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Cylindrocladium blight of *Eucalyptus grandis* in Colombia

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Abstract. Cylindrocladium leaf blight is one of the most important diseases affecting *Eucalyptus grandis* plantations in Colombia. Disease symptoms include both leaf blotch and shoot blight and these can lead to severe defoliation. This reduces the productivity of *E. grandis* in forestry zones with high humidity. The objective of this study was to identify the *Cylindrocladium* spp. associated with Cylindrocladium leaf blight of *E. grandis* in three important forestry regions of Colombia. Isolates were obtained from samples collected within these areas and morphology as well as DNA sequence data were used for identification. Results of both morphological comparisons and analysis of β-tubulin gene sequences showed that only *C. spathulatum* was associated with the disease symptoms in the evaluated areas. 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 future.

Introduction

Colombia has a large and growing forestry industry with ~140 000 ha planted mainly to various species of *Pinus* and *Eucalyptus.* These trees are used to produce structural timber, pulpwood and paper. Approximately 47700 ha (34%) of the forestry areas are planted to *Eucalyptus* species, which are rapidly becoming a major component of the forestry industry in Colombia (Osorio *et al*. 1995). However, as in other *Eucalyptus* plantations world-wide, new pests and pathogens are appearing 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. are recognised as one of the most important threats to *Eucalyptus grandis*. *Cylindrocladium* spp. represent an important group of pathogens associated with diverse hosts in tropical and subtropical regions of the world (Crous 2002). A possible reason for the common occurrence of these fungi on *E. grandis* in Colombia is the high humidity in areas where plantations have been established. During the last 6 years, leaf spot symptoms and defoliation caused by *Cylindrocladium* spp. have occurred in young plantations located in the Caldas, Quindio, Risaralda and Valle provinces of Colombia.

The most common symptoms of *Cylindrocladium* infection on *E. grandis* in commercial plantations in Colombia are similar to those found elsewhere in the world (Sharma *et al*. 1985; Crous 2002; Old *et al*. 2003). Leaf and shoot blight, which develop upwards from the base of trees, occur on young trees. On *E. grandis*, leaf spots that form initially on the mature leaves on the lower branches of young trees (1- to 2-years-old) are most prominent. Defoliation moves upwards from the base and centres 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.

Preliminary surveys (Crous and Kang 2001; Wingfield, unpublished) between 1993 and 1995 led to the identification of a number of *Cylindrocladium* spp. from *Eucalyptus* plantations in Colombia. These include *C. candelabrum*, *C. gracile*, *C. parasiticum*, *C. spathulatum* and *C. reteaudii*. These *Cylindrocladium* spp. originated from soils collected in plantations of various *Eucalyptus* spp. (Crous 2002). Although the presence of these species was of interest, the work was largely of a taxonomic nature and their relative importance as *Eucalyptus* pathogens was not considered.

Cylindrocladium spp. 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 (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 (Crous 2002). Morphological features of species of the *Calonectria* teleomorph tend to be more conserved and

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species identification based on these characters alone is generally not possible (Crous and Wingfield 1994; Crous 2002). To further aid in identification, DNA sequences exist to compare isolates. The most extensive of these datasets originates from the study by Schoch *et al*. (2001) that employed sequences of the β-tubulin genes for more than 30 *Calonectria* spp.

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

Methods

Isolates

Isolates were obtained from leaf spots on *E. grandis* in plantations displaying Cylindrocladium leaf blight symptoms (Fig. 1). Samples were collected from 14 farms located in three different geographic areas of Colombia (Table 1). Twenty diseased leaves from each of ten randomly selected trees were collected at each of the 14 farms sampled. These collections 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. Three leaves, most representative of the disease symptoms, from each selected tree (thus a total of 420 leaves) were placed in moist chambers and incubated at 25°C for ~10 days to promote sporulation. Conidia produced on typical *Cylindrocladium* conidiophores (Fig. 2*a*) were present on virtually all symptomatic leaves. Conidia from these leaves were then transferred onto 2% malt-extract agar (MEA; Biolab, Midrand, South Africa) in Petri dishes. Isolations were made only from *Cylindrocladium* structures and other fungi on symptomatic leaves were not considered. Dishes were incubated for 8 days at 25°C under continuous near-ultraviolet light. From this larger collection, a representative set of 19 cultures (Table 1) has been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Dried cultures of representative isolates have been lodged with the National Collection of Fungi (PREM) in Pretoria, South Africa (Table 2).

Morphological characteristics

Growth characteristics of the cultures obtained from the diseased tissue were compared on 2% MEA to identify different cultural groups. Of the total number of isolates (420), a subset of similar-looking isolates (19) was chosen for further in-depth identifications. These isolates were plated onto carnation-leaf agar (CLA) (Crous 2002) to induce production of both anamorph and teleomorph structures. These plates were incubated at 25°C under near-ultraviolet light and examined after 7 days. Cultural and morphological characteristics were determined as described by Crous (2002). Conidiophores on the surface of carnation leaves were mounted on microscope slides in lactophenol and 20 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 − s.d.) − (average + s.d.) (-max).

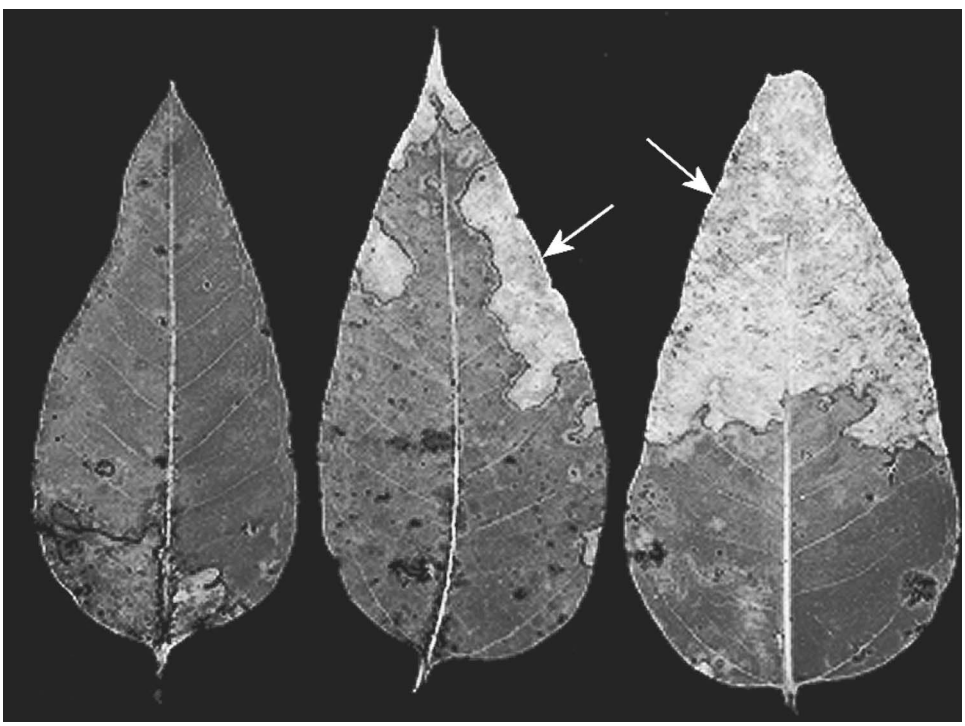

Fig. 1. Leaf spot symptoms (indicated with arrows) at different stages of development associated with *Cylindrocladium spathulatum* infection on leaves of *Eucalyptus grandis* in Colombia.

Table 1. *Cylindrocladium* isolates from *Eucalyptus grandis* 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	Angela 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	Angela 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.

Deoxyribonucleic acid sequence comparisons

Five isolates (Table 2) were used in the DNA sequencing and subsequent phylogenetic analyses. These isolates (CMW 10369 and CMW 10357 from Valle, CMW 10363 and CMW 10367 from Cauca, and CMW 10374 from Andina) were selected from those collected from different farms in the three different geographic areas in Colombia and were included in the 19 isolates selected for the morphological studies. 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. Deoxyribonucleic acid was extracted using the technique described by Möller *et al*. (1992).

A 473 bp fragment of the β-tubulin gene was amplified using primers T1 (5′ AACATGCGTGAGATTGTAAGT 3′) (O'Donnell and Cigelnik 1997) and Bt2b (5′ ACCCTCAGTGTAGTGACCCTTGGC 3′) (Glass and Donaldson 1995). The PCR reactions of 25 µL comprised of 2.5 units of Taq (Roche Molecular Biochemicals, Almeda, California, USA), 10 × buffer, 1 mM $MgCl_2$ (as supplied by the manufacturer), 0.25 mM deoxynucleotide triphosphates, 0.5 µM primers and ~30 ng of fungal genomic DNA as target. 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 3100 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). A heuristic search was executed on the aligned dataset using the TBR (tree-bisection-reconnection) algorithm (MulTrees option effective, saving all optimal trees). Gaps were treated as missing data 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* 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 2-year-old trees planted at Angela Maria farm in Andina, 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, was 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 isolations were made from a random sample of leaves for identifications. Statistical analysis of the infection data was carried out using SAS Statistical Software (1990). Analysis of variance tables were produced, as well as graphs of means with the 95% confidence limits for each mean.

Results

Morphological characteristics

White conidiophores typical of those of *Cylindrocladium* spp. (Fig. 2*a*) 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 19 isolates collected from the 14 farms and they all represented a single morphological entity. A *Calonectria* state was common on the carnation leaves and in culture (Fig. 2*b*).

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*, 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 diameter (Fig. 2*c* and *d*). Each terminal branch of the fertile branches produced approximately five phialides (Fig. 2*c* and *d*). Phialides were cylindrical, straight, doliiform to reniform, hyaline and aseptate (Fig. 2*d*). The conidia were cylindrical, rounded at the ends, straight, 3-septate (Fig. 2*e*).

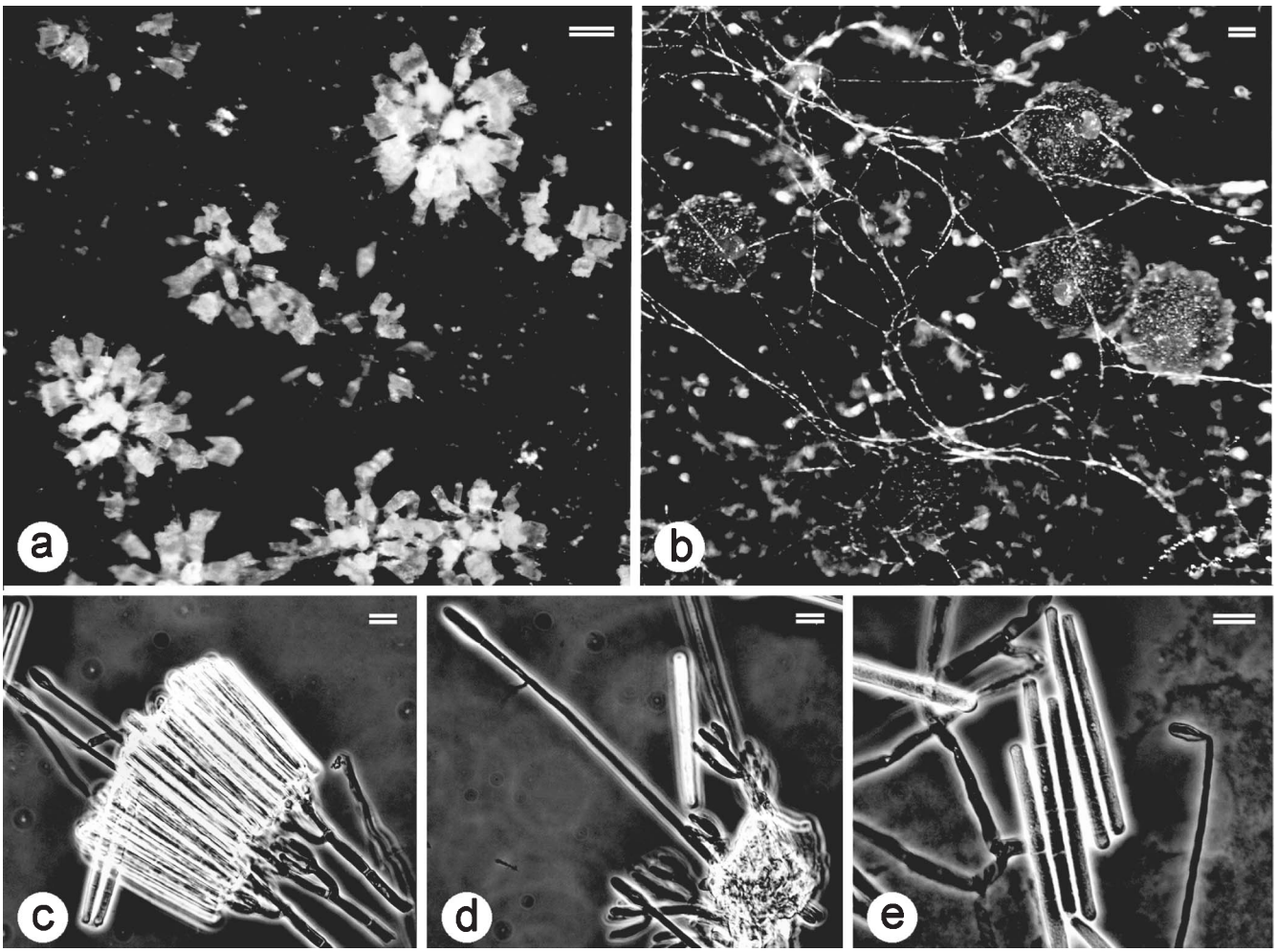

Fig. 2. Morphology of *Cylindrocladium spathulatum.* (*a*) Superficial sporulation of *C. spathulatum* on a *Eucalyptus grandis* leaf. (*b*) Perithecia of the *Calononectria* state produced on MEA medium. (*c*) Macroconidiophore with attached conidia. (*d*) Conidiophore with extending stipe and terminal vesicle. (*e*) Conidia. Bars a–b = 100 µm, c–e = 20 µm.

The size variation computed for 90 conidia was (48–)53–73(–90) × (3–)4–6(–8) µm (average = 63 × 5.5 µm).

Deoxyribonucleic acid 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 dataset of 473 characters of which 278 were constant, and of the variable characters 99 were parsimony-uninformative and 96 parsimony-informative. Due to variation in relationships between the *C. candelabrum*, *C. scoparium* and *C. insulare* isolates, 54 most parsimonious trees were obtained. There was no variation in the grouping of the *Cylindrocladium* isolates from Colombia and one tree from the 54 trees was chosen for presentation (Fig. 3). 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. 3) 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. 4). There was a clear continuum of levels of susceptibility of clones, but clones could be classified as highly susceptible and highly tolerant to *C. spathulatum* at the upper and lower limits. 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

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

Isolate number[A]	Species identity	GenBank numbers
CMW 10359[B]	*C. spathulatum*	–
CMW 10357	*C. spathulatum*	AY700590
CMW 10374[B]	*C. spathulatum*	AY700594
CMW 10367[B]	*C. spathulatum*	AY700592
CMW 10369	*C. spathulatum*	AY700593
CMW 10363[B]	*C. spathulatum*	AY700591
CMW 10370[B]	*C. spathulatum*	–
STE U 2712	*C. spathulatum*	AF308463
STE U 599	*C. spathulatum*	AF308464
STE U 925	*C. pauciramosum*	AF210470
STE U 416	*C. pauciramosum*	AF210869
STE U 1677	*C. candelabrum*	AF210858
STE U 1674	*C. candelabrum*	AF210857
ATCC 46300	*C. scoparium*	AF210873
ATCC 38227	*C. scoparium*	AF210872
STE U 616	*C. insulare*	AF210860
STE U 768	*C. insulare*	AF210853
STE U 1237	*C colhounii*	AF231953
STE U 1339	*C. colhounii*	AF232851
STE U 516	*C. reteaudii*	AF232870
ATCC 16550	*C. reteaudii*	AF232868
STE U 941	*C. mexicanum*	AF210864
STE U 927	*C. mexicanum*	AF210863
NRRL 22016	*Fusarium circinatum*	AF434472

[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 (PREM), Pretoria under the following numbers: PREM 57504 (= CMW 10359), PREM 57501 (= CMW 10374), PREM 57503 (= CMW 10367), PREM 57502 (= CMW 10363), PREM 7505 (= CMW 10370).

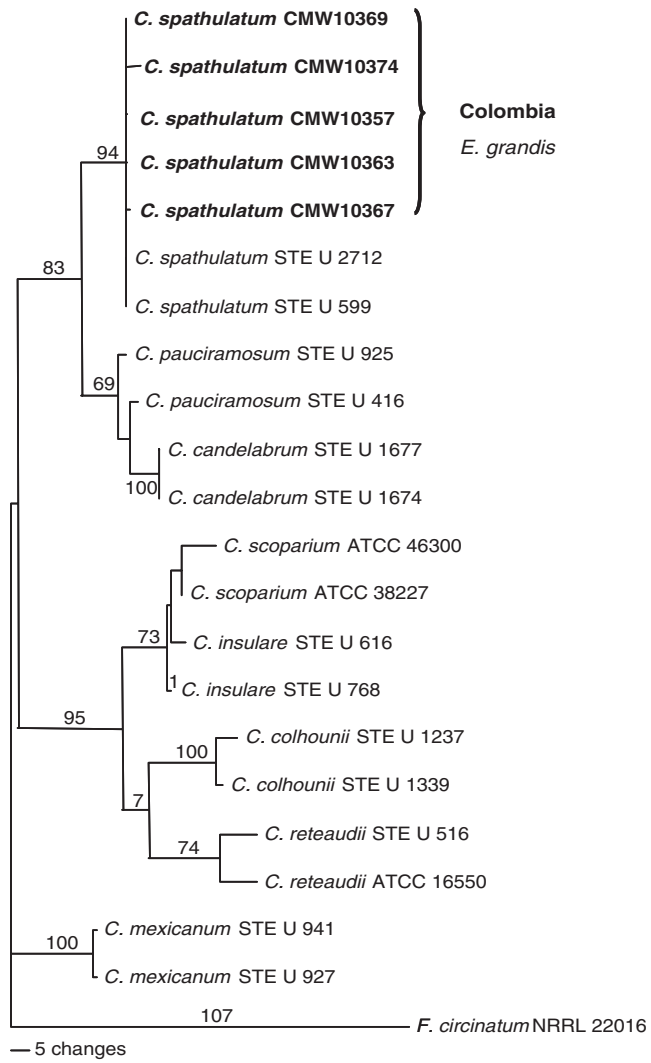

Fig. 3. Phylogenetic tree obtained from β-tubulin gene sequences. Confidence levels >50% determined by a bootstrap analysis (1000 replicates) of the tree branch nodes are shown. Isolates sequenced in this study are presented in bold. A *Fusarium circinatum* isolate was used as the outgroup taxon.

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 showed no differences in susceptibility based on position of the branches either lower or 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 associated with Cylindrocladium leaf blight on *E. grandis* in four different areas of Colombia. *C. spathulatum* was the only *Cylindrocladium* sp. found amongst a large collection of isolates from leaves. This result is interesting as previous preliminary surveys (Crous and Kang 2001; Wingfield, unpublished) also identified four other species of *Cylindrocladium* in Colombian plantations. None of these species was isolated during this study and the fact that they originated from soil samples suggests that they are probably not important pathogens of *Eucalyptus* in Colombia.

There are two possible explanations for the fact that only *C. spathulatum* was isolated from Cylindrocladium leaf blight symptoms in Colombia in this survey. One is that the previous surveys were more random, were made from several hosts, included soil samples and were not necessarily linked to major outbreaks of leaf blight. In this study, we specifically focused on isolating *Cylindrocladium* species associated with leaf blight from several locations in Colombia. It is also known that *Cylindrocladium* spp. responsible for

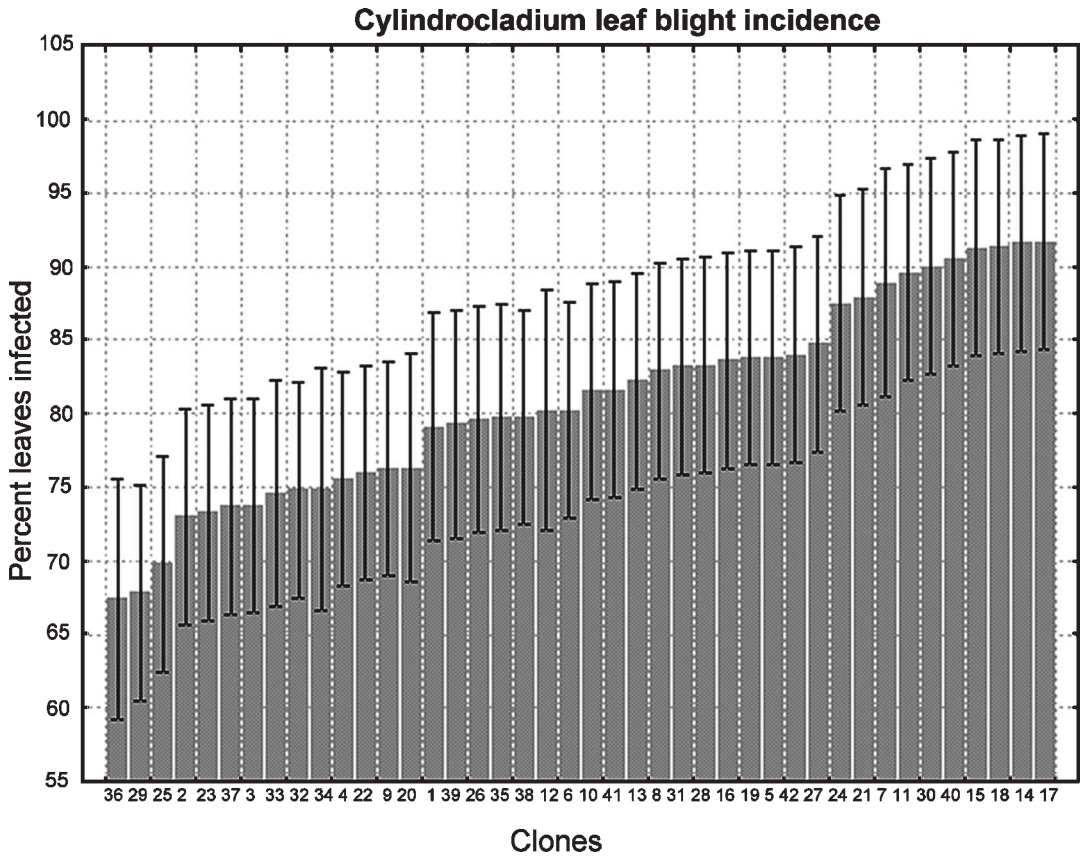

Fig. 4. Graphical presentation of percentage leaves infected by *Cylindrocladium spathulatum* on 42 *Eucalyptus grandis* clones at the Angela Maria farm in Andina, Colombia. Data are presented as percentage leaves infected and 95% confidence limits are also shown.

leaf blight in *Eucalyptus* plantations can change over time (Crous 2002), and it is possible that other species of *Cylindrocladium* could be isolated from diseased leaves in the future.

C. 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 and 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 and Kang 2001; Crous 2002). Results of this study also indicate that *C. spathulatum* is a serious pathogen in Colombia. 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 Colombia.

We have shown that the species responsible for leaf blight in Colombia, *C. spathulatum*, is the only species present in three planting zones that differ markedly in climate. This is contrary to previous results (Booth *et al*. 2000) where climate was shown to strongly influence Cylindrocladium leaf blight occurrence. However, all four sites are typified by humid conditions that clearly facilitate infection (Sharma and Mohanan 1991). 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 (Old *et al*. 2003; 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 (Sharma *et al*. 1985; Blum and Dianese 1993; Crous 2002). Our results are encouraging from a management perspective as it should be possible to start with a selection programme to develop planting stock with high levels of resistance to Cylindrocladium leaf blight in Colombia. Furthermore, breeding using trees with such resistance is likely to reduce the impact of Cylindrocladium leaf blight in Colombia in the longer term.

Acknowledgements

We thank Dr B. E. Eisenberg for assistance with the statistical analyses of the pathogenicity data and Liliana Perafan for providing additional statistical support. We are grateful to Dr Luis F. Osorio and Mr Ramon Arbelaez who provided technical assistance during sampling and field trials in Colombia. Financial support of Smurfit Carton de Colombia, the National Research Foundation (NRF), members of the Tree Protection Co-operative Programme (TPCP), and the THRIP support programme of the Department of Trade and Industry from South Africa, made this study possible.

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Received 25 February 2004, accepted 13 October 2004