesew.	AF 232870
ATCC 16550	*C. reteaudii*	AF 232868
STE U 941	*C. mexicanum* C. L. Schoch & Crous	AF 210864
STE U 927	*C. mexicanum*	AF 210863
NRRL 22016	*Fusarium circinatum*	AF 434472

[a] Culture collection designations: CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, S.A. STE U = culture collection of the Department of Plant Pathology, University of Stellenbosch, S.A. ATCC = American Type Culture Collection (ATCC), Manassas, Virginia. NRRL = Agricultural Research Service Culture Collection, Peoria, Illinois, USA. All CMW cultures were sequenced in this study and DNA sequences for the other isolates came from GenBank.

[b] Dried cultures of the isolates from Colombia have been deposited in the National Collection of Fungi Pretoria (PREM) under the following numbers: PREM 57504 (= CMW10359), PREM 57501 (= CMW 10374), PREM 57503 (= CMW10367), PREM 57502 (= CMW10363), PREM 7505 (= CMW10370).

Table 3. Analysis of variance table for evaluation of healthy leaves vs. leaves infected with *Cylindrocladium spathulatum* in a trial containing 42 *Eucalyptus* clones.

Source	DF	MS	F value	*P* value
Blocks	4	50461.8	181.46	0.0001
Clones	41	870.6	3.13	0.0001
Position	1	422.5	1.52	0.2181
Clone x position	41	110.7	0.40	1.0
Error	726	278.0		

Blocks = number of blocks in the trial

Position = refers to the evaluated position of branches on the trees

Clone x position = refers to the position of the clones in the trial

Fig. 1. Disease symptoms associated with *Cylindrocladium spathulatum* on *Eucalyptus grandis* in Colombia. **(a)** Defoliation of a one-year-old *E. grandis* tree with most severe symptoms at the base of the tree. **(b)** Leaf spots on leaves at different stages of development.

Fig. 2. Map showing the different geographic areas in Colombia in which samples were collected.

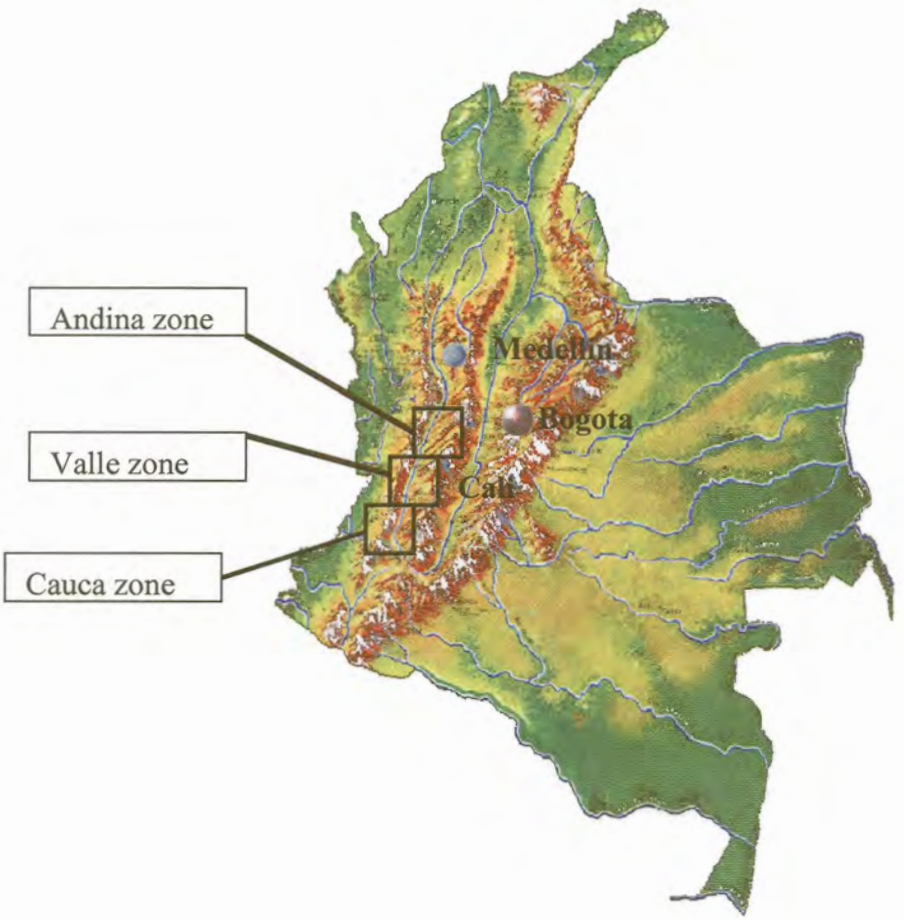


Fig. 3. Morphology of *Cylindrocladium spathulatum*. **(a)** Superficial sporulation on a *Eucalyptus grandis* leaf. **(b-d)** Colony morphology on MEA at (left to right) three days, five days and eight days. **(e)** Perithecia produced on carnation leaf in CLA. **(f)** Perithecia produced on MEA medium. **(g)** Ascus and ascospores. **(h)** Ascospores. (i) Macroconidiophore with attached conidia. **(j)** Conidiophore with extending stipe and terminal vesicle. **(k)** Conidia. Bars Figs. g-h, k = 10 µm, i-j = 20 µm.

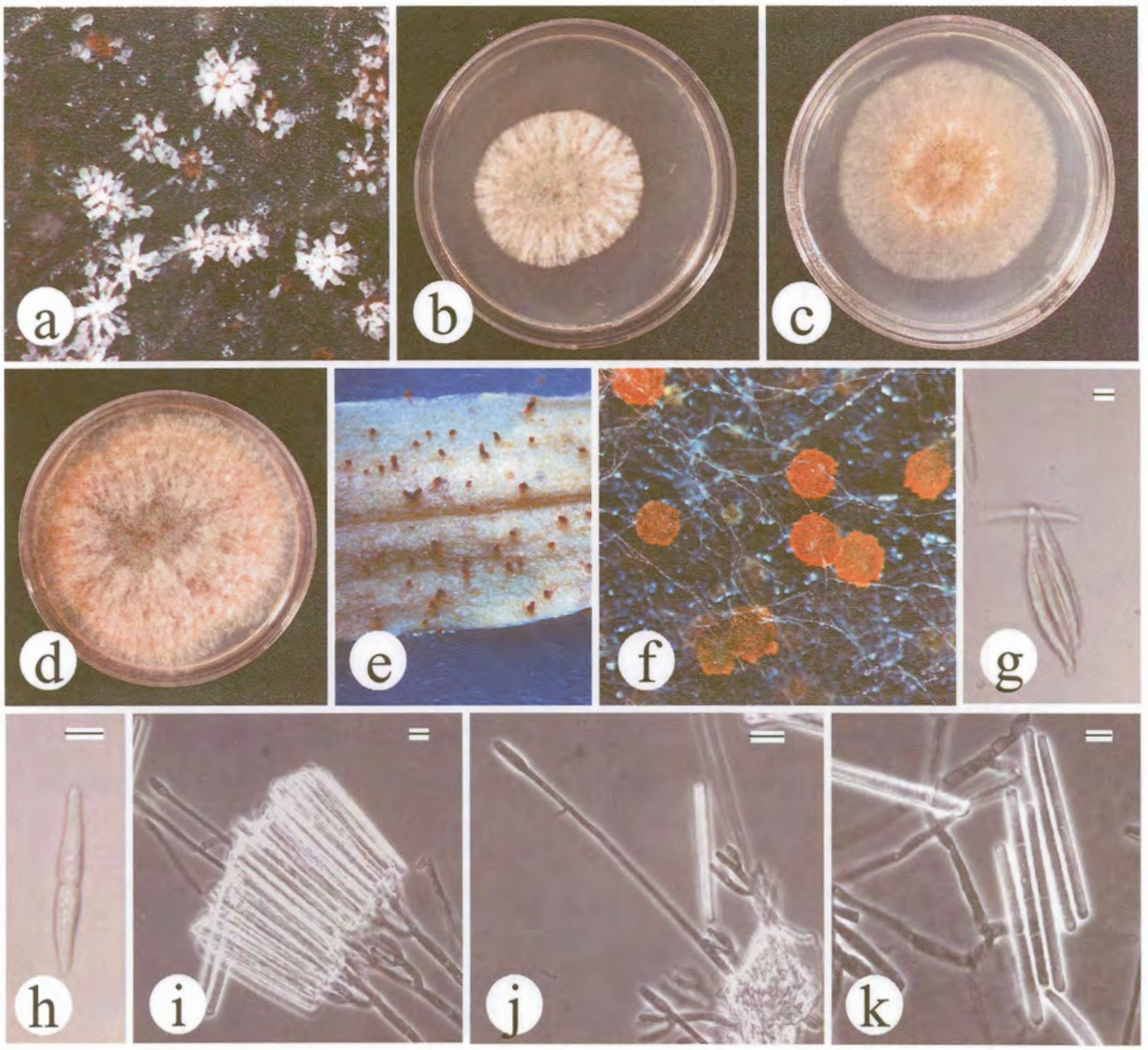


Fig. 4. Raw sequence data of the β-tubulin gene. Unknown sequence characters are indicated with a "**N**", while gaps inserted to achieve sequences alignment are indicated with "-". Bases matching those of STE-U 2712 are indicated with a ".".

```
[                    10         20         30         40         50]

STE_U_2712     TTGTTGCT-G CCCCT-GATT CTACCCCGCC GCCCCGGTTT --CCACCGCT
STE_U_599      .........- .....-.... .......... .-........ --........
CMW10369       .........- .....-.... .......... .......... --........
CMW10374       ...-....-. ...-.-...- .......C.- .......-.. --.-......
CMW10357       .........- .....-.... .......... .......... --........
CMW10363       NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
CMW10367       .........- .....TC... .......... .......... --........
STE_U_925      .........T .....-.... .......... .......... --........
STE_U_416      .........T .....-.... .......... .......... --........
ATCC_46300     .G......T. .....-.... .......... .......... --......A.A
ATCC_38227     .........- .....-.... .......... .......... --......A.A
STE_U_1237     ........-T .....-.... .......... .A........ --........
STE_U_1339     .........- .....-.... .......... .A........ --........
STE_U_1677     ........T. .....-.... .......... .......... --...T....
STE_U_1674     ........T. .....-.... .......... .......... --...T....
STE_U_941      ........T. .....-.... .......... .T........ --........
STE_U_927      ........T. .....-.G.. .......... .T........ --........
STE_U_516      .........- .....-.... .......... .A........ --........
ATCC_16550     .G......-. .....-.... .......... .A........ --........
STE_U_768      ........T. .....-.... .......... .......... --......A.C
STE_U_616      ........T. .....-.... .......... .......... --......A.C
NRRL_22016     ..A.-.G.-. .....-.... .........T .-GG....GG CAG.T.AA.G

 [                   60         70         80         90
100]

STE_U_2712     TCGA-CGACA ACAAAGCCGC AGCCTC-A-C GATCATGACG A-GATATCAG
STE_U_599      ....-..... .......... .......-.- .......... .-........
CMW10369       ....-..... .......... .......-.- .......... .-........
CMW10374       G...----.. ........-. .......C-. .......... .-........
CMW10357       ....-..... .......... .......-.- .......... .-........
CMW10363       NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NN........ .-........
CMW10367       ....-..... .......... .......-.- .......... .-........
STE_U_925      .G..-..... .......... ..G...-.-. .......... .-........
STE_U_416      ....-..... .......... .......-.- ......A... .-........
ATCC_46300     ....-...A. .......... .......-.- ..A.....T. T-........
ATCC_38227     ....-...A. .......... .......-.- ..A.....T. T-........
STE_U_1237     ....-..... .......... .......-.- ..G....... .-.......GA
STE_U_1339     ....-..... .......... .......-.- ..G....... .-.......GA
STE_U_1677     ....-..... .......... .......-.- .......... .-........
STE_U_1674     ....-..... .......... .......-.- .......... .-........
STE_U_941      A.-.A..... .......... .......G.- -.A.....GC .A........
STE_U_927      A.-.A..... .......... .......G.- -.A.....GC .A........
STE_U_516      ....-..... .......... .A.A..-.T- ..A..A.... .-.......G.
ATCC_16550     ....-..... .......... .-....GT-- ..A....... .-.......G.
STE_U_768      ....-..... .......... .......-.- ..A.....T. T-........
STE_U_616      ....-..... .......... .......-.- ..A.....T. T-........
NRRL_22016     A.A.TGC..G .T.GC--T-- .-G.A-G-CT TTAA..AC.T TCTG.CAAGA
```

```
[                    110         120         130         140         150]

STE_U_2712     AACAAGATT- GCTAACCGTG TGCTTCTTTC TCGATTATAG GTCCACCTCC
STE_U_599      .........- .......... .......... .......... ..........
CMW10369       .........- .......... .......... .......... ..........
CMW10374       .........- .......... .......... .......... ..........
CMW10357       .........- .......... .......... .......... ..........
CMW10363       .........- .......... .......... .......... ..........
CMW10367       .........- .......... .......... .......... ..........
STE_U_925      .........- .......... .......... .......... ..........
STE_U_416      .........- .......... .......... .......... ..........
ATCC_46300     .........- .......... .......... .......... ..........
ATCC_38227     .........- .......... .......... .......... ..........
STE_U_1237     .........T ...G...A.. .........T ..A....... ..........
STE_U_1339     .........T ...G...A.. .........T ..A....... ..........
STE_U_1677     .........- .......... .........T .......... ..........
STE_U_1674     .........- .......... .........T .......... ..........
STE_U_941      G.T.T...G- .......... ..T.....C. ..A...C... ..T.......
STE_U_927      G.T.T...G- .......... ..T.....C. ..A...C... ..T.......
STE_U_516      .........- .......A.. .......... .......... ..........
ATCC_16550     .........- .......A.. .......... .......... ..........
STE_U_768      .........- .......... .......... .......... ..........
STE_U_616      .........- .......... ...C...... .......... ..........
NRRL_22016     TGA.GA.--- .....T.A-. AT...T.C.. .GCG--.... ..T.......

[                    160         170         180         190         200]

STE_U_2712     AGACCGGTCA GTGCGTAAGT ACTTTTCTCA ACTCCAACAA AATTCTCACG
STE_U_599      .......... .......... .......... .......... ..........
CMW10369       .......... .......... .......... .......... ..........
CMW10374       .......... .......... .......... .......... ..A.......
CMW10357       .......... .......... .......... .......... ..........
CMW10363       .......... .......... .......... .......... ..........
CMW10367       .......... .......... .......... .......... ..........
STE_U_925      .......... .......... ...C...... .........C. ..........
STE_U_416      .......... .......... ...C...... .......... ..........
ATCC_46300     .......... .......... G..C..--.. .......... ..........
ATCC_38227     .......... .......... G..C-.T-.. .......... ..........
STE_U_1237     .......C.. .......... G..C..G... .......... T...A.....
STE_U_1339     .......... A......... G..C..G... .......... T...A...--
STE_U_1677     .......... .......... ..CC...... .....G..C. ..........
STE_U_1674     .......... .......... ..CC...... .....G..C. ..........
STE_U_941      .......... .......... ...CC.TG.. .......... CT........
STE_U_927      .......... .......... ...CC.TG.. .......... CT........
STE_U_516      .......... .......... G..CC....G .T.....AT. TG......T.
ATCC_16550     .......... .......... G..CC..--G -....G.A.. TG......T.
STE_U_768      .......... .......... G..C..--.. .......... ..........
STE_U_616      .......... .......... G..C..--.. ........GG ..........
NRRL_22016     .......... .......... G..CA..G.T --...TCG.C G--..G..T.
```

```
 [                    210         220         230         240
250]

STE_U_2712     ACGAGATTCA CTGACAGTTG TCGATAGGGT AACCAAATTG GTGCTGCTTT
STE_U_599      .......... .......... .......... .......... ..........
CMW10369       .......... .......... .......... .......... ..........
CMW10374       .......... .......... .......... .......... ..........
CMW10357       .......... .......... .......... .......... ..........
CMW10363       .......... .......... .......... .......... ..........
CMW10367       .......... .......... .......... .......... ..........
STE_U_925      .....G...G .......... .......... .......... ..........
STE_U_416      .......... .......... .....  ... .......... ..........
ATCC_46300     ..CG...... .........A ....C..... .......... ..........
ATCC_38227     ..CG.. ... .........A ....C..... .......... ..........
STE_U_1237     .---....T. ......TA.. .......... .......... ..........
STE_U_1339     -.......T. ......CA.. .......... .......... ..........
STE_U_1677     .......... .......... ..C....... .......... ..........
STE_U_1674     .......... .......... ..C....... .......... ..........
STE_U_941      G.C.TGA..T .......AC. .......... .......... ..........
STE_U_927      G.C.TGA..G .......AC. .......... .......... ..........
STE_U_516      ..A....... .......... .......... .......... ..........
ATCC_16550     ..A.CG.... .......... .......... .......... ..........
STE_U_768      ..C....... .........A ....C..... .......... ..........
STE_U_616      ..C....... .........A ....C..... .......... ..........
NRRL_22016     -T.G.GGATG ..C..GA.GT .TATC..... .......... ..........

 [                  260         270         280         290         300]

STE_U_2712     CTGGCAGACC ATTTCTGGCG AGCACGGCCT CGACAGCAAT GGCGTCTACG
STE_U_599      .......... .......... .......... .......... ..........
CMW10369       .......... .......... .......... .......... ..........
CMW10374       .......... .......... .......... .......... ..........
CMW10357       .......... .......... .......... .......... ..........
CMW10363       .......... .......... .......... .......... ..........
CMW10367       .......... .......... .... ...  .......... ..........
STE_U_925      .......... .......... .......T.. .......... ..T.......
STE_U_416      .......... .......... .......T.. .......... ..T.......
ATCC_46300     .......... .......... .......T.. .......... ..T.......
ATCC_38227     .......... .......... .......T.. .......... ..T.......
STE_U_1237     .......... ..C....... .......T.. .......... ..T.......
STE_U_1339     .......... ..C....... .......T.. .......... ..T.......
STE_U_1677     .......... ..C.-..... .......T.. .......... ..T.......
STE_U_1674     .......... ..C....... .......T.. .......... ..T.......
STE_U_941      .......... .......... .......T.. .......... ..T.......
STE_U_927      .......... .......... .......T.. .......... ..T.......
STE_U_516      T......... ..C....... .......T.. ....  ... ..T.......
ATCC_16550     .......... ..C....... .......... .......... ..T.......
STE_U_768      .......... .......... .......T.. .......... ..T.......
STE_U_616      .......... .......... .......T.. .......... ..T.......
NRRL_22016     ......A... ..C....... .......... .......... ..T......A
```

```
[                    310         320         330         340         350]

STE_U_2712     CCGGTACCTC CGAGCTCCAG CTCGAGCGTA TGAACGTCTA CTTCAACGAG
STE_U_599      .......... .......... .......... .......... ..........
CMW10369       .......... .......... .......... .......... ..........
CMW10374       .......... .......... .......... .......... ..........
CMW10357       .......... .......... .......... .......... ..........
CMW10363       .......... .......... .......... .......... ..........
CMW10367       .-........ .......... .......... .......... ..........
STE_U_925      .......... .......... .......... .......... ..........
STE_U_416      .......... .......... .......... .......... ..........
ATCC_46300     .T........ .......... .......... .......... ....C.....
ATCC_38227     .T........ .......... .......... .......... ..........
STE_U_1237     .T........ .......... .......... .......... ..........
STE_U_1339     .T........ .......... .......... .......... ..........
STE_U_1677     .......... .......... .......... .......... ..........
STE_U_1674     .......... .......... .......... .......... ..........
STE_U_941      .......... .......... .......... .......... ..........
STE_U_927      .......... .......... .......... .......... ..........
STE_U_516      .T........ .......... .......... .......... ..........
ATCC_16550     .T.....T.. .......... .......... .......... ..........
STE_U_768      .T........ .......... .......... .......... ..........
STE_U_616      .T........ .......... .......... .......... ..........
NRRL_22016     A......... .......... .......... ...GT..... ..........

[                    360         370         380         390         400]

STE_U_2712     GTATGTGAAA ACCACTCGAA GCACTCCCTT GGCCGAGAAG CA-CAAGCCA
STE_U_599      .......... .......... .......... .......... ..-.......
CMW10369       .......... .......... .......... .......... ..-.......
CMW10374       .......... .......... .......... .......... ..-.......
CMW10357       .......... .......... .......... .......... ..-.......
CMW10363       .......... -......... .......... .......... ..-.......
CMW10367       .......... .......... .......... .......... ..-.......
STE_U_925      .......... .......... .......... .A........ ..-.......
STE_U_416      .......... .......... .......... .A........ ..-.......
ATCC_46300     .......... .....G..GT .TT...A.AC .-.......G. ..-.....A.
ATCC_38227     .......... .....G..GT .T....A.AC .-.......G. ..-.....A.
STE_U_1237     .......... G.TG..T.GT .T-.C..TA. .T.-....GA .G-...CTA.
STE_U_1339     .......... G.TG..T.GT .T..C..TA. .T.-....GA .G-.....A.
STE_U_1677     .......... .......... .......... .A........ ..-...T..G
STE_U_1674     .......... .......... .......... .A........ ..-...T..G
STE_U_941      ......A... ...G...C.. .A.A.TT... T.T..G..C. .C-...A.A.
STE_U_927      ......A... ...G...C.. .A.A.TT... T.T..G..C. .C-...A.A.
STE_U_516      .....C.... .A.CA.GCCT ..G...G... T.T...A..A GCA.....A.
ATCC_16550     .....-CA... .A.-A.GCGT ..G...A... T.A......A T.-..G..A.
STE_U_768      .......... ...G.G..GT .T..C.A.AC .-.......G. ..-.....A.
STE_U_616      .......... .T...G..GT .T....A.AC .-.......G. ..-.....A.
NRRL_22016     .....CTTT. ..-.G----- -----..A--A T...A....T TCC.----A.
```

```
[                    410         420         430         440         450]

STE_U_2712     ACTCACACAC T-CATGT--A GGCTTCCGGC AACAAGTTCG TTCCTCGCGC
STE_U_599      .......... .-.....--. .......... .......... ..........
CMW10369       .......... .-.....--. .......... .......... ..........
CMW10374       .......... .-.....--. .......... .......... ..........
CMW10357       .......... .-.....--. .........- .......... ..........
CMW10363       .......... .-.....--. .......... .......... ..........
CMW10367       .......... .-.....--. .......... .......... ..........
STE_U_925      .........A .-.....--. .......... .......... ..........
STE_U_416      ........CA .-.....--. .......... .......... ..........
ATCC_46300     ...G....-- --.....--. ......T... .........C G....T.T..
ATCC_38227     ...G....-- --.....--. ......T... .......... ..........
STE_U_1237     ...G....-- --.....--. ...C.T.... .......... ..........
STE_U_1339     ...G..G.-- --.....--. .......... .......... ..........
STE_U_1677     ........CA .-.....--. .......... .......... ..........
STE_U_1674     ........CA .-.....--. .......... .......... ..........
STE_U_941      .......... GT.....--. .......... .......... ..........
STE_U_927      .......--- -T.....--. .......... .......... ..........
STE_U_516      ...G...... --.....--. .......... .......... ..........
ATCC_16550     ...G...... --.....GT. .......... .......... ..........
STE_U_768      ...G....-- --.-...--. ......T... .......... ..........
STE_U_616      ...G....-- --.....--. ......T... .......... ..........
NRRL_22016     G..-CACAC- -----AACT. ...C..T... .......AT. ....C..A..

[                    460         470     ]

STE_U_2712     TGTCCTCGTC GATCTTGAGC CCG
STE_U_599      ..-....... .......... ...
CMW10369       .......... .......... ...
CMW10374       .........- .....-.... ...
CMW10357       .......... .......... ...
CMW10363       .......... .......... ...
CMW10367       .......... .......... ...
STE_U_925      .......... .......... ...
STE_U_416      .......... .......... ...
ATCC_46300     ....G..... .......... ...
ATCC_38227     .......... .....C.... ...
STE_U_1237     .......... .......... ...
STE_U_1339     .........- .......... ...
STE_U_1677     .......... .......... ...
STE_U_1674     .......... .......... ...
STE_U_941      .......... .......... ...
STE_U_927      .......... .......... ...
STE_U_516      .......... .......... ...
ATCC_16550     .......... .......... ...
STE_U_768      .......... .....C.... ...
STE_U_616      .......... .....C.... ...
NRRL_22016     C......... .......... .T.
```

Fig. 5. The phylogenetic tree obtained from β-tubulin gene sequences. Confidence levels >50% determined by a bootstrap analysis (1000 replicates) of the tree branch nodes are shown. Isolates sequenced in this study are bolded. A *Fusarium circinatum* isolate was defined as the outgroup taxon.

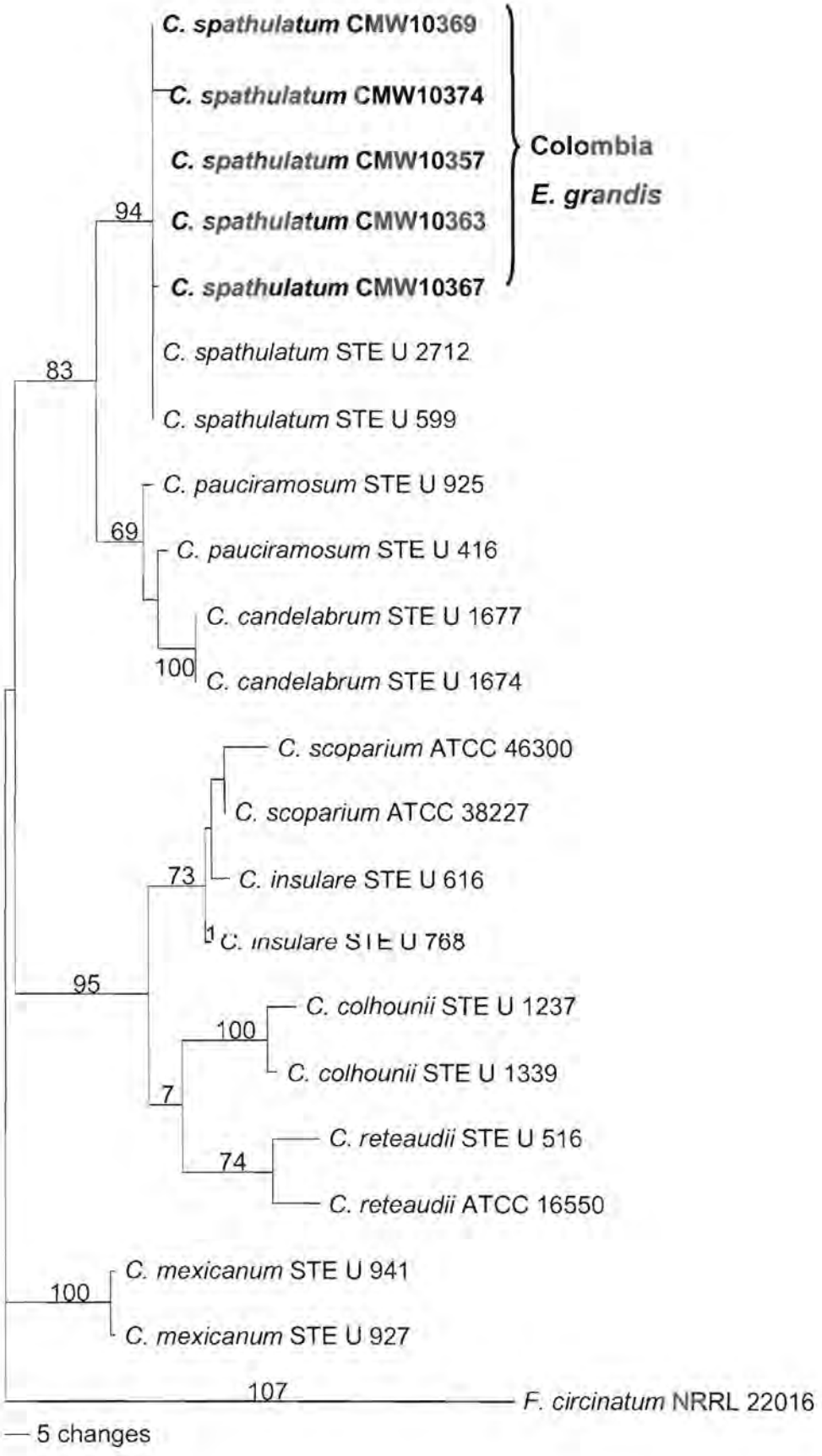
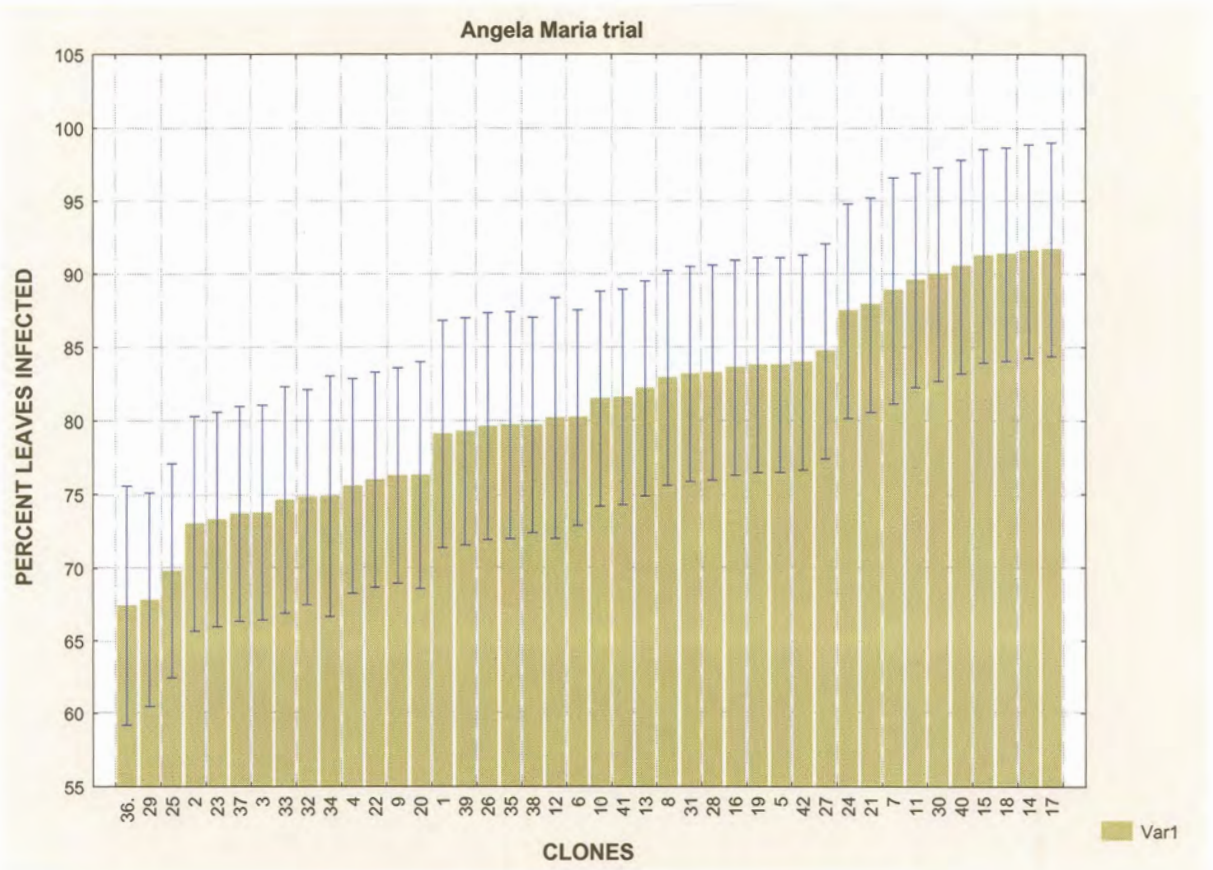
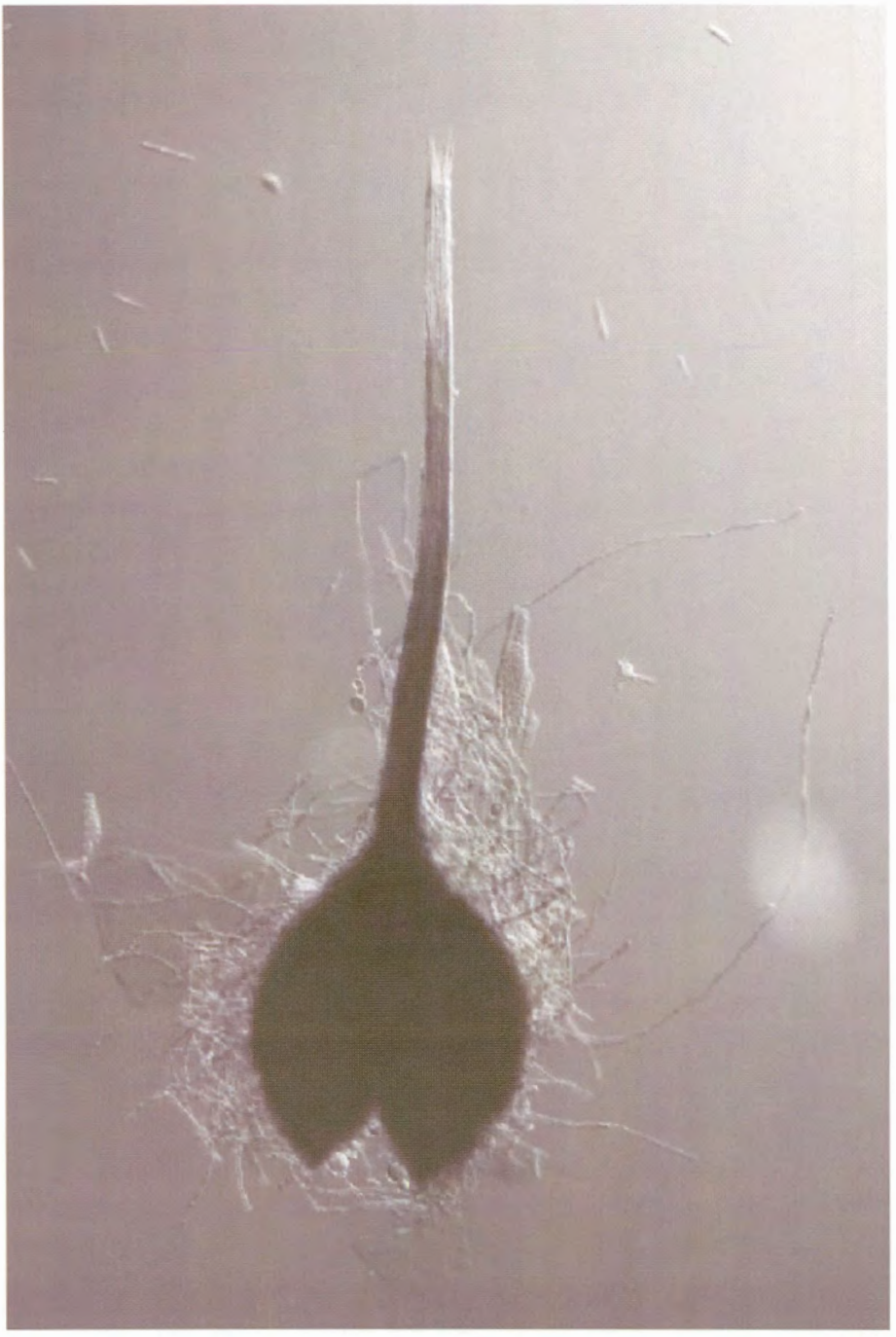


Fig. 6. Graphical presentation of leaves of 42 *Eucalyptus grandis* clones infected by *Cylindrocladium spathulatum* on the Angela Maria farm in Colombia. Data are presented as percentage leaves infected and 95% confidence limits are also shown.



CHAPTER 5



First report of *Ceratocystis fimbriata* on *Eucalyptus grandis* in Colombia

ABSTRACT

Ceratocystis fimbriata is a serious canker pathogen of woody plants and has recently been discovered on *Eucalyptus* spp. in Central Africa as well as in Brazil and Uruguay. In Colombia, the fungus causes cankers on coffee trees, but has not previously been detected on *Eucalyptus* spp. The aim of this study was to investigate whether *C. fimbriata* occurs on *Eucalyptus* trees, and to assess the possible impact that it might have on the forestry industry in Colombia. *Eucalyptus grandis* trees were artificially wounded in three different geographic zones of Colombia, after which isolations were made for *Ceratocystis* spp. These species were identified based on morphology and through sequence comparison of the ITS regions of the rDNA operon. Only two *Ceratocystis* isolates were obtained from wounds on *Eucalyptus* stems. Morphological and DNA sequence comparisons showed that these isolates represented *C. fimbriata*. The two isolates from *Eucalyptus* and one previously collected from *Schizolobium parahybum* were used in field pathogenicity trials. Two isolates were shown to differ in their ability to cause lesions on *Eucalyptus*, with one isolate from *Eucalyptus* highly pathogenic on this host. The different clones of *E. grandis* also differed in their susceptibility to the pathogen. These differences could now be used in plant breeding programs aimed to reduce losses that might occur due to infections by *C. fimbriata* on *Eucalyptus* spp. in Colombia.

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. Exotic species of *Eucalyptus* and *Pinus* are the most common trees grown, and these currently make up approximately 145 759 hectares of plantations (SITEP 1999). In the case of *Eucalyptus*, large areas have been planted to clones of *E. grandis* W. Hill. ex 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.) Sacc. is one of the most important pathogens of woody plants (Kile 1993). This pathogen has a wide host range and cosmopolitan distribution, causing either cankers or vascular wilt diseases. *Eucalyptus* has not been known as a host of *C. fimbriata* in the past. However, this pathogen was recently discovered killing *Eucalyptus* spp. in plantations in Brazil and Central Africa (Roux *et al.* 1999, Roux *et al.* 2001). *Ceratocystis fimbriata* has also recently been found associated with deaths of pruned *E. grandis* in Uruguay (Barnes *et al.* 2003a). Thus, this fungus is emerging as a major threat to *Eucalyptus* plantings.

Ceratocystis fimbriata is a serious pathogen of coffee (*Coffea arabica* L.) in Colombia (Pontis 1951, Mourichon 1994). The importance of coffee to the Colombian economy validates *C. fimbriata* as one of the most important agricultural pathogens (Castro 1998). The fungus infects trees via wounds made at the bases of coffee trees during farming operations. Its common occurrence in soil as chlamydospores (Kile 1993) provides ample opportunity for infection through wounds.

It has been showed that *C. fimbriata* can kill *Eucalyptus* (Roux *et al.* 1999).The wide spread occurrence of this pathogen on coffee in Colombia, often in areas in close proximity to *Eucalyptus* plantations, has thus been a matter of concern. The aim of this study was, therefore, to determine whether *C. fimbriata* might occur on *Eucalyptus* spp. in this country. Furthermore, the potential threat of *C. fimbriata* to *Eucalyptus* forestry in Colombia was considered.

MATERIALS AND METHODS

Collection of isolates

To determine whether *C. fimbriata* occurs in *Eucalyptus* trees in Colombia, wounds were made on trees at two farms in each of three different forestry zones of Colombia. These were at the San Jose and Vanessa farms in the Cauca zone, the Suiza and Samaria farms located in the Valle zone, and the Carolina and Angela Maria farms in the Andina zone. At each of the two farms in the three zones, twenty trees were selected for wounding, thus 120 trees were wounded in total. Wounds were made in June 2002 by cutting a patch of bark 10 cm x 10 cm from the stems of trees, to expose the cambium.

After eight weeks, wood samples were collected from the wounds, placed in paper packets and transported back to the laboratory for analysis. Isolations were made from discoloured wood by wrapping pieces of wood (~ 2 cm^2) tightly between two slices (~ 2 cm thick) of carrot that were surface disinfested with 74 % Ethanol (Moller & DeVay 1968). Carrot baits were incubated at 25 °C for two weeks and regularly inspected for the presence of *Ceratocystis* ascocarps. When present, the ascospore masses were removed from the apices of the ascoscarps and transferred to 2 % malt extract agar plates (MEA: 20g malt extract, 15g agar in 1 l distilled water; Biolab Diagnostics Ltd, Midrand, South Africa) and incubated at 25 °C. The isolates obtained were lodged in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Morphological characteristics

The obtained *Ceratocystis* isolates were grown on MEA and identified based on morphological characteristics. Ten measurements of ascocarps, ascospores, conidiophores and conidia were made from structures mounted in lactophenol on microscope slides. Measurements are presented as (min-)(average - std. dev.) (average + std. dev.)(-max). Microscope slides bearing structures and dried down cultures have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa (Table 1).

DNA sequence comparisons

DNA was isolated from isolates CMW 11285 and CMW 11284 from *E. grandis* studied in the morphological comparisons and pathogenicity tests. An additional isolate, CMW 8858 from *Schizolobium parahybum* S. F. Blake provided by B.L. Castro (Cenicafé, Colombia), that was used in the pathogenicity tests, were also sequenced. The variable internal transcribed spacer (ITS) regions and the 5.8S rDNA of the ribosomal operon were sequenced using primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.* 1990). The obtained sequences were compared with sequences from Barnes *et al.* (2003a) of *C. fimbriata* isolates from various hosts in Colombia, as well as *C. fimbriata* sequences from *Eucalyptus* spp. in other parts of the world (Table 1). Additional *Ceratocystis* species (Barnes *et al.* 2003a) were also included (Table 1).

For DNA extraction, a single mass of ascospores from a single ascoma was incubated for 5 days at room temperature in a 1.5 ml Eppendorf tube containing 800 µl of 2% malt extract broth and kept at room temperature. DNA extraction was performed as described by Barnes *et al.* (2001). Ten ng of 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 Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme) and 1.25 U of Expand High Fidelity PCR system enzyme mix (Roche Molecular Biochemicals, Almeda, California, USA). The PCR amplification consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 50 s at 58 °C and 2 min at 72 °C. Final chain elongation was achieved at 72 °C for 5 min.

PCR products were visualized using UV light after separation on a 1.5% agarose gel containing ethidium bromide. The products were then purified using the High pure PCR product purification kit (Roche Molecular Biochemicals) and sequenced using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems, Foster City, CA). Sequencing reactions were run on an ABI Prism 310 DNA sequencer (Applied BioSystems).

Sequences were aligned using the program Sequence Navigator version 1.0.1 (Applied Biosystems). The alignments were analysed using Phylogenetic Analysis Using

Parsimony (PAUP) software, version 3.1.1. (Swofford 2002). The heuristic search option based on parsimony with random stepwise addition and tree bisection reconnection was used. Gaps were treated as fifth character state "Ns" missing and confidence intervals using 1000 bootstrap replicates were calculated. Trees were rooted with additional sequences from *Petriella setifera* (Schmidt) Curzi (ATCC 26490) from Barnes *et al.* (2003a).

Pathogenicity tests

Three inoculation trials were conducted in commercial *E. grandis* plantations in the Valle zone of Colombia. The three plantations were situated in the Buenos Aires Farm, Trujillo, Valle (1973 masl, 2312 mm/y of precipitation, located at 76° 21' 36" W, 4° 14'10" N), the Cedral farm in Darien, Valle (1825 masl, with an average precipitation of 1825 mm/y, located at 76° 26' 06" W, 3° 57' 06" N) and the La Suiza farm in Restrepo, Valle (precipitation 1067 mm/y, located at 1469 masl, 76° 29' 49" W, 3° 51' 45" N). At each of the three sites, two clones (clone 301, clone 2) and one seed sources (seed lot 211) of *E. grandis* were inoculated with each of three isolates of *C. fimbriata*. These isolates were the same as those used in the morphological and DNA sequence comparisons (CMW 11285, CMW 11284, CMW 8858). At each site, ten trees of each of the *E. grandis* clones or the seed source were inoculated with uninoculated 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, ten trees of each of the *E. grandis* clones 301 and 2 were inoculated with the three isolates of *C. fimbriata* and the control respectively. At Cedral, the same number of trees was inoculated but only clone 301 was available. Thus, instead of clone 2, ten 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 these were distributed in four blocks with ten 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 mm 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 taken from the edge of a rapidly growing 11-days-

old colony was 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 from inoculated trees were recorded in mm after 12 weeks. Statistical analysis of the measurements were carried out using SAS (1990). Analysis of variance as well as graphical presentations of means with 95% confidence limits was produced.

RESULTS

Morphological characteristics

From the 120 wounded trees, only two isolates of *Ceratocystis* were obtained from two trees. These were CMW 11285 from the Suiza farm and CMW 11284 from the Buenos Aires farm. Morphological characteristics (Table 2) of these isolates were typical of *C. fimbriata* (Upadhyay 1981, Webster & Butler 1967). Cultures covered the plates in eight to 15 days and had a strong fruity aroma characteristic of *C. fimbriata*. The ascocarps were black and had typical long necks with convergent ostiolar hyphae (Figs 1a-b) and produced hat-shaped ascospores (Fig. 1c). *Thielaviopsis* anamorphs (Fig. 1d) were common in cultures and both cylindrical conidia (Fig. 1e) and chlamydospores (Fig. 1f) were present (Table 2).

DNA sequence comparisons

The data set consisted of 22 ingroup taxa, with the sequence of *P. setifera* defined as the outgroup taxon (Fig. 2). This data set consisted of 510 sequence characters of which 173 were constant, 106 were parsimony-uninformative and 231 were parsimony-informative. Two trees were obtained from the heuristic search and one phylogenetic tree was chosen for presentation in Fig. 3 (tree length = 788 steps, CI = 0.77288, RI = 0.7926).

The two isolates from *E. grandis* in Colombia grouped within the one lineage of *C. fimbriata* isolates (Fig. 3; Bootstrap support 91 %) characterised previously from Colombia (Barnes *et al.* 2003a, Marin *et al.* 2003). This clade also contained the isolate

from *Schizolobium* (CMW 8858) and isolates from *Coffea* sp (CMW 4844, CMW 4824). The second clade contained the other *C. fimbriata* isolates from *Eucalyptus* spp. in Brazil (CMW 4903), Uruguay (CMW 7383, CMW 7387, CMW 7389), Congo (CMW 4793) and Uganda (CMW 5312). This second clade also contained isolates from *Coffea* sp. (CMW 4835) and citrus (CMW 4829) in Colombia.

Pathogenicity tests

The three *C. fimbriata* isolates used in the inoculations gave rise to lesions (Fig. 4) of varying length on inoculated *E. grandis* trees (Figs 5-7). Although average lesion lengths exceeded those of the controls (Figs 5-7; Tables 3-5), for most isolates these differences were not statistically significant. However, one isolate (CMW 11285) from wounds on *E. grandis* at La Suiza, was highly pathogenic and produced extensive lesions significantly different ($P = 0.0001$) to all others (Figs 5-7).

Eucalyptus grandis clone 301 planted at all three sites was most susceptible to isolate CMW 11285 with lesions extending up to 350 mm (Fig. 5-7). Lesions on Clone 2 planted at Cedral and La Suiza were not significantly different from each other (Figs 5-6), irrespective of the isolate used for inoculation. Clone 2 was clearly resistant to even the most pathogenic isolate of *C. fimbriata*. Trees representing the seed lot *E. grandis* 211 were significantly more susceptible to the highly pathogenic *C. fimbriata* isolate CMW 11285, but not to isolates CMW 11284 or CMW 8858 (Fig. 7; Table 5).

DISCUSSION

Results of this study have shown clearly that *C. fimbriata* is able to infect wounds on *E. grandis* trees in Colombia. These infections, however, appear not to be common and occurred only on two trees cultivate in two different zones considered in this study. Although wounds became infected with *C. fimbriata*, no indication was found of trees dying due to these infections. This may be due to the fact that trees were inspected once after eigth weeks, which might not have been enough time for symptoms expresion. A more likely explanation would be that environment not favourable or trees were not highly susceptible to infection by *C. fimbriata*.

The two isolates from wounds on *E. grandis* in Colombia, grouped inside one of the two distinct phylogenetic lineages previously described for isolates of *C. fimbriata* from Colombia (Barnes *et al.* 2003a, Marin *et al.* 2003). Other *C. fimbriata* isolates from *Eucalyptus* in South America and Africa, however, grouped in the other lineage. Various authors (Barnes *et al.* 2003a, Marin *et al.* 2003, Webster & Butler 1967) have suggested that *C. fimbriata* could represent a complex of distinct species that are morphologically similar. Results from this study, therefore, suggest that at least two species of *Ceratocystis* occur on *Eucalyptus* spp. in the world.

Ceratocystis fimbriata is a virulent pathogen of a wide range of hosts (Kile 1993) including *Eucalyptus* (Barnes *et al.* 2003a; Roux *et al.* 1999, Roux *et al.* 2001). Although the fungus was not found associated with naturally infected trees in this study, we were able to show that wounds on *Eucalyptus* can be infected by the fungus. Furthermore, pathogenicity tests showed clearly that one *E. grandis* clone deployed in Colombian plantations is highly susceptible to infection by this fungus. Certainly these results have shown that *C. fimbriata* is a potentially important *Eucalyptus* pathogen in Colombia. Previously unexplained deaths of trees at Smurfit Carton de Colombia plantations could well have been due to this fungus, which can also be difficult to isolate.

In this study, artificially infected wounds were made on trees to determine whether these might become infected by *C. fimbriata*. Similar wounding studies have previously been used on *Eucalyptus* in Australia (Barnes *et al.* 2003b; Kile *et al.* 1996) and these have led to the discovery of new species of *Ceratocystis*. *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 & French 1983, Teviotdale & Harper 1991). We believe that *C. fimbriata* 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. fimbriata* from wounds on *Eucalyptus,* was significantly more pathogenic than two other isolates 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 (Wolfe & McDermott 1994). If this isolate

had not been included in the trials, *C. fimbriata* would not have been recognised as a potentially important pathogen of *Eucalyptus* in Colombia.

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. fimbriata*. Thus, clone 301 was highly susceptible to infection by the most pathogenic isolate of *C. fimbriata* at all three sites where this clone was tested. This is in contrast to clone 2 that was not susceptible to any of the isolates tested. The fact that the trees generated from seed were significantly more susceptible to infection to the most pathogenic isolate than clone 2 but less so than clone 301, is typical of results found with other *Eucalyptus* pathogens (Keane, Kile, Podger & Brown 2000). Thus seedling material harbours a wide range of susceptibility to pathogens and display wide variability in their response to infection.

Results of this study have shown that *C. fimbriata* 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 *C. fimbriata* infections should be included. Results have also shown that clones differ markedly in their susceptibility to infection. If *C. fimbriata* becomes an important pathogen in the future, there will be excellent opportunities to reduce losses through the selection of disease tolerant planting stock.

REFERENCES

Barnes, I., Roux, J., Wingfield, M. J., Coetzee, M. P. A. & Wingfield, B. D. (2001) Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and Histone sequences. *Plant Disease* **85**: 317-321.

Barnes, I., Roux, J., Wingfield, B. D., O'Neill, M. & Wingfield, M. J. (2003a) *Ceratocystis fimbriata* infecting *Eucalyptus grandis* in Uruguay. *Australasian Plant Pathology* (accepted).

Barnes, I., Roux, J., Wingfield, B. D., Dudzinski, M. J., Old, K. M. & Wingfield, M. J. (2003b) *Ceratocystis pirilliformis*, a new species from *Eucalyptus nitens* in Australia. *Mycologia* (in press).

Castro, B. (1998) Evaluación del daño económico ocasionado por la llaga macana del cafeto. In Informe anual de actividades 1997-1998. Centro Nacional de Investigaciones de Café, Cenicafé, Chinchiná, Colombia.

Hinds, T. E. (1972) Insect transmission of *Ceratocystis* species associated with aspen cankers. *Phytopathology* **62**: 221-225.

Juzwik, J. & French, D. W. (1983) *Ceratocystis fagacearum* and *C. piceae* on the surfaces of free-flying and fungus-mat-inhabiting nitidulids. *Phytopathology* **73**: 1164-1168.

Keane, P. J., Kile, G. A., Podger, F. D. & Brown, B. N. (2000) Diseases and pathogens of Eucalyptus. CSIRO 2000, 565.

Kile, G. A. (1993) Plant diseases caused by species of *Ceratocystis sensu stricto* and *Chalara*. In Ceratocystis *and* Ophiostoma: Taxonomy, Ecology and Pathogenicity. (M. J. Wingfield, K. A. Seifert & J. A. Webber, eds.): 173-183. APS Press, St. Paul, Minnesota.

Kile, G. A., Harrington, T. C., Yuan, Z. Q., Dudzinski, M. J. & Old, K. M. (1996) *Ceratocystis eucalypti* sp. nov., a vascular stain fungus from eucalypts in Australia. *Mycological Research* **100**: 571-579.

Marin, M., Castro, B., Gaitan, A., Preisig, O., Wingfield, B. D. & Wingfield M. J. (2003) Relationships of *Ceratocystis fimbriata* isolates from Colombian coffee-growing regions based on molecular data and pathogenicity. *Journal of Phytopathology* (in press).

Moller, W. J. & DeVay, J. E. (1968) Carrot as a species-selective isolation medium for *Ceratocystis fimbriata*. *Phytopathology* **58**: 123-126.

Mourichon, X. (1994) Serious citrus die-back in Colombia caused by *Ceratocystis fimbriata*. *Fruits* **49**: 415-416.

Pontis, R. E. (1951) A canker disease of the coffee tree in Colombia and Venezuela. *Phytopathology* **41**: 179-184.

Roux, J., Wingfield, M. J., Wingfield, B. D., Bouillett, J. P. & Alfenas, A. C. (1999) A serious new disease of *Eucalyptus* caused by *Ceratocystis fimbriata* in Central Africa. *Forest Pathology* **30**: 175-184.

Roux, J., Coutinho, T. A., Mujuni Byabashaija, D. & Wingfield, M. J. (2001) Diseases of plantation *Eucalyptus* in Uganda. *South African Journal of Science* **97**: 16-18.

SAS Statistical Software (1990) *SAS/STAT Users Guide*. Version 6. 4th edn. 2 vol. 1. SAS Institute, Cary, North Carolina, USA.

SITEP (1999) Sistema Tecnico Estadistico para Plantaciones Forestales Industriales en Colombia, Programa para el manejo de recursos naturales – BIRF (Banco Internacional de Reconstruccion y Fomento) - *Investigacion Ambiental* Año 3 No 5, 12p.

Swofford, D. L. (2002) *PAUP: Phylogenetic Analysis Using Parsimony (* and other methods)* version 4. Sinauer Associates, Sunderland, MA.

Teviotdale, B. L. & Harper, D. H. (1991) Infection of pruning and small bark wounds in almond by *Ceratocystis fimbriata*. *Plant Disease* **75**: 1026-1030.

Upadhyay, H. P. (1981) *A monograph of* Ceratocystis *and* Ceratocystiopsis. University of Georgia Press, Athens.

Webster, R. K. & Butler, E. E. (1967) A morphological and biological concept of the species *Ceratocystis fimbriata*. *Canadian Journal of Botany* **45**: 1457-1468.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal rRNA genes for phylogenetics. In *PCR Protocols: A guide to methods and applications* (A. M. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315-322. Academic Press, San Diego, USA.

Wolfe, M. S. & McDermott, J. M. (1994) Population genetics of plant pathogen interactions: The example of the *Erysiphe graminis* – Hordeum vulgare Pathosystem. *Annual Review of Phytopathology* **32**: 89-113.

Table 1. Isolates of *Ceratocystis fimbriata* obtained from different hosts for which the internal transcribed spacer (ITS) regions and the 5.8S rDNA, data were sequenced or obtained from the GenBank database.

Isolates	Culture number[a]	Host	Country	GenBank number
Ceratocystis fimbriata	CMW 11284[b]	*Eucalyptus grandis*	Buenos Aires, Colombia	n.a.
C. fimbriata	CMW 11285[b]	*E. grandis*	La Suiza, Colombia	n.a.
C. fimbriata	CMW 5312	*E. grandis.*	Uganda	AF395687
C. fimbriata	CMW 4793	*Eucalyptus* sp.	Congo	AF395684
C. fimbriata	CMW 4903	*Eucalyptus* sp.	Brazil	AF395683
C. fimbriata	CMW 1896	*Platanus* sp.	Switzerland	AF395681
C. fimbriata	CMW 2242	*Platanus* sp.	Italy	AF264903
C. fimbriata	CMW 4844	*Coffea* sp.	Colombia	AF 395691
C. fimbriata	CMW 4824	*Coffea* sp.	Colombia	AF 395692
C. fimbriata	CMW 4835	*Coffea* sp.	Colombia	AF 395689
C. fimbriata	CMW 8858[b]	*Schizolobium parahybum*	Colombia	AY233865
C. fimbriata	CMW 4829	*Citrus* sp.	Colombia	AF395688
C. fimbriata	CMW 7383	*E. grandis*	Uruguay	AF453488
C. fimbriata	CMW 7387	*E. grandis*	Uruguay	AF453439
C. fimbriata	CMW 7389	*E. grandis*	Uruguay	AF453440
C. albofundus Wingfield, De Beer & Morris	CMW 2475	*Acacia mearnsii*	South Africa	AF043605
C. albofundus	CMW 2148	*A. mearnsii*	South Africa	AF264910
C. coerulescens (Münch.) Bakshi	CBS 140.37	*Picea abies*	Germany	U75615
C. eucalypti Z. Q. Yuan & Kile	CMW 3254	*E. sieberi*	Australia	U75627
C. virescens (Davidson) C. Moreau	CMW 0460	*Quercus* sp.	USA	AF043603
C. fagacearum (Bretz) Hunt	CMW 2651	*Quercus* sp.	USA	AF043598
C. moniliformis (Hedgc.) C. Moreau	CMW 3782	*Erythrina* sp.	South Africa	AF043597
Petriella setifera	ATCC 24690	*Rock hyrax* dung	Kenya	AF043596

[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.

[b] Isolates CMW 1185, CMW 11284 and CMW 8858 correspond to isolates that were sequenced in this study. Dried cultures representing CMW 1185 (PREM 57511) and CMW 11284 (PREM 57512) were also deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Table 2. Morphological characteristics of *Ceratocystis fimbriata* isolates from *Eucalyptus grandis* in Colombia.

Morphological characteristics	*Ceratocystis fimbriata* from Colombia
Ascomatal base	
Diameter	195-277(-282) µm
Ascomatal neck	
Length	(381-)383-638 µm
Ostiolar hyphae	
Colour	Hyaline
Orientation	Convergent
Ascospores	
Shape	Oblong-ellipsoidal
Appearance	Hat-shaped
Length	5.5-6.5 µm
Width	(4-)4.5-5 µm
Conidiophores	
Shape	Cylindrical, unbranched
Length	33-61(-67) µm
Conidia	
Shape	Cylindrical
Colour	hyaline
Length	20-24(-25.5) µm
Width	3-4.5(-5)µm
Chlamydospores	Present

Table 3. One way ANOVA analysis for lesion length measurement of two isolates of *Ceratocystis fimbriata* from *E. grandis* (CMW 11285 and CMW 11284) and an isolate of *Schizolobium parahybum* (CMW 8858) inoculted in *E. grandis* trees in the Buenos Aires farm.

Source	**Df**	**MS**	**F**	**Pr > F**
Block	4	27220.3	2.16	0.0732
Host	1	850360.6	67.38	0.0001
Isolate	3	748743.7	59.33	0.0001
Host x Isolate	3	688233.2	54.53	0.0001
Error	388	12620.5		

R-Square = 0.52

CV = 144.1

Table 4. One way ANOVA analysis for lesion length measurements of two isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and an isolate of *Schizolobium parahybum* (CMW 8858) inoculted in *E. grandis* trees in La Suiza farm.

Source	**Df**	**MS**	**F**	**Pr > F**
Block	4	15036.0	7.74	0.0001
Host	1	60565.2	31.18	0.0001
Isolate	3	109989.5	56.63	0.0001
Error	388	1942.3		

R-Square = 0.48

CV = 102.1

Table 5. One way ANOVA analysis for lesion length measurements of two isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and an isolate of *Schizolobium parahybum* (CMW 8858) inoculated in *E. grandis* trees in the Cedral farm.

Source	**Df**	**MS**	**F**	**Pr > F**
Block	4	23251.2	2.31	0.0574
Host	1	198742.4	19.75	0.0001
Isolate	3	745467.4	74.07	0.0001
Host x Isolate	3	150542.9	14.96	0.0001
Error	367	10063.0		

R-Square = 0.45

CV = 138.6

Fig. 1. Morphological features of *Ceratocystis fimbriata* from *Eucalyptus grandis* in Colombia. **(a).** Ascoma. **(b).** Ostiolar hyphae. **(c).** Ascospores (hat shape indicated with arrows). **(d).** Phialide. **(e).** Cylindrical conidia. **(f).** Chlamydospores. Bars a-b = 100 μm; c-f = 10 μm.

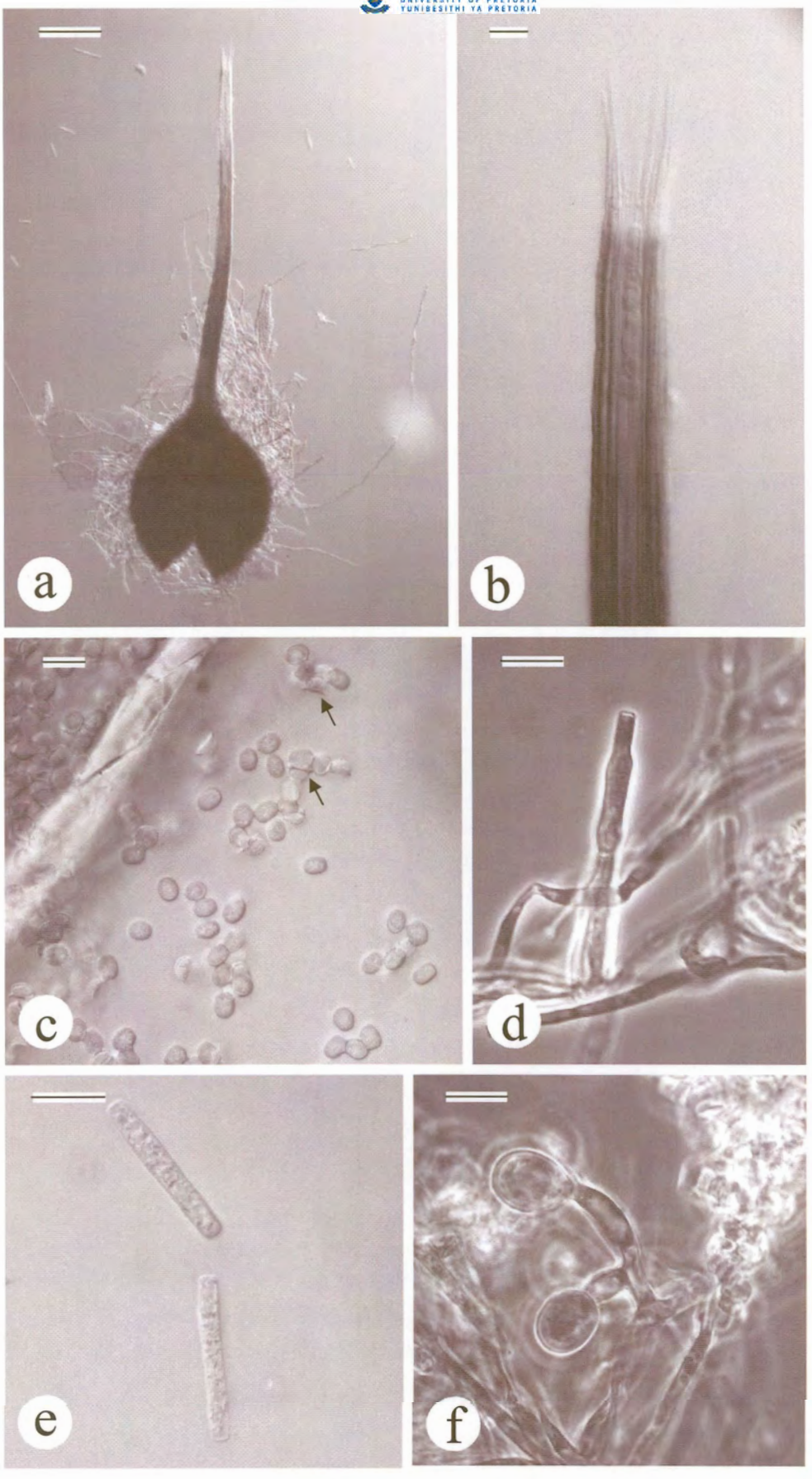


Fig. 2. Raw sequence data of the ITS1 and ITS2 regions and 5.8S gene of the ribosomal operon for various species of *Ceratocystis*. Unknown sequence characters are depicted with an "**N**", while gaps inserted to achieve sequence alignment are shown as "-". Bases matching those of CMW 2242 are indicated with a ".".

```
[                  10         20         30         40         50]

CMW_2242       CCATGTGTGA ACGT-ACC-T ATCTTGTAGT GA-GATGAAT GCTGTTTT-G
CMW_1896       .......... ....-...-. .......... ..-....... .........-
CMW_4903       .......... ....-...C. .......... ..-....... .........-
CMW_7383       .......... ....-...C. .......... ..-....... .........-
CMW_7387       .......... ....-...C. .......... ..-....... .........-
CMW_7389       .......... ....-...C. .......... ..-....... .........-
CMW_4829       .......... ..A.-...C. .......... ..-....... .........-
CMW_4835       .......... ..A.-T..C. .......... ..A....... .........-
CMW_4844       .......... ....-...-. .......... ..-....... .........-
CMW_4824       .......... ....-...-. .......... ..-....... .........-
CMW_11285      .......... ....-...-. G....A.... ..-....... .........-
CMW_11284      .......... ....-...C. .......... ..-....... .........-
CMW_8858       .......... ....-...-. .......... ..-....... .........-
CMW_4793       .......... ....-...-. .......... ..-....... .........-
CMW_5312       .......... ....-...-. .......... ..-....... .........-
CMW_2475       G.TGCCT..G TG.G-----. G...-..... .GT.T.-..C -..C......
CMW_2148       G.TGCCT..G TG.G-----. G...-..... .GT.T.-..C -..C....--
CBS_140.37     ....A..... ..A.--..-. T.T.---..- ---------- -...C....-
CMW_2651       ....T..... ..A.-...GA T.T..T.TTC TC----T... A...C....-
CMW_3254       ....A..... ..A.-...-. TCT-----AG ---------- -...C....-
CMW_0460       ....A..... ..A.-...-. ..T-----AG ---------- -...C....-
CMW_3782       ....T..... .TT.--..-A CAAACA.C.A A--------- -...CGA.T.
ATCC_26490     ..C.T..... ..C.T...A. TGT--TA--- ---------- TG.TGCC.C.

[                  60         70         80         90        100]

CMW_2242       GTGGT-AGGG CCCTTCTGAA GGG------- -CACCGCTGC CAGCAGTAT-
CMW_1896       .....-.... .......... ...------- -......... .........-
CMW_4903       .....-.... .......... .A.AGGG--- -......... .........T
CMW_7383       .....-.... .......... .A.AGGG--- -......... .........T
CMW_7387       .....-.... .......... .A.AGGG--- -......... .........T
CMW_7389       .....-.... .......... .A.AGGG--- -......... .........T
CMW_4829       .....T.... .......... A..GGGGG-- -......... .........-
CMW_4835       .....T.... .......... A..GGGGG-- -......... .........-
CMW_4844       .....-.... .......... ...T------ -A........ .........-
CMW_4824       .....-.... .......... ...------- -......... .G.......-
CMW_11285      .....-.... .......... ...------- -......... .G.......-
CMW_11284      .....-.... .......... ...------- -......... .........-
CMW_8858       .....-.... .......... ...-----TA- --------- .........-
CMW_4793       .....-.... .......... .A.AGGG--- -......... .........-
CMW_5312       .....-.... .......... .A.AGGG--- -......... .........T
CMW_2475       -.AT--.A.. GGGCAGCCC. CTACCGCTAG C....AGCAG ..TACAAG.C
CMW_2148       -.AT--.A.. GGGCAGCCC. CTACCGCTAG C....AGCAG ..TACAAG.C
CBS_140.37     .C-------.- --...GGTTT .AAAAAAACA AGT------. .G.......-
CMW_2651       .C-------. A-...-CTTT CTTCAGGGGA TGTTT-.... ---.....-T
CMW_3254       .C-------.- --...GGT.. CA---CAAGT CT-------.. .G.------T
CMW_0460       .C-------.- --...GGT.. CA---CAA-- ----GT.... .G.------T
CMW_3782       .C----G..T .T.CC.GCCC ..CAGT---- ---------- ---------A
ATCC_26490     .C----G... TTAGC.CCC. AA--GC-TTC T.C.GC.G.- ---...C.--
```

```
[                    110         120         130         140         150]

CMW_2242       AGTCT-CGCC ACTGTAAA-- ---CTCTT-- ---AT-ATTT TT-CCAGA--
CMW_1896       .....-.... ........-- ---.....-- ---..-.... ..-.....--
CMW_4903       .....T.... ........-- ---.....TT T---.T.... .--.T...--
CMW_7383       .....T.... ........-- ---.....TT T---.T.... .--.T...--
CMW_7387       .....T.... ........-- ---.....TT T---.T.... .--.T...--
CMW_7389       .....T.... ........-- ---.....TT T---.T.... .--.T...--
CMW_4829       .AGTCTTCA. CACTGT..AA ---.....TT T--..-.... .--.TTA.--
CMW_4835       .AGTCTTCA. CACTGG..AA ---.....TT T-T..-.... .--.T...--
CMW_4844       .....-.... ........-- ---.....TT TCT..--... ---.TTA.--
CMW_4824       .....-.... ........-- ---.....TT TCT..--... ---.TTA.--
CMW_11285      ....C-.A.. ...--...-- ---.....TT TCT..--... ---.TTA.--
CMW_11284      ....C-.A.. ........A- ---.....TT TCT..--... ---.TTA.--
CMW_8858       .....-.... ........-- ---.....TT TCT..--... ---.TTA.--
CMW_4793       .....-.... ........AA AAA.....TT ---..T.C.. .--.T...--
CMW_5312       .....T.... ........A- ---.....TT T---.T.... .--.T...--
CMW_2475       TT.TA---.. ...A....-- ---.CT.CT- --GTAT.... ..T--.A.A-
CMW_2148       TT.TA---.. ...A....-- ---.CT.CT- --GTAT.... ..T--.A.A-
CBS_140.37     ---------- --.A..T.AA AAA.A..CTT T----A..A. ..-.T...GA
CMW_2651       ..-------- --.A.TT.CA AA-.....TT T----A.... ---.T...GA
CMW_3254       ..-------- --.A.TT.CA AA-.....TT T-----.... ---.T...GA
CMW_0460       ..-------- --.A.TTT-A AAA.....TT TTTT--...- ---.T.A.GA
CMW_3782       CTCT.----- ---------G AACTCG..TT ----.ATA-- ---TA.AGAA
ATCC_26490     ----CTAAAT T..TA.TTTT -ATA--GCGG ATT--ATAC. .----CTGAA

[                    160         170         180         190         200]

CMW_2242       -TTTTTT--- ----CATT-G CTGAGTGGCA T--AACTATA AAAAA---GT
CMW_1896       -.......--- ----....-. .......... .--..... .....---..
CMW_4903       -.....---- ----....-. .......... .--..... .....A--..
CMW_7383       -.....---- ----....-. .......... .--..... .....A--..
CMW_7387       -.....---- ----....-. .......... .--..... .....A--..
CMW_7389       -.....---- ----....-. .......... .--..... .....A--..
CMW_4829       -.....---- ----....-. .......... .--..... .....AA-..
CMW_4835       -.....---- ----....-. .......... .--..... .....AAA..
CMW_4844       -...C----- ----....T. .......... .--..... .....AA-..
CMW_4824       -...C----- ----....T. .......... .--..... .....AA-..
CMW_11285      -...C----- ----....T. .......... .--..... .........-..
CMW_11284      -...C----- ----....T. .......... .--..... .........-..
CMW_8858       -...C----- ----....T. .......... .--..... .....AA-..
CMW_4793       -.....---- ----....-. .......... .--..... .....A--..
CMW_5312       -.....---- ----....-. .......... .--..... .........-..
CMW_2475       -.....AAAA -----...-. .......... .--..... .....A--..
CMW_2148       -.....AAAA -----...-. .......... .--..... .....A--..
CBS_140.37     A.......-A TTTT....-. .......... .T-...ATA. T..-----..
CMW_2651       A..------A TT--....-. ......T... .TT...A.A. T.------..
CMW_3254       A..------A TT--....-. .......... .T-...ATA. T..-----..
CMW_0460       A..------A TT--....-. .......... .T-...ATA. T..-----..
CMW_3782       T..------A TT--....-. ......A... .TTT.TA.AT ------GTA.
ATCC_26490     TACAA.AC-- ---------- ---------- -AA...A.A- ---------.
```

```
[                    210         220         230         240         250]

CMW_2242        TAAAACTTTC AACAACGGAT CTCTTGGCTC TAGCATCGAT GAAGAACGCA
CMW_1896        .......... .......... .......... .......... ..........
CMW_4903        .......... .......... .......... .......... ..........
CMW_7383        .......... .......... .......... .......... ..........
CMW_7387        .......... .......... .......... .......... ..........
CMW_7389        .......... .......... .......... .......... ..........
CMW_4829        .......... .......... .......... .......... ..........
CMW_4835        .......... .......... .......... .......... ..........
CMW_4844        .......... .......... .......... .......... ..........
CMW_4824        .......... .......... .......... .......... ..........
CMW_11285       .......... .......... .......... .......... ..........
CMW_11284       .......... .C........ .......... .......... ..........
CMW_8858        .......... .......... .......... .......... ..........
CMW_4793        .......... .......... .......... .......... ..........
CMW_5312        .......... .......... .......... .......... ..........
CMW_2475        .......... .......... .......... .......... ..........
CMW_2148        .......... .......... .......... .......... ..........
CBS_140.37      .......... .......... .......... .......... ..........
CMW_2651        .......... .......... .......... .......... ..........
CMW_3254        .......... .......... .......... .......... ..........
CMW_0460        .......... .......... .......... .......... ..........
CMW_3782        .......... .......... .......... .......... ..........
ATCC_26490      A......... .......... .......T.. .G........ ..........

[                    260         270         280         290         300]

CMW_2242        GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA ATCATCGAAT
CMW_1896        .......... .......... .......... .......... ..........
CMW_4903        .......... .......... .......... .......... ..........
CMW_7383        .......... .......... .......... .......... ..........
CMW_7387        .......... .......... .......... .......... ..........
CMW_7389        .......... .......... .......... .......... ..........
CMW_4829        .......... .......... .......... .......... ..........
CMW_4835        .......... .......... .......... .......... ..........
CMW_4844        .......... .......... .......... .......... ..........
CMW_4824        .......... .......... .......... .......... ..........
CMW_11285       .......... .......... .......... .......... ..........
CMW_11284       .......... .......... .......... .......... ..........
CMW_8858        .......... .......... .......... .......... ..........
CMW_4793        .......... .......... .......... .......... ..........
CMW_5312        .......... .......... .......... .......... ..........
CMW_2475        .......... .......... .......... .......... ..........
CMW_2148        .......... .......... .......... .......... ..........
CBS_140.37      .......... ...C...... .......... .......... ..........
CMW_2651        .......... .......... .......... .......... ..........
CMW_3254        .......... ...C...... .......... .......... ..........
CMW_0460        .......... ...C...... .......... .......... ..........
CMW_3782        .......... .......... .......... .......... ..........
ATCC_26490      .......... .......... .......... .......... ..........
```

```
[                    310          320          330          340
350]

CMW_2242       CTTTGAACGC ACATTGCGCC TGGCAGTATT CTGCCAGGCA TGCCTGTCCG
CMW_1896       .......... .......... .......... .......... ..........
CMW_4903       .......... .......... .......... .......... ..........
CMW_7383       .......... .......... .......... .......... ..........
CMW_7387       .......... .......... .......... .......... ..........
CMW_7389       .......... .......... .......... .......... ..........
CMW_4829       .......... .......... .......... .......... ..........
CMW_4835       .......... .......... .......... .......... ..........
CMW_4844       .......... .......... .......... .......... ..........
CMW_4824       .......... .......... .......... .......... ..........
CMW_11285      .......... .......... .......... .......... ..........
CMW_11284      .......... .......... .......... .......... ..........
CMW_8858       .......... .......... .......... .......... ..........
CMW_4793       .......... .......... .......... .......... ..........
CMW_5312       .......... .......... .......... .......... ..........
CMW_2475       .......... .......C.. ...T...... .......... ..........
CMW_2148       .......... .......... .......... .......... ..........
CBS_140.37     .......... ......GC.. .......... .......... ..........
CMW_2651       .......... .......... .A........ ....T..... ..........
CMW_3254       .......... .......... .......... .......... ..........
CMW_0460       .......... .......... .......... .......... ..........
CMW_3782       .......... .......... CA......C. ....TG.... ..........
ATCC_26490     .......... .......... C.......A. .....G.... ..........

[                  360          370          380          390          400]

CMW_2242       AGCGTCATTT CACCACTCAA GGACTC---C TTT-GTTCTT GGCGTTGGAG
CMW_1896       .......... .......... .......---. ...-...... ..........
CMW_4903       .......... .......... -.......---. ...T...... ..........
CMW_7383       .......... .......... -.......---. ...T...... ..........
CMW_7387       .......... .......... -.......---. ...T...... ..........
CMW_7389       .......... .......... -.......---. ...T...... ..........
CMW_4829       .......... .......... -...CT---. ...T...... ..........
CMW_4835       .......... .......... -...CT---. ...T...... ..........
CMW_4844       .......... .......... -.....C--. ...-...... ..........
CMW_4824       .......... .......... -.....C--. ...-...... ..........
CMW_11285      .......... .......... -.....C--- ...-...... ..........
CMW_11284      .......... .......... -.....C--- ...-...... ..........
CMW_8858       .......... .......... -.....C--. ...-...... ..........
CMW_4793       .......... .......... -..-.TATT. ...----... ..........
CMW_5312       .......... .......... -..-.TATT. ...----... ..........
CMW_2475       .......... .......... -....T--G. ...A...T.- ..T.......
CMW_2148       .......... .......... -....T--G. ...A...T.- ..T.......
CBS_140.37     .......... .......... -.-...TG-- --------C. T.GTG.T.GA
CMW_2651       .......... .......... -.-.CTT-G. ..--------- ..T.......
CMW_3254       .......... .......... -.-...C-G. ..--------- ..T.......
CMW_0460       .......... .......... -.-...C-G. ..--------- ..T.......
CMW_3782       .......... .......... -.-...T-G. ..--------- .TT.......
ATCC_26490     .......... ....CTCG.G ---.CTAAGT ...TTAAAC. ------.A..
```

```
[                    410         420         430         440         450]

CMW_2242       GTCCTGTTCT CCCC----TG AACAGGCCGC CGAAATGTAT CGGCTGTTA-
CMW_1896       .......... ....----.. .......... .......... .........-
CMW_4903       .......... ....----.. .......... .......... .........-
CMW_7383       .......... ....----.. .......... .......... .........-
CMW_7387       .......... ....----.. .......... .......... .........-
CMW_7389       .......... ....----.. .......... .......... .........-
CMW_4829       .......... ....----.. .......... .......... .........-
CMW_4835       .......... ....----.. .......... .......... .........-
CMW_4844       .......... ....C---.. .......... .......... .........-
CMW_4824       .......... ....C---.. .......... .......... .........-
CMW_11285      .......... ....----.. .......... .......... .........-
CMW_11284      .......... ....----.. .......... .......... .........-
CMW_8858       .......... ....C---.. .......... .......... .........-
CMW_4793       .......... ....----.. .......... .......... .........-
CMW_5312       .......... ....----.. .......... .......C.. .........-
CMW_2475       .......... TA..CTTC.. .......... .......C.. .........T
CMW_2148       .......... TA..CTTCC. .......... .......C.. .........T
CBS_140.37     .GA.CCGCG. .TTTTTTG.T TG.G...... .......C.. .........T
CMW_2651       .A..CCA-.. TGT.ACAAG- --.G.C.A-. .......C.. .......AGT
CMW_3254       .A.TC.CATC TTA----TGA TG.G...... .......C.. .........T
CMW_0460       .A......G. TT-------.C .........A. .......C.. .........T
CMW_3782       AG.....G.. AT-------. CG.G....T. T......C.. .......GTT
ATCC_26490     .AT.G.---. GTTGGGGCGC T....--..G TTCTTC.G.G .A.....A--

[                    460         470         480         490         500]

CMW_2242       ---TACTTGC C-AACTCCCC TGTGTAGTAT AAAA-TTTCT -AATTTTTAC
CMW_1896       ---....... .-........ .......... ....-..... -.........
CMW_4903       ---....... .-........ .......... ....-..... -.........
CMW_7383       ---....... .-........ .......... ....-..... -.........
CMW_7387       ---....... .-........ .......... ....-..... -.........
CMW_7389       ---....... .-........ .......... ....-..... -.........
CMW_4829       ---....... .C........ .......... ....-..... -.........
CMW_4835       ---....... .C........ .......... ....-..... -.........
CMW_4844       ---....... .-........ .......... ....-..... -.........
CMW_4824       ---....... .-........ .......... ....-..... -.........
CMW_11285      ---....... .-........ .......... ....-..... -.........
CMW_11284      ---....... .-........ .......... ....-..... -.........
CMW_8858       ---....... .-........ .......... ....-..... -.........
CMW_4793       ---....... .-........ .......... ....-..... -.........
CMW_5312       ---....... .-........ .......... ....-..... -.........
CMW_2475       TTT....... .-........ .........C ..G.T...T. A.........
CMW_2148       TTT....... .-........ .........C ..G.T...T. A.........
CBS_140.37     ----.T.... --.G..T... .........A ----TA..T- --.......-..
CMW_2651       ----.T.... --.G..T... ..C......A ..C-T...-G TG-.----..
CMW_3254       ----...... --.G..T... .........A ----TA..T- --.....--..
CMW_0460       ----...... --.G..T... .........A ----TA.--C T-.....--..
CMW_3782       -----..A... --.GT.T... .........A ..C-------- --...G..G.
ATCC_26490     ---------- -GGC.CTG-- ---------.A .T.CAG.GGC GGTCCCGCCG
```

```
[                      510]

CMW_2242       ACTTTGAAGT
CMW_1896       ..........
CMW_4903       ..........
CMW_7383       ..........
CMW_7387       ..........
CMW_7389       ..........
CMW_4829       ..........
CMW_4835       ..........
CMW_4844       ..........
CMW_4824       ..........
CMW_11285      ..........
CMW_11284      ..........
CMW_8858       ..........
CMW_4793       ..........
CMW_5312       ..........
CMW_2475       G.....G...
CMW_2148       G.....G...
CBS_140.37     G.......AC
CMW_2651       G...C...AC
CMW_3254       G.......AC
CMW_0460       ........AC
CMW_3782       ........AC
ATCC_26490     CGGCGCNNNN
```

Fig. 3. The phylogenetic tree (tree length = 788 steps, consistency index/CI = 0.7728, retention index/RI = 0.7926) generated from DNA sequences of the ITS1/2 regions of the ribosomal DNA for various *Ceratocystis* species. Bootstrap values >50% (1000 replicates) are indicated below the branches in bold. An isolate of *Petriella setifera* (ATCC 26490) was used as the outgroup taxon.

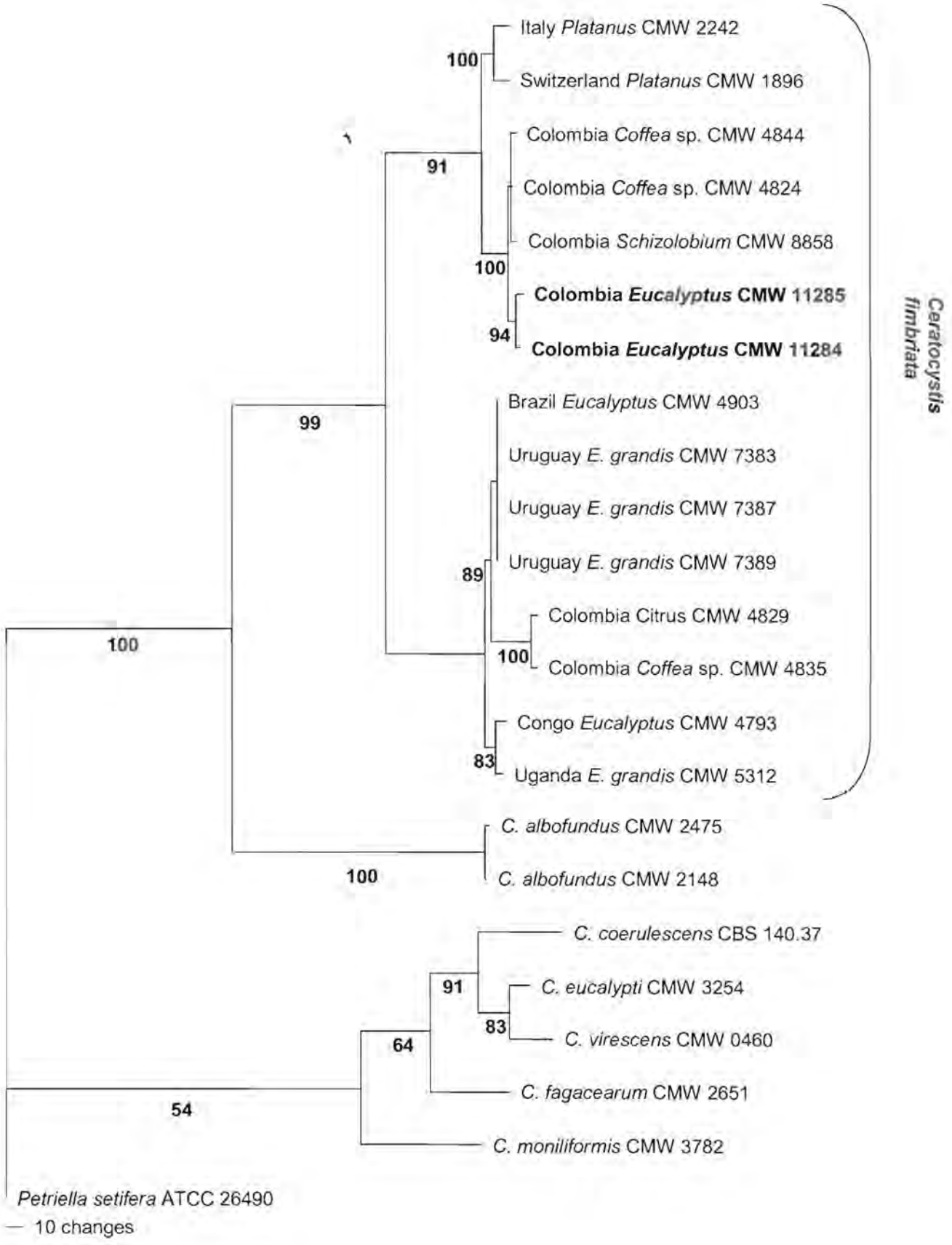


Fig. 4. Lesions produced by isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and an isolate of *Schizolobium parahybum* (CMW 8858) on various clones or seedling trees inoculated in field trials in Colombia.

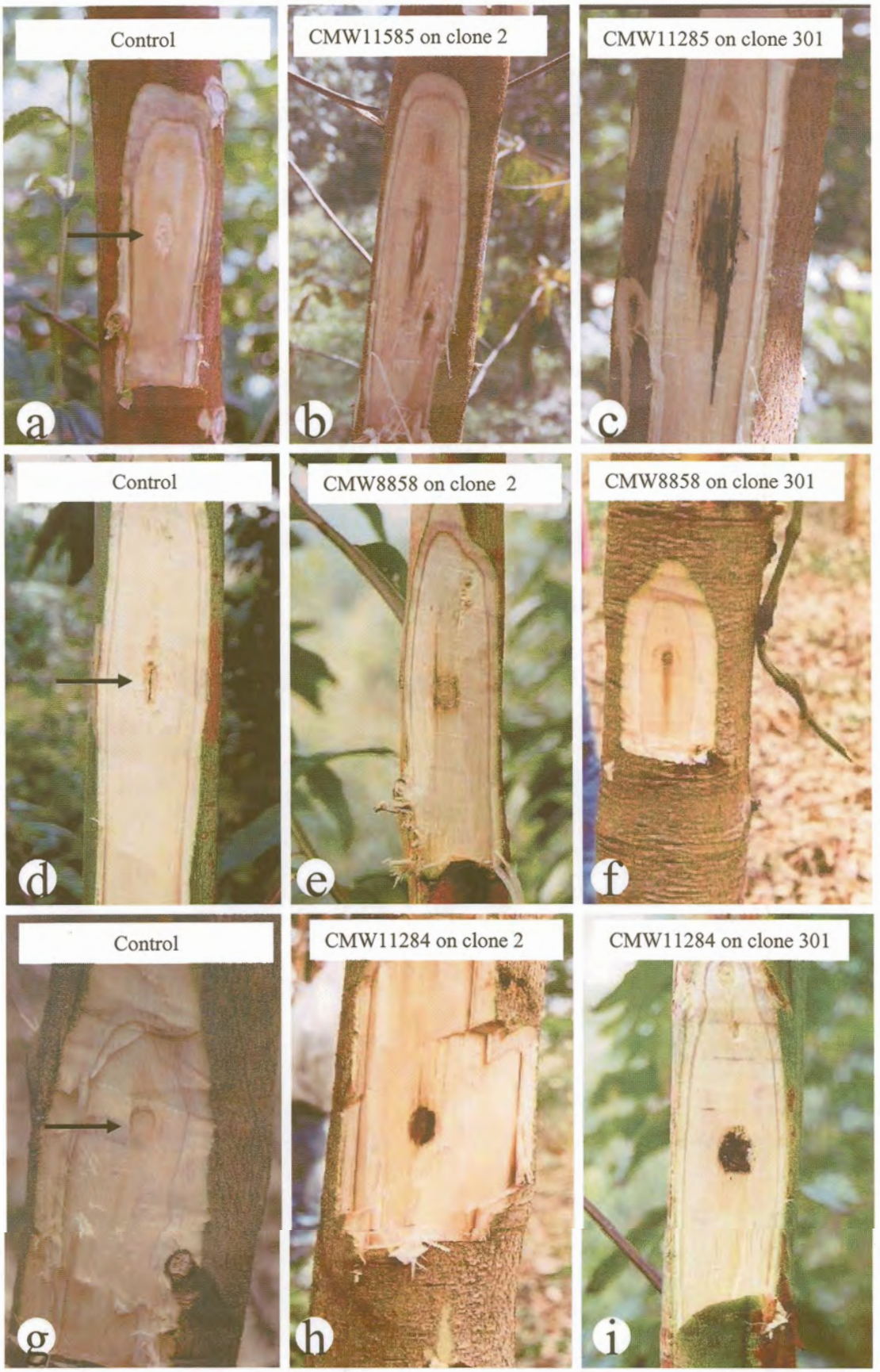


Fig. 5. Results of an inoculation trial with isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and *Schizolobium parahybum* (CMW 8858) from Colombia and a negative control. Inoculations were done on *E. grandis* clones 301 and 2 at Buenos Aires farm, Trujillo Valle. Mean length of lesions is shown with 95% confidence limits.

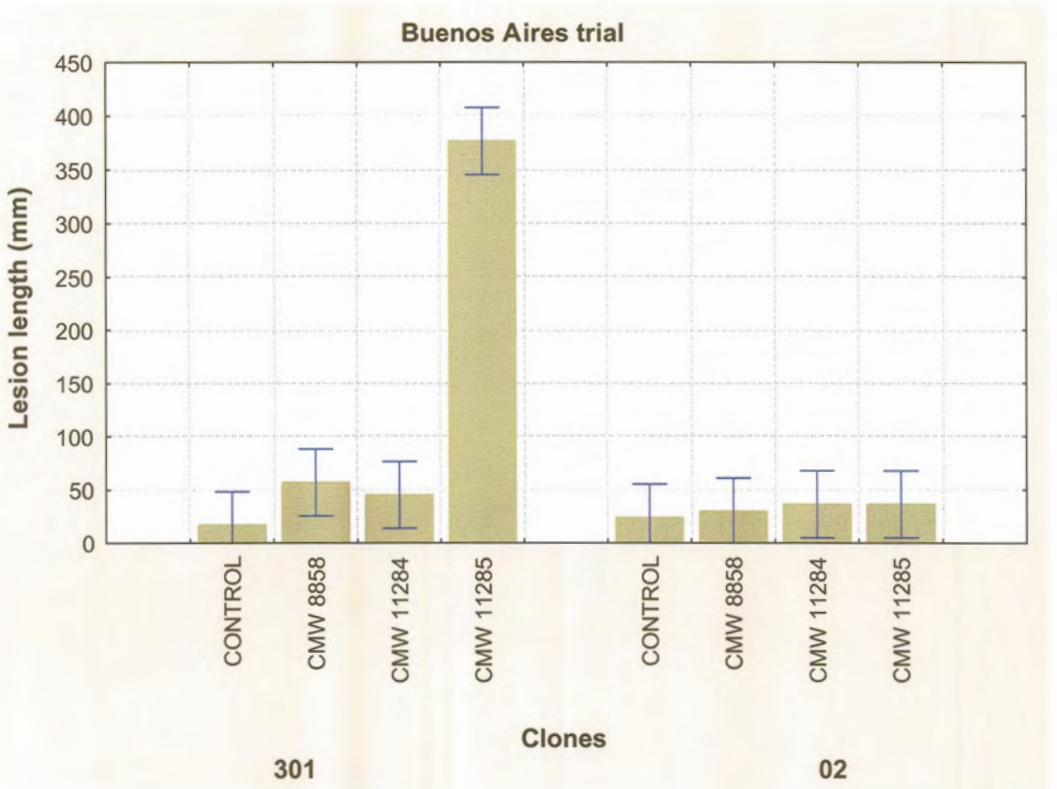

Buenos Aires trial
Lesion length (mm)
450
400
350
300
250
200
150
100
50
0
CONTROL
CMW 8858
CMW 11284
CMW 11285
CONTROL
CMW 8858
CMW 11284
CMW 11285
Clones
301
02

Fig. 6. Results of an inoculation trial with isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and *Schizolobium parahybum* (CMW 8858) from Colombia and a negative control. Inoculations were done on *E. grandis* clones 301 and 2 at La Suiza farm, Restrepo Valle. Mean length of lesions is shown with 95% confidence limits.

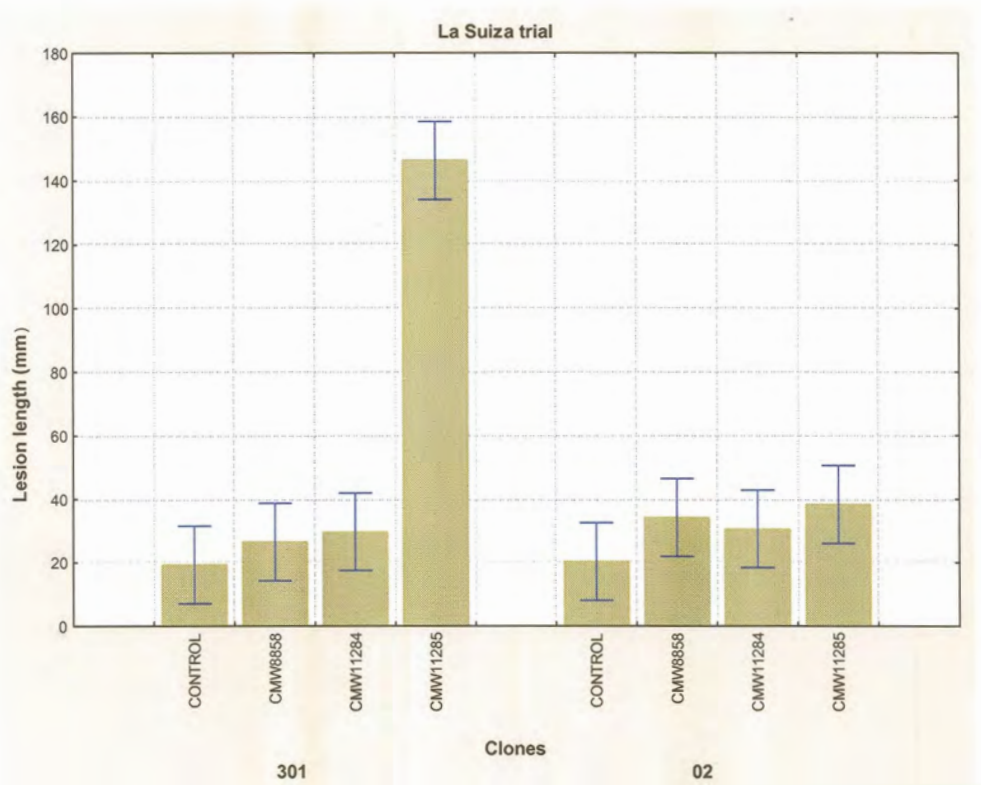
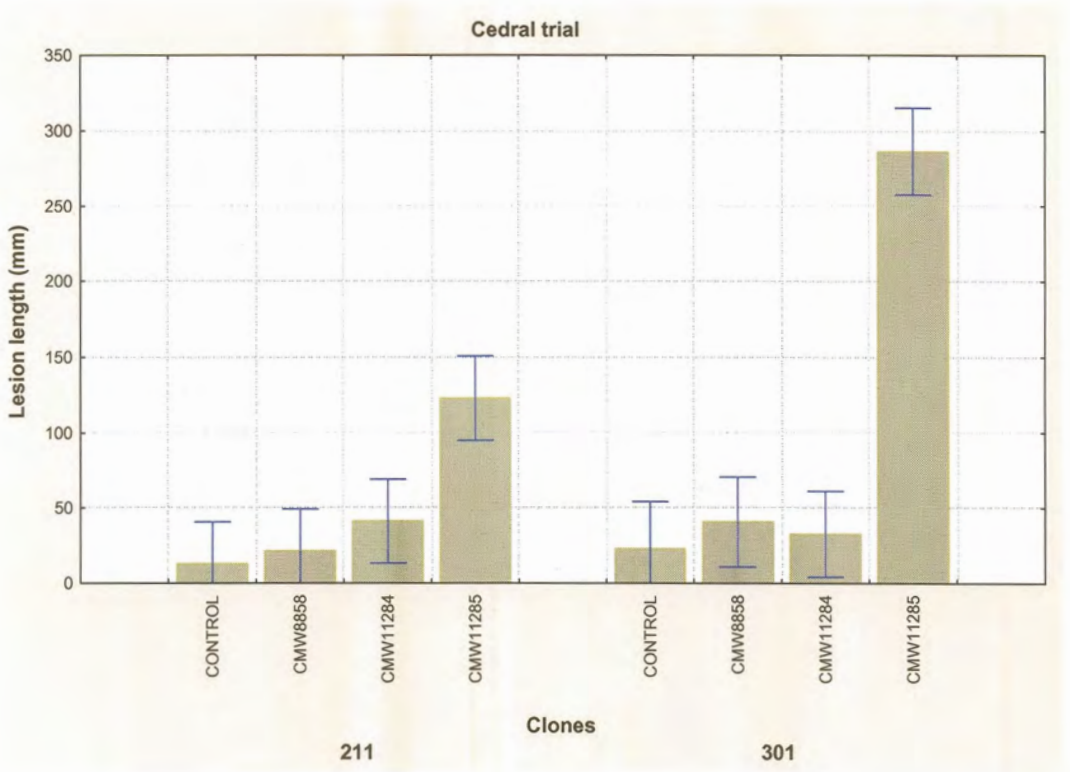

La Suiza trial
Lesion length (mm)
180
160
140
120
100
80
60
40
20
0
CONTROL
CMW8858
CMW11284
CMW11285
CONTROL
CMW8858
CMW11284
CMW11285
Clones
301
02

Fig. 7. Results of an inoculation trial with isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and *Schizolobium parahybum* (CMW 8858) from Colombia, and a negative control. Inoculations were done on clone 301 and seed 211 at Cedral farm, Darien, Valle. Length of lesions is shown with 95% confidence limits.


Cedral trial
Lesion length (mm)
350
300
250
200
150
100
50
0
CONTROL
CMW8858
CMW11284
CMW11285
CONTROL
CMW8858
CMW11284
CMW11285
Clones
211
301