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Botryosphaeriaceae associated with *Eucalyptus* canker diseases in Colombia

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Summary

The identities of Botryosphaeriaceae causing cankers on *Eucalyptus* in Colombia were investigated using morphological and DNA sequence comparisons. The pathogenicity of the species was also assessed on 42 *Eucalyptus grandis* clones planted at four different sites. Two species of the Botryosphaeriaceae were found to occur on *E. grandis* in Colombia. *Neofusicoccum ribis* was the more common species, and also the most pathogenic. *Botryosphaeria dothidea* was found only in one zone of Colombia, and was also less pathogenic than *N. ribis*. These two species could be distinguished easily based on DNA sequences of the ITS1/ITS2 rDNA region and EF1-α, in addition to conidial sizes. Significant differences in resistance of clones to these pathogens were also evident from the various trials.

1 Introduction

Species of the Botryosphaeriaceae include an important group of opportunistic and pathogenic fungi that infect a wide range of woody plants world-wide (Slippers and Wingfield 2007). These fungi are well-adapted facultative parasites or saprophytes on dead wood and other plant material. Botryosphaeriaceae also infect healthy plant tissue and can exist for extended periods of time in a latent form (Fisher et al. 1993; Smith et al. 1996). Under certain environmental conditions, they can penetrate their hosts through wounds, open stomata and lenticels, infecting twigs, stems, roots and leaves. With the onset of stress, they then become active and cause serious disease (Schoeneweiss 1980; Old et al. 1990). Disease symptoms include stem and branch cankers, die-back, bleeding necrosis, coppice failure and seed capsule abortion (Webb 1983; Barnard et al. 1987; Smith et al. 1994; Neely 1996).

The taxonomy of the Botryosphaeriaceae has been confused and complicated for many decades. Thousands of species have been described in teleomorph and anamorph taxa linked to this group (Denman et al. 2000; Crous et al. 2006; www.indexfungorum.org). Anamorphs are more common in nature and in culture than the teleomorph states, and are thus used more frequently to identify species. The morphology of both states however can be confusing, because characters, such as conidial pigmentation, septation and stromatal morphology, show extensive plasticity and can be influenced by substrate and growth conditions (Von Arx and Müller 1954; Butin 1993). Nevertheless, Botryosphaeriaceae anamorphs appear to provide the best means of morphological identification of these species (Denman et al. 2000; Slippers et al. 2004a; b). Recently, DNA-based techniques

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have been applied to the taxonomy of Botryosphaeriaceae (Jacobs and Rehner 1998; Zhou and Stanosz 2001; Phillips et al. 2002; Slippers et al. 2004a; Crous et al. 2006). This has made it possible to differentiate more accurately between species and to evaluate the taxonomic value of various identification procedures.

Botryosphaeriaceae constitute an important group of pathogens of non-native plantation trees, especially of *Eucalyptus* (Smith et al. 1994, 2001; Slippers et al. 2004b). The names for fungi, such as *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not. and *Neofusicoccum ribis* Grossenb. & Dugg. have however been used loosely and interchangeably (Denman et al. 2000; Slippers et al. 2004a). Previous reports of *B. dothidea* and *N. ribis* on *Eucalyptus* could be incorrect as DNA sequence comparisons have now shown that these species are rare on *Eucalyptus* and that earlier reports probably represent other Botryosphaeriaceae, such as *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum eucalyptorum* (Crous, H. Smith & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum eucalypticola* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips and other species (Slippers et al. 2004a,b; Burgess et al. 2005).

Eucalyptus plantations represent an important renewable resource for the forestry industry in Colombia. One of the most important species presently planted is *Eucalyptus grandis.* This species is used in reforestation and clonal programmes by private companies and in government projects for the production of timber, pulp and protection of eroded soils.

Botryosphaeriaceae have been recognized as an important constraint to the productivity of *E. grandis* plantations in Colombia. Since 1994, diseases caused by Botryosphaeriaceae have been recorded on *E. grandis* trees in different geographic zones managed by the company Smurfit Carton de Colombia (Wingfield and Rodas, unpublished data). *Eucalyptus grandis* plantations affected by this disease commonly range in age from 6 to 36 months, with the most susceptible trees being those between 18 to 26 months. Common symptoms (Fig. 1) include small necrotic lesions at the nodes on the shoots, which develop to form large irregular cankers causing shoot die-back. Cankers located on branches and main stems give rise to abundant production of kino, degrading the wood and weakening the stems. Wind then leads to stem breakage and considerable loss.

Despite their importance, almost nothing is known regarding the identity or occurrence of Botryosphaeriaceae in Colombia. The aims of this study were therefore to identify the Botryosphaeriaceae associated with cankers on *E. grandis* in Colombian plantations and test their pathogenicity to *Eucalyptus* in the country.

2 Materials and methods

2.1 Symptoms and collection of samples

Isolations were made from plant material showing various symptoms, including die-back of shoots and twigs, small necrotic lesions at nodes on the shoots and irregular cankers formed on stems and branches (Fig. 1). In addition, isolations were made from pseudothecia or pycnidia on the bark of diseased *Eucalyptus* branches, when present.

Samples of diseased *E. grandis* tissue were collected from three distinct geographical areas, namely the Andina, Valle del Cauca and Cauca zones. From these areas, 17 farms belonging to Smurfit Carton de Colombia were included in the collections. All samples included in this study were collected between May 2000 and May 2001.

Tissue samples from diseased stems and branches were surface-sterilized in 70% ethanol for 30 s and thereafter washed in sterile distilled water. This material was placed in moist

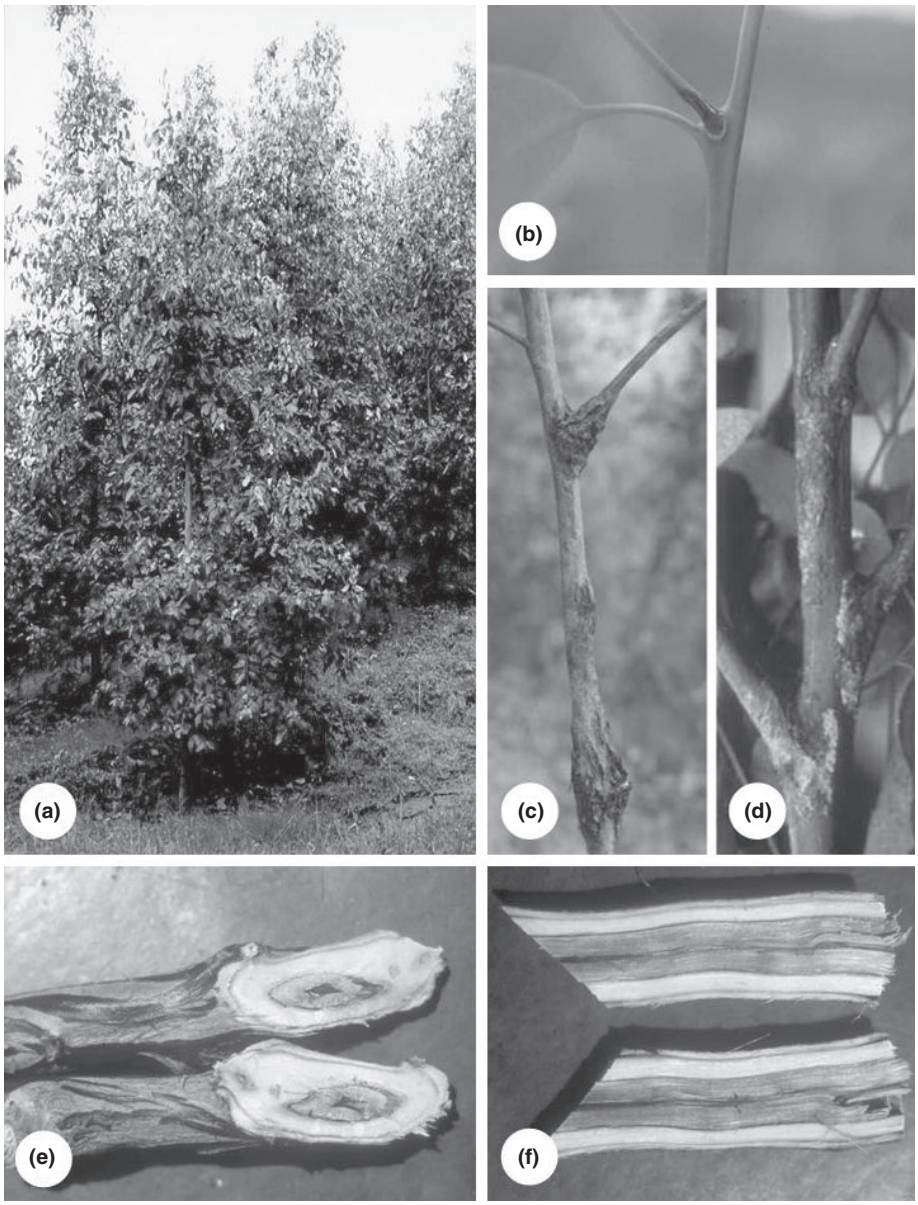

Fig. 1. Disease symptoms associated with Botryosphaeriaceae infection on *Eucalyptus grandis*. (a) Die-back of the leader shoots. (b) Lesion beginning at branching points of twigs. (c–d) Cankers on the stems and branches. (e–f) Internal lesions in sapwood associated with die-back.

chambers and incubated at 24°C until fungi began to sporulate on the surface of the samples. In addition, after surface sterilization, small (1–2 mm) pieces of tissue were placed on the surface of malt-yeast extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab, Midrand, South Africa) and incubated at 25°C for 7 days.

Forty-four isolates of Botryosphaeriaceae were obtained from preliminary isolations and single conidial sub-cultures were made of all these isolates. In order to produce single conidial isolates, fungi were grown on water agar (WA) (2% agar; Biolab) with sterilized pine needles (autoclaved twice) placed on the surface. After incubation for 7–14 days at 25°C under continuous fluorescent light, spore masses began to exude from pycnidia. Conidial masses were collected, diluted in sterile water and streaked out onto the surface of WA. After 7 h, germinating conidia were selected and transferred to MYA. Isolates were maintained on 2% malt extract agar (MEA) (Biolab) slants and stored at 4°C. The cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (CMW 8922–CMW 8966).

2.2 Morphological characteristics

Conidia from pycnidia that formed on pine needles on WA (as described earlier) were mounted on glass microscope slides in a drop of lactophenol. Length and width measurements were made for 10 conidia per isolate using a light microscope with an Axiocam digital camera and accompanying Axiovision 3.1 software (Carl Zeiss, Mannheim, Germany). The measurements were subjected to statistical analyses and are presented as (min–)(average − SD) − (average + SD)(–max).

2.3 DNA extraction, amplification and sequence analysis

Five isolates were chosen to represent the morphological groups identified as described earlier. DNA was obtained using a modified version of the method of Raeder and Broda (1985) as described in Slippers et al. (2004a). The DNA concentration was determined by UV light visualization after electrophoresis on a 1% agarose gel, which was stained with ethidium bromide.

The PCR amplification of a part of the nuclear rRNA operon was achieved using the primers ITS1 and ITS4 (White et al. 1990). In addition, a part of the translation elongation factor 1α (EF1-α) gene was amplified using the primers EF1-728F and EF1-986R (Carbone et al. 1999). The PCR reactions followed the protocols outlined by Slippers et al. (2004a). All PCR products were run on 1% agarose gels, stained with ethidium bromide, and visualized under UV light. DNA concentration was determined by comparison with 100 bp or standard λ size markers.

The PCR-amplified fragments were purified using a HIGH PURE PCR product Purification Kit (Roche Molecular Biochemicals, Almeda, CA, USA). PCR products were sequenced in both directions with the same primers used for amplification. Sequencing reactions were carried out using the ABI PRISM^{TM} Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, CA, USA) and capillary electrophoresis on an ABI PRISM^{TM} 3100 DNA Autosequencer (Applied BioSystems). All the reactions were carried out using protocols recommended by the manufacturers.

Sequence data were processed using Sequence Navigator version 1.0.1^{TM} (Applied BioSystems). The nucleotide sequences were aligned manually by inserting gaps and phylogenetic relationships determined from the aligned sequences using phylogenetic analysis using parsimony (paup) version 4.0b10 (Swofford 2002).

In order to establish the phylogenetic relationships and the identities of the Botryosphaeriaceae used in this study, 14 sequences of known Botryosphaeriaceae from GenBank were included in the alignment (Table 1), including *N. ribis, N. eucalyptorum, N. luteum, B. dothidea, Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Diplodia seriata* De Not. (= '*B.*' obtusa) and *Diplodia mutila* (Fr.) Mont. Trees were rooted using a sequence of a *Bionectria* sp. as outgroup taxon (Table 1).

Table 1. **Isolates of different *Neofusicoccum*, *Botryosphaeria*, *Lasiodiplodia* and *Diplodia* species used in the phylogenetic studies.**

Isolate no.[1]	Species	Host	Origin	Isolator	GenBank ITS	GenBank EF1-α
CMW 7772	*N. ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236935	AY236877
CMW 7773	*N. ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236936	AY236878
CMW 8961	*N. ribis*	*Eucalyptus grandis*	Estrella, Colombia	C. A. Rodas	FJ153798	FJ153799
CMW 8959	*N. ribis*	*E. grandis*	Ignacia, Colombia	C. A. Rodas	FJ153796	FJ153797
CMW 8949	*N. ribis*	*E. grandis*	Libano, Colombia	C. A. Rodas	FJ153800	FJ153801
CMW 9078	*N. parvum*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236940	AY236885
CMW 9080	*N. parvum*	*Populus nigra*	New Zealand	G. J. Samuels	AY236942	AY236887
CMW 10125	*N. eucalyptorum*	*E. grandis*	Mpumalanga, South Africa	H. Smith	AF283686	AY236891
CMW10126	*N. eucalyptorum*	*E. grandis*	Mpumalanga, South Africa	H. Smith	AF283687	AY236892
CMW 9076	*N. luteum*	*Malus* x *domestica*	New Zealand	S. R. Pennycook	AY236946	AY236893
CMW 992	*N. luteum*	*A. deliciosa*	New Zealand	G. J. Samuels	AF027745	AY236894
CMW 8922	*B. dothidea*	*E. grandis*	Andes, Colombia	C. A. Rodas	FJ153802	FJ153803
CMW 8929	*B. dothidea*	*E. grandis*	Andes, Colombia	C. A. Rodas	FJ153804	FJ153805
CMW 8000	*B. dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236898
CMW 7999	*B. dothidea*	*Ostrya* sp.	Crocifisso, Switzerland	B. Slippers	AY236948	AY236897
CMW 0130	*L. theobromae*	*Vitex doniana*	Uganda	J. Roux	AY236952	AY236901
CMW 9074	*L. theobromae*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236901
CMW 7774	*D. seriata*	*Ribes* sp.	New York	B. Slippers/G. Hudler	AY236953	AY236902
CMW 7060	*D. mutila*	*Fraxinus excelsior*	Netherlands	H. A van der Aa	AY236955	AY236904
CMW 7063	*Bionectria* sp.	Unknown	Netherlands	Unknown	AY236956	AY236905

N., Neofusicoccum; *B., Botryosphaeria*; *L., Lasiodiplodia*; *D., Diplodia*.
Isolates in italics were sequenced in this study.
[1]CMW, culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Nucleotide substitutions were treated as unordered, unweighted characters. Maximum parsimony trees were found using the heuristic search option with unlimited random addition replicates with the tree bisection reconstruction (TBR) as branch swapping algorithm. Gaps were treated as fifth character. Support for clades was assessed by 1000 bootstrap replicates (Felsenstein 1985). Statistical congruence between ITS rDNA and EF1-α sequence data sets was tested using the partition homogeneity test (Farris et al. 1995; Huelsenbeck et al. 1996) in paup. These tests revealed that the data were congruent and subsequently were analysed together.

2.4 Virulence and tolerance tests

2.4.1 Greenhouse trial

A preliminary virulence test was conducted in a greenhouse maintained at approximately 25°C with natural light. In this trial, six isolates representing two distinct morphological and phylogenetic groups identified in the prior taxonomic studies and referred to as group A (CMW 8922, CMW 8925, CMW 8929) and group B (CMW 8949, CMW 8961, CMW 8956) were used for the inoculations. The inoculations were conducted on 18-month-old trees of an *E. grandis* clone (ZG14), known to be susceptible to infection by Botryosphaeriaceae. After 2 weeks of acclimatization in the greenhouse, 10 trees were inoculated with each isolate. Inoculum was derived from 1-week-old cultures growing on MYA. Ten trees were inoculated with sterile agar medium as negative controls.

For inoculations, wounds were made on the stems of trees using a cork borer (6-mm diameter) to expose the cambium. Wounds were inoculated with an agar disc of the same size bearing mycelium (or not), and sealed with Parafilm (American National Can, Chicago, USA) to prevent desiccation. Lesion lengths (mm) were assessed 6 weeks after inoculation.

2.4.2 Field trials

Four inoculation trials were conducted in the field in Colombia. Four different sites were chosen to represent a wide range of climatic and humidity conditions where Botryosphaeria cankers are found naturally. The trials were as follows:

Trial N°1: La Suiza farm in Restrepo, Valle del Cauca, at 1469 m a.s.l., 1067 mm year^{-1} of precipitation, and located at 76°29′49″ W, 3°51′45″ N. Trial N°2: Cecilia farm near Darien, Valle del Cauca, at 1825 m a.s.l., 2414 mm year^{-1} of precipitation, and located at 76°26′06″ W, 3°57′06″ N. Trial N°3: Libano farm near Pereira, Risaralda, at 2102 m a.s.l., 3143 mm year^{-1} of precipitation, and located at 75°35′49″ W, 4°43′13″ N. Trial N° 4: Angela Maria farm near Santa Rosa, Risaralda, at 1864 m a.s.l., 2437 mm year^{-1} of precipitation, and located at 75°36′2″ W, 4°49′10″ N.

Isolates CMW 8922 and CMW 8961, representing the two different Botryosphaeriaceae morphological groups and shown to be most virulent in the greenhouse trial, were used in the field inoculations. These inoculations were on a total of 560, 30-month-old *E. grandis* trees. These trees consisted of 10 clones (2, 4, 11, 12, 18, 20, 23, 27, 301, 303) and four seed lots (210, T210, 211, T211) used in all four trials. Forty trees of each clone or seed lot, distributed in 10 different blocks (four trees per block), were inoculated with each isolate. The block design consisted of two lines of trees per block, in which each clone or seed lot was planted twice. Each isolate was thus inoculated onto trees in one line of each block. In order to avoid any border effect, the trial sites were surrounded by two rows of border trees. The same design was used in all four trials.

Inoculations were made as described earlier for the greenhouse inoculations except that the cork borer size for the inoculations was 4 mm (diameter) and the inoculated areas were

covered with tissue paper moistened with sterile water and secured with masking tape. Trials were initiated during June 2002 and the resultant external and internal lesions (lengths and widths) were measured after 12 weeks in September 2002.

Statistical analyses of greenhouse and field trial data were carried out using SAS ANALYTICAL PROGRAMS (1990). Analysis of variance tables were produced in addition to tables of means with the 95% confidence limits (CL) for each mean.

3 Results

3.1 Morphological characteristics

Isolates were similar in colour when grown on MYA medium at 24°C. Initially (2–4 days), the mycelium was white and gradually darkened from the centres of the colonies, first having a dark green colour and gradually becoming dark grey to black with age.

The 44 isolates used in this study could be separated into two groups based on conidial differences. Eight isolates belonged in group A and 36 isolates resided in group B. All isolates belonging to group A were collected from the Andina zone, while isolates of group B were found in all three zones from which isolates were collected.

Both groups of isolates had Fusicoccum-type conidia that were hyaline, thin-walled, aseptate and smooth. The conidia of these groups however differed in size and shape. Conidia of group A isolates were fusiform to fusiform–elliptical with obtuse to pointed ends and were (25.0–)27.0 – 29.0(–30.0) × 5.0(–7.0) μm (average 27.9 × 5.2 μm). Conidia of isolates belonging to group B were elliptical to fusiform with pointed ends, having granular contents and (18.0–)20.0 – 21.0(–23.0) × (5.0–)6.0(–7.0) μm (average 20.3 × 5.6 μm).

3.2 DNA sequencing and analysis

Sequences of approximately 560 bp were obtained from the amplified ITS 1/2 rDNA region and approximately 330 bp for EF1-α. A partition homogeneity test of the full data set indicated that these datasets were congruent ($p = 0.60$). Alignment of the combined sequences gave rise to a data set of 889 characters. Of these, 601 characters were parsimony uninformative and 288 were parsimony informative. Phylogenetic analyses were performed on the combined data set of ribosomal DNA and the EF1-α intron region sequences. Two most parsimonious trees were obtained [length = 642 steps, consistency index (CI) = 0.822, Retension Index (RI) = 0.899, Rescaled Consistency Index (RC) = 0.739, and g1 = –0.821], with no differences between them in overall clade topography, but having rearrangements within the clades (Fig. 2). The isolates from Colombia (CMW 8922, CMW 8929) representing morphological group A resided in the *B. dothidea* clade with 100% bootstrap support. The three isolates representing morphological group B (CMW 8961, CMW 8959, CMW 8949), grouped closely with *N. ribis* with a 74% bootstrap support.

3.3 Virulence and tolerance tests

3.3.1 Greenhouse trial

The mean lesion length for isolates in the *N. ribis* (group B) group was significantly ($p = 0.0001$) greater than that for the *B. dothidea* group (group A) (Table 2; Fig. 3). Of the *N. ribis* group isolates, CMW 8961 gave rise to the longest lesions (average lesion length 81.7 mm). Of the *B. dothidea* group isolates, CMW 8922 produced the longest lesions (average lesion length 40.7 mm). These two isolates were therefore selected for subsequent field virulence and tolerance trials.

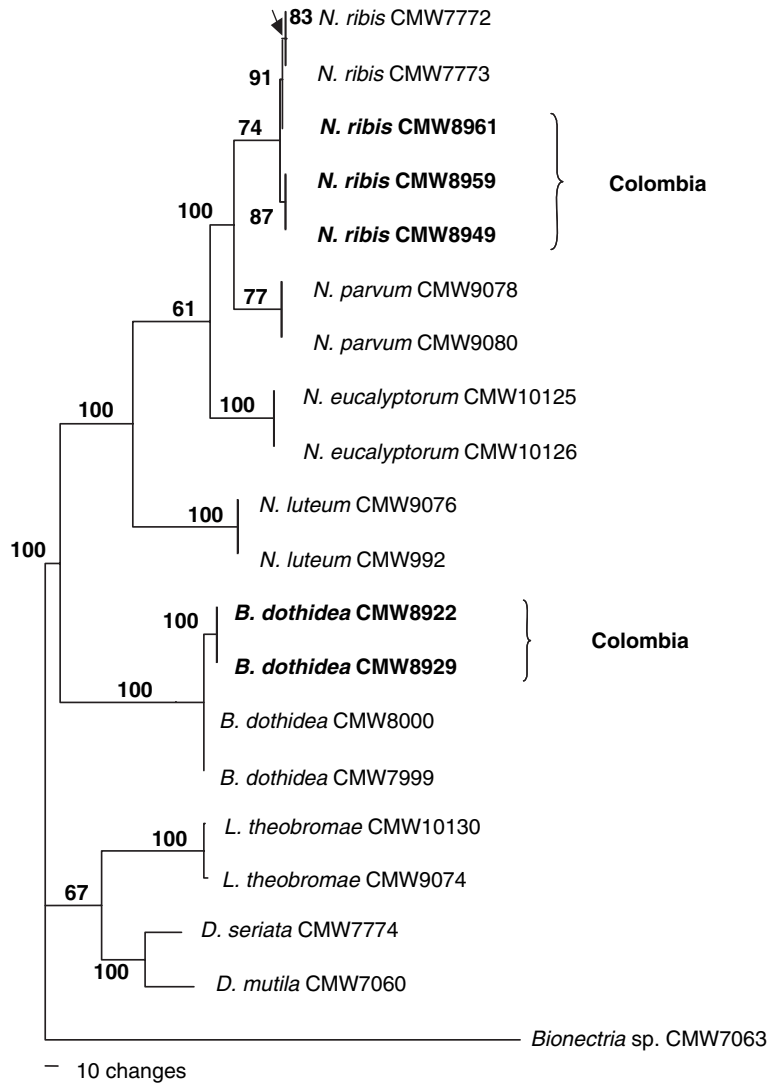

Fig. 2. One of the most parsimonious trees generated from a combined data set of the ITS 1/2 region and 5.8S rRNA gene and EF1-α gene and intron region for various Botryosphaeriaceae The bootstrap values (1000 replications) >50% are indicated above the branches. The tree includes 19 ingroup taxa and the outgroup taxon *Bionectria* sp.

Table 2. **Analysis of variance of internal lesion lengths on an *Eucalyptus grandis* clone (ZG 14) inoculated with six isolates of *Neofusicoccum ribis* and *Botryosphaeria dothidea* in the greenhouse.**

Source	Degrees of Freedom (DF)	Mean Square (MS)	*F* value	p value
Isolates	6	7207.8	7.96	0.0001
Error	61	905.8		

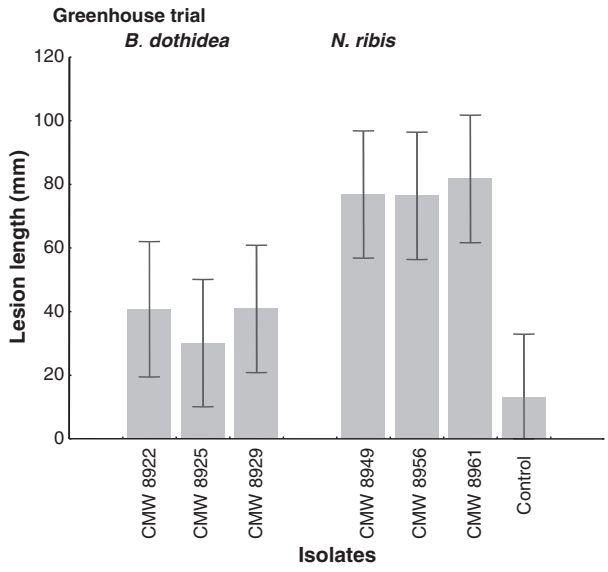

Fig. 3. Mean lesion length after inoculation with *Botryosphaeria dothidea* and *Neofusicoccum ribis* on *Eucalyptus grandis,* clone ZG14 in a greenhouse trial. Bars represent 95% confidence limits for each isolate.

3.3.2 Field trials

In general, results derived from the greenhouse and the four field virulence trials were consistent in showing that the isolate of *N. ribis* was significantly more virulent than the isolate of *B. dothidea.* This fact was clearly seen in the significantly smaller ($p = 0.0001$) lesion lengths associated with *B. dothidea* than those caused by *N. ribis* (Table 3; Fig. 4). Lesions caused by *N. ribis* were also more variable in size on the different clones, while the lesions caused by *B. dothidea* were all similar in size (Fig. 4). Lesions produced by *N. ribis* were the largest on average at La Suiza and the smallest at Angela Maria, while *B. dothidea* produced the largest lesions at Cecilia and the smallest at Libano.

Lesions on the different clones inoculated in the different trials, formed a continuum of values, not differing significantly from those directly above or below in the ranking. Lesions with a low ranking however differed significantly from lesions with a high ranking, especially in the case of the *N. ribis* inoculations (Table 3; Fig. 4).

The clones that were the least and the most tolerant were however different in each trial (Table 3; Fig. 4). For example, the most tolerant clone to *N. ribis* in the La Suiza farm trial was clone 20, but this clone was the least tolerant in the Angela Maria trial (Fig. 4). Some clones were however consistently among the most tolerant (e.g. clones T210 and T211) or less tolerant (e.g. clone 303). In contrast, for clones inoculated with *B. dothidea*, clone 23 was often the least (or among the most) tolerant and clone 303 was often more tolerant (Fig. 4). Clones T210 and T211 could not be included in the La Suiza trial, because an undue number of these trees had died owing to other causes prior to the completion of the experiment.

4 Discussion

This study represents the first detailed investigation of Botryosphaeriaceae on *Eucalyptus* in Colombia. Results have shown that two species, *N. ribis* and *B. dothidea* are associated

Table 3. **Analysis of variance resulting from inoculation of *Eucalyptus grandis* clones with isolates of *Botryosphaeria dothidea* and *Neofusicoccum ribis* at the farms La Suiza, Cecilia, Libano and Angela Maria.**

Source	DF	MS	F value	p value
La Suiza				
Blocks	9	3836.9	1.33	0.2191
Isolates	1	1 025 195.5	355.4	0.0001
Clones	11	12 440.0	4.31	0.0001
Isolates × clones	11	15 845.9	5.49	0.0001
Error	408	2884.7		
Cecilia				
Blocks	9	10 924.5	5.53	0.0001
Isolates	1	602 729.7	305.0	0.0001
Clones	13	7500.8	380	0.0001
Isolates × clones	13	7615.14	390	0.0001
Error	370	1976.2		
Libano				
Blocks	9	2929.2	8.03	0.0001
Isolates	1	44 605.3	122.27	0.0001
Clones	13	869.5	2.38	0.0042
Isolates × clones	13	695.9	1.91	0.0273
Error	473	364.8		
Angela Maria				
Blocks	9	1442.80	1.27	0.2525
Isolates	1	112 951.9	233.74	0.0001
Clones	13	3041.65	395	0.0001
Isolates × clones	13	3693.31	3.82	0.0001
Error	459	890.96		

The significant interaction between isolates and clones indicates that the clones which are susceptible to *B. dothidea* are not necessarily also susceptible to *N. ribis.*

with stem cankers and shoot die-back of *Eucalyptus* in this country. The study also showed and there is considerable variation in the virulence of isolates of the two species on different *E. grandis* clones in different areas. This information will be useful for future control strategies of Botryosphaeria canker in *Eucalyptus* plantations in Colombia.

DNA sequence data were essential to identify the Botryosphaeriaceae recognized in this study. This has also been true in a number of recent studies considering species of Botryosphaeriaceae (Jacobs and Rehner 1998; Zhou and Stanosz 2001; Slippers et al. 2004a; b; Burgess et al. 2005). However, once these species had been clearly defined, it was also possible to show that they could be distinguished based on morphology. This was best achieved based on conidial size, as *B. dothidea* (anamorph *F. aesculi*) has longer spores than those of *N. ribis*. These data also correlate with known conidial sizes for these species (Slippers et al. 2004a). This morphological characteristic should facilitate rapid identification of the two species in the future.

Neofusicoccum ribis is more common than *B. dothidea* on *E. grandis* in Colombia. To the best of our knowledge, this is the first definitive record of *N. ribis*, confirmed by sequence data, causing serious disease of this important plantation species outside Australia (Slippers et al. 2004b). Despite the strong support for the grouping of Colombian isolates with *N. ribis* type isolates, there is also a strongly supported sub-structure within this clade. This is similar to the findings in other studies where significant variation was demonstrated within *N. ribis* and *N. parvum* (Slippers et al. 2004b, 2005; Slippers, unpublished). It was outside the scope of this study to determine whether this variation represents speciation

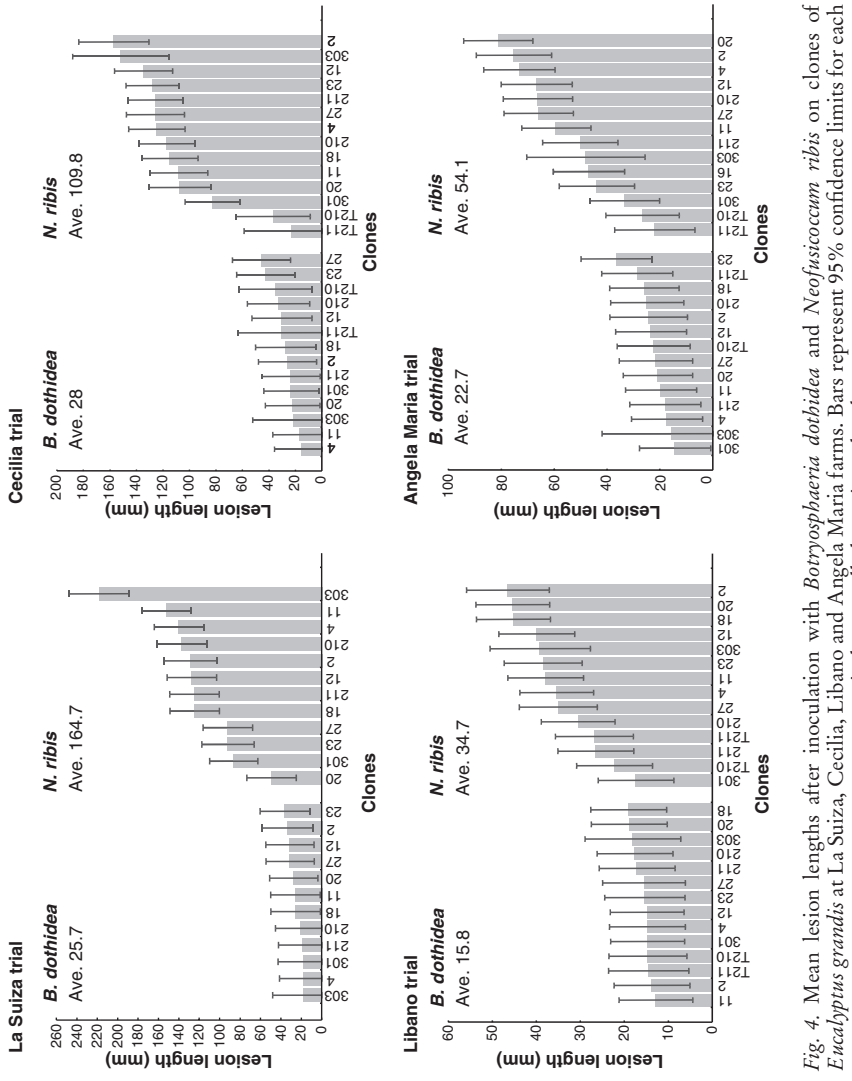

Fig. 4. Mean lesion lengths after inoculation with *Botryosphaeria dothidea* and *Neofusicoccum ribis* on clones of *Eucalyptus grandis* at La Suiza, Cecilia, Libano and Angela Maria farms. Bars represent 95% confidence limits for each isolate on all clones inoculated.

events or population variation within species. We thus consider our isolates as representing *N. ribis sensu lato* (Slippers 2003).

Although *B. dothidea* has been viewed as an important pathogen of *Eucalyptus* in the past, it seems to be relatively unimportant in most areas outside Colombia (Slippers et al. 2004b). Webb (1983) reported the presence of *B. dothidea* in commercial seed of *Eucalyptus camaldulensis* in South Florida. Shearer et al. (1987) showed that this fungus was responsible for the death of *E. radiata* in selection trials in Australia. Davison and Tay (1983) reported the natural occurrence of *B. dothidea* cankers in *E. marginata* forests in Australia. Likewise, Barnard et al. (1987) showed that *B. dothidea* is involved as one of a complex of organisms that cause coppice failure of *E. grandis* in Florida. Smith et al. (1994) also report that *B. dothidea* is associated with cankers on *Eucalyptus* in South Africa. Caution is needed however when interpreting these reports, as subsequent studies have shown that the name *B. dothidea* has frequently been incorrectly used (Slippers et al. 2004a,b).

In this study, we found significant differences in virulence between selected isolates of *B. dothidea* and *N. ribis*. Lesion lengths associated with *B. dothidea* inoculations were significantly smaller than those associated with *N. ribis* in both greenhouse and field trials. In addition, this result was confirmed at sites across three major climatic zones of Colombia, where *E. grandis* and its hybrids are currently being planted. The consistently greater virulence of *N. ribis* in all areas shows that it has the capacity to infect trees and cause disease, assuming climatic conditions are conducive to infection. Future research aimed at reducing the impact of Botryosphaeria canker in Colombia should clearly focus on the more common and more virulent species, *N. ribis*.

This study included isolates from three different zones (Cauca, Valle del Cauca and Andina) of Colombia, where Botryosphaeria canker is an important disease of *E. grandis*. These areas differ substantially in climate, but *N. ribis* was found to cause disease in both cooler and warmer areas. Temperature therefore does not appear to be an important factor limiting the occurrence of the fungus in Colombia. However, because Botryosphaeriaceae are typically stress-related pathogens (Schoeneweiss 1980), the difference in climate may be important when it affects the susceptibility of trees. For example, the difference in tolerance observed in clone 20 to *N. ribis* isolate CMW 8961 (most tolerant at La Suiza, but least tolerant at Angela Maria) appears to be the result of the response of the clone to the environment, rather than the fungus. The interaction between the trees, the fungi and the environment, which might cause such variation, is not entirely clear and deserves future attention.

Results of this study clearly show that different *E. grandis* clones differ substantially in their tolerance to infection by *B. dothidea* and, especially, *N. ribis*. This is also consistent with observations of the natural occurrence of Botryosphaeria canker on different clones. Our results suggest that it will be possible to conduct field inoculation trials to select clones that are tolerant to infection by the fungus. This would have many advantages for *Eucalyptus* forestry in Colombia.

The *Botryosphaeria* species composition on *Eucalyptus* in Colombia is unusual, compared with similar studies conducted elsewhere. For example, in Chile and South Africa, *N. parvum*, *N. eucalyptorum* and *N. eucalypticola* were associated with Botryosphaeria canker and die-back diseases of *Eucalyptus* (Smith et al. 2001; Ahumada 2003; Slippers et al. 2004a; b) and *N. australe* and *Neofusicoccum macroclavatum* in western Australia (Burgess et al. 2005). The species composition in Chile and South Africa reflect that of eastern Australia (Slippers et al. 2004b), which is a part of the native range of *Eucalyptus*. Unlike Chile and South Africa, Colombia seems to have been less affected by introduced native Australian Botryosphaeriaceae. On the other hand, *B. dothidea* and *N. ribis* also occur elsewhere in the world and might well also be an introduced species in Colombia (Slippers et al. 2004a; Slippers and Wingfield 2007). The origin of these

species is worth further investigation. The observations from this study confirm the importance of thorough identification of Botryosphaeriaceae involved in causing specific disease symptoms on *Eucalyptus* in different areas and at different times rather than extrapolating data from studies in other settings.

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