Diseases of *Eucalyptus* in Colombia

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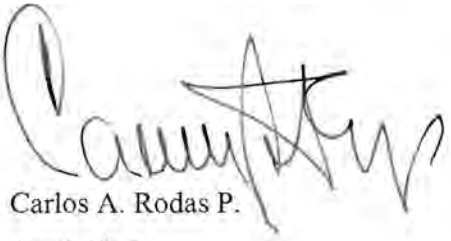
DECLARATION

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Magister Scientiae to the University of Pretoria, contain my own independent work and has hitherto not been submitted for any degree at any other University.



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PREFACE

Colombia is a tropical country located in South America. The size of Colombia is 114.1 million hectares, of which the potential forestry area has been estimated at 83 million hectares. Indiscriminate deforestation is a continuous problem and comprises of approximately 190 000 ha/year. To provide an alternative source of timber, 145 759 ha have been planted with different species of *Pinus, Cupressus* and *Eucalyptus* spp. during the course of the last 50 years. Private companies and government projects are developing reforestation programs in order to compensate for solid and pulpwood needs of the country. These plantations are particularly prone to damage by insect pests and diseases. Identification of diseases and subsequent management of pathogens is becoming essential in Colombia. The aim of this study was to develop a foundation for future research and particularly for management of *Eucalyptus* diseases in Colombia.

Chapter One of this dissertation is a report of the occurrence of *Eucalyptus* diseases in Colombia. This is based on three general surveys conducted in a variety of age classes of trees, represented by different seed sources and clones. A wide range of sites was also considered for plantations in Colombia, ranging from the low altitude tropics to those in the cooler Andean regions.

In **Chapter Two**, I characterise species of *Botryosphaeria* associated with cankers on *E. grandis* in plantations. This is achieved based on comparisons using morphological and DNA-based methods. Isolates were collected from three different and representative geographical zones, where *Eucalyptus grandis* has been planted in Colombia. Isolates of *Botryosphaeria* spp. identified in these studies are also tested for pathogenicity in greenhouse and field trials.

Cryphonectria cubensis is a well-known pathogen associated with basal cankers in *Eucalyptus* and considered a serious pathogen of *E. grandis* around the world. In **Chapter Three**, native Colombian Melastomataceae are reported as hosts of this important pathogen. These include *Miconia rubiginosa* and *M. theaezans*. The role of these trees as alternative hosts of *C. cubensis* is also considered.

Cylindrocladium blight of *Eucalyptus* is one of the most important diseases of *E. grandis* in young plantations. This disease has not been fully characterised in Colombia where it has previously been recognised as important. The aim of the study presented in **Chapter Four** is to identify the species of *Cylindrocladium* responsible for the disease. Isolates of the pathogen were, therefore, collected from three geographical zones and these are identified based on morphology and DNA based comparisons.

In **Chapter Five** of this dissertation, the presence of *Ceratocystis fimbriata* on *E. grandis* in Colombia is reported for the first time. The fungus is a serious pathogen of other crops such as coffee in the country, but it has not as yet been recognised as a threat to *Eucalyptus* in Colombia.

Studies that make up this dissertation were conducted both in Pretoria and in Colombia. This has to some extent limited the range of investigations that were possible. However, the overall aim of providing a background to the future studies of *Eucalyptus* diseases in Colombia was achieved. Chapters making up this document were developed over a period of two years and they should be read as entirely independent entities. This format has necessitated some duplication between chapters.

SUMMARY

The forestry industry in Colombia comprises of 145 759 ha of commercial plantations. Of these, 47 700 ha are planted to *Eucalyptus* that is used mainly for the production of pulp, construction timber and paper. Eight years ago, an extensive breeding program to develop clones of *Eucalyptus* was started in Colombia. One of the objectives was to reduce the incidence of disease and insect pests. Little is, however, known regarding the diseases that occur on *Eucalyptus* spp. in Colombia, and this was a serious impediment to the breeding and tree improvement programme. The studies presented in this thesis were undertaken to improve our knowledge of *Eucalyptus* diseases in Colombia. Although most detailed experimentation has emerged during the course of the last two years, many collections, preliminary trials and observations extend back to 1995.

Chapter One of this thesis presents the results of extensive surveys on *Eucalyptus,* carried out to evaluate the diseases present in Colombia. A number of diseases were recorded. The most serious of these are Cryphonectria canker caused by *C. cubensis*, and Botryosphaeria canker, now known to be caused by *B. ribis* and *B. dothidea*. Cylindrocladium shoot and leaf blight disease was also important on *Eucalyptus* spp. This disease is associated with several *Cylindrocladium* species in Colombia, namely *C. reteaudii*, *C. candelabrum*, *C. parasiticum*, *C. gracile* and *C. spathulatum*. Other less important diseases present included Mycosphaerella leaf blotch disease (MLB) caused by *M. suberosa*, *M. parkii*, *M. africana*, *M. colombiensis*, *M. flexuosa* and *M. lateralis*. Phaeoseptoria leaf spot caused by *Phaeophleospora epicoccoides*, and corky leaf spot caused by *Aulographina eucalypti* also occurred on *Eucalyptus* leaves. *Puccinia psidii*, a serious rust pathogen on *Eucalyptus* spp. in Brazil and other countries in South America, was found on native *Eugenia jambos* and *Psidium guajava* (Myrtaceae) in Colombia, but never on *Eucalyptus*. Background literature pertaining to these diseases and *Eucalyptus* diseases in general is also presented in this chapter, which provides a foundation for many studies presented in subsequent parts of the thesis.

In the chapters subsequent to the first, the identity and relative importance of the most important pathogens found in the disease surveys, was considered in greater detail. Isolates of these fungi were also used in pathogenicity trials. In **Chapter Two**, the

identity of the species associated with Botryosphaeria canker was considered. Previously, only the name *B. dothidea* had been used for collections from *Eucalyptus* in Colombia. In this study, however, it was shown that in addition to *B. dothidea*, *B. ribis* was also present. The latter species was also the more common. This was based on morphological comparisons and DNA sequence comparisons for the ITS1/2 region of the ribosomal operon (rDNA) and the elongation factor (EF) 1-α genes. In pathogenicity trials on *E. grandis*, *B. ribis* was found to be the more pathogenic species and clones were shown to vary considerably in their susceptibility to infection.

Cryphonectria cubensis is one of the most important canker pathogens of *Eucalyptus* world-wide. Recently this pathogen was discovered on native *Tibouchina* spp. in Colombia. These trees are members of the Melastomataceae, which is recognized as relatively closely related to the Myrtaceae. In **Chapter Three**, I report on additional native Melastomataceous hosts of *C. cubensis*. These include *Miconia theaezans* and *M. rubiginosa*. The identity of the fungus occurring on the native trees was confirmed based on comparisons of morphology and DNA sequences for the ITS1/2 rDNA regions and two regions of the β-tubulin gene. Pathogenicity trials were also conducted with isolates from *M. rubiginosa* and *M. theaezans* on *E. grandis* clones, *T. urvilleana*, *T. lepidota*, *T. semidecandra*, *M. theaezans* and *M. rubiginosa*. The isolates from the native hosts were mildly pathogenic on most of these tree species. Differences in resistance was also seen in various of these trees. This study provided further evidence that some pathogens of *Eucalyptus* in Colombia are probably native and have originated from Colombian Myrtaceae and Melastomataceae.

The aim of the studies presented in **Chapter Four** was to identify species associated with Cylindrocladium shoot and leaf blight disease. Based on morphological studies and DNA sequence comparisons for the β-tubulin gene, only one species, *C. spathulatum*, was found to occur in the regions sampled. A *Eucalyptus* clonal trial was also assessed for the presence of the disease. Results showed that different clones differed greatly in susceptibility to *C. spathulatum*.

Ceratocystis fimbriata is one of the most serious pathogens of coffee trees in Colombia. Recently, this pathogen has also begun to emerge as a serious threat to *Eucalyptus* trees

in Brazil, Uruguay and parts of Africa. This fungus has, however, not been found on *Eucalyptus* trees in Colombia. In **Chapter Five**, we considered whether *C. fimbriata* occurs on *Eucalyptus* spp. in Colombia. *Eucalyptus grandis* trees in plantations were artificially wounded, and checked for the presence of *Ceratocystis* spp. after eight weeks. Only two *C. fimbriata* isolates were obtained from two zones. The identification was based on morphology and DNA sequences of the ITS1/2 rDNA region. Two of the isolates from *E. grandis* and a known *C. fimbriata* isolate from *Schizolobium parahybum* (a native tree to Colombia), were inoculated into trees of two clones of *E. grandis* and a commonly grown *E. grandis* seed source. One of the *Eucalyptus* isolates was found to be highly pathogenic on all of the hosts. The second *Eucalyptus* isolate produced lesions that were not statistically different from those of the control inoculations. Differences in susceptibility were also found between the *E. grandis* clones. Our results confirm that *C. fimbriata* presents a serious threat to *Eucalyptus* in Colombia, but that breeding and selection of disease-tolerant clones and hybrids will reduce this risk.

Diseases of *Eucalyptus* trees can result in serious economic losses to the forestry industry in Colombia. Such experiences are being felt elsewhere in the world where these trees are used in exotic plantation forestry. Through the studies presented in this thesis, I hope to have established a firm understanding of the diseases present on *Eucalyptus* trees in Colombia. This was based on disease surveys, as well as more detailed taxonomic studies to confirm identifications. In the various studies, it was shown that some of these pathogens have originated on native plants and have adapted to infect *Eucalyptus* trees. It was also evident from the various pathogenicity trials that differences in resistance against these fungal pathogens exist in currently used *E. grandis* clones. This is encouraging as these differences can now be exploited in breeding programs aimed at reducing losses due to disease.

OPSOMMING

Die bosbou industrie in Colombië beslaan 145 759 ha kommersiële plantasies. Hiervan is 47 700 ha beplant met *Eucalyptus* wat hoofsaaklik gebruik word vir die produksie van pulp, konstruksiehout en papier. Agt jaar gelede is 'n omvangryke telingsprogram begin om *Eucalyptus* klone te ontwikkel. Een van die doelwitte was om die voorkoms van siektes en insekpeste te verminder. Min is egter bekend oor die siektes wat op *Eucalyptus* spp. in Colombië voorkom, en dit was 'n ernstige hindernis in die telings- en boomveredelingsprogram. Die studies voorgedra in hierdie tesis was onderneem om ons kennis oor *Eucalyptus* siektes in Colombië te vermeerder. Alhoewel die meeste werk die afgelope twee jaar gedoen is, dateer baie versamelings, voorbereidende proewe en waarnemings terug tot 1995.

Hoofstuk Een van die tesis bevat die resultate van uitgebreide opnames op *Eucalyptus* om vas te stel watter siektes voorkom in Colombië. Heelwat siektes is gevind. Die ernstigste siektes is Cryphonectria kanker wat veroorsaak word deur *C. cubensis*, en Botryosphaeria kanker, wat ons nou weet veroorsaak word deur *B. ribis* en *B. dothidea*. Cylindrocladium-loot-en-blaar-skimmelsiekte was ook belangrik op *Eucalyptus* spp. Hierdie siekte word geassosieer met verskeie *Cylindrocladium* spesies in Colombië, naamlik *C. reteaudii*, *C. candelabrum*, *C. parasiticum*, *C. gracile* en *C. spathulatum*. Ander minder belangrike siektes wat teenwoordig is, is Mycosphaerella blaarvleksiekte (MLB) wat veroorsaak word deur *M. suberosa*, *M. parkii*, *M. africana*, *M. colombiensis*, *M. flexuosa* en *M. lateralis*. Phaeoseptoria blaarvlek veroorsaak deur *Phaeophleospora epicoccoides*, en kurkblaarvlek veroorsaak deur *Aulographina eucalypti* kom ook voor op *Eucalyptus* blare. *Puccina psidii*, 'n ernstige roespatogeen van *Eualyptus* spp. in Brazil en ander lande in Suid Amerika, was gevind op inheemse *Eugenia jambos* en *Psidium guajava* (Myrtaceae) in Colombië, maar nooit op *Eucalyptus* nie. Agtergrondliteratuur oor hierdie siektes en oor *Eucalyptus* siektes in die algemeen, is ook opgeneem in die hoofstuk, wat 'n fondasie lê vir baie studies voorgedra in opvolgende dele van die tesis.

In die daaropvolgende hoofstukke, is die identiteit en relatiewe belangrikheid van die belangrikste patogene wat gevind is in die siekteopnames, in groter detail ondersoek. Isolate van hierdie swamme was ook in patogenisiteitstoetse gebruik. In **Hoofstuk Twee**, is die identiteit van die spesies verbind met Botryosphaeria kanker, ondersoek. Voorheen

is slegs die naam *B. dothidea* gebruik vir versamelings vanaf *Eucalyptus* in Colombië. In hierdie studie, het ek egter gevind dat behalwe *B. dothidea*, ook *B. ribis* teenwoordig is. Laasgenoemde spesie kom ook die algemeenste voor. Hierdie bevindinge was gebaseer op morfologiese vergelykings en DNS volgordebepalings van die ITS1/2 gebied van die ribosomale operon (rDNA) en die 'elongation factor' (EF) 1-α geen. In die patogenisiteitsproewe op *E. grandis*, is daar gevind dat *B. ribis* die meer patogeniese spesie was, en dat klone heelwat gevarieer het ten opsigte van hul vatbaarheid vir infeksie.

Cryphonectria cubensis is een van die belangrikste kankerpatogene van *Eucalyptus* wêreldwyd. Hierdie patogeen is onlangs op inheemse *Tibouchina* spp. in Colombië ontdek. Hierdie bome is lid van die Melastomataceae, wat relatief naby verwant is aan die Myrtaceae waarbinne *Eucalyptus* resorteer. In **Hoofstuk Drie**, raporteer ek addisionele Melastomataceae gashere vir *C. cubensis*. Hierdie sluit in *Miconia theaezans* en *M. rubiginosa*. Die identiteit van die swam op hierdie inheemse bome was bevestig met morfologie en vergelykings van DNS volgordes van die ITS1/2 rDNA gebied en twee dele van die β-tubulin gene. Patogenisiteitsproewe was ook met isolate van *M. rubiginosa* and *M. theaezans* gedoen op *E. grandis* klone, *T. urvilleana*, *T. lepidota*, *T. semidecandra*, *M. theaezans* en *M. rubiginosa*. Die isolate van die inheemse gashere was matig patogenies op die meeste van hierdie bome. Verskille in weerstand was ook waargeneem in verskeie van hierdie bome. Hierdie studie het verder bewys dat sommige patogene van *Eucalyptus* moontlik in Colombië inheems kan wees en ontstaan het op Colombiaanse Myrtaceae en Melastomataceae.

Die doelwit van die studies in **Hoofstuk Vier** was om spesies geassosieer met Cylindrocladium-loot-en-blaar-skimmelsiekte, te identifiseer. Morfologiese studies en DNS volgordebepaling van die β-tubulin gene, het gewys dat slegs een spesie, *C. spathulatum*, voorkom in die areas waar opnames gedoen was. 'n *Eucalyptus* klonale proef was ook ge-evalueer vir die teenwordigheid van die siekte. Resultate het gewys dat die onderskeie klone heelwat verskil ten opsigte van hul vatbaarheid vir *C. spathulatum*.

Ceratocystis fimbriata is een van die ernstigste patogene van koffiebome in Colombië. Hierdie patogeen het ook onlangs 'n ernstige bedreiging begin word vir *Eucalyptus* bome

in Brazil, Uruguay en dele van Afrika. Die swam is egter nie gevind op *Eucalyptus* bome in Colombië nie. In **Hoofstuk Vyf**, het ons probeer vasstel of *C. fimbriata* op *Eucalyptus* spp. in Colombië voorkom. *Eucalyptus grandis* bome in plantasies was kunsmatig gewond, en spesifieke isolasies vir *Ceratocystis* spp. was na agt weke gemaak. Slegs twee *C. fimbriata* isolate is verkry vanaf twee areas in Colombië. Hierdie identifikasie was gebaseer op morfologie en DNS volgordes van die ITS1/2 rDNA gebied. Twee van die isolate van *E. grandis* en 'n bekende *C. fimbriata* isolaat van *Schizolobium parahybum* ('n inheemse boom in Colombië), was geïnokuleer in twee klone van *E. grandis* en bome van saad wat algemeen gebruik word. Die tweede Eucalyptus isolaat het egter letsels geprodusser wat nie van die kontrole inokulasies verskil het nie. Vatbaarheidsverskille was ook tussen die *E. grandis* klone gekry. Ons resultate bevestig dat *C. fimbriata* 'n ernstige bedreiging inhou vir *Eucalyptus* in Colombië, maar dat teling en seleksie van siekteweerstandbiedende klone en hibriede die risiko sal verminder.

Siektes van *Eucalyptus* bome kan ernstige ekonomiese verliese veroorsaak vir die bosbou industrie in Colombië. Soortgelyke bevindings word ook in ander dele van die wêreld ondervind waar die bome in uitheemse plantasie bosbou gebruik word. Ek hoop om deur die studies opgevat in hierdie tesis, 'n waardevolle bydrae te lewer oor die teenwoordigheid van siekes op *Eucalyptus* bome in Colombië. Hierdie inligting is verkry deur siekteopnames, asook meer gedetaileerde taksonomiese studies om die identifikasie van patogene te bevestig. Ek het ook in die onderskeie studies ontdek dat sommige van hierdie patogene ontstaan het op inheemse plante en aangepas het om *Eucalyptus* bome te infekteer. Dit was verder duidelik uit die verskeie patogenisiteitsproewe dat verskille in weerstand in *E. grandis* klone teen hierdie swampatogene bestaan. Dit is bemoedigend, omdat hierdie verskille gebruik kan word in telingsprogramme wat ontwikkel word om verliese as gevolg van siektes te verminder.

CHAPTER 1

Diseases of *Eucalyptus* in Colombia

ABSTRACT

Eucalyptus propagation encompasses approximately 33% of the Colombian forestry industry. Significant losses are, however, regularly experienced due to fungal diseases in plantations. Prior to 1993, virtually nothing was known regarding the causal agents of these diseases and consequently, detailed assessments of diseases in *Eucalyptus* plantations in Colombia have been undertaken. The most serious diseases recorded to date are Cryphonectria canker caused by *Cryphonectria cubensis*, Botryosphaeria canker caused by *Botryosphaeria dothidea* and *B. ribis*, Cylindrocladium leaf blight caused by *Cylindrocladium spathulatum* and Mycosphaerella leaf disease caused by various *Mycosphaerella* spp. A number of other less serious diseases have also been recorded. Selection and development of clonal *Eucalyptus grandis* and its hybrids with *E. urophylla* are underway to reduce the impact to these diseases. Monitoring of current disease incidence continues as does research into the interactions of pathogens with species, clones, sites and sylvicultural practices. This intensive and emerging pathology programme should ensure that diseases do not impair the future of intensively managed plantations in Colombia.

INTRODUCTION

Eucalyptus species are being extensively propagated as exotics in monocultures in many parts of the world. Turnbull (2000) estimates that there are more than ten million hectares of *Eucalyptus* spp. in plantations outside Australia. Some of the most extensive forestry operations utilising these trees are in Asia, Africa and South America.

Diseases pose a serious threat to the productivity of *Eucalyptus* plantations (Wingfield 1990). Although these trees were initially separated from their natural enemies, there are numerous records of diseases that have caused substantial damage during the early period of *Eucalyptus* plantation forestry. These diseases were caused not only by pathogens known to occur on the trees in their native range, but also by pathogens native to the areas where these exotic plants were established (Wingfield *et al.* 2001a, b). In South Africa, for example, Mycosphaerella leaf blotch caused by *M. juvenis* Crous & M. J. Wingf., which has not been recorded from Australia, led to the abandonment of *E. globulus* Labill during the early phases of *Eucalyptus* propagation and continues to be an important disease on *E. nitens* Maiden (Crous & Wingfield 1996).

Propagation of *Eucalyptus* spp. in industrial plantations in Colombia is rapidly becoming a major component of this country's forestry industry (Osorio, Wright & White 1995). The most important species presently planted is *E. grandis* W. Hil ex Maiden, although hybrids of this and other species are likely to become important in future (Wright & Osorio 1996). Although seedling stands were initially established, these are rapidly being replaced with clones selected for optimum local performance. Diseases have already been recognised as an important constraint to *Eucalyptus* production in Colombia and these are being seriously considered in selection and breeding programmes (Wright 1997). In this study, we report on the occurrence of *Eucalyptus* diseases in Colombia based on three surveys conducted in May 1995, 1997 and 1998, and further observations between 2000 and 2002. The relative importance of the various pathogens is also discussed. The causal agent of Eucalyptus rust, namely *Puccinii psidii* Winter, which occurs on native hosts in Colombia, is also discussed.

MATERIALS AND METHODS

Plantations chosen for inspection were primarily those reported by field foresters to include trees showing symptoms of disease. An effort was also made to inspect trees of a variety of age classes, those representing different seed sources or clones as well as those on a variety of different sites. Although most trees examined were those of *E. grandis*, trees of other species growing in species trials were also inspected. In some instances, stands thought to have disease symptoms had nutritional deficiencies due to site or sylviculture. This is important where pathogens that are favoured by stress.

Diseased leaf and stem tissues were collected for laboratory examination and confirmation of diagnoses. Soil samples were also collected to augment observations on the presence of *Cylindrocladium* spp. that were associated with leaf and twig blight symptoms. Where necessary for diagnosis, isolations from plant tissue were made on malt extract agar (MEA; 20 g Merck malt extract, 20 g Merck agar/ litre distilled water). Isolations from soil samples were made by placing a small amount of a representative soil sample in a Petri dish, and baiting the soil with surface-disinfested alfalfa seeds (Crous *et al.* 1997a). After 14 days, the germinating seedlings were removed, submerged in 1 % NaOCl for 30 s, rinsed in sterile H_2O, and plated onto MEA, amended with streptomycin sulphate (0.05 g/litre). Petri dishes were incubated for seven days at 25 °C under continuous near-ultraviolet light, after which single spores of sporulating *Cylindrocladium* isolates were plated onto carnation leaf agar (CLA) (Crous, Phillips & Wingfield 1992), and identified using the keys of Crous & Wingfield (1994) and Crous (2002).

Leaves with Mycosphaerella leaf blotch (MLB) symptoms were collected from several plantations in Colombia. Lesions were excised from leaves, and single ascospore cultures were established on MEA using the technique described by Crous (1998). Germinating ascospores were examined after 24 h, germination patterns were determined, and they were then transferred to MEA. Colonies were subcultured onto divided plates with one half containing CLA and the other MEA and incubated at 25 °C under continuous near-ultraviolet light.

RESULTS AND DISCUSSION

Various important or potentially important *Eucalyptus* pathogens were identified in this study. These included leaf, shoot and stem pathogens. Their relative importance is deduced from the intensity of their incidence in Colombia as well as their significance in other countries.

Cryphonectria canker

Basal stem cankers caused by *C. cubensis* (Bruner) Hodges (Figs 1a-c) were observed on one-year-old trees of *E. grandis* at low altitude and areas with high humidity and temperature. The incidence of this disease was relatively limited and only scattered trees had been girdled at the bases resulting in tree death. Perithecia and pycnidia of the pathogen occurred abundantly on the dead bark at the crown of dead or dying trees (Fig. 1d). As far as we are aware, this (Van der Merwe *et al.* 2001) was the first confirmed report of *C. cubensis* from Colombia.

Cankers caused by *C. cubensis* higher on the stems of older trees such as those found in Brazil (Ferreira 1989) and India (Sharma, Mohanan & Florence 1985) were also observed on older trees. This was especially in areas experiencing high temperatures and rainfall and particularly between 900 and 1400 masl. The basal girdling cankers on young trees were more typical of those commonly encountered in South Africa (Wingfield, Swart & Abear 1989) and in Colombia appear to be of minor importance. However, the larger stem cankers observed in high temperature sites are extremely damaging with a high proportion of trees severely damaged or killed. Strategies to select *Cryphonectria* tolerant clones for planting on these sites are being developed to avoid further damage by this pathogen.

Recently in Colombia, C. *cubensis* was found on *Tibouchina urvilleana* (DC). Logn., native to Brazil, and *T. lepidota* Baill., which is native to Colombia (Wingfield *et al.* 2001a). This discovery on Melastomataceae was unusual, as it was previously believed that the pathogen was restricted to members of the Myrtaceae. However, contemporary phylogenetic data have shown that the Melastomataceae are relatively closely related to the Myrtaceae within the order Myrtales (Conti, Litt & Sytsma 1996, Conti *et al.* 1997)

and this association might not be unusual. Consequent surveys for *C. cubensis* on native hosts in Colombia, also revealed that the fungus occurs on native *Miconia theaezans* (Bonpl.) Cogn. and *M. rubiginosa* (Bonpl.) DC. (Melastomataceae) (Chapter 3, this dissertation). The occurrence of *C. cubensis* on native hosts in Colombia suggests that the fungus might have originated in Central and South America (Wingfield *et al.* 2001a, Wingfield 2003).

Representative specimens (PREM) and living cultures used in this study: **Colombia**: *C. cubensis* on *E. grandis* bark, Vanessa, M. J. Wingfield, 2000, PREM 57294, CMW 10639; on *M. rubiginosa* bark, Vanessa, C. A. Rodas, 2001, PREM 57517, CMW 2357, CMW 9996, CMW 10022, CMW 10024, CMW 10025, CMW 10026, CMW 10028.

Botryosphaeria canker and die-back

Shoot blight and stem cankers were commonly observed on *E. grandis* trees of a variety of different age classes and on many different sites (Fig. 2). The species most commonly associated with this disease was *B. ribis* Grossenb. & Duggar (anamorph *Fusicoccum ribis* Slippers, Crous & Wingfield, sp. prov.) and *B. dothidea* (Moug.) Ces. & De Not. (anamorph *F. aesculi* Corda apud Sturm) (Chapter 2, this dissertation). *B. ribis* is also known to infect *Eucalyptus* spp. (Shearer, Tippett & Bartle 1987, Webb 1983) elsewhere in the world and appears to be the primary causal agent of the symptoms observed in Colombia. A similar disease of *Eucalyptus* spp. is caused by *B. dothidea* and *B. eucalyptorum* Crous, H. Smith & M.J. Wingf. in temperate and mediterranean areas of the world (Gibson 1979, Smith, Kemp & Wingfield 1994, Smith *et al.* 2001), although the relative importance of these fungi is not known.

Botryosphaeria dothidea is a well-recognised endophyte on *Eucalyptus* spp. that commonly causes disease on stressed trees (Smith *et al.* 1994). The fungus is a latent pathogen (Smith, Wingfield & Petrini 1996) and although this has not been shown experimentally, we assume that the same is true of *B. ribis.* In most cases, trees infected with this pathogen in Colombia had not been subjected to any noticeable stress, although high rainfall might, in some cases, have contributed to disease development. In South Africa, there is clear evidence of variation in the susceptibility of different clones of *E. grandis* to infection by *B. dothidea,* which is also considered to be one of

the most damaging pathogens of *Eucalyptus* in the country (Smith *et al.* 2001). Factors associated with disease development and the relative susceptibility of *E. grandis* clones to *B. ribis* are being considered in clonal deployment and breeding strategies for Colombia (Wright 1997).

Representative dried cultures (PREM) collected in this study: **Colombia**: *B. ribis* on *E. grandis* branches, La Estrella, C. A. Rodas, Aug. 2001, PREM 57509; La Ignacia, C. A. Rodas, Aug. 2001, PREM 57510; El Líbano, C. A. Rodas, Aug. 2001, PREM 57506. *B. dothidea* on *E grandis,* Andes, M. J. Wingfield, May. 2000, PREM 57508.

Eucalyptus rust

Puccinia psidii was commonly found in this survey in Colombia on native Myrtaceae such as *Eugenia jambos* L. (Fig. 3). The fungus has, however, not been found infecting *Eucalyptus* spp. as is commonly the case in Brazil (Ferreira 1983). The climate in Colombia is highly conducive to rust development and given the presence of the pathogen, this disease could become important in the future. This is especially if susceptible *Eucalyptus* spp. or clones are propagated.

Eucalyptus rust caused by *P. psidii* is potentially one of the most important diseases of *Eucalyptus* spp. in the world. The pathogen is known primarily from countries in South and Central America where it commonly occurs on native Myrtaceae (Coutinho *et al.* 1998). The disease is best known on *Eucalyptus* spp. such as *E. cloeziana* F. Muell. and *E. saligna* Sm. in Brazil, where the pathogen also occurs on several native plants (Coutinho *et al.* 1998). It has also been reported from *E. camaldulensis* Dehnhardt in Taiwan (Coutinho *et al.* 1998, Wang 1992), although it is uncertain whether this fungus is the same as that occurring in South America.

Cylindrocladium shoot and leaf blight

Numerous species of *Cylindrocladium* have been associated with nursery and plantation diseases of *Eucalyptus* spp. (Crous & Wingfield 1994). Cylindrocladium shoot and leaf blight disease of *E. grandis* plantation trees in Colombia (Fig 4) was predominantly associated with *C. reteaudii* (Bugnic.) Boesew. and *C. candelabrum*

Viégas in the past (Crous 2002). These two species, and especially *C. reteaudii*, were also the most dominant species isolated from the collected soil samples. Other species that were baited from soil under *Eucalyptus* trees, and that have been linked to diseases of this host in the past include *C. parasiticum* Crous, M.J. Wingf. & A.C. Alfenas and *C. gracile* (Bugnic.) Boesew. (Crous 2002, Ferreira 1989, Hodges & May 1972).

Cylindrocladium leaf blight on *E. grandis* in Colombia is commonly found in one-year-old plantations and the disease is prevalent in areas associated with high stand density and high rainfall and humidity. Most recent collections from leaf blight affected areas have all been of *C. spathulatum* El–Gholl, Kimbr., E. L. Barnard, Alfieri & Schoult, while none of the other species previously reported has been found (Chapter 4, this dissertation). The pathogen has not been reported in nurseries as it occurs in other parts of the world.

Representative living (STE-U) and dried cultures (PREM) used in this study: **Colombia**: *C. candelabrum* on *E. grandis* leaves, La Selva, M. J. Wingfield, Jun. 1995, STE-U 1151-1152. *C. gracile*, soil under *E. grandis*, La Paz Rodal, M. J. Wingfield, 1993, STE-U 726. *C. parasiticum*, soil under *E. grandis*, La Selva, M. J. Wingfield, 1993, STE-U 723-725. *C. reteaudii* on *E. grandis* leaves, La Selva, M. J. Wingfield, Jun. 1995, STE-U 1069-1070. *C. spathulatum* on *E. grandis* leaves, La Suiza, C. A. Rodas, July 2002, PREM 57504; Samaria, C. A. Rodas, July 2002, PREM 57505; Don Miguel, C. A. Rodas, July 2002, PREM 57502; Sta Maria, C. A. Rodas, July 2002, PREM 57503; Libano, C. A. Rodas, July 2002, PREM 57500; A. Maria, C. A. Rodas, July 2002, PREM 57505.

Mycosphaerella leaf blotch

Mycosphaerella leaf blotch disease (MLB) (Fig. 5) of *Eucalyptus* spp. is caused by a complex of *Mycosphaerella* spp. There are currently 29 *Mycosphaerella* spp. found on *Eucalyptus* spp., including various anamorph states for which no *Mycosphaerella* state has yet been recorded (Crous 1998). Although several species have been recorded from most countries where *Eucalyptus* spp. are grown, recent collections have shown that these identifications were mostly incorrect, and that only a few species occur on more than one continent (Crous 1998).

Several *Mycosphaerella* spp. were identified from *Eucalyptus* spp. in Colombia. *Mycosphaerella suberosa* Crous, F.A. Ferreira, Alfenas & M.J. Wingf. was associated with corky leaf spots. This fungus has also been found on *E. grandis*, *E. dunnii* Maiden and *E. molluccana* Roxb. in Brazil (Crous *et al.* 1993a), Indonesia (Crous & Wingfield 1997) and Western Australia (Carnegie, Keane & Podger 1997). In Colombia, it was observed on young and older mature foliage of *E. globulus* (M. J. Wingfield, personal communication). *Mycosphaerella parkii* Crous, M.J. Wingf., F.A. Ferreira & Alfenas, known from *E. grandis* and *E. globulus* in Brazil (Crous *et al.* 1993b) and *E. grandis* in Indonesia (Crous & Alfenas 1995), was isolated from leaf spots on juvenile leaves of *E. grandis*, and appeared to be fairly common in Colombian plantations. *Mycosphaerella africana* Crous & M.J. Wingf. that has been described from several *Eucalyptus* spp. in South Africa (Crous & Wingfield 1996) and has also been recorded from *E. globulus* in Portugal, was found on *E. grandis* in Colombia (Crous & Wingfield 1997). Other species that occur in Colombia, is *M. flexuosa* Crous & M. J. Wingf. and *M. lateralis* Crous & M. J. Wingf. (M. J. Wingfield, personal communication).

Sonderhenia eucalypticola (A. R. Davis) H. Swart & J. Walker (teleomorph: *Mycosphaerella walkeri* R.F. Park & Keane) was found on juvenile leaves of *E. globulus*. This fungus is known from several *Eucalyptus* spp. in Australia and New Zealand (Park *et al.* 2000). However, the teleomorph occurs on *E. globulus* and another *Eucalyptus* sp. from Australia (Peredo,1995), and has recently also been recorded on a *Eucalyptus* sp. from Chile (Wingfield, Crous & Peredo 1995). It has also recently been collected on *E. globulus* leaves from Ecuador (Crous and Wingfield, unpublished), suggesting that it occurs widely throughout South America.

Representative specimens used in this study: **Colombia**: *M. africana*, on *E. grandis* leaves, Sinai, M. J. Wingfield, Jun. 1995, PREM 54978. *M. colombiensis* on leaves of *E. urophylla,* Pinal farm, M. J. Wingfield, May 1995, PREM 54396, PREM 54397. *M. flexuosa,* on leaves of *E. globulus,* Selva, M. J. Wingfield, May 1995, PREM 54401. *M. lateralis* on leaves of *E. grandis,* Selva, M. J. Wingfield, Jun. 1994, PREM 54403; on leaves of *E. grandis,* Sinai, M. J. Wingfield, Jun. 1995, PREM 54404.

Phaeoseptoria leaf spot and Corky leaf spot

Phaeoseptoria leaf spot, caused by *Phaeophleospora epicoccoides* Cooke & Massee (Crous *et al.* 1997b) (Figs 6a-c) and corky leaf spot, caused by *Aulographina eucalypti* Cooke & Massee (Wall & Keane 1984) (Figs 6d-e) occur commonly and widespread on *Eucalyptus* spp. in Colombia. These pathogens are found in most areas of the world where *Eucalyptus* spp. are propagated, and they are also known in Australia (Sankaran, Sutton & Winter 1995). *Phaeophleospora epicoccoides* generally infects mature and senescent leaves, especially under conditions of severe stress. It is not considered an important pathogen (Knipscheer, Wingfield & Swart 1990).

Aulographina eucalypti causes a disease commonly known as corky leaf disease and is most common on mature leaves as well as on petioles and twigs. The pathogen is generally not considered to be particularly important although a single report of serious defoliation in Australia (Stefanatos 1993) might be cause for some concern (Crous & Wingfield 1997, Crous 1998).

CONCLUSIONS

Diseases pose a serious threat to productivity of *Eucalyptus* plantations. In Colombia, knowledge of disease presence and biology has led to significant changes in sylviculture and clonal deployment. Trials to select disease tolerant planting stock have been established and have already shown positive results. Furthermore, monitoring of plantations for disease problems is actively pursued and should make it possible to reduce the impact of diseases in the future.

The use of conventional methods is not likely to eliminate the threat of diseases in plantations in Colombia. However, with sufficient research effort and the utilisation of this information, losses to plantations can be minimized. Sustainable development of *Eucalyptus* plantations will require that diseases strategies are strongly considered in all management plans.

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Fig. 1. Typical disease symptoms associated with infection by *Cryphonectria cubensis*. **(a)** Sudden wilt and death of trees. **(b)** Basal stem canker and cracked bark. **(c)** Section through a stem canker showing discoloured and dead sapwood (indicated with arrow). **(d)** Abundant presence of sexual and asexual fruiting structures on bark surrounding canker.

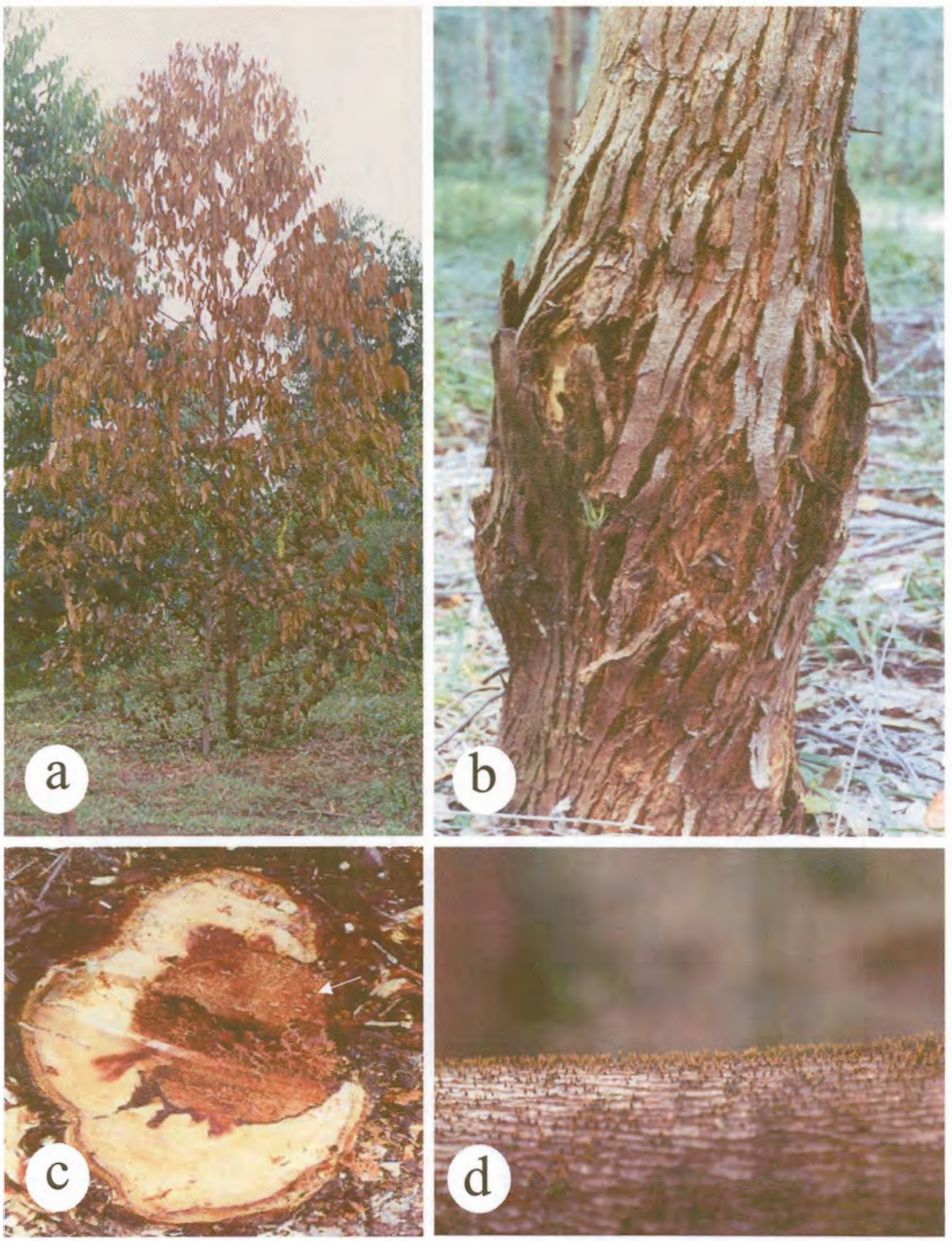
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Fig. 2. Symptoms associated with infections by *Botryosphaeria* spp. on *Eucalyptus grandis*. **(a)** Die-back of the leader shoots. **(b-c)** Shoot blight and twig canker (indicated with arrows). **(d)** Stem and branch cankers. **(e)** Gum like kino exudation.

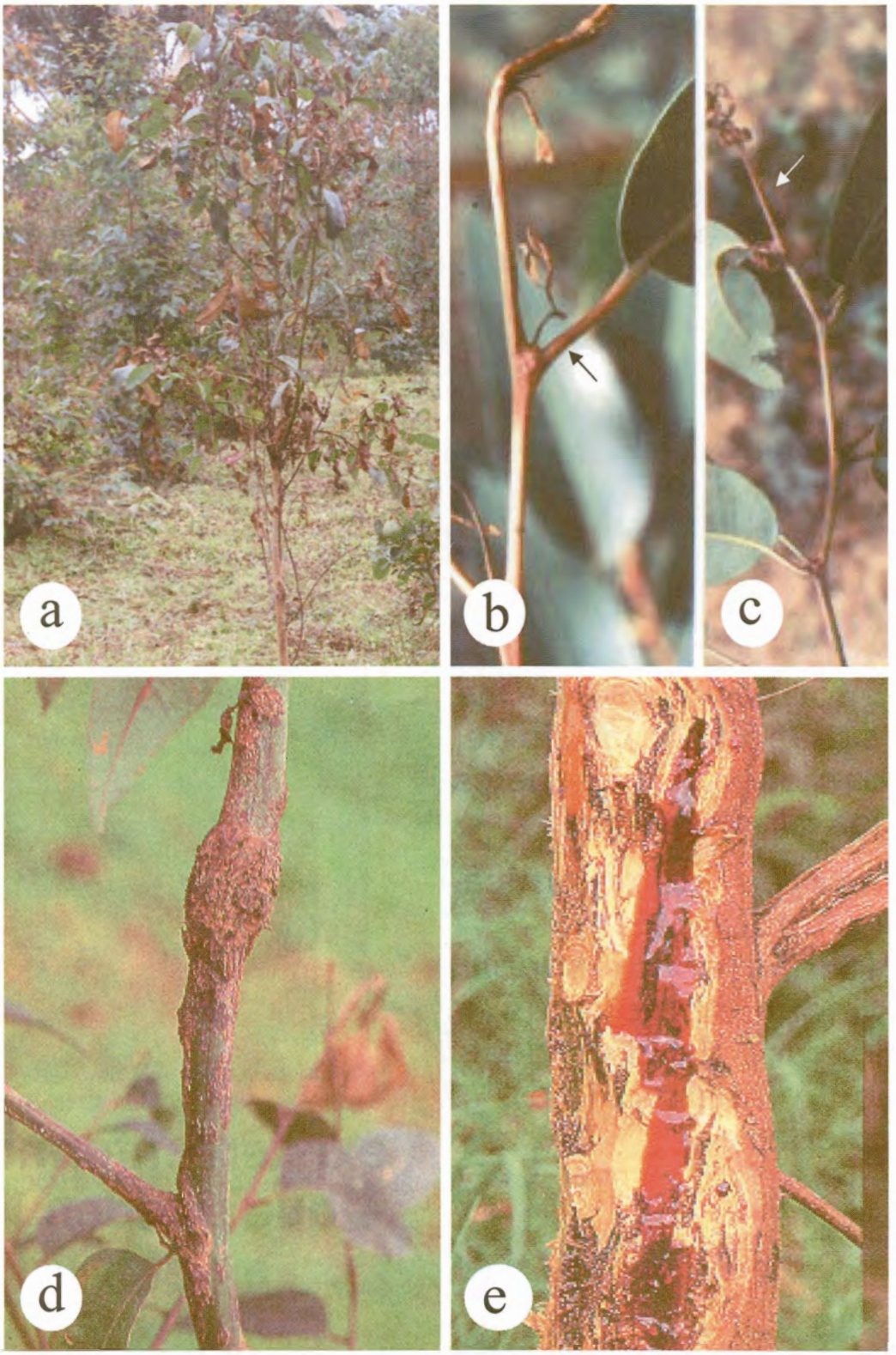

Fig. 3. Symptoms associated with rust caused by *Puccinia psidii*. **(a)** Uredinia formed on leaves of *Eugenia jambos*. **(b)** Characteristic egg-yolk yellow pustules developing on the top and bottom of leaf surfaces. **(c)** Leaf deformation and shoot death.

Fig. 4. Symptoms of infection by *Cylindrocladium* spp. on leaves of *Eucalyptus grandis*. **(a-b)** Early stage of development of leaf spots on the top and bottom of leaves. **(c-d)** Well-developed leaf spots on the top and bottom of leaves showing deformation and necrosis of substantial areas of the leaf surfaces.

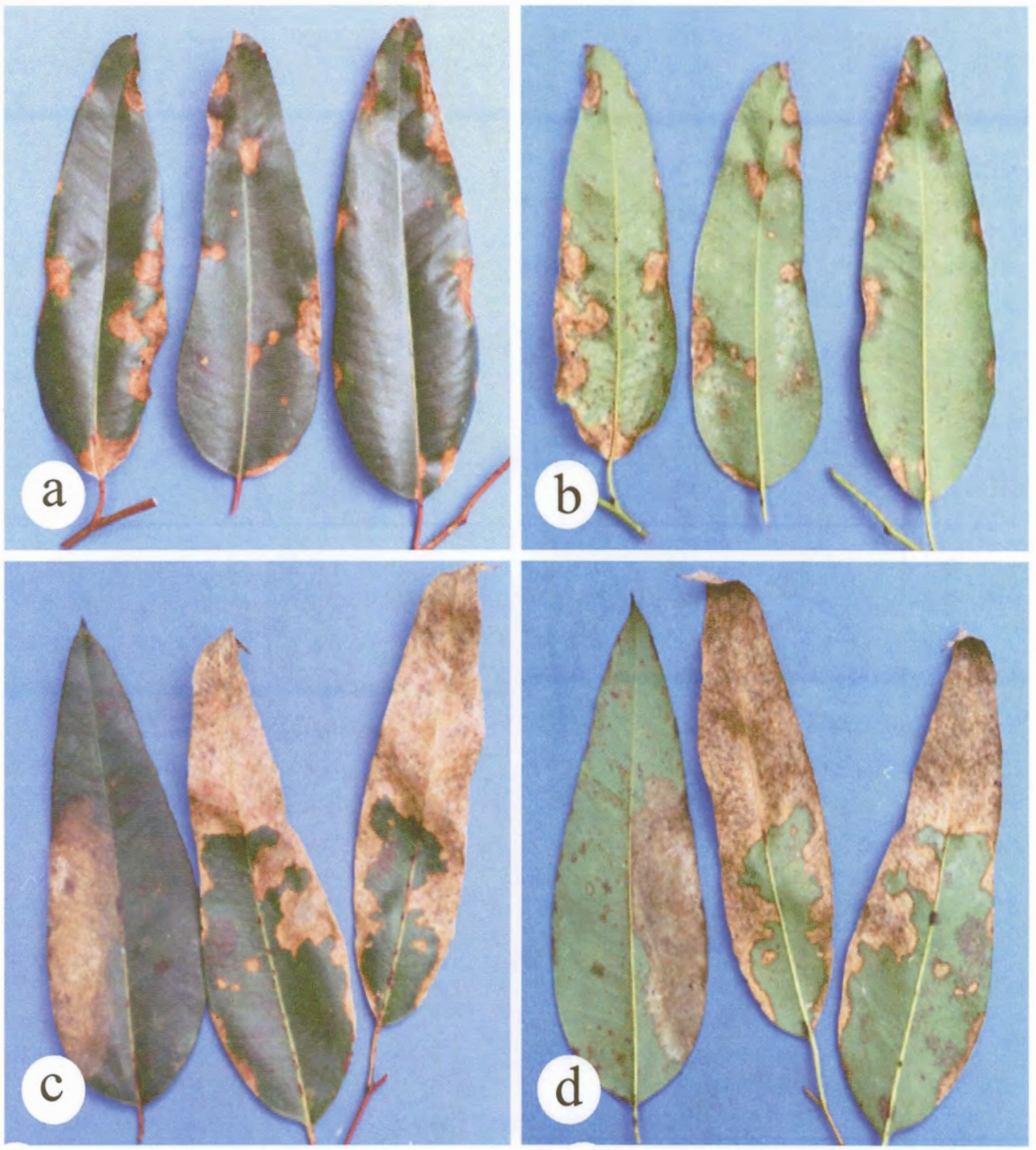
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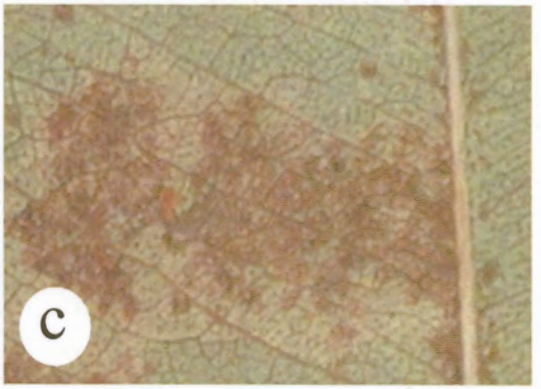
Fig. 5. Symptoms associated with infection by *Mycosphaerella* spp. **(a-b)** Irregular to sub-circular leaf spots on the top and bottom of leaves of 16-month-old *Eucalyptus grandis* trees.

Fig. 6. (a-c) Small dark and red lesions on *Eucalyptus grandis* leaves typical of infections by *Phaeophleospora epicoccoides*. **(d-e)** Corky lesions caused by *Aulographina eucalypti* on the abaxial surfaces of *E. grandis* leaves.


a


b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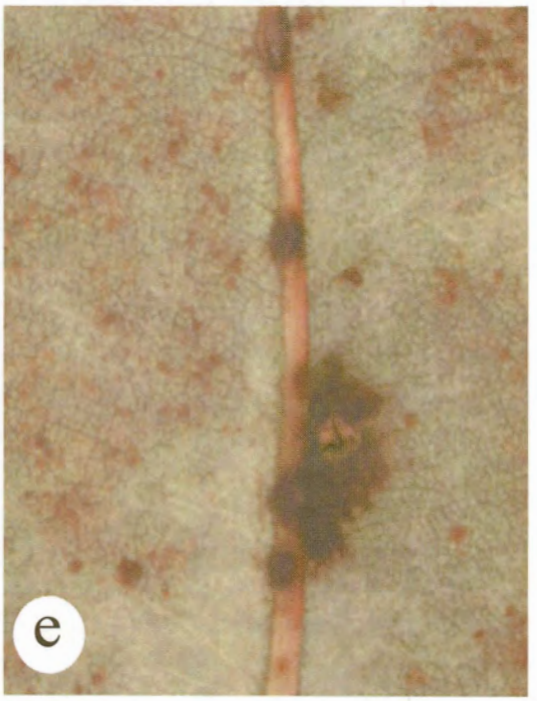
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CHAPTER 2

Diseases of *Eucalyptus* associated with *Botryosphaeria* spp. in Colombia

ABSTRACT

Botryosphaeria canker is a serious disease of *Eucalyptus* trees in all major *Eucalyptus* growing areas of the world. Although Botryosphaeria canker has been known to occur in Colombia, the species associated with the disease, as well as the impact they have on *Eucalyptus* plantations in Colombia, have not been studied. In this study, the identity of the *Botryosphaeria* species were investigated through morphological and DNA sequence studies. The pathogenicity of these species was also assessed on 42 clones planted in three different sites. Two species of *Botryosphaeria* were found to occur on *E. grandis* in Colombia. *Botryosphaeria ribis* was the more common species, and also more aggressive. *Botryosphaeria dothidea* was only found in one zone, and was also athogenic than *B. ribis*. These two species could be distinguished easily based on DNA sequences of the ITS1/ITS2 rDNA region and EF1-α, as well as conidial sizes. Significant differences in resistance of clones to these pathogens were also evident from the various trials. This study has shown that of the two species present in Colombia, *B. ribis* should be the focus of future efforts to reduce the impact of this disease, and that a resistance breeding program against the pathogen will be possible.

INTRODUCTION

Species of *Botryosphaeria* include an important group of pathogenic fungi that infect a wide range of woody plants. These fungi have a cosmopolitan distribution and are also found on many woody plants in the tropics and sub-tropics. Disease symptoms include stem and branch cankers, die-back, bleeding necrosis, coppice failure and seed capsule abortion (Smith, Kemp & Wingfield 1994, Neely 1996, Barnard *et al.* 1987, Webb 1983).

Botryosphaeria spp. are members of the family Botryosphaeriaceae and order Dothideales (Hawksworth *et al.* 1995). Identification of these fungi is often difficult, because teleomorph states are rarely encountered in nature. The taxonomy of anamorphs is confusing, because it is based on characters such as conidial pigmentation, septation and stromatal morphology, which show extensive plasticity and can be influenced by substrate and growth conditions (Butin 1993, Sutton 1980). More than 140 *Botryosphaeria* spp. have been described, mainly based on the hosts on which they occur (Denman *et al.* 2000). Many of these are likely to be synonyms, while others most likely represent species complexes.

The taxonomy of *Botryosphaeria* spp. has been confused and complicated for many decades. New species have tended to be described based on new hosts on which they occur. Another problem has been that names for these fungi, such as *Botryosphaeria dothidea* (Moug.) Ces. & De Not. and *B. ribis* Grossenb. & Dugg. have been used loosely and interchangeably (Denman *et al.* 2000, Slippers *et al.* 2003). Previous reports of *B. dothidea* and *B. ribis* on *Eucalyptus* could be wrong as molecular taxonomy have now shown that these species are rare on *Eucalyptus* and many new species have been described. Recently, DNA based techniques have been applied to the taxonomy of *Botryosphaeria* spp. (Jacobs & Rhener 1998, Slippers *et al.* 2003, Zhou & Stanosz 2001). This has made it possible to more accurately differentiate between species and to evaluate the taxonomic value of various identification procedures.

Botryosphaeria anamorphs appear to provide the best means to identify species. These states occur in two distinct groups that also have strong phylogenetic support (Crous & Palm 1999, Denman, Crous & Wingfield 1999, Denman *et al.* 2000, Slippers *et al.*

2003, Zhou, Smith & Stanosz 2001, Zhou & Stanosz 2001). These include those with hyaline conidia that are treated in *Fusicoccum* and others with dark, thick-walled conidia that are best placed in *Diplodia.*

Botryosphaeria spp. are well recognized as opportunistic wound and stress related pathogens (Schoeneweiss 1981). These fungi are also well adapted to live as facultative parasites or saprophytes on dead wood and other plant material (Sivanesan 1984). Under certain environmental conditions they can be virulent pathogens infecting twigs, stems, roots and leaves, which penetrate their hosts through wounds, open stomata and lenticels (Luttrell 1950, Wene & Schoeneweiss 1980). A recent understanding of these fungi is that they infect healthy plant tissue and can exist for extended periods of time in a latent form (Fisher, Petrini & Sutton 1993, Roux 1998, Smith, Wingfield & Petrini 1996). With the onset of stress, they then become active and cause serious disease.

Botryosphaeria spp. constitute an important group of pathogens of many hosts throughout the world (Von Arx & Muller 1954). In terms of exotic plantation forestry these species are also pathogens of *Eucalyptus* (Smith 1995, Roux 1998). *Botryosphaeria dothidea* has been reported to cause canker and die-back diseases of *Eucalyptus* species in Australia and the United States (Barnard *et al.* 1987, Davison & Tay 1983, Webb 1983). Webb (1983) reported the presence of *B. dothidea* in commercial seed of *Eucalyptus camaldulensis* Dehnh. in South Florida. Shearer *et al.* (1987) showed that this fungus was responsible for the death of *Eucalyptus radiata* Sieb. ex DC. in selection trials in Australia. Davison & Tay (1983) reported the natural occurrence of *B. dothidea* cankers in *Eucalyptus marginata* Donn. ex Sm. 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* W. Hill ex Maiden in Florida. Smith *et al.* (1994) found that two species of *Botryosphaeria* are associated with cankers on *Eucalyptus* in South Africa namely *B. dothidea* and *B. eucalyptorum* Crous, H. Smith & M. J. Wingf.

Eucalyptus plantations represent an important renewable resource for the forestry industry in Colombia. One of the most important species presently planted is *E. grandis.* This species has been used in reforestation and clonal programs by private

companies and government projects for the production of timber, pulp and protection of eroded soils.

Botryosphaeria spp. have been recognized as an important constraint to the productivity of *E. grandis* plantations in Colombia. Since 1994, diseases caused by *Botryosphaeria* spp. have been recorded in *E. grandis* trees in different geographic zones at the Smurfit Carton de Colombia and, *Eucalyptus grandis* plantations commonly affected by this disease range in age from 6 to 36 months, with the most susceptible trees being those between 18 to 26 months. Common symptoms include small necrotic lesions at the point of the insertion of twigs on the shoots and these develop to form large irregular cankers causing die-back of shoots. Cankers located on branches and main stems give rise to abundant production of kino which degrades the wood and weakens the stems. Wind or other external agents then lead to stem breakage and considerable loss.

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

MATERIALS AND METHODS

Symptoms and collection of samples

Isolations were made from a wide range of symptoms. These included die-back of shoots and twigs, small necrotic lesions at the insertion points of twigs on the shoots and irregular cankers formed on stems and branches (Fig. 1). In addition, isolations were made from pseudothecia on the bark of diseased *Eucalyptus* branches.

Samples of diseased *E. grandis* tissue were collected from three distinct geographical areas, namely the Andina, Valle and Cauca zones (Fig. 2). From these areas, 17 farms belonging to Smurfit Carton de Colombia were included in the collections. Diseased tissue was collected and transferred to the laboratory for detailed examination. All samples included in this study were collected between May 2000 and May 2001.

Tissue samples from diseased stems and branches were surface sterilised in 70 % ethanol for 30 s and thereafter washed in sterile distilled water. This material was placed in moisture chambers and incubated at 24 °C until fungi began to sporulate on the surface of the samples. In addition, after surface sterilisation, 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, S.A.) and incubated at 25 °C for seven days.

Forty four isolates of *Botryosphaeria* spp. were obtained from preliminary isolations and single conidial subcultures were made for all of these isolates. In order to produce single conidial isolates, fungi were grown on water agar (WA) (2 % agar; Biolab) with sterilized pine needles 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 seven hours, germinating conidia were selected and transferred to MYA.

No differences in the morphology of cultures were observed between samples. All 44 single conidial isolates were transferred to 2% malt extract agar (MEA) (Biolab) slants and stored at 4 °C. These have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Morphological characteristics

The obtained isolates were inoculated on sterile pine needles on WA to induce sporulations. Conidia from pycnidia 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 – std. dev.) – (average + std. dev)(-max).

DNA extraction and amplification

DNA was obtained using a modified version of the method of Raeder & Broda (1985). Mycelium was scraped off the surface of single conidial isolates and transferred to sterile Eppendorf tubes (1.5 ml). An amount of 400 µl of DNA extraction buffer (200 mM Tris-HCl pH 8.0, 150 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) were added to each tube, and cell walls were broken using a pestle. When a homogeneous solution was obtained an additional 400 µl of DNA extraction buffer was added as well as 500 µl of phenol and 300 µl of chloroform. These were then mixed using a vortex mixer and centrifuged at 10000 rpm for 60 min and at 4 (C. The DNA-containing aqueous layer was transferred to new tubes and extraction and washing were repeated until the white interface disappeared. Precipitation of DNA was achieved by adding 0.1 volume of 3 M NaAc (pH 5 to 5.5) and 2 volumes of absolute ethanol which was then centrifuged for 30 min at 4 (C and the EtOH was removed. The pelleted DNA was washed with 70% EtOH, dried under vacuum and re-suspended in sterile SABAX water (100 µl). An amount of 5 µl Rnase was added to the DNA solution that was incubated for two hours at room temperature. 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 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCG GCTTATTGATATGC3') (White et al. 1990). The amplified region included the 3' end of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS 1) the complete 5.8S rRNA gene, the second ITS (ITS 2) region and the 5' end of the 26S (large subunit) of the rRNA gene. In addition, a part of the translation elongation factor 1α (EF1-α) gene was amplified using the primers EF1-728F (5'CATCGAGAAGTTCGAGAAGG) and EF1-986R (5'TACTTGAAGGAACCCTT ACC) (Carbone, Anderson & Khon 1999). The PCR reaction mixture for all was done according to Slippers et al. (2003) and contained 1X PCR reaction Buffer and MgCl2 (10 mM Tris-CHl, pH 8, 1.5 mM MgCl2, 50 mM KCL, pH 8.3) (Roche Diagnostics, Randburg, South Africa), 0.2 mM of each dNTP (Roche Diagnostics), 0.15 µM of each primer, 2.5 Units Expand High Fidelity Taq DNA polymerase (Roche Molecular

Biochemicals, Almeda, California, USA) and 10 ng of DNA. The reaction mixture was made up to a final volume of 50 µl by adding sterile deionized Sabax water. The amplification of the EF1-α region was done using the same protocol as described above, except that ExpandTM High Fidelity Taq polymerase was used (Roche Molecular Biochemicals).

The PCR reaction conditions for both regions, comprised of an initial denaturation of the DNA at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, elongation at 72 °C for 1 $^{1}/_{2}$ min, and concluding with a final elongation at 72 °C for 5 min. 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.

DNA sequencing and analysis

The PCR amplified fragments were purified using a High Pure PCR product Purification Kit (Roche Molecular Biochemicals). Each strand of the PCR products was 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, California, USA) and capillary electrophoresis on an ABI PRISM ^{TM} 310 DNA Autosequencer (Applied BioSystems). All the reactions were done 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 were determined from the aligned sequences using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2002).

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 and "Ns" are mising. 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 partition homogeneity test (Farris *et al.* 1995, Huelsenbeck, Bull & Cunningham 1996) in PAUP. These tests revealed that the data were combinable and all DNA sequence sets were subsequently analysed together. In order to establish the phylogenetic relationships and the identities of the *Botryosphaeria* spp. used in this study, sequences of known *Botryosphaeria* spp. from GenBank and Slippers *et al.* (2003) were included in the alignment (Table 2). Trees were rooted using a sequence of *Guignardia philoprina* (Ellis) Viala & Ravaz as outgroup taxon (Table 2).

Pathogenicity Tests

Greenhouse trial. A preliminary pathogenicity test was conducted in a greenhouse maintained at approximately 25 °C with natural light. In this trial, six isolates representing two distinct morphological 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 trees of a *E. grandis* clone (ZG14), 18 months old, known to be susceptible to infection by *Botryosphaeria* spp. After two weeks of acclimatization in the greenhouse, ten trees were inoculated with each isolate. Inoculum was derived from one-week-old cultures growing on MYA. Ten trees were inoculated with sterile agar medium as negative control.

For inoculations, wounds were made in the stems of trees using a cork borer (6 mm diam) 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 six weeks after inoculation.

Field trials. Four inoculation trials were conducted in the field in Colombia. These were at four different sites chosen to represent a wide range of climate and humidity conditions where *Botryosphaeria* cankers are found. The trials were as follows:

Trial N°1. La Suiza farm in Restrepo, Valle, located at 1469 masl, 76° 29' 49" W, 3° 51' 45" N. This site receives an average annual precipitation of 1067 mm/y. **Trial N°2.** Cecilia farm near Darien, Valle, at 1825 masl, 1825 mm/y of precipitation, located at 76° 26' 06" W, 3° 57' 06" N. **Trial N°3.** Libano farm near Pereira, Risaralda, with 2102 masl, precipitation of 3143 mm/y and 75° 35' 49" W, 75° 35' 49" N. **Trial N° 4.** Angela Maria farm near Santa Rosa, Risaralda located at 1864 masl, with an average of 2437 mm/y precipitation and located at 75° 11' 14" W, 6° 8' 46" N.

Isolates CMW 8922 and CMW 8961 representing the two different *Botryosphaeria* morphological groups and previously shown to be most pathogenic 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 ten clones (2, 4, 11, 12, 18, 20, 23, 27, 301, 303) and four seed lots (210, T210, 211, T 211) used in all four trials. Forty trees of each clone or seed lot, distributed in ten 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 in 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 above for the greenhouse inoculations except that the cork borer size for the inoculations was 4 mm and the inoculated areas were covered with tissue paper moistened with sterile water and secured with masking tape (Fig. 3). Trials were initiated during June 2002 and 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, as well as tables of means with the 95% confidence limits (C.L.) for each mean. Analysis of interactions between *Botryosphaeria* isolates, *E. grandis* clones and localities of the trials was assessed using an AMMI analysis (Eisenberg *et al.* 1996), because this method is uniquely able to focus completely on the interaction sums of squares.

RESULTS

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 center of colonies, first having a dark-green colour and gradually becoming black with age (Figs 4a, c). No obvious or consistent differences could be detected in the colour of the two groups of isolates (Fig. 4).

Of the 44 isolates used in this study, two morphological groups were found based on conidial differences. Eight isolates belonged in one group, which we have designated Group A, and the remaining 36 isolates resided in Group B. All isolates belonging to Group A were collected from only one farm in the Andina zone while isolates of Group B were found in all three evaluated zones.

Both groups had *Fusicoccum*-type conidia that were hyaline, thin-walled, aseptate and smooth (Figs 4b, d). The conidia of these groups, however, differed in size and shape. Conidia of Group A were fusiform to fusiform-elliptical with obtuse to pointed ends and were (25.0-)27.0 – 29.0(-30.0) x 5.0(-7.0) µm (average 27.9 x 5.2 µm) (Fig. 4d, Table 3). Conidia of isolates belonging to Group B were elliptical to fusiform with pointed ends, granular contents and (18.0-)20.0 – 21.0(-23.0) x (5.0-)6.0(-7.0) µm (average length x width 20.3 x 5.6 µm) (Fig. 4b, Table 3).

DNA sequencing and analysis

Sequences of approximately 557 bp were obtained from the amplified ITS 1/2 rDNA region and approximately 332 bp for EF1-α. A partition homogeneity test of the full data set indicated that they could be combined ($p = 0.60$). Alignment of the combined sequences gave rise to a data set of 889 characters (Fig. 5). Of these, 601 characters were parsimony-uninformative and 288 were parsimony-informative. Phylogenetic analyses were done on the combined data set of ribosomal DNA and the EF1-α intron region of five *Botryosphaeria* isolates from Colombia, and 14 isolates from GenBank

including *B. ribis, B. eucalyptorum, B. lutea* Phillips, *B. dothidea, B. rhodina* (Cooke) von Arx, *B. obtusa* (Schw.) Shoemaker and *B. stevensii* Shoemaker. The dataset thus consisted of 19 ingroup taxa and the outgroup taxon, *G. philoprina*. Two most parsimonious trees were obtained (length = 642 steps, consistency index = 0.822, retention index = 0.899, rescaled consistency index = 0.739, and g1 = -0.821) (Fig. 6). It showed that of the five *Botryosphaeria* isolates from Colombia, two (CMW 8922, CMW 8929), belonging to Group A, grouped in the *B. dothidea* clade with 100 % bootstrap support. The remaining three grouped closely with *B. ribis* with a 74 % bootstrap support. These three isolates (CMW 8961, CMW 8959, CMW 8949) belonged to the morphological Group B.

Pathogenicity Tests

Greenhouse trial. Six weeks after inoculation of *E. grandis* with six isolates of *Botryosphaeria* spp. representing *B. dothidea* (CMW 8922, CMW 8925, CMW 8929) and *B. ribis* (CMW 8949, CMW 8956, CMW 8961), the mean lesion lengths for isolates in the *B. ribis* group was significantly (P = 0.05) greater than that for the *B. dothidea* group (Table 4a-b, Figs 7-8). Of the *B. 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 pathogenicity trials.

Field trials. In general, results derived from the greenhouse and the four field pathogenicity trials were consistent in showing that the isolate of *B. ribis* was significantly more pathogenic 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 *B. ribis* (Figs 9-13). Lesions caused by *B. ribis* were also more variable in size on the different clones, while the lesions caused by *B. dothidea* were all similar in size (Figs. 9-13).

Lesions on the different clones inoculated in the different trials, formed a continuum of values. The smaller lesions, however, usually differed significantly from the larger lesions especially in the case of the *B. ribis* inoculations (Figs 10-13). The clones that were the least and the most resistant, however, was different in each trial. In the La

Suiza farm trial, clone 303 had the largest lesions while clone 20 was most resistant when inoculated with *B. ribis*. In contrast, for clones inoculated with *B. dothidea* clone 23 had the longest lesions and clone 303 had the shortest lesions (Table 5a-b, Fig. 10). Unfortunately, clones T210 and T211 inoculated with *B. dothidea*, and T210 and T211 inoculated with *B. ribis*, could not be included in this trial because an undue number of these trees had died due to other causes prior to the completion of the experiment. In the Cecilia farm trial, clone 2 was most susceptible while clone T211 was the most resistant to *B. ribis*. For inoculation with *B. dothidea* clone 4 was more susceptible than clone 27 (Table 6a-b, Fig. 11). In the Libano farm trial, lesion length in clone 2 was significantly higher than in clone 301 when they were inoculated with *B. ribis*. In addition, clone 18 was more susceptible than clone 11 when inoculated with *B. dothidea* (Table 7a-b, Fig. 12). In the Angela Maria farm trial, the susceptibility of clone 020 was significantly greater than that of clone T211 when inoculated with *B. ribis*. For inoculations with *B. dothidea,* clone 301 was more resistant than clone 23 (Table 8a-b, Fig. 13).

Results from the AMMI analysis of the interaction between *Botryosphaeria* isolates, *E. grandis* clones and sites, showed that for the four localities (La Suiza, Cecilia, Libano and Angela Maria) studied, differences were present in the amount of interaction at each trial site. Thus, Libano and Angela Maria had the lowest lesion lengths, but were nevertheless highly interactive as seen from their high positive scores (Fig. 14). In the analysis Cecilia is situated closer to the zero score line and this locality is expected to exhibit more stable pathogenicity around the 60 to 80 mm length. La Suiza had the highest average lesion lengths and was also highly interactive. Therefore, it can be expected to produce more variable results.

Data for the inoculated clones are scattered in a line from smaller lesions and relatively low scores through increasing lesion length and higher scores (Fig. 14). Clones inoculated with *B. dothidea* had the smallest lesions and their positive scores may be multiplied with the positive scores at Libano and Angela Maria to give a greater positive value. This implies that *B. dothidea* may result in greater pathogenicity at these localities. Conversely, the scores for these clones inoculated with *B. dothidea,* when multiplied with the negative scores of Cecilia and La Suiza would result in negative values. This implies that their lesion development is expected to be reduced in these

environments; therefore *B. dothidea* is not expected to become an important pathogen in the Cecilia and La Suiza environments. The same can be applied to the score values obtained for the different clones inoculated with *B. ribis* (Fig. 14) at the various locations.

DISCUSSION

Botryosphaeria spp. were first recognised to be associated with cankers on *Eucalyptus* in Colombia approximately ten years prior to this study. However, the identity of the fungus had not been determined conclusively. Pathogenicity tests to determine the role of the fungus in disease had also not been conducted. This study represents the first detailed investigation of *Botryosphaeria* spp. on *Eucalyptus* in Colombia. Results have shown that two species of the fungus are associated with serious die-back of planted *Eucalyptus* in this country. They have also provided an enhanced understanding of the role of these fungi in disease.

Botryosphaeria ribis and *B. dothidea* were shown to both be associated with stem cankers and shoot die-back. These species have previously been associated with *Eucalyptus* diseases (Barnard *et al.* 1987, Davison & Tay 1983, Shearer *et al.* 1987, Smith *et al.* 2001, Webb 1983). Previous studies have, however, treated *B. dothidea* and *B. ribis* as aggregate species and the identity of the causal agent might not have been consistent with modern taxonomic treatments of the group (Slippers *et al.* 2003).

This study has shown that *B. ribis* is more common and substantially more pathogenic than *B. dothidea* on *Eucalyptus* in Colombia. To the best of our knowledge, this is the first definitive record of *B. ribis* causing serious disease of this important plantation species. Although it was previously recognized as an important pathogen of *Eucalyptus* in the past, it now seems that *B. dothidea* is relatively unimportant (Slippers *et al.* 2003). Future research aimed at reducing the impact of *Botryosphaeria* canker in Colombia should clearly focus on *B. ribis*.

In this study, we have included isolates from three different zones (Cauca, Valle and Andina) in Colombia, in which *Botryosphaeria* canker is an important disease of *E. grandis*. These areas differ substantially in climate but *B. ribis* was found to occur in

both cooler and warmer areas. Temperature, therefore, does not appear to be an important factor limiting the occurrence of the fungus. However, because *Botryosphaeria* spp. are typically stress related pathogens (Schoeneweiss 1980), climate may affect susceptibility of trees.

The AMMI analyses have shown that there are distinct interactions between site, clones inoculated and the two *Botryosphaeria* spp. present. Thus, at La Suiza and Cecilia *B. ribis* is clearly the most severe pathogen across clones. Although *B. dothidea* was, in general, only mildly pathogenic at Angela Maria, the high environmental score for this site indicates that it does seem to have the capacity to increase pathogenicity of the disease should conditions or the fungus change.

DNA sequence data were essential to identify the *Botryosphaeria* spp. recognised in this study. This has also been true in a number of recent studies considering species of the fungus (Jacobs & Rhener 1998, Slippers *et al.* 2003, Zhou & Stanosz 2001). 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, where *B. dothidea* has longer spores than those of *B. ribis*. This morphological characteristic should facilitate rapid identification of the two species in the future.

In this study, we found significant differences in pathogenicity between *B. dothidea* and *B. ribis*. Lesion lengths associated with *B. dothidea* inoculations were significantly smaller than those associated with *B. ribis,* in both greenhouse and field trials. In addition, this result was confirmed at sites across three major climatic zones where *E. grandis* and its hybrids are currently being planted. The consistent pathogenicity of *B. ribis* in all areas shows that it has the capacity to infect trees, assuming climatic conditions are conducive to infection.

Results of this study clearly show that different clones differ substantially in their susceptibility to infection by *B. 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.

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Table 1. *Botryosphaeria* isolates from *Eucalyptus grandis* used in this study.

Isolate number (CMW) [a]	Locality / Zone	Altitude (masl)	Collector
8922	Andes / Andina	2102	M. J. Wingfield
8923	Andes / Andina	2102	M. J. Wingfield
8924	Andes / Andina	2102	M. J. Wingfield
8925	Andes / Andina	2102	M. J. Wingfield
8926	Andes / Andina	2102	M. J. Wingfield
8927	Andes / Andina	2102	M. J. Wingfield
8928	Andes / Andina	2102	M. J. Wingfield
8929	Andes / Andina	2102	M. J. Wingfield
8930	Carolina / Andina	1700	C. A. Rodas
8931	Carolina / Andina	1700	C. A. Rodas
8932	Selva / Andina	2048	C. A. Rodas
8933	Selva / Andina	2048	C. A. Rodas
8934	Selva / Andina	2048	C. A. Rodas
8935	Carolina / Andina	1700	C. A. Rodas
8936	Carolina / Andina	1700	C. A. Rodas
8937	Carolina / Andina	1700	C. A. Rodas
8938	Carolina / Andina	1700	C. A. Rodas
8939	Carolina / Andina	1700	C. A. Rodas
8940	Carolina / Andina	1700	C. A. Rodas
8941	Carolina / Andina	1700	C. A. Rodas
8942	Carolina / Andina	1700	C. A. Rodas
8943	Carolina / Andina	1700	C. A. Rodas
8944	Carolina / Andina	1700	C. A. Rodas
8945	Carolina / Andina	1700	C. A. Rodas
8946	Carolina / Andina	1700	C. A. Rodas
8947	Carolina / Andina	1700	C. A. Rodas
8948	Carolina / Andina	1700	C. A. Rodas
8949	Libano / Andina	2102	C. A. Rodas
8950	Sta Rosa / Cauca	1750	C. A. Rodas
8951	Cabuyerita / Cauca	1750	C. A. Rodas
8952	Guineal / Cauca	1650	C. A. Rodas
8953	Sta Maria / Cauca	1850	C. A. Rodas
8955	HatoFrio / Cauca	2000	C. A. Rodas
8956	HatoFrio / Cauca	2000	C. A. Rodas
8957	Vanessa / Cauca	1000	C. A. Rodas
8958	Ignacia / Cauca	2000	C. A. Rodas
8959	Ignacia / Cauca	2000	C. A. Rodas
8960	Alaska / Valle	1870	C. A. Rodas
8961	Estrella / Valle	1469	C. A. Rodas
8962	Volconda / Valle	1700	C. A. Rodas
8963	Cedral / Andina	1839	C. A. Rodas
8964	Carolina / Andina	1700	C. A. Rodas
8965	Aguabonita / Andina	1950	C. A. Rodas
8966	Buenos Aires/ Andina	1900	C. A. Rodas

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

Table 2. Isolates of different *Botryosphaeria* species used in the phylogenetic studies. Isolates sequenced in this study are in bold.

Isolate number [a]	Species	Host	Origin	Isolator	Genbank ITS	EF1-α
CMW 7772	*Botryosphaeria ribis*	*Ribes* sp.	New York	B. Slippers/ G. Hudler	AY236935	AY236877
CMW 7773	*B. ribis*	*Ribes* sp.	New York	B. Slippers/ G. Hudler	AY236936	AY236878
CMW 8961	***B. ribis***	***Eucalyptus grandis***	**Estrella, Colombia**	**C. A. Rodas**	**n.a.**	**n.a.**
CMW 8959	***B. ribis***	***E. grandis***	**Ignacia, Colombia**	**C. A. Rodas**	**n.a.**	**n.a.**
CMW 8949	***B. ribis***	***E. grandis***	**Libano, Colombia**	**C. A. Rodas**	**n.a.**	**n.a.**
CMW 9078	*B. parva*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236940	AY236885
CMW 9080	*B. parva*	*Populus nigra*	New Zealand	G. J. Samuels	AY236942	AY236887
CMW 10125	*B. eucalyptorum*	*E. grandis*	Mpumalanga, S. A.	H. Smith	AF283686	AY236891
CMW10126	*B .eucalyptorum*	*E. grandis*	Mpumalanga, S. A.	H. Smith	AF283687	AY236892
CMW 9076	*B. lutea*	*Malus x domestica*	New Zeland	S. R. Pennycook	AY236946	AY236893
CMW 992	*F. luteum*	*A. deliciosa*	New Zeland	G. J. Samuels	AF027745	AY236894
CMW 8922	***B. dothidea***	***E. grandis***	**Andes, Colombia**	**C. A. Rodas**	**n.a.**	**n.a.**
CMW 8929	***B. dothidea***	***E. grandis***	**Andes, Colombia**	**C. A. Rodas**	**n.a.**	**n.a.**
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	*B. rhodina*	*Vitex doniana*	Uganda	J. Roux	AY236952	AY236901
CMW 9074	*B. rhodina*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236901
CMW 7774	B. obtusa	*Ribes* sp.	New York	B. Slippers/ G. Hudler	AY236953	AY236902
CMW 7060	B. stevensii	*Fraxinus excelsior*	Netherlands	H. A van der Aa	AY236955	AY236904
CMW 7063	Guignardia philoprina	*Taxus baccata*	Netherlands	H. A. van der Aa	AY236956	AY236905

[a] Designation of culture collections: CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, (FABI), University of Pretoria, Pretoria, S.A.

Table 3. Differences in conidial size of isolates of *Botryosphaeria* spp. from Colombia.

Isolate[a] Group	CMW[b]	Length Min.	Length Average	Length Max.	Width Min	Width Average	Width Max.
Botryosphaeria dothidea	8922	27.5	29.2	30.0	4.1	5.0	6.8
(Group A)	8923	25.0	27.0	30.0	5.0	5.5	7.5
	8924	25.0	26.0	27.5	5.0	5.0	5.0
	8925	25.0	26.7	27.5	5.0	5.0	5.0
	8926	27.5	29.0	32.5	5.0	5.5	7.5
	8927	27.5	31.2	35.0	5.0	5.2	7.5
	8928	25.0	28.2	32.5	5.0	5.5	7.5
	8929	17.5	26.5	27.5	5.0	5.2	7.5
Average of 80 conidia		**25.0**	**27.9**	**30.3**	**4.8**	**5.2**	**6.7**
B. ribis **(Group B)**	8930	17.5	19.5	22.5	5.0	5.4	5.1
	8932	20.9	22.7	25.2	5.1	5.4	5.2
	8933	16.0	16.8	19.0	4.6	5.3	6.6
	8934	22.6	25.2	28.6	5.0	5.8	6.6
	8935	18.8	20.1	21.4	4.7	5.4	6.0
	8936	18.1	19.5	22.1	4.9	5.5	6.3
	8937	15.6	17.5	18.5	4.9	5.3	6.1
	8938	15.6	19.0	21.5	4.3	5.6	6.8
	8939	17.0	19.3	21.4	3.7	4.3	5.0
	8940	17.2	18.9	20.6	3.7	4.7	5.1
	8941	23.4	26.1	30.8	6.1	5.6	9.1
	8942	20.7	22.9	25.5	5.1	6.6	7.6
	8944	19.9	22.9	24.7	5.3	5.8	6.4
	8945	17.6	19.5	22.1	5.2	5.6	6.2
	8946	16.6	19.2	20.5	4.3	5.6	6.8
	8947	17.2	19.8	21.2	5.1	5.8	6.7
	8948	15.0	16.5	17.5	5.0	5.2	7.5
	8949	17.5	19.2	22.5	5.0	5.5	7.5
	8950	22.5	24.5	30.0	5.0	6.0	7.5
	8951	20.0	23.5	25.0	5.0	5.3	7.5
	8952	20.0	24.0	25.0	5.0	6.2	7.5
	8956	22.5	24	25.0	5.0	7.0	7.5
	8957	20.0	21.2	22.5	5.0	6.0	7.5
	8958	17.5	21.0	22.5	4.7	5.8	7.5
	8959	15.6	17.6	20.0	4.5	5.3	6.4
	8960	15.0	18.5	22.5	5.0	5.2	7.5
	8961	17.3	18.6	19.8	4.0	5.0	5.7
	8962	17.5	19.7	22.5	5.0	7.0	7.5
	8963	17.5	19.0	22.5	5.0	6.2	7.5
	8965	12.5	15.5	20.0	5.0	5.5	7.5
	8966	17.5	18.7	22.5	5.0	6.7	7.5
Average of 310 conidia		**18.1**	**20.3**	**22.7**	**4.8**	**5.6**	**6.8**

[a] Ten conidia were measured for each isolate.

[b] Designation of the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, (FABI), University of Pretoria.

Table 4. Comparison of lesion lengths (mm) **(a)** and analysis of variance **(b)** of internal lesion lengths on an *Eucalyptus grandis* clone (ZG 14) inoculated with six isolates of *Botryosphaeria ribis* and *B. dothidea* in the greenhouse.

(a)

Botryosphaeria dothidea		***Botryosphaeria ribis***	
Isolate	**Mean**	**Isolate**	**Mean**
CMW 8922	40.7	CMW 8949	76.8
CMW 8925	30.1	CMW 8956	76.4
CMW 8929	40.9	CMW 8961	81.7
Average	**37.2**		**78.3**
Control	12.9		12.9
S.E.M of trial	13.46		

The difference between average of *B. dothidea* and *B. ribis* is statistically significant at $P < 0.05$.

(b)

Source	**DF**	**MS**	**F value**	***P* value**
Isolates	6	7207.8	7.96	0.0001
Error	61	905.8		

Table 5. Mean lesion lengths (mm) **(a)** and analysis of variance **(b)** resulting from inoculation of *Eucalyptus grandis* clones with isolates of *Botryosphaeria dothidea* and *B. ribis* at the farm La Suiza.

(a)

Botryosphaeria dothidea			*Botryosphaeria ribis*		
Clone	**Mean**	**% Susceptibility [a]**	**Clone**	**Mean**	**% Susceptibility [a]**
23	35.8	100	303	217.6	100
2	34.8	97.2	4	195.5	89.8
27	32.8	91.5	2	191.4	88.0
12	31.3	87.3	12	189.9	87.3
20	27.5	76.8	20	177.3	81.5
18	26.3	73.4	11	172.1	79.1
11	25.9	72.4	210	167.3	76.9
210	20.8	58.2	23	165.0	75.8
211	18.9	52.8	211	162.0	74.4
303	18.9	52.7	18	137.9	63.4
301	17.9	50.0	27	108.2	49.7
4	17.9	49.8	301	86.3	39.7
S.E.M	**4.22**		**S.E.M**	**17.7**	

[a] % Susceptibility = lesion lengths as a percent of most susceptible clone. eg Clone N^0 23 = 35.8 / 35.8 = 100%.

(b)

Source	**DF**	**MS**	**F value**	***P* value**
Blocks	9	3836.9	1.33	0.2191
Isolates	1	1025195.5	355.4	0.0001
Clones	11	12440.0	4.31	0.0001
Isolates x clones	11	15845.9	5.49	0.0001
Error	408	2884.7		

Table 6. Mean lesion lengths (mm) **(a)** and analysis of variance **(b)** resulting from inoculation of *Eucalyptus grandis* clones with isolates of *Botryosphaeria dothidea* and *B. ribis* at the farm Cecilia.

(a)

Botryosphaeria dothidea			*Botryosphaeria ribis*		
Clone	**Mean**	**% Susceptibility [a]**	**Clone**	**Mean**	**% Susceptibility [a]**
27	45.6	100	2	157.0	100
23	42.5	93.2	303	152.0	96.8
T210	35.0	76.8	12	134.6	85.7
210	32.8	71.8	23	127.8	81.4
12	30.2	66.3	211	125.7	80.1
T211	30.2	66.1	27	125.7	80.1
18	27.5	60.3	4	124.6	79.4
2	26.0	57.1	210	116.9	74.4
211	23.4	51.2	18	114.7	73.1
301	23.3	51.0	11	108.1	68.9
20	22.1	48.5	20	107.2	68.3
303	21.2	46.5	301	82.7	52.7
11	17.1	37.5	T210	36.7	23.4
4	15.2	33.3	T211	22.9	14.6
S.E.M	**3.56**		**S.E.M**	**4.83**	

[a] % Susceptibility = lesion lengths as a percent of most susceptible clone.

(b)

Source	**DF**	**MS**	**F value**	***P* value**
Blocks	9	10924.5	5.53	0.0001
Isolates	1	602729.7	305.0	0.0001
Clones	13	7500.8	380	0.0001
Isolates x clones	13	7615.14	390	0.0001
Error	370	1976.2		

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

Table 7. Mean lesion lengths (mm) **(a)** and analysis of variance **(b)** resulting from inoculation of *Eucalyptus grandis* clones with isolates of *Botryosphaeria dothidea* and *B. ribis* at the farm Libano.

(a)

Botryosphaeria dothidea			*Botryosphaeria ribis*		
Clone	**Mean**	**% Susceptibility [a]**	**Clone**	**Mean**	**% Susceptibility [a]**
18	19.0	100	2	46.5	100
20	18.9	99.4	20	45.4	97.6
303	18.0	94.9	18	45.2	97.3
210	17.6	92.4	12	39.9	85.9
211	17.1	90.0	303	39.1	84.2
27	15.5	81.7	23	38.5	82.8
23	15.3	80.7	11	37.9	81.5
12	14.8	77.9	4	35.4	76.1
4	14.7	77.6	27	35.0	75.3
301	14.7	77.3	210	30.5	65.6
T210	14.6	77.0	T211	26.8	57.7
T211	14.5	76.1	211	26.5	57.0
2	13.7	72.1	T210	22.2	47.8
11	12.8	67.3	301	17.3	37.3
S.E.M	**1.73**		**S.E.M**	**5.43**	

[a] % Susceptibility = lesion lengths as a percent of most susceptible clone.

(b)

Source	**DF**	**MS**	**F value**	***P* value**
Blocks	9	2929.2	8.03	0.0001
Isolates	1	44605.3	122.27	0.0001
Clones	13	869.5	2.38	0.0042
Isolates x clones	13	695.9	1.91	0.0273
Error	473	364.8		

Isolates and clones interaction significant because of rank differences of clones under two isolates.

Table 8. Mean lesion lengths (mm) **(a)** and analysis of variance **(b)** resulting from inoculation of *Eucalyptus grandis* clones with isolates of *Botryosphaeria dothidea* and *B. ribis* at the farm Angela Maria.

(a)

Botryosphaeria dothidea			*Botryosphaeria ribis*		
Clone	**Mean**	**% Susceptibility [a]**	**Clone**	**Mean**	**% Susceptibility [a]**
23	36.4	100	20	81.2	100
T211	28.6	78.5	2	75.2	92.6
18	25.9	71.1	4	73.1	90.1
210	24.8	68.1	12	66.6	82.1
2	24.3	66.6	210	66.2	81.5
12	23.3	64.1	27	65.9	81.1
T210	22.4	61.4	11	59.2	73
27	21.5	59.0	211	50.1	61.7
20	20.8	57.1	3	48.0	59.2
11	19.5	53.6	18	46.9	57.7
211	18.0	49.3	23	43.9	54.1
4	17.3	47.6	301	33.2	40.9
303	15.5	42.4	T-210	26.6	32.7
301	14.2	39.1	T-211	21.9	27
S.E.M	**3.86**		**S.E.M**	**8.52**	

[a] % Susceptibility = lesion lengths as a percent of most susceptible clone.

(b)

Source	**DF**	**MS**	**F value**	***P* value**
Blocks	9	1442.80	1.27	0.2525
Isolates	1	112951.9	233.74	0.0001
Clones	13	3041.65	395	0.0001
Isolates x clones	13	3693.31	3.82	0.0001
Error	459	890.96		

The interaction is highly significant indicating that the rank of clones under one isolate differs from that under the other isolate.

Fig. 1. Disease symptoms associated with *Botryosphaeria* 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.

Fig. 2. Geographic areas in Colombia where samples of diseased *Eucalyptus grandis* were collected.


Andina Zone
Valle Zone
Cauca Zone
50 0 50 100 150 200 250 Kilometers

Fig. 3. Procedures involved in the inoculation of *Botryosphaeria* spp. on *Eucalyptus grandis*. **(a)** Wounding of stem with cork borer. **(b)** Culture of fungus to be inoculated showing discs of mycelium of equal size to wounds on stem. **(c)** Mycelial disc being placed in a wound. **(d)** Inoculated area covered with moistened filter paper and secured with masking tape.

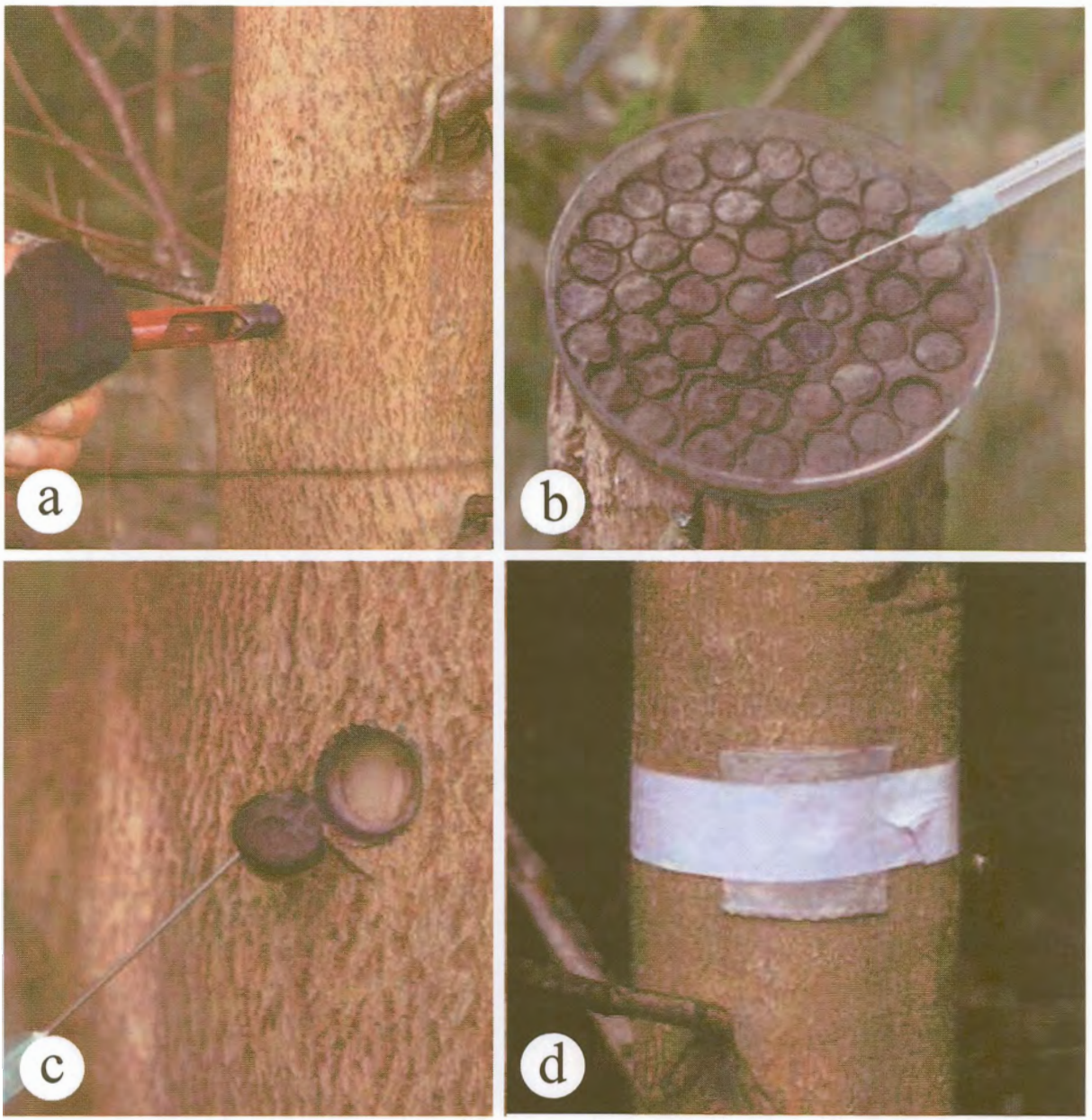

Fig. 4. Cultural and conidial characteristics of two *Botryosphaeria* spp. from *Eucalyptus grandis* in Colombia. **(a)** Growth of *B. ribis* in culture after 4 days (left) and 20 days (right). **(b)** Conidia of *B. ribis*. **(c)** Growth of *B. dothidea* in culture after 4 days (left) and 20 days (right). **(d)** Conidia of *B. dothidea*. Bars Figs. b, d = 10 µm.

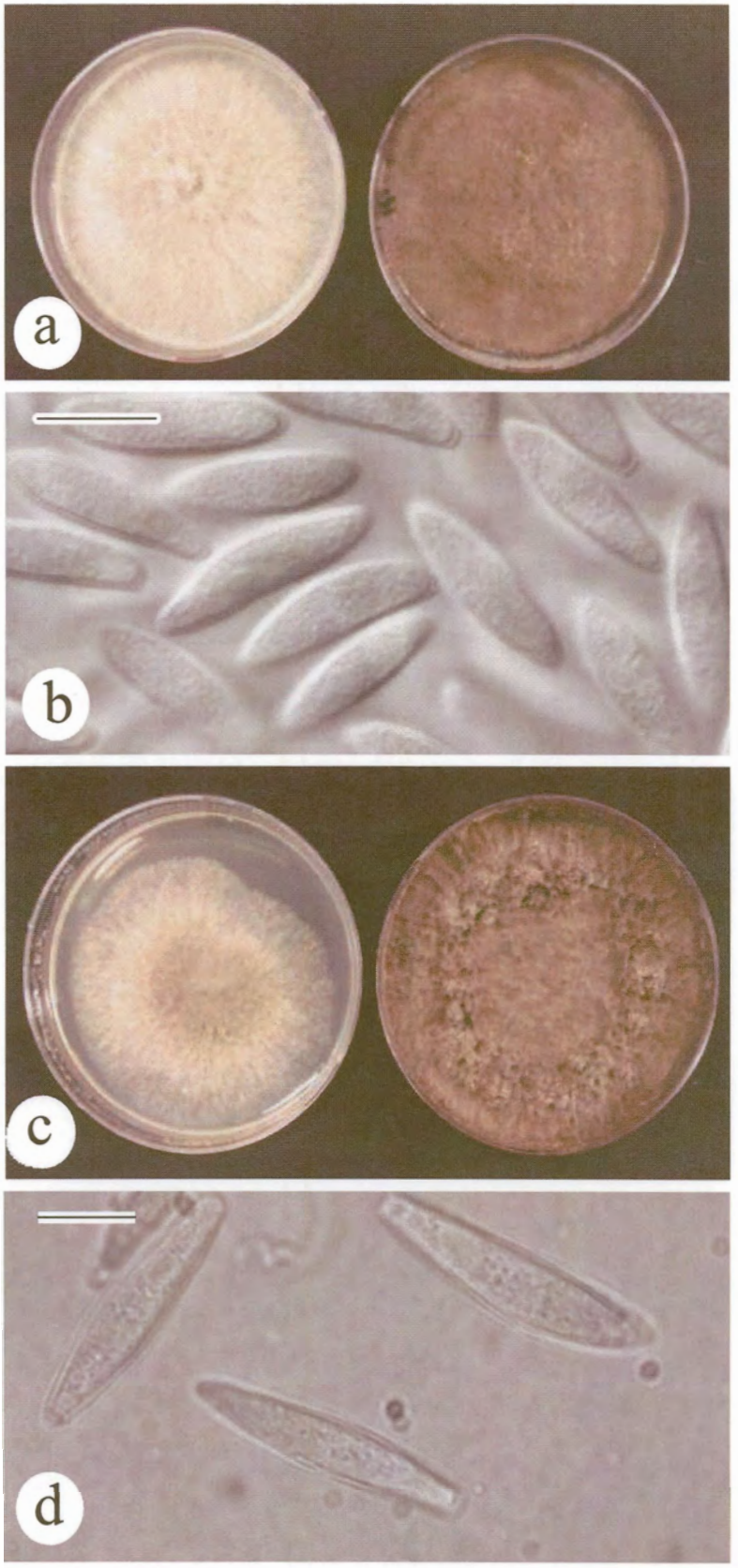
a
b
c
d

Fig. 5. Raw sequence data of the ITS 1/2 region and 5.8S rRNA gene and EF1-α gene and intron region for various *Botryosphaeria* spp. Unknown sequence characters are indicated with a "**N**", while gaps inserted to achieve sequences alignment are indicated with "-". Bases matching those of CMW 7772 are indicated with a ".".

```
[                  10         20         30         40         50]

CMW7772     GAAGGATCAT TACCGAGTTG ATTCGAGCTC CGGCTCGACN -TC-TCCCAC
CMW7773     .......... .......... .......... .........N ..........
CMW8959     .......... .......... .......... .........N ..........
CMW8961     NNNNNNNNNN NNNNNNNN.. .......... .........N ..........
CMW8949     NNNNNNN... .......... .......... .........N ..........
CMW9078     .......... .......... .......... .........N ..........
CMW9080     .......... .......... .......... .........N ..........
CMW10125    .......... .......... .C........ .........N ..........
CMW10126    .......... .......... .C........ .........N ..........
CMW9076     .......... .......... .......... .........N ..........
CMW992      .......... .......... .......... .........N ..........
CMW8922     .......... .......... .....G.... ....C...-N ...C......
CMW8929     .......... .......... .....G.... ....C...-N ...C......
CMW8000     .......... .......... .....G.... ....C...-N ...C......
CMW7999     .......... .......... .....G.... ....C...-N ...C......
CMW10130    .......... .........- -....G...T .........N .....N....
CMW9074     .......... .........- -......... .........N ..........
CMW7774     ....N..... .........- C.-..G...T .........AN ..........
CMW7060     .......... .........- C.-.......T .........AN ..........
CMW7063     .......... .........- ---------- -----.AC.AN C...-..A.A

[                  60         70         80         90        100]

CMW7772     CCAATGTGTA CCTACCTCTG TTGCTTTGGC GGGCCGCGGT CCT--CCGC-
CMW7773     .......... .......... .......... .......... ..........
CMW8959     ........C. .......... .......... .......... ..........
CMW8961     .......... .......... .......... .......... ..........
CMW8949     .......... .......... .......... .......... ..........
CMW9078     ..T....... .......... .......... .......... ..........
CMW9080     ..T....... .......... .......... .......... ..........
CMW10125    ..T....... .......... .......... .......... ..........
CMW10126    ..T....... .......... .......... .......... ..........
CMW9076     ..C....... .......... .......... .......... ..........
CMW992      ..C....... .......... .......... .......... ..........
CMW8922     ..TT...... .......... .......... .......... T........G
CMW8929     ..TT...... .......... .......... .......... .........G
CMW8000     ..TT...... .......... .......... .......... .........G
CMW7999     ..TT...... .......... .......... .......... .........G
CMW10130    ..TT....A. .G........ .......... ..-.-----. .--..---.N
CMW9074     ..TT....A. .G........ .......... ..-.-----. .--..---.G
CMW7774     ..TT....A. .A........ .......... ..-.-----. .T.TG....G
CMW7060     ..TT....A. .A........ .......... ..-.-----. .T..G....G
CMW7063     ..C.....A. .A.....A.T G.TGC..C.G C..G------ AT..G..C.G
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[                  110         120         130         140         150]

CMW7772      ACCGG-CGCC C-TT--CG-G GGGGGCTGGC CA----GCGC ---CCGCCAG
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... ....-..... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... ....-..... .......... ..........
CMW9078      .......... .......... ....-..... .......... ..........
CMW9080      .......... .......... ....-..... .......... ..........
CMW10125     .......T.. .T....-... ....-..... .........T ..........
CMW10126     .......T.. .T....-... ....-..... .........T ..........
CMW9076      ....AC.C.. G......... .....-.C... .......... ..........
CMW992       ....AC.C.. G......... .....-.C... .......... ..........
CMW8922      G...-C.C.. ...CCC.... .....--...T .......... ..........
CMW8929      G...-C.C.. ...CCC.... .....--...T .......... ..........
CMW8000      G....C.C.. ...CCC.... .....--.... .......... ..........
CMW7999      G....C.C.. ...CCC.... .....--.... .......... ..........
CMW10130     G----.---- -.--.--.- ---------- --......... .........A
CMW9074      G----.---- -.--.--.- ---------- --......... .........A
CMW7774      .--..AG... ...-....C. ..CC--CCC. .-..GC.... TTT.......
CMW7060      T--..AG... ...-...AAA AA.C--CCC. .C..GT.... T.T.......
CMW7063      ---..C-... -..CGTGT.- --.C--CCCG G.TCAG.... .........TA

[                  160         170         180         190         200]

CMW7772      AGGACCAT-A AAACTCCAGT CAGTGAAC-T TCGCAGTCTG AAAAA-C-AA
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......-C. .......... ....A...G. .-........ ..........
CMW10126     .......-C. .......... ....A...G. .-........ ..........
CMW9076      .......-C. .......... ....A...G- .......... .G........
CMW992       .......-C. .......... ....A...G- .......... .G........
CMW8922      ........C. ..-....... ....A...GA C-........ .....A...T
CMW8929      ........C. ..-....... ....A...GA C-........ .....A...T
CMW8000      ........C. ..-....... ....A...GA .-........ .........T
CMW7999      ........C. ..-....... ....A...GA .-........ .........T
CMW10130     ......T.C. ..-....... ....A...GC A-.AC..... .T........
CMW9074      ......T.C. ..-....... ....A...GC A-.AC..... .T........
CMW7774      ......T.C. ..-....... ....A...G- ...AC..... .T........
CMW7060      ......T.C. ..-....... ....A...G- ...AC..... ..........
CMW7063      G.A.A.T..- ..-...TT.. TTTATTTTG- GAATCT.... .GT.GTTTTT
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[                  210         220         230         240         250]

CMW7772      GTTAATAAAC TAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......... .......... .......... .......... ..........
CMW10126     .......... .......... .......... .......... ..........
CMW9076      .......... .......... .......... .......... ..........
CMW992       .......... .......... .......... .......... ..........
CMW8922      -......... .......... .......... .......... ..........
CMW8929      -......... .......... .......... .......... ..........
CMW8000      -......... .......... .......... .......... ..........
CMW7999      -......... .......... .......... .......... ..........
CMW10130     .......... .......... .......... .......... ..........
CMW9074      .......... .......... .......... .......... ..........
CMW7774      .......... .......... .......... .......... ..........
CMW7060      .......... .......... .......... .......... ..........
CMW7063      ACA......T A......... .......... .......... ..........

[                  260         270         280         290         300]

CMW7772      GAAGAACGCA GCGAAATGCG ATAAGTAATG TGAATTGCAG AATTCAGTGA
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......... .......... .......... .......... ..........
CMW10126     .......... .......... .......... .......... ..........
CMW9076      .......... .......... .......... .......... ..........
CMW992       .......... .......... .......... .......... ..........
CMW8922      .......... .......... .......... .......... ..........
CMW8929      .......... .......... .......... .......... ..........
CMW8000      .......... .......... .......... .......... ..........
CMW7999      .......... .......... .......... .......... ..........
CMW10130     .......... .......... .......... .......... ..........
CMW9074      .......... .......... .......... .......... ..........
CMW7774      .......... .......... .......... .......... ..........
CMW7060      .......... .......... .......... .......... ..........
CMW7063      .......... .......... .......... .......... ..........
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[                  310         320         330         340         350]

CMW7772      ATCATCGAAT CTTTGAACGC ACATTGCGCC CCTTGGTATT CCGAGGGGCA
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......... .......... .......... .......... ..........
CMW10126     .......... .......... .......... .......... ..........
CMW9076      .......... .......... .......... .......... ..........
CMW992       .......... .......... .......... .......... ..........
CMW8922      .......... .......... .......... .T........ ....A.....
CMW8929      .......... .......... .......... .T........ ....A.....
CMW8000      .......... .......... .......... .T........ ....A.....
CMW7999      .......... .......... .......... .T........ ....A.....
CMW10130     .......... .......... .......... .......... ...G......
CMW9074      .......... .......... .......... .......... ...G......
CMW7774      .......... .......... .......... ..C...C... ...G......
CMW7060      .......... .......... .......... ......C... ..........
CMW7063      .......... .......... .......... .GCCA..... .T.GC.....

[                  360         370         380         390         400]

CMW7772      TGCCTGTTCG AGCGTCATTT CAACCCTCAA GCT-CT---G CTTGGTATTG
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......... .......... .......... ...T-..... ..........
CMW10126     .......... .......... .......... ...T-..... ..........
CMW9076      .......... .......... .......... .......... ..........
CMW992       .......... .......... .......... .......... ..........
CMW8922      .......... .........A .......... .......... ..........
CMW8929      .......... .........A .......... .......... ..........
CMW8000      .......... .........A .......... .......... ..........
CMW7999      .......... .........A .......... .......... ..........
CMW10130     .......... .........A .......... .......... .....A....
CMW9074      .......... .........A .......... .......... .....A....
CMW7774      .......... .........A .......... .......... ..........
CMW7060      .......... .........A .......... .......... ..........
CMW7063      .......CT. .......... .........T ..CC..AGG. .G....G...
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[                  410         420         430         440         450]

CMW7772      GGCTCCGTCC TCCA---CGG ACGCGCC--T TAAAGACCTC GGCGGTGGC-
CMW7773      .......... .......... .......... .......... ..........
CMW8959      ....T..... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      ....T..... .......... .......... .......... ..........
CMW9078      ...C...... .......... .......... .......... ..........
CMW9080      ...C...... .......... .......... .......... ..........
CMW10125     ...C...... .-.T..GT.. .......... C......... ..........
CMW10126     ...C...... .-.T..GT.. .......... C......... ..........
CMW9076      .......... .-.T..GT.. .......... CG........ ..........
CMW992       .......... .-.T..GT.. .......... CG........ ..........
CMW8922      ...A...... .--T.TG... G......... C......... ..........
CMW8929      ...A...... .--T.TG... G......... C......... ..........
CMW8000      ...A...... .--T.TG... G......... C......... ..........
CMW7999      ...A...... .--T.TG... G......... C......... ..........
CMW10130     ...A...... .-..CTG... .......... C......... .........T
CMW9074      ...A...... .-..CTG... .......... C......... .........T
CMW7774      ...G...... .-.TCTG... .......... .......... .........T
CMW7060      ...GA..... .-.TCTG... .......... C......... .........T
CMW7063      ..GAT..G.. AAAGCCCGC. .G.GA.GGCC GGCCCCTAAA TCTA.....G

[                  460         470         480         490         500]

CMW7772      GTCTTGCC-T CAAGCGTAGT AGAAAA--CA CCTCGCTTTG GAGCGCACGG
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......... .......... ....-.T... .......... .......T..
CMW10126     .......... .......... ....-.T... .......... .......T..
CMW9076      .......... .......... .......... .......... ..........
CMW992       .......... .......... .......... .......... ..........
CMW8922      .......... .......... ....C.TA.. T.......C. .......G..
CMW8929      .......... .......... ....C.TA.. T.......C. .......G..
CMW8000      .......... .......... ....C.TA.. T.......C. .......G..
CMW7999      .......... .......... ....C.TA.. T.......C. .......G..
CMW10130     ..TCA...C. .......... .....--TA.. .......... .....GTT..
CMW9074      ..TCA...C. .......... .....--TA.. .......... .....GTT..
CMW7774      ..TCA...C. .......... .....--TA.. .......... .....GTT..
CMW7060      ..TCA...C. ........A. .....--TA.. .......... .....GTT..
CMW7063      .A.CC.T.G. GGCCTCCTC. GCG..GTAGT GA.ATTCCGC ATCG.AGA.C
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[                  510         520         530         540         550]

CMW7772      -CGTCG-CCC GCCGGACGAA CCTT-TGAAT TATTTCTCAA GGTTGACCTC
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... ...NNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......... .......... .......... .-........ ..........
CMW10126     .......... .......... .......... .-........ ..........
CMW9076      .......... .......... .......... .-........ ..........
CMW992       .......... .......... .......... .-........ ..........
CMW8922      .......... .......... ....C....C .-........ ..........
CMW8929      .......... .......... ....C....C .-........ ..........
CMW8000      .......... .......... ....C....C .-........ ..........
CMW7999      .......... .......... ....C....C .-........ ..........
CMW10130     .......... .......... ....C....C .-........ ..........
CMW9074      .......... .......... ....C....C .-........ ..........
CMW7774      .......... .......... ....C....C .-........ ..........
CMW7060      .......... .......... ....C....C .-........ ..........
CMW7063      GACGA.C... TG.C.TTA.. ..CCC--..C .-..-.-... ..........

[                  560         570         580         590         600]

CMW7772      GGATGAAGTT CGAGAAGGTA AGA------- -A-AG-TTTT TCC-TTCC-G
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      NNNN...... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ..........
CMW9080      .......... .......... .......... .......... ..........
CMW10125     .......... .......... .......... .....-A.... ..........
CMW10126     .......... .......... .......... .....-A.... ..........
CMW9076      .......... .......... .......... .-...A.... ..-G......
CMW992       .......... .......... .......... .-...A.... ..-G......
CMW8922      ....NNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNN.. -.TA.G..T.
CMW8929      ....NNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNN.. -.TA.G..T.
CMW8000      .......... .......... ..-.CA..CA .-.-CA.... -.TG.G..T.
CMW7999      .......... .......... ..-.CA..CA .-.-CA.... -.TG.G..T.
CMW10130     .......... .........C C.TGCACGCA TGTC...... .-TAAC..CT
CMW9074      .....G.... .........C C.TGCACGCA TGTC...... .-TAAC..CT
CMW7774      .......... .......... ...G...... .-.-.A.... ...TGCT.CC
CMW7060      .......... .......... ...G...... .-.-.A.... ...CG.T.CC
CMW7063      A......... .......... ...ACAGC.. .-.-CAC.CC .TTGA.A.CC
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[                610        620        630        640        650]

CMW7772     -CTGCACGCG C--TGGGTGC TGGGTGCTGG GTGCTGGGTG CTGGGTTCCC
CMW7773     .......... .......... .......... .......... ..........
CMW8959     .......... .......... CA........ ..-------- ------....
CMW8961     .......... .......... .......... ..-------- ------....
CMW8949     .......... .......... CA........ ..-------- ------....
CMW9078     .......... .......... CA........ ..-------- ------....
CMW9080     .......... .......... CA........ ..-------- ------....
CMW10125    .........T TCC....... ---------... ..-------- ------....
CMW10126    .........T TCC....... ---------... ..-------- ------....
CMW9076     .....C.... .GA...-... ---------... .A-------- ------.G..
CMW992      .....C.... .GA...-... ---------... .A-------- ------.G..
CMW8922     .---....T- -...---... ---------... ..-------- ------...T
CMW8929     .---....T- -...---... ---------... ..-------- ------...T
CMW8000     .---....-- -...---... ---------... ..-------- ------...T
CMW7999     .---....-- -...---... ---------... ..-------- ------...T
CMW10130    ATC.AC-TTC GGCGC--... A--.C....C .---------- ----------
CMW9074     CTC.AC-TTC GGCGC--... A--.C....C .---------- ----------
CMW7774     G.AC.G..T- -..------- ---------- ---------- ----------
CMW7060     G.AC-GT.T. .AG..C-... ..C-----..C .---------- ----------
CMW7063     AGAT.GT... GCG.CTCGCA .CTCACACCT .GCA.TCTGT GCCCC.NTTA

[                660        670        680        690        700]

CMW7772     GCACTCAATT TGCCTTATC- GCTTC---GG TGAGGGGCA- TTT--TGGTG
CMW7773     .......... .......... .......... .......... ..........
CMW8959     .......... .......... .......... .......... ..........
CMW8961     .......... .......... .......... .......... ..........
CMW8949     .......... .......... .......... .......... ..........
CMW9078     .......... .......... .......... .......... ..........
CMW9080     .......... .......... .......... .......... ..........
CMW10125    .......... .......... ........A. .......... ..........
CMW10126    .......... .......... ........A. .......... ..........
CMW9076     .......... ..T....... .......... .......... ..........
CMW992      .......... ..T....... .......... .......... ..........
CMW8922     ..G.CG.... .........A -..-...T.. .........A ...CT.....
CMW8929     ..G.CG.... .........A -..-...T.. .........A ...CT.....
CMW8000     ..G.CG.... .........A -..-...T.. .........A ...CT.....
CMW7999     ..G.CG.... .........A -..-...T.. .........A ...CT.....
CMW10130    ..G.A..G.C C......... ...-...T.. .......... ....T.C...
CMW9074     ..G.A..G.C C......... ...-...T.. .......... ....T.C...
CMW7774     --G.A..GCC CA........ ...-...... .......... ....T.C...
CMW7060     ---------- ---......T ..CG.CCT.. .......... ....T.C...
CMW7063     C.C...CT-- ----------. ------...-- ----------. ---..-----
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[                  710         720         730         740         750]

CMW7772      GTGGGGT-TG GCCCGCGCTA AGCCTCGTTC GGGCT-CGGC AAAATGTCCG
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .........T .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .........T .......... ..........
CMW9078      .......... .......... .........T .......... ..........
CMW9080      .......... .......... .........T .......... ..........
CMW10125     .---...C-. .......... .T...G...T .T...T.... .....C....
CMW10126     .---...C-. .......... .T...G...T .T...T.... .....C....
CMW9076      .......C-. .......... .........T .T...T.... .....C....
CMW992       .......C-. .......... .........T .T...T.... .....C....
CMW8922      .-..C--... .......... .........T ..T..T.... .....C....
CMW8929      .-..C--... .......... .........T ..T..T.... .....C....
CMW8000      .-..C--... .......... .........T ..T..T.... .....C....
CMW7999      .-..C--... .......... .........T ..T..T.... .....C....
CMW10130     .......T.. .......... -........CT ...-.T.... ......A...
CMW9074      .......T.. .......... -........CT ...-.T.... ......A...
CMW7774      .......T.. .......... .A......CT .A........ ...C..G...
CMW7060      .......T.. .......... .A......CT .A........ ...C..G...
CMW7063      --------.-- ---------- ---------- -----.---. ...--------

[                  760         770         780         790         800]

CMW7772      CATCTGGTTT TTTTGCGACC GGCGTGCGAC CGAAGCG--C ACCCCTCGCC
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... G.........
CMW8961      .......... .......... .......... .......... G.........
CMW8949      .......... .......... .......... .......... G.........
CMW9078      .......... .......... .......... .......... G.........
CMW9080      .......... .......... .......... .......... G.........
CMW10125     T......... ...-...... .......... ....AT.... G.........
CMW10126     T......... ...-...... .......... ....AT.... G.........
CMW9076      .......... ...-...... .......... ...C...... ..........
CMW992       .......... ...-...... .......... ...C...... ..........
CMW8922      .......A.. .....T.... .........G. ...C...AA. ......TA..
CMW8929      .......A.. .....T.... .........G. ...C...AA. ......TA..
CMW8000      .......A.. .....T.... .......... ...C...AA. .......A..
CMW7999      .......A.. .....T.... .......... ...C...AA. .......A..
CMW10130     ..CT...... .......... .....CT.G. ...C...CCT C...ACTAG.
CMW9074      ..CT...... .......... .....CT.G. ...C...CCT C...ACTAG.
CMW7774      ..CT...... ...-...... .....CA.G. ...T...CC. C-T.ACTAG.
CMW7060      ..CT...... ..--...... ..T...A.G. ...T...CC. C.T.ACTAG.
CMW7063      -.ATCAA... ....TGTGG. CCTT.TTAGT G.GGC.ACAA C...GC.AGA
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```
[                  810         820         830         840         850]

CMW7772      AGA-----CA CG--CCACGC A--------T GTGCGACCAG ACGCTAACGG
CMW7773      .......... .......... .......... .......... ..........
CMW8959      .......... .......... .......... .......... ..........
CMW8961      .......... .......... .......... .......... ..........
CMW8949      .......... .......... .......... .......... ..........
CMW9078      .......... .......... .......... .......... ........A.
CMW9080      .......... .......... .......... .......... ........A.
CMW10125     .--..T.G.. --.------- -......... .C...G.... .......T.C
CMW10126     .--..T.G.. --.------- -......... .C...G.... .......T.C
CMW9076      ....CTCG.. -.......A. -.........- ......T.G. ..........A
CMW992       ....CTCG.. -.......A. -.........- ......T.G. ..........A
CMW8922      .ACGCTTC.. G-......T. .CGTTCGTC. A........T .T......CA
CMW8929      .ACGCTTC.. G-......T. .CGTTCGTC. A........T .T......CA
CMW8000      .ACGCTTC.. G-......T. .CGTTCGTC. A........T .T......CA
CMW7999      .ACGCTTC.. G-......T. .CGTTCGTC. A........T .T......CA
CMW10130     GA.AAATG.T .TGA....T. .TGTACCGTC ..A...G..A .G.......C
CMW9074      GA.AAATG.T .TGA....T. .TGTACCGTC ..A...G..A .G.......C
CMW7774      GAGCAATG.C .CGA....T. .TGTGCTCTC .........C .G.......C
CMW7060      GA.AAATG.C .CGA....T. .TGTGCTCTC ..A......C .G.......C
CMW7063      GTTCTCGAT. GCAT.TCAAG GAAGGCACGC .CTGACAGTC C.AAA.T---

[                  860         870         880          ]

CMW7772      CCATCC---- CAGGAAGCCG CCGAGCTCGG TAAGGGTTC
CMW7773      .......... .......... .......... .........
CMW8959      ..C....... .......... .......... .........
CMW8961      .......... .......... .......... .........
CMW8949      ..C....... .NNNNNNNNN NNNNNNNNNN NNNNNNNNN
CMW9078      .......... .......... .......... .........
CMW9080      .......... .......... .......... .........
CMW10125     .......... .......... ....A..... .........
CMW10126     .......... .......... ....A..... .........
CMW9076      ..G..T.... .......... .......... .........
CMW992       ..G..T.... .......... N......... .........
CMW8922      ..G-..ACAA .......... .T........ .........
CMW8929      ..G-..ACAA .......... .T........ .........
CMW8000      ..G-..ACAA .......... .T........ .........
CMW7999      ..G-..ACAA .......... .T........ .........
CMW10130     G.C-..ACTA .......... .T........ .........
CMW9074      G.C-..ACTA .......... .T........ .........
CMW7774      A.G-..ACAA .......... .......... .........
CMW7060      G.G-..ACAA .......... .T........ .........
CMW7063      ---------- -......... ....A..... .........
```

Fig. 6. Phylogenetic tree 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 *Botryosphaeria* spp. One most parsimonious tree was generated using the heuristic search option. The bootstrap values (1000 replications) > 50 % are indicate above the branches. The tree includes 19 ingroup taxa and the outgroup taxon *G. philoprina*.

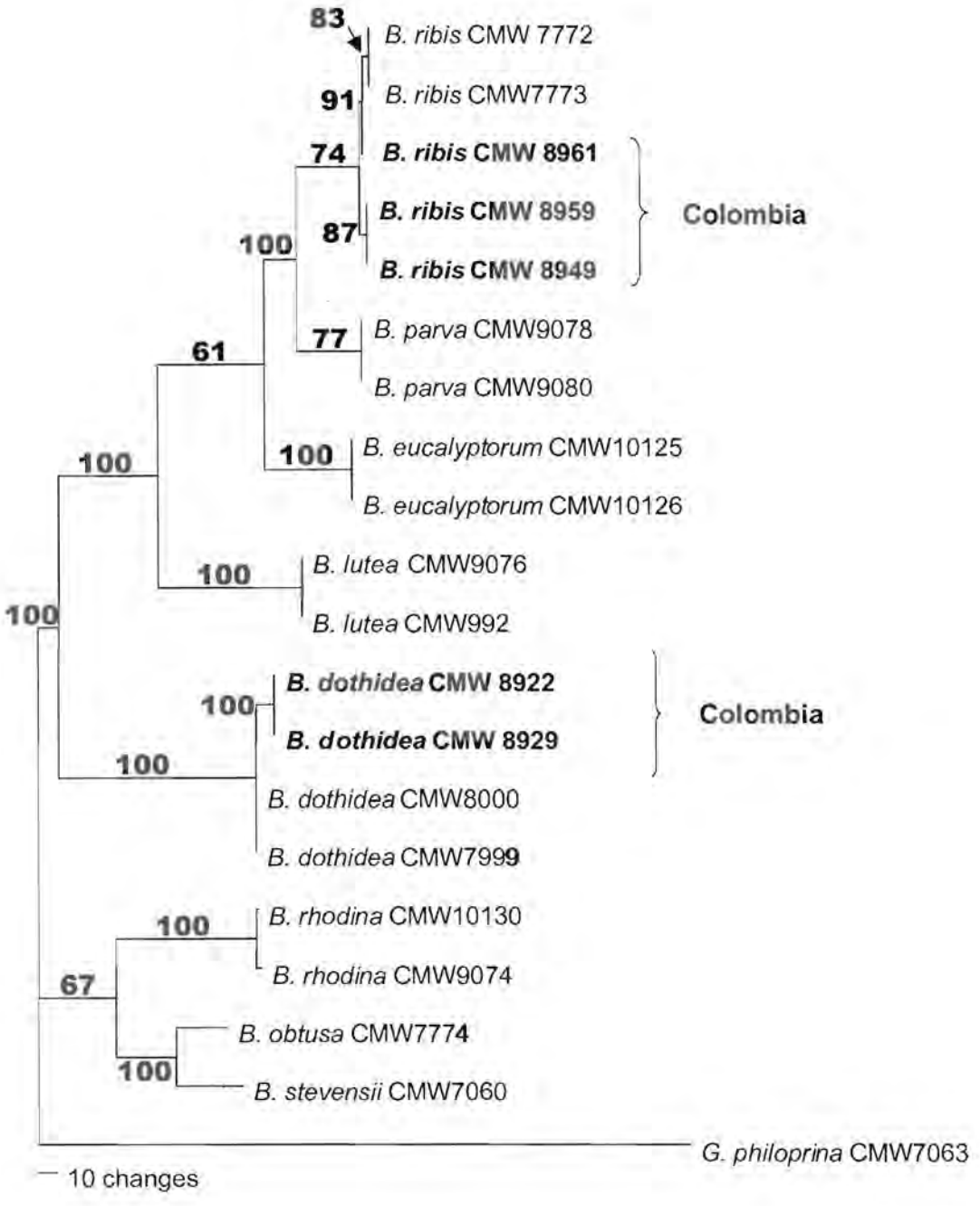

83
B. ribis CMW 7772
91
B. ribis CMW7773
74
B. ribis CMW 8961
B. ribis CMW 8959
87
B. ribis CMW 8949
Colombia
100
77
B. parva CMW9078
61
B. parva CMW9080
100
B. eucalyptorum CMW10125
100
B. eucalyptorum CMW10126
100
B. lutea CMW9076
100
B. lutea CMW992
B. dothidea CMW 8922
100
B. dothidea CMW 8929
Colombia
100
B. dothidea CMW8000
B. dothidea CMW7999
100
B. rhodina CMW10130
67
B. rhodina CMW9074
B. obtusa CMW7774
100
B. stevensii CMW7060
G. philoprina CMW7063
10 changes

Fig. 7. Lesions formed after inoculation with six isolates of *Botryosphaeria* spp. and one control on a *Eucalyptus grandis* clone (ZG14) in the greenhouse.

Fig. 8. Mean lesion length after inoculation with *Botryosphaeria dothidea* and *B. ribis* on *Eucalyptus grandis,* clone ZG14 in a greenhouse trial. Bars represent confidence limits for each isolate.

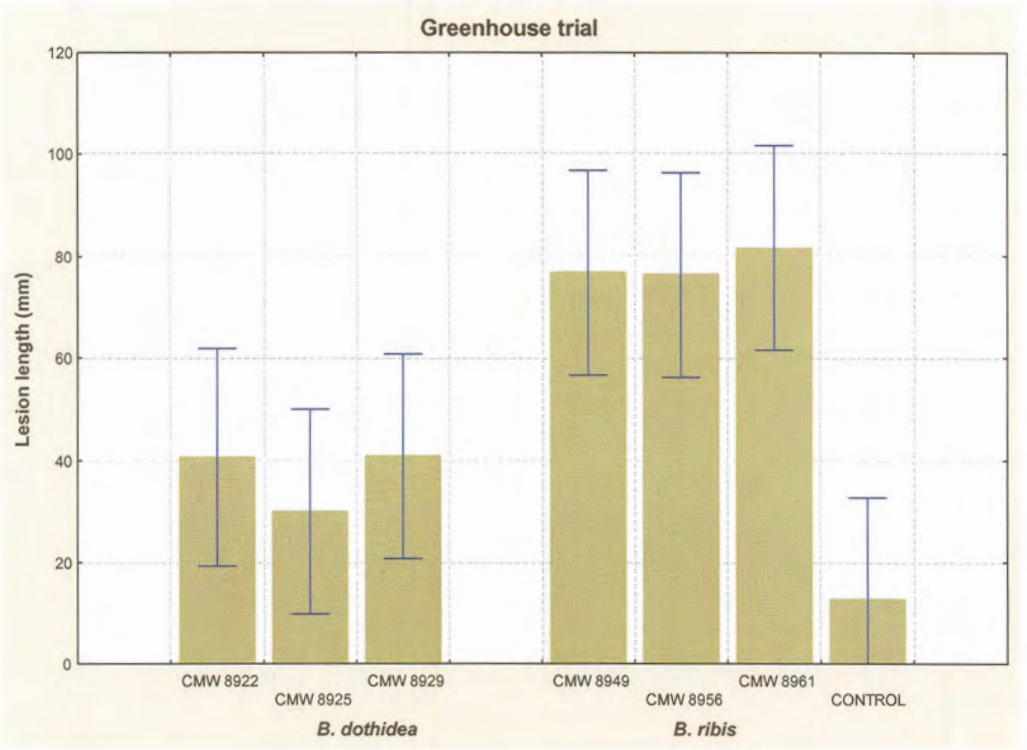

Greenhouse trial
Lesion length (mm)
120
100
80
60
40
20
0
CMW 8922
CMW 8925
CMW 8929
CMW 8949
CMW 8956
CMW 8961
CONTROL
B. dothidea
B. ribis

Fig. 9. Lesions associated with inoculation of *Botryosphaeria* spp. on old *Eucalyptus grandis* trees at La Suiza farm. **(a)** Control (arrow shows part of inoculation). **(b)** Lesion associated with *B. dothidea*. **(c)** Lesion associated with *B. ribis*.

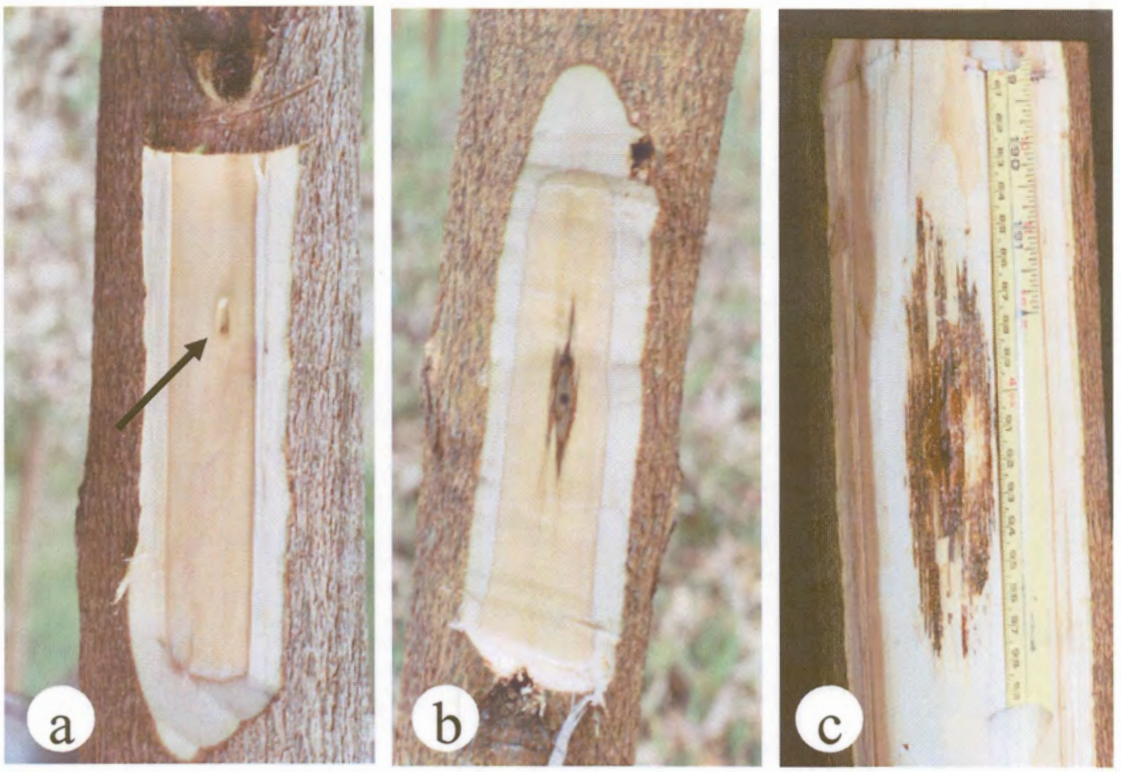

a
b
c

Fig. 10. Mean lesion length after inoculation with *Botryosphaeria dothidea* and *B. ribis* on 12 clones of *Eucalyptus grandis* at La Suiza farm. Bars represent confidence limits for each isolate on all clones inoculated.

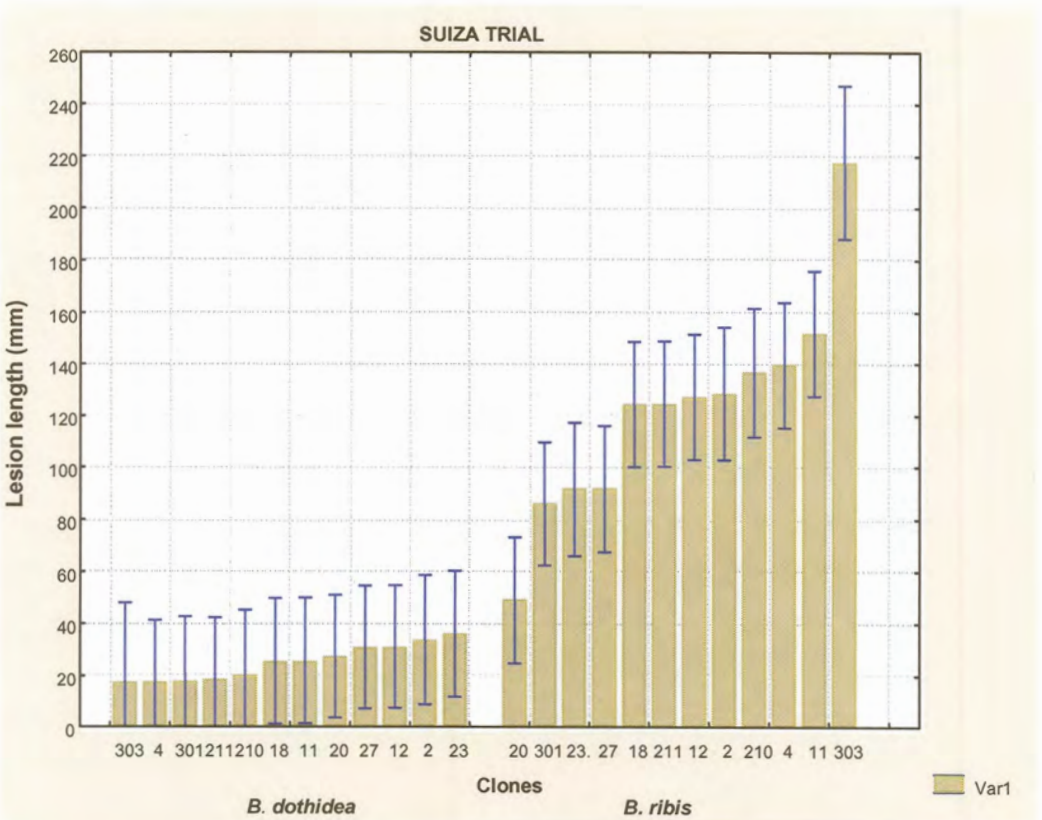

SUIZA TRIAL
Lesion length (mm)
260
240
220
200
180
160
140
120
100
80
60
40
20
0
303 4 301 211 210 18 11 20 27 12 2 23
20 301 23. 27 18 211 12 2 210 4 11 303
Clones
B. dothidea
B. ribis
Var1

Fig. 11. Mean lesion lengths after inoculation with *Botryosphaeria dothidea* and *B. ribis* on 14 clones of *Eucalyptus grandis* at Cecilia farm. Bars represent confidence limits for each isolate on all clones inoculated.

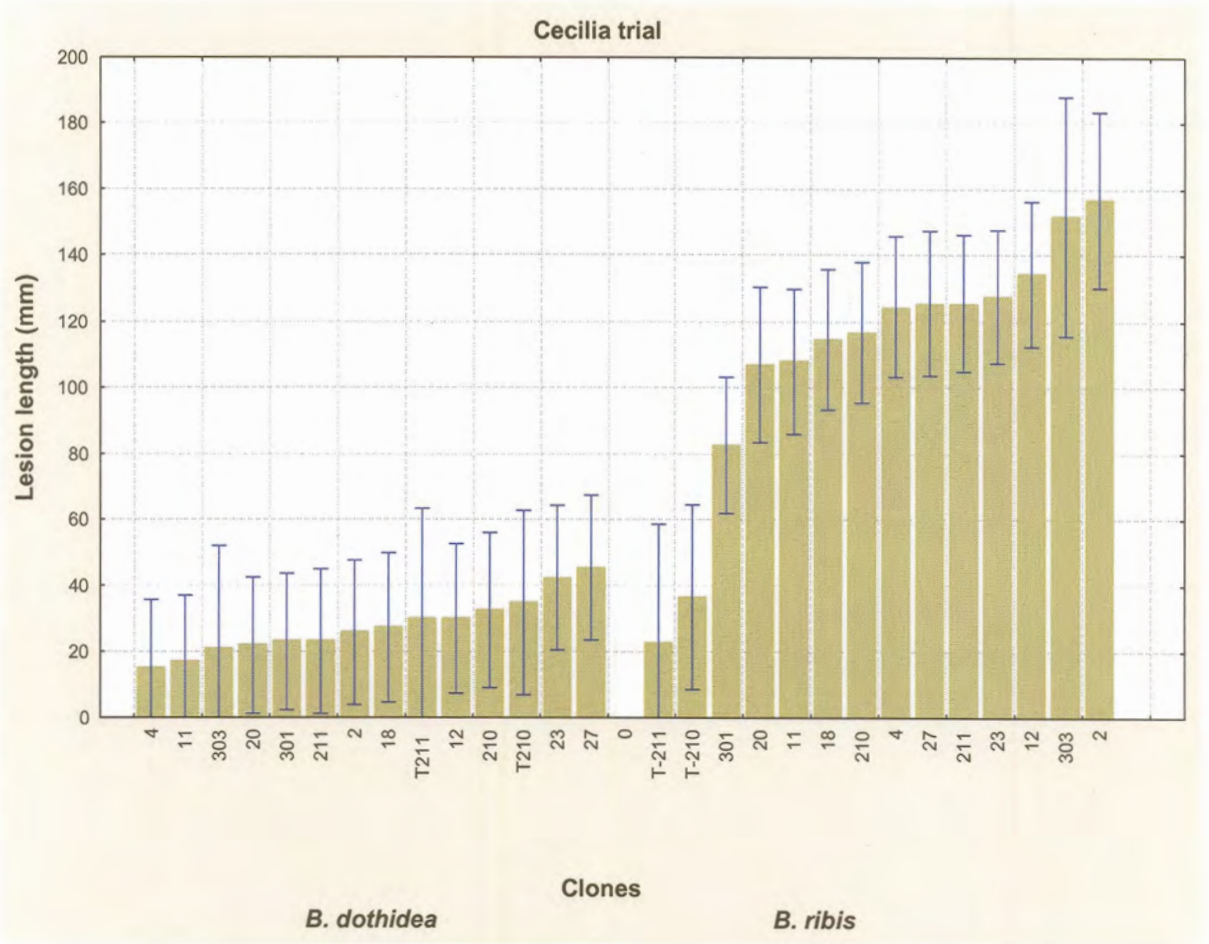

Cecilia trial
Lesion length (mm)
200
180
160
140
120
100
80
60
40
20
0
4
11
303
20
301
211
2
18
T211
12
210
T210
23
27
0
T-211
T-210
301
20
11
18
210
4
27
211
23
12
303
2
Clones
B. dothidea
B. ribis

Fig. 12. Mean lesion lengths after inoculation with *Botryosphaeria dothidea* and *B. ribis* on 14 clones of *Eucalyptus grandis* at Libano farm. Bars represent confidence limits for each isolate on all clones inoculated.

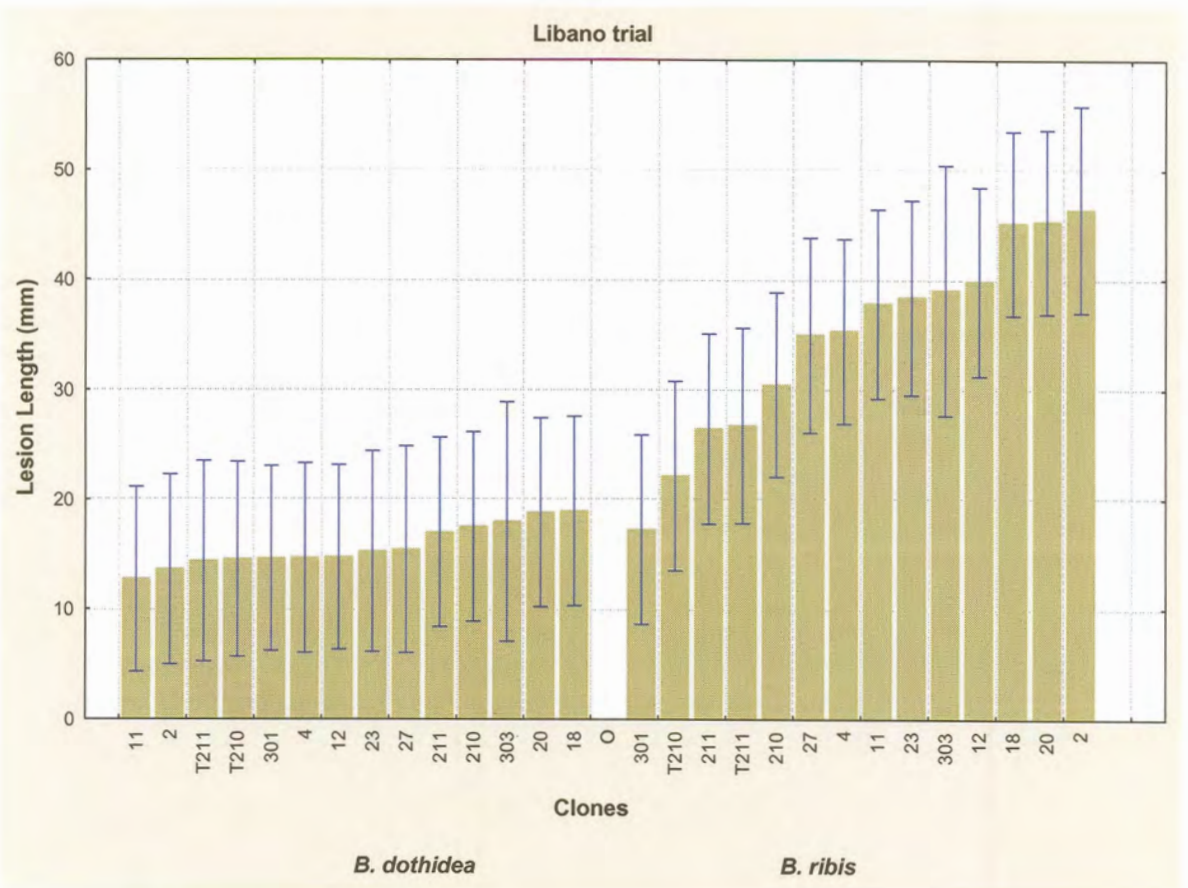

Libano trial
Lesion Length (mm)
0
10
20
30
40
50
60
11
2
T211
T210
301
4
12
23
27
211
210
303
20
18
O
301
T210
211
T211
210
27
4
11
23
303
12
18
20
2
Clones
B. dothidea
B. ribis

Fig. 13. Mean lesion lengths after inoculation with *Botryosphaeria dothidea* and *B. ribis* on 14 clones of *Eucalyptus grandis* at Angela Maria farm. Bars represent confidence limits for each isolate on all clones inoculated.

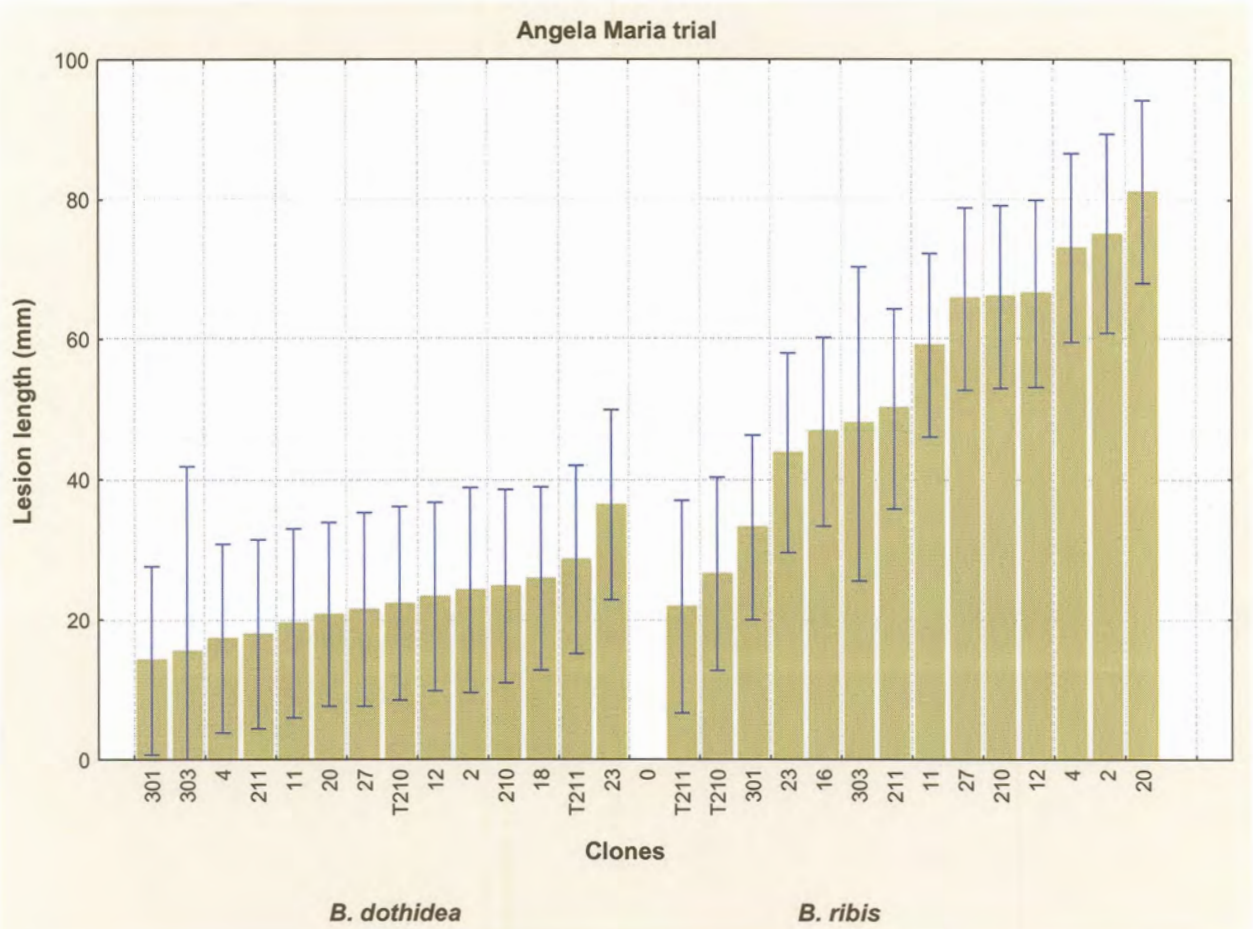
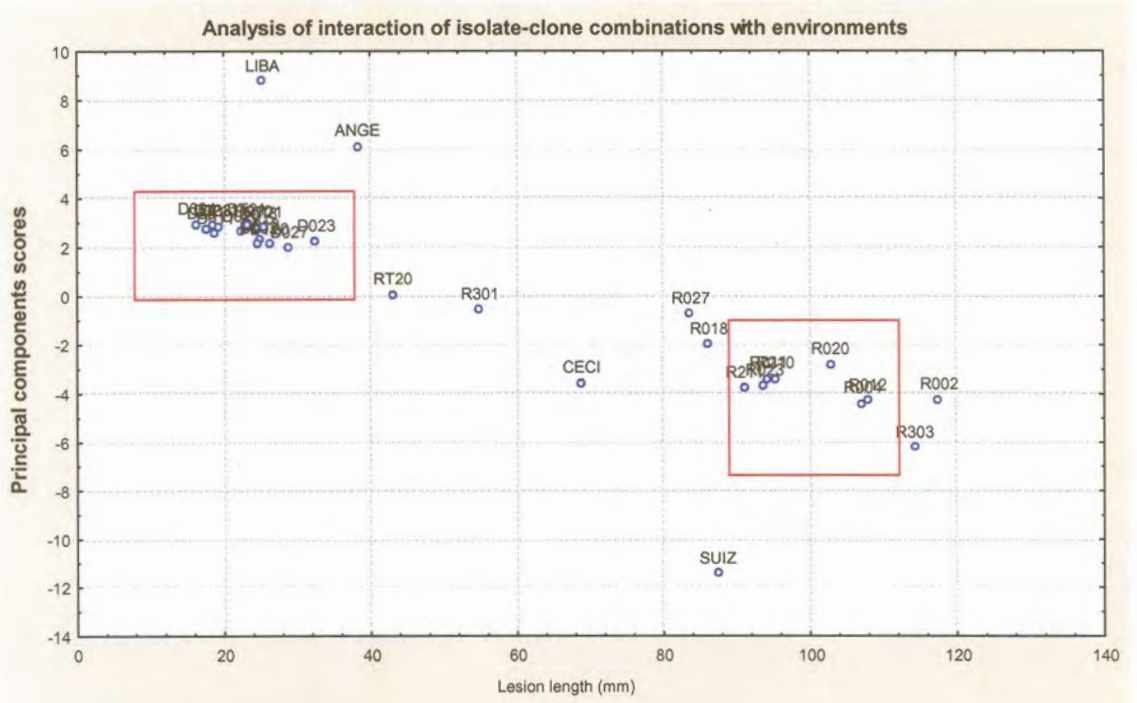

Angela Maria trial
Lesion length (mm)
100
80
60
40
20
0
301
303
4
211
11
20
27
T210
12
2
210
18
T211
23
0
T211
T210
301
23
16
303
211
11
27
210
12
4
2
20
Clones
B. dothidea
B. ribis

Fig. 14. Score values obtained from an AMMI analysis plotted against the overall lesion length for isolates of *Botryosphaeria dothidea* and *B. ribis*, susceptibility of clones and location interaction.



Clones marked with a 'D' = *B. dothidea*
Clones marked with a 'R' = *B. ribis*
The rectangle al lef marks clone exclusively infected with *B. dothidea*
The square al right marks clone exclusively infected with *B. ribis*

CHAPTER 3

Discovery of the *Eucalyptus* canker pathogen, *Cryphonectria cubensis*, on *Miconia* (Melastomataceae) in Colombia

ABSTRACT

Cryphonectria cubensis is a serious canker pathogen on commercially grown *Eucalyptus* species in the tropics and subtropics. During recent surveys for native hosts of *C. cubensis* in Colombia, a fungus with fruiting structures similar to those of *C. cubensis* was found on native *Miconia theaezans* and *M. rubiginosa,* both members of the Melastomataceae. The morphology of this fungus was studied and DNA sequences were obtained for the ITS1/ITS2 region of the rDNA operon and the β-tubulin genes. Pathogenicity of the fungus was also assessed on various Melastomataceae. Isolates from *M. theaezans* and *M. rubiginosa* grouped together with other South American *C. cubensis* isolates from *Eucalyptus* species and *Syzygium aromaticum.* Fruiting structures on *M. rubiginosa* also resembled those of *C. cubensis* on *E. grandis*. *Cryphonectria cubensis* isolates from *E. grandis* and *M. theaezans* were mildly pathogenic on the various hosts, although *Tibouchina* spp. and *M. rubiginosa* appeared to be more susceptible to *C. cubensis* than a number of *Eucalyptus* clones and *M. theaezans*. The occurrence of *C. cubensis* on native *Miconia* spp. supports the view that this pathogen is native to South and Central America.

INTRODUCTION

Cryphonectria cubensis (Bruner) Hodges is one of the most serious pathogens of *Eucalyptus* spp. (Myrtaceae) in South America (Boerboom & Maas 1970, Hodges *et al.* 1976, Hodges, Geary & Cordell 1979, Hodges 1980), including Colombia (Van der Merwe *et al.* 2001). The associated canker disease has also been reported from other parts of the world with tropical, sub-tropical or temperate climates, mostly Africa (Gibson 1981, Roux *et al.* 2003, Wingfield, Swart & Abear 1989), Southeast Asia (Florence, Sharma & Mohanan 1986, Hodges, Alfenas & Cordell 1986, Sharma, Mohanan & Florence 1985) and Australia (Davison & Coates 1991). In these regions, Cryphonectria canker is most severe in areas with high rainfall and temperature (Boerboom & Maas 1970; Hodges *et al.* 1976, 1979, Sharma *et al.* 1985).

Cankers caused by *C. cubensis* are usually found at the base or lower stems of trees, but may also occur higher up on the trunks (Sharma *et al.* 1985, Hodges *et al.* 1976, 1979). The pathogen kills the cambium and in severe cases, can result in tree death (Sharma *et al.* 1985, Hodges *et al.* 1976, 1979). The only practical management option for the disease is planting resistant *Eucalyptus* species and clones (Alfenas, Jeng & Hubbes 1983, Hodges *et al.* 1976, Sharma *et al.* 1985, Van Heerden & Wingfield 2002).

Until recently, *C. cubensis* has been known only to occur on trees belonging to the Myrtaceae. These hosts are predominantly species of *Eucalyptus* but also include clove (*Syzygium aromaticum* (L.) Merr. & Perry) (Hodges *et al.* 1986) and strawberry guava (*Psidium cattleianum* Sabine) (Hodges 1988). The recent discovery of *C. cubensis* on *Tibouchina urvilleana* (DC). Logn. (Fig. 1a) and *T. lepidota* Baill. (Fig. 1b), which are members of the Melastomataceae native to South America, was thus considered intriguing (Wingfield *et al.* 2001). The report of Wingfield *et al.* (2001) has led to subsequent disease surveys and the discovery of the fungus on ornamental *T. granulosa* in South Africa (Myburg *et al.* 2002a).

The possible origin of *C. cubensis* presents an interesting question that is also important in terms of disease management. One hypothesis is that the pathogen originated on clove, also a member of the Myrtaceae, in Indonesia (Hodges *et al.* 1986). The world-wide distribution of this fungus would then have occurred through the establishment of clove

plantations linked to the spice trade (Hodges *et al.* 1986). The discovery of *C. cubensis* on native *Tibouchina* spp. in South America has, however, raised the alternative hypothesis that *C. cubensis* could have originated in that part of the world (Wingfield *et al.* 2001).

Results from phylogenetic studies, based on DNA sequence for three gene regions (Myburg, Wingfield & Wingfield 1999, Myburg *et al.* 2002b), have shown that *C. cubensis* from South America and Southeast Asia resolve into two distinct phylogenetic sub-clades. This suggests that *C. cubensis* in these areas are different from one another and was not introduced into one area from another. Equally intriguing is the recent discovery based on comparisons of β-tubulin and histone *H3* gene sequences (Myburg *et al.* 2002b), that South African isolates of *C. cubensis* are distinct from those of South American and Southeast Asian origin, and probably represent a distinct taxon.

During recent surveys for *C. cubensis* on native Melastomataceae in Colombia, a fungus resembling *C. cubensis* was found on a number of new hosts in the Melastomataceae. The aim of this study was to identify the fungus based on morphology and DNA sequences. Pathogenicity of the isolates originating from the new hosts was also tested on these hosts and on *E. grandis* W. Hill ex Maiden.

MATERIALS AND METHODS

Symptoms and collection of samples

Disease surveys were conducted in various areas of Colombia with different altitudes and precipitation (Fig. 2). Specimens were collected from *Miconia theaezans* (Bonpl.) Cogn. (Fig. 1c) in a natural forest from the La Selva farm of Smurfit Carton de Colombia near the city Pereira in the Risaralda province. Cankers covered in fruiting structures were also found on *M. rubiginosa* (Bonpl.) DC. trees (Fig. 1d) of different ages on the farm Vanessa, near Timba in the Cauca province. These trees occurred within a *Eucalyptus* plantation where *C. cubensis* has previously been collected.

Disease symptoms on the *Miconia* spp. included branch die-back, and cankers on branches, trunks or the tree bases that often resulted in the death of trees or tree parts.

The cankers were generally associated with physical wounds to branches and stems. Fruiting structures were produced abundantly around the edges of the actively growing canker margins.

Specimens collected from cankers were transported to the laboratory for further analysis. Single conidial isolations were made from the fruiting structures by suspending spore masses in sterile water and plating the resulting suspensions on malt extract agar MEA (20 g/l Biolab malt extract agar). Single germinating conidia were then transferred to fresh MEA plates. Representative isolates have been preserved at 5 °C in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa (Table 1). The original bark specimens from whom isolations were made have been deposited (Table 2) in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

DNA sequence comparisons

Isolates from *Miconia* spp. and *E. grandis* were included in the DNA sequence comparisons (Table 1). Previously characterised *C. cubensis* isolates from *Eucalyptus* spp. (Myburg *et al.* 2002b) and *S. aromaticum* (Myburg *et al.* 1999, 2003) from different parts of the world were included for comparative purposes. In addition, representative species of *Cryphonectria* and *Endothia*, namely *C. parasitica* (Murr.) Barr, *C. radicalis* (Schw.: Fr.) Barr, *C. nitschkei* (Otth.) Barr, *C. macrospora* (Kobayashi & Ito) Barr and *E. gyrosa* (Schw.: Fr.) Fr. were sequenced by Venter *et al.* (2002). Two *Diaporthe ambigua* Nitschkei isolates were included as outgroup taxa to root the phylogenetic trees.

Isolates for DNA sequence comparisons were grown in Malt Extract Broth (20 g/l Biolab malt extract). DNA was extracted from mycelium as described in Myburg *et al.* (1999). The internal transcribed spacer (ITS) regions ITS1 and ITS2, as well as the conserved 5.8S gene of the ribosomal RNA (rRNA) operon, were amplified using the primer pair ITS 1 and ITS 4 (White *et al.* 1990). Two regions within the β-tubulin gene were amplified with primer pairs Bt1a/Bt1b and Bt2a/Bt2b respectively (Glass & Donaldson 1995). The reaction conditions for amplifying these gene regions were the same as those given by Myburg *et al.* (1999) and Myburg *et al.* (2002b) respectively. PCR products were visualised on 1% agarose (ethidium bromide stained) gels using a UV light.

Purification of PCR products was done using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany).

The purified PCR products were sequenced with the same primers that were used to amplify the respective DNA regions. An ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, United Kingdom) was used to sequence the amplification products on an ABI PRISM 3100™ automated DNA sequencer.

The resulting raw nucleotide sequences were edited using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) software. Sequences were manually aligned. Phylogenetic trees were inferred using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b (Swofford 2002). A Templeton Nonparametric Wilcoxon Signed Ranked test (Kellogg, Appels & Mason-Gamer 1996) was applied to the rRNA and β-tubulin gene sequence data sets to determine whether they could be analysed collectively in the parsimony analysis.

A phylogenetic tree was inferred from maximum parsimony (MP) using the heuristic search option with the tree-bisection-reconnection (TBR) branch swapping and MULTREES options (saving all optimal trees) effective. Gaps inserted during manual sequence alignment were treated as fifth character (NEWSTATE) in the heuristic searches. A 1000 replicate bootstrap was executed to assess the confidence levels of the branch nodes of the phylogenetic tree. The sequence data generated in this study have been deposited in GenBank and accession numbers are listed in Table 1.

Morphology

Conidiomata from the bark specimens were rehydrated for one min in boiling water. The structures were then sectioned at –20 °C to a thickness of 12-14 µm with a Leica CM1100 cryostat after embedding them in Leica mountant (Setpoint Premier, Johannesburg, South Africa). Sections were mounted on microscope slides in lactophenol. Structures were also sectioned by hand to observe the morphology of the conidiophores. Twenty measurements, presented as (min-)(mean-SD) – (mean+SD)(-max) µm, of ascospores, asci, conidia and conidiophores suspended in lactophenol and

3% KOH, were taken for the specimens. A measurement range from two structures was obtained for the eustromata and perithecia. Colour notations of Rayner (1970) were used.

Pathogenicity tests

Greenhouse inoculation trials. Three isolates from *E. grandis* in Colombia (CMW 10638, CMW 10639, CMW 10640) and two isolates from *M. theaezans* (CMW 10625, CMW 10626) were screened for pathogenicity on *T. urvilleana* (seven months old) plants in a greenhouse with natural light at ~25 °C. Five trees per isolate were inoculated and an equal number of trees were inoculated with sterile water agar (WA) (20 g/l Biolab agar) plugs as controls. Inoculations were made with a cork borer (9 mm diam). Agar discs of the same size were taken from the edges of actively growing cultures and placed inside the wounds with the mycelium facing downwards. The agar discs were covered with tissue paper moistened with sterile water, and secured with masking tape. The masking tape was removed after ten days.

Trees were inoculated in October 2001 and lesion development was evaluated after four weeks. Lesions were exposed by scraping away the bark and the lengths of the lesions were measured. The most pathogenic isolates from *E. grandis* and *M. theaezans* (CMW 10639 and CMW 10625 respectively) were selected for subsequent field inoculation trials.

In a second greenhouse trial, two isolates from *M. rubiginosa* (CMW 10022 and CMW 10024) were inoculated on *T. urvilleana* and *E. grandis* (clone ZG14), which were 17-24 months old and up to 1.8 m high. A highly pathogenic isolate of *C. cubensis* from South Africa (CMW 2113), used in previous pathogenicity studies (Myburg *et al.* 2002a, Van Heerden & Wingfield 2001, 2002) was included for comparative purposes. Inoculation procedures were the same as those in the first trial and ten trees were inoculated for each of the three isolates and for the negative control using WA discs. Inoculations were done as described above except that a cork borer with a diameter of 6 mm was used. The trial was inoculated in May 2002, and evaluated in June 2002.

Field inoculation trials. The first inoculation trial was conducted at Rancho Grande farm, Restrepo, Valle (76° 30' 49" W and 3° 51' 43" N, 1067 mm/y, 1469 masl). This trial included reciprocal inoculations with isolates from *E. grandis* (CMW 10639) and *M. theaezans* (CMW 10625) selected in the first greenhouse trial. Five tree species were used, namely *T. semidecandra* Cogn. (Fig. 1f), *T. lepidota*, *T. urvilleana*, *M. theaezans* and a clone of *E. grandis* (clone 274). These trees were one year old and were growing in plastic planting containers. Twenty trees of each species were inoculated per isolate, and an equal number of trees were inoculated with WA discs to serve as negative controls. Inoculations were conducted in a similar way to greenhouse inoculations but the diameter of the wound was 4 mm. Trees were inoculated in May 2002 and lesion development was evaluated after twelve weeks. Internal lesion length in the cambium was measured for all field trials.

The second field trial was at the Vanessa farm (Fig. 2), Timba, Cauca province (76° 35' 15" W and 3° 5' 42" N, 3143 mm/y, 2048 msal). Isolate CMW 10022 from *M. rubiginosa*, shown to be pathogenic in the preliminary greenhouse trial, was used. Twenty three-year-old *E. grandis* trees (clone 275), 20 trees from seeds of a cross between *E. grandis* and *E. urophylla* (*E. "urogandis"* clone 212), and 20 *M. rubiginosa* trees were inoculated. The *M. rubiginosa* trees were approximately six years old and formed part of the native vegetation surrounding the commercial plantations. Ten trees of each host were inoculated with MEA to serve as negative controls. The trial was initiated in June 2002 and lesion lengths were measured after 12 weeks in late September 2002. The same inoculation techniques used in greenhouse and other field trials were applied, except that the inoculation wounds were six mm in diameter. The data for the pathogenicity trials were analysed using a one-way Analysis of Variance (ANOVA) with SAS (1990).

RESULTS

DNA sequence comparisons

Amplification of the ITS1, 5.8S and ITS2 rRNA regions as well as the two regions in the β-tubulin gene resulted in PCR products of approximately 600bp and 550bp respectively. The Templeton Nonparametric Wilcoxon Signed Ranked test (Kellogg *et al.* 1996)

showed that the rRNA and the β-tubulin sequence data sets could be combined in the phylogenetic analyses. The combined data set consisted of 32 taxa with the *D. ambigua* isolates as the outgroup (Fig. 3). This data set consisted of 1498 sequence characters of which 886 were constant, 44 were variable parsimony-uninformative and 568 were variable parsimony-informative.

The phylogenetic tree generated from the heuristic search (Fig. 4, tree length = 1198 steps, consistency index/CI = 0.8, retention index/RI = 0.9) resolved the taxa into three clades separately from the outgroup taxa. The largest of the three clades represented *C. cubensis*, while the other two included representative species of *Cryphonectria* (*C. parasitica*, *C. radicalis*, *C. nitschkei* and *C. macrospora)* grouping in the one clade, and *E. gyrosa* in the other (bootstrap support = 100% respectively).

The *C. cubensis* clade represented this fungus isolated from a variety of hosts originating from South America, Southeast Asia and South Africa. All three geographical areas are represented as three well supported clades in the phylogenetic tree (Fig.4). The Southeast Asian group (bootstrap 98%) included *C. cubensis* isolated from clove and *Eucalyptus* species. The South African group is characterised by *C. cubensis* isolated from *E. grandis* (bootstrap 95%). The South American group (bootstrap 72%) include *C. cubensis* isolated from *Eucalyptus* spp. and *S. aromaticum* as reported previously (Myburg *et al.* 1999, 2002b, 2003). Isolates originating from *M. theaezans* (CMW 9980, CMW 9993, CMW 10626, CMW 10639) and *M. rubiginosa* (CMW 9970, CMW 9996, CMW 10022, CMW 10024, CMW 10025, CMW 10026, CMW 10028), grouped within the South American sub-clade.

Morphology

Specimens from *M. rubiginosa* (PREM 57517) had ascomata similar to those of *C. cubensis* found on *E. grandis* in Colombia (PREM 57294). They could be distinguished from conidiomata since only one to three fuscous-black (13''''m), cylindrical necks (380-720 µm long) emerged from the bark (Fig. 5a). Orange (15) stromatic tissue was sometimes visible at the base of the necks (Figs 5a-b). Longitudinal sections revealed umber (15m), *textura porrecta* tissue surrounding the black perithecial necks (Figs 5b-c) and reduced prosenchymatous stromatic tissue present at the base of the neck (Figs 5b,

5d). Asci were fusoid, eight-spored with a refractive apical ring, (19.5-)20.5-24.5(-27.0) µm long and (4.5-)5.0-6.5(-7.0) µm wide (Fig. 5e). Ascopores were fusoid to oval, hyaline with a single septum in the center of the spores, (5.0-)5.5-7.0(-8.5) µm long and 2.0-2.5 µm wide (Figs 5f). The ascomata also resembled those previously described from South America (Bruner 1917, Hodges *et al.* 1979, Hodges 1980) and ascomata previously described from other parts of the world (Heath *et al.* 2003, Myburg *et al.* 2002a, 2003).

Conidiomata of the fungus on *M. rubiginosa* (PREM 57517) were similar in shape to those of *C. cubensis* occurring on *E. grandis* (PREM 57294). Structures were pyriform, superficial and fuscous-black (13''''m) with a single attenuated neck and luteous (19) spore drops or tendrils (Figs 5g-h). The tissue of the conidiomatal base was umber (15m), *textura globulosa* but the neck tissue was *textura porrecta* (Fig. 5i). Conidiophores were branched, and conidiogenous cells enteroblastic phialidic, cylindrical with inflated bases and attenuated apices (Figs 5j-k). Conidia were hyaline, oblong to oval, aseptate, 3.0-4.0 µm long, 1.5-2.0 µm wide (Fig. 5l). These characteristics were also similar to those described previously (Bruner 1917, Hodges *et al.* 1979, Hodges 1980, Myburg *et al.* 2002b, 2003).

A few morphological differences exist between structures on *E. grandis* and *M. rubiginosa*. The stromatic tissues of the ascomata on *E. grandis* were slightly more distinctly erumpent than those on *M. rubiginosa*. Conidiomata on *M. rubiginosa* were much smaller (25-400 µm long in total above surface of bark) than those on *E. grandis* (420-960 µm long in total above surface of bark). Conidiomata on *E. grandis* were also better developed with wide bases (210-420 µm wide above surface of bark) and long, strongly attenuated necks (220-440 µm long), unlike conidiomata on *M. rubiginosa* that had narrow bases (140-260 µm wide above surface of bark) and shorter necks (140-180 µm long).

Pathogenicity tests

Greenhouse inoculations. In the first greenhouse trial (Table 3), inoculation with *C. cubensis* isolates from *E. grandis* (CMW 10638, CMW 10639, CMW 10640) and *M.*

theaezans (CMW 10625, CMW 10626) resulted in lesion formation (Fig. 6). The more pathogenic isolates (CMW 10625, CMW 10638, CMW 10639) were not significantly different from each other (Fig. 6), but differed significantly ($P < 0.0014$) from the control inoculation (Table 3). Isolates CMW 10639 from *E. grandis* and CMW 10625 from *M. theaezans* were chosen for field inoculations (Fig. 6) because they were most pathogenic for each isolate group from a particular host.

In the second greenhouse trial (Table 4), isolates from *M. rubiginosa* (CMW 10022, CMW 10024) and the South African isolate of *C. cubensis* (CMW 2113) resulted in different size lesions (Fig. 7). The South African isolate was more pathogenic on the *E. grandis* clone than the other isolates tested (Fig. 7). This isolate was also less pathogenic on *T. urvilleana* (Fig. 7) than on the *E. grandis* clone. An isolate from *M. rubiginosa* (CMW 10024) was more pathogenic on *E. grandis* than on *T. urvilleana* (Fig. 7) and it was also more pathogenic on *E. grandis* than the other isolate from *M. rubiginosa* (CMW 10022). Isolate CMW 10022 was equally pathogenic on *E. grandis* and *T. urvilleana* (Fig. 7). All isolates produced lesions significantly larger ($P = 0.001$) than the control inoculations (Table 4). Only *E. grandis* trees infected by the South African isolate (CMW 2113) produced epicormic shoots below the inoculation points, indicating that the inoculated stems were being girdled.

Field inoculation trials. In the first field trial (Table 5), lesions were produced on all tree species (*T. urvilleana*, *T. lepidota*, *T. semidecandra*, *M. theaezans*, *E. grandis*) in response to inoculation with isolates CMW 10693 from *E. grandis* and CMW 10625 from *M. theaezans*. The longest lesions were produced on *T. urvilleana* (Fig. 8a) and *T. lepidota* (Fig. 8b), while lesions on *T. semidecandra* (Fig. 8c), although smaller, also differed significantly ($P = 0.001$) from control inoculations (Fig. 9). Lesions on *M. theaezans* (Fig. 8d) and the *E. grandis* clone (Fig. 8e) were only slightly longer than the control inoculations (Fig. 9). Lesions produced by the two isolates (CMW 10639, CMW 10625) were similar in size on each tree species (Fig. 9).

In the second field trial (Table 6), trees of *M. rubiginosa* (Figs 8f, 10) were more susceptible ($P = 0.0001$) to the *C. cubensis* isolate from *M. rubiginosa* (CMW 10022) than the *E. grandis* trees tested (Figs 8g-h, 10). Inoculations with isolate CMW 10022 on

the susceptible *E. grandis* clone 275 and the hybrid clone 212 gave rise to lesions that did not differ from those of the control inoculations (Fig. 10).

DISCUSSION

This study reports on the discovery of the serious *Eucalyptus* pathogen *C. cubensis* on native *Miconia* species (Melastomataceae) in Colombia. Isolates of the fungus from *M. theaezans* and *M. rubiginosa* grouped in the sub-clade that characterises *C. cubensis* occurring in South America, as defined in previous studies (Myburg *et al.* 1999, 2002a, 2002b, 2003, Roux *et al.* 2003). Structures on herbarium specimens linked to these isolates had conidiomata and ascomata typical of *C. cubensis* and spores were similar in size to those previously reported for this fungus (Hodges 1980, Myburg *et al.* 2002b, 2003).

Different host bark and environmental conditions have in the past been shown to result in variable morphology of *C. cubensis* structures (Bruner 1917, Hodges *et al.* 1986, Myburg *et al.* 2003). This complicates morphological comparisons between samples from different hosts. For instance, conidiomata on *M. rubiginosa* were much smaller than those on *E. grandis*, but the isolates originating from the specimens of *M. rubiginosa*, were shown to be identical to those from *E. grandis* based on DNA sequences. These differences observed between the conidiomata on *M. rubiginosa* and *E. grandis*, complicates identification. DNA sequences should thus accompany morphological identifications to verify identifications.

Native Melastomataceae in Colombia differed in their susceptibility to *C. cubensis* in the field inoculation trials. In the field trial where five different host species were tested, *T. urvilleana* and *T. lepidota* were highly susceptible to the two isolates of *C. cubensis*. This is in contrast to *M. theaezans* that was highly tolerant to infection. *Tibouchina semidecandra* was less susceptible than the other two species of *Tibouchina*, but more susceptible than *M. theaezans*.

Results of the different pathogenicity trials suggest that in the field, *C. cubensis* is more pathogenic on *M. rubiginosa* than on *E. grandis*. It was previously suggested that *C. cubensis* could have an origin in South America on native Melastomataceae (Wingfield

et al. 2001). It is generally believed that pathogens are less pathogenic on their native hosts than exotic species (Leppik 1970, Newhouse 1990). Therefore, the *E. grandis* clones used in the trials were expected to be more susceptible to *C. cubensis* than *M. rubiginosa*. However, these commercially grown clones have been subjected to intensive selection for resistance to disease over the past few years. It is thus possible that the clones or seed lots chosen for these trials have high degrees of tolerance to the pathogen. The fact that disease is not commonly seen on native Melastomataceae might also imply that the artificial inoculation techniques used to test pathogenicity, breach barriers that limit infection under natural conditions.

In this study we have shown that *C. cubensis* from South America occurs on *M. theaezans* and *M. rubiginosa*, two species of a genus not previously known as a host of the pathogen. The other recently recognised native hosts of the fungus in this country are *Tibouchina* spp. (Wingfield *et al.* 2001). The first discovery of *C. cubensis* on *M. theaezans*, was in native vegetation far removed from *Eucalyptus* plantations. It thus seems likely that *C. cubensis* occurs naturally on this host. In the case of *M. rubiginosa*, the trees were felled during the establishment of a *Eucalyptus* compartment. The *M. rubiginosa* trees, however, recovered and *C. cubensis* was found on these trees, as well as on the *Eucalyptus* trees in the adjacent compartment. It is unclear in which direction *C. cubensis* spread in this case, although it most likely was already present on *M. rubiginosa*. Further studies will be required to resolve this question.

Members of the Melastomataceae are common in South America, Central America, the Caribbean islands and Hawaii (Everett 1981). The occurrence of *C. cubensis* on species belonging to this family supports the hypothesis that the fungus occurred widely through South and Central America and the Caribbean prior to the commercial planting of *Eucalyptus* species. Detailed population studies will shed more light on the origin or origins of *C. cubensis*, and its movement throughout the world.

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Table 1. Isolates included in this study.

Isolate number[a]	Species identity	Host	Origin	GenBank accession numbers
CMW 2113	*Cryphonectria cubensis*	*Eucalyptus grandis*	South Africa	AF 046892, AF 273067, AF 273462
CMW 62	*C. cubensis*	*E. grandis*	South Africa	AF 292041, AF 273063, AF 273458
CMW 8755	*C. cubensis*	*E. grandis*	South Africa	AF 292040, AF 273064, AF 273459
CMW 8757	*C. cubensis*	*Eucalyptus*	Venezuela	AF 046897, AF 273069, AF 273464
CMW 8758	*C. cubensis*	*Eucalyptus*	Venezuela	AF 046898, AF 273068, AF 273463
CMW 9970	*C. cubensis*	*Miconia rubiginosa*	Colombia	AY 214291, AY 214219, AY 214255
CMW 9996	*C. cubensis*	*M. rubiginosa*	Colombia	AY 214292, AY 214220, AY 214256
CMW 10022	*C. cubensis*	*M. rubiginosa*	Colombia	AY 262389, AY 262393, AY 262397
CMW 10024	*C. cubensis*	*M. rubiginosa*	Colombia	AY 262390, AY 262394, AY 262398
CMW 10025	*C. cubensis*	*M. rubiginosa*	Colombia	AY 214293, AY 214221, AY 214257
CMW 10026	*C. cubensis*	*M. rubiginosa*	Colombia	AY 214294, AY 214222, AY 214258
CMW 10028	*C. cubensis*	*M. rubiginosa*	Colombia	AY 214295, AY 214223, AY 214259
CMW 9980	*C. cubensis*	*M. theaezans*	Colombia	AY 214297, AY 214225, AY 214261
CMW 9993	*C. cubensis*	*M. theaezans*	Colombia	AY 214298, AY 214226, AY 214262
CMW 10625	*C. cubensis*	*M. theaezans*	Colombia	---
CMW 10626	*C. cubensis*	*M. theaezans*	Colombia	AY 262392, AY 262396, AY 262400
CMW 10639	*C. cubensis*	*M. theaezans*	Colombia	AY 263419, AY 263420, AY 263421
CMW 10775	*C. cubensis*	*Syzygium aromaticum*	Brazil	AY 084003, AY 084015, AY 084027

CMW 10776	*C. cubensis*	*S. aromaticum*	Brazil	AY 084004, AY 084016, AY 084028
CMW 10777	*C. cubensis*	*S. aromaticum*	Brazil	AY 084005, AY 084017, AY 084029
CMW 8756	*C. cubensis*	*E. marginata*	Indonesia	AF 046896, AF 273077, AF 375606
CMW 2632	*C. cubensis*	*E. marginata*	Australia	AF 046893, AF 273078, AF 375607
CMW 3839	*C. cubensis*	*S. aromaticum*	Indonesia	AF 046904, AY 084011, AY 084023
CMW 1651	*C. parasitica*	*Castanea dentata*	USA	AF 046901, AF 273074, AF 273467
CMW 1652	*C. parasitica*	*C. dentate*	USA	AF 046902, AF 273075, AF 273468
CMW 10455	*C. radicalis*	*C. dentate*	Italy	AF 452113, AF 525705, AF 525712
CMW 10477	*C. radicalis*	*C. dentate*	Italy	AF 368328, AF 368347, AF 368346
CMW 10463	*C. macrospora*	*Castanopsis cuspidata*	Japan	AF 368331, AF 368351, AF 368350
CMW 10518	*C. nitschkei*	*Quercus sp.*	Japan	AF 452118, AF 525706, AF 525713
CMW 10435	*Endothia gyrosa*	*Q. palustris*	USA	AF 368325, AF 368337, AF 368336
CMW 10442	*E. gyrosa*	*Q. palustris*	USA	AF 368326, AF368339, AF368338
CMW 5288	*Diaporthe ambigua*	*Malus domestica*	South Africa	AF 543817, AF 543819, AF 543821
CMW 5587	*D. ambigua*	*M. domestica*	South Africa	AF 543818,AF 543820,AF 543822

[a] CMW refers to the culture collection of the Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Table 2. Specimens used in morphological comparisons.

Identity	Herbarium no.[a]	Linked culture[b]	Host	Origin	Date	Collector
Cryphonectria cubensis	PREM 57294	CMW 10639[c]	*Eucalyptus grandis*	Vanessa, Colombia	2000	M. J. Wingfield
C. cubensis	PREM 57517	CMW 2357 CMW 9996 CMW 10025 CMW 10026 CMW 10028 CMW 10022 CMW 10024	*Miconia rubiginosa*	Vanessa, Colombia	2001	C. A. Rodas

[a] PREM, National Collection of Fungi, Pretoria, South Africa.

[b] CMW refers to the culture collections of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa.

[c] Isolate CMW 10639 did not originate from PREM 57294, but were collected from the same location.

Table 3. One way ANOVA analysis for lesion length measurements of Colombian *Cryphonectria cubensis* isolates from *Eucalyptus grandis* (CMW 10638, CMW 10639, CMW 10640), *Miconia theaezans* (CMW 10625, CMW 10626) and a negative control inoculated on *Tibouchina urvilleana* seedlings in the greenhouse.

Source	SS	df	MS	F	Pr > F
Isolate	33267.36	5	6653.47	5.66	0.0014
Error	28219.6	24	1175.81		

R-Square = 0.541047

CV = 55.63578

Table 4. One way ANOVA analysis for lesion length measurements of Colombian *Cryphonectria cubensis* isolates from *Miconia rubiginosa* (CMW 10022 and CMW 10024), a South African *C. cubensis* isolate (CMW 2113) and a negative control inoculated on *Tibouchina urvilleana* and *Eucalyptus grandis* (clone ZG14) seedlings in the greenhouse.

Source	SS	df	MS	F	Pr > F
Host	310624.68	7	44374.95	39.58	0.0001
Error	80714.3	72	1121.03		

R-Square = 0.793748

CV = 35.99711

Table 5. One way ANOVA analysis for lesion length measurements of Colombian *Cryphonectria cubensis* isolates from *Eucalyptus grandis* (CMW 10639), *Miconia theaezans* (CMW 10625) and a negative control inoculated on one-year-old *Tibouchina semidecandra*, *T. lepidota*, *T. urvilleana*, *M. theaezans* and *E. grandis* (clone 274) seedlings in Colombia.

Source	**SS**	**df**	**MS**	**F**	**Pr > F**
Isolate	380936.00	2	190468.00	65.59	0.0001
Host	645144.98	4	161286.24	55.54	0.0001
Isolate*Host	349986.03	8	43748.12	15.07	0.0001
Error	792756.03	273	2903.86		

R-Square = 0.629927

CV = 95.18900

Table 6. One way ANOVA analysis for lesion length measurements of a Colombian *Cryphonectria cubensis* isolate from *Miconia rubiginosa* (CMW 10022) and a negative control inoculated on three-year-old *Eucalyptus grandis* trees (clone 275), trees from a *E. grandis* and *E. urophylla* cross (*E. urograndis* 212) and six-year-old *M. rubiginosa* trees in Colombia.

Source	**SS**	**Df**	**MS**	**F**	**Pr > F**
Isolate	43156.02	2	21578.01	60.43	0.0001
Host	58174.31	2	29087.15	81.50	0.0001
Isolate*Host	24.37	1	24.37	0.07	0.7945
Error	29622.25	83	356.89		

R-Square = 0.728547

CV = 53.02294

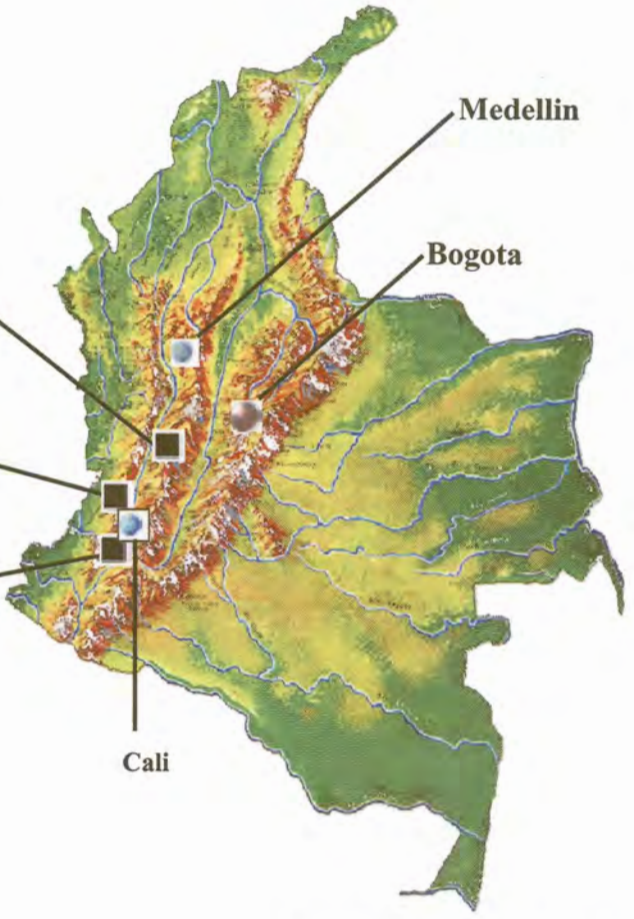
Fig. 1. Native Melastomataceae on which *Cryphonectria cubensis* was found and that were used in pathogenicity trials. **(a)**. *Tibouchina urvilleana*. **(b)**. *T. lepidota*. **(c)**. *Miconia theaezans*. **(d)**. *M. rubiginosa*. **(e)**. *T. semidecandra*.

Fig. 2. Map of Colombia showing co-ordinates, altitude and precipitation of the locations where *Cryphonectria cubensis* was discovered on various Melastomataceae, and where field trials were conducted.

Discovery of *Miconia theaezans*
Selva, Pereira, Risaralda
2048 masl, 3143 mm/y
75^0 35' 34" W and 4^0 47' 26" N

First field inoculation trial
Rancho Grande farm, Restrepo, Valle,
2102 masl, 2414 mm/y
76^0 26' 39" W and 4^0 1' 51" N

Discovery of *Miconia rubiginosa*
and second field inoculation trial
Vanessa, Timba, Cauca
1000 masl, 2365 mm/y
76^0 35' 15" W and 3^0 5' 42" N



Source: Instituto Geografico Agustin Codazzi

Fig. 3. Raw sequence data of the two regions sequenced within the β-tubulin gene (designated as β-tub 1a/1b and β-tub 2a/2b) and the ITS1, conserved 5.8S and ITS2 regions of the rDNA operon. The start of each region is indicated above the alignment. The exon regions of the β-tubulin gene, as well as the conserved 5.8S region of the rDNA operon, are indicated in red. Unknown sequence characters are indicated with a "**N**", while gaps inserted to achieve sequences alignment are indicated with "-". Bases matching those of **CMW 2113** are indicated with a ".".

```
[                10        20        30        40        50        60        70        80        90]
[ β-tub 1a/1b →  .         .         .         .         .         .         .         .         .]
CMW 2113         TGACCAGCCG TGGCGCCCAC TCCTTCCGCG CTGTCACGGT GCCCGAGTTG ACCCAGCAGA TGTTCGACCC CAAGAACATG ATGGCTGCCT
CMW 62           .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 8755         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 8756         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 2632         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 3839         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 8758         GA........ .......... ..A....... .......C.. .......... .......... .......... .......... ..........
CMW 8757         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 9970         .......... .......... .......... .......C.. ..TA...... .......... .......... .......... ..........
CMW 9996         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10025        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10026        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10028        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 9980         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 9993         .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10626        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10022        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10024        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10639        NNNNNNNNNN NNNG...... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10775        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10776        .......... .......... .......... .......C.. .......... .......... .......... .......... ..........
CMW 10777        .......... .......... .......... .......C.. .......... ........A. .......... .......... ..........
CMW 1651         .......... .......... .......... .CC.A..C.. C......... .......... .......... .......... ..........
CMW 1652         .......... .......... .......... .CC....C.. C......... .......... .......... .......... ..........
CMW 10455        .......... .......T.. .......... .CC....C.. T......... .......... .......... .......... ..........
CMW 10477        .......... .......T.. .......... .CC....C.. T......... .......... .......... .......... ..........
CMW 10463        .......... .......T.. .......... .CC....C.. T......... .......... .......... .......... ..........
CMW 10518        .......... .......T.. .......... .CC.A..C.. T......... .......... .......... .......... ..........
CMW 10435        .......... ......TT.. .......... .C.....C.. C......... .......... .......... .......... .....C....
CMW 10442        .......... ......TT.. .......... .C.....C.. C......... .......... .......... .......... .....C....
CMW 5288         .......... C......... ........T. .C.....C.. .......C.C .......... .......... .......... .....C....
CMW 5587         .......... C......... ........T. .C.....C.. .......C.C .......... .......... .......... .....C....
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```
[                100       110       120       130       140       150       160       170       180]
[                .         .         .         .         .         .         .         .         .]
CMW 2113    CTGACTTCCG CAACGGTCGC TACCTGACGT GCTCCGCCAT CTTGTAAGTC CCCCGCCCCT CGCGCCTCGG GGCGCCTCGG CCGAAGCTCG
CMW 62      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755    .......... .......... .......... .......... .......... .......... .......... ..A..A.... ..........
CMW 8756    .......... .......... .......... .......... .......... .......... .......... ..A..A.... ........T.
CMW 2632    .......... .......... .......... .......... .......... .......... .......... ..A..A.... ........T.
CMW 3839    .......... .......... .......... .......... .......... .......... .......... ..A..A.... ........T.
CMW 8758    .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 8757    .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 9970    .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 9996    .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 10025   .......... .......... .......... .......G.. .......... T......... .......... A.A..A.... ........T.
CMW 10026   .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 10028   .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 9980    .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 9993    .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 10626   .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 10022   .......... .......... .......... ...A...... .......... T......... .......... A.A..A.... ........T.
CMW 10024   .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 10639   .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 10775   .......... .........T .....A.... .......... .......... T.......T. .........A A.A..A.... ........T.
CMW 10776   .......... .......... .......... .......... .......... T......... .......... A.A..A.... ........T.
CMW 10777   .......... .........T .......... .......... .......... T......... ........G.. A.A..A.... ........T.
CMW 1651    .......... .......... .........A. ....T..... ..........T TT.TTGT.T. T--------- ---------T ..TCGCAAGT
CMW 1652    .......... .......... .........A. ....T..... ..........T TT.TTG..T. T--------- ---------T ..TCGCAAGT
CMW 10455   .......... .......... .......... ....T..... ..........G GTTTTTTTT. TT.TT..TTC CC.CTTG.CT ..TCGCAAGT
CMW 10477   .......... .......... .......... ....T..... ..........G GTTTTTTTT. TT.TT..TTC CC.CTTG.CT ..TCGCAAGT
CMW 10463   .......... T......... .........A. ....T..... ..........T TT.TATA... T--------- ---------- -.TCGCAAGC
CMW 10518   .......... .........T .........T. ....T..... ..........T TT.T.TGT.. TCT------- ---------- ..TCGCAGGC
CMW 10435   .......... ......C..T .......... ....T..... ..........G GT-TC....C ACACA.C..C T.G-.GC.TT TG.GG.GCT.
CMW 10442   .......... ......C..T .......... ....T..... ..........G GT-TC....C ACACA.C..T T.G-.GC.TT TG.GG.GCT.
CMW 5288    .......... T..T...... .......... ....T..... ..........- ---------- GAGCATCT-- -----.CACA .GACCCAAGT
CMW 5587    .......... T..T...... .......... ....T..... ..........- ---------- GAGCATCT-- -----.CACA .GACCCAAGT
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[                 190        200        210        220        230        240          250        260        270]
[                  .          .          .          .          .          .           .          .          .]
CMW 2113     TCTGCTAACC CTCATCGTC- ---------- ---------- ---------- ---------- ---------- CAGCCGTGGC AAGGTCTCCA
CMW 62       .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 2632     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 3839     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 8758     ....T....T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 8757     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 9970     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 9996     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10025    .........T ..T....... .......... .......... .......... .......... .......... ..T....... ..........
CMW 10026    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10028    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 9980     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 9993     .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10626    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10022    .......G.T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10024    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10639    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10775    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10776    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 10777    .........T ..T....... .......... .......... .......... .......... .......... .......... ..........
CMW 1651     C---------T .GAC-GAA.G TCTTG....G GCTGTTTGGC TAACCCTGTC TTTCTCTCTT CCCCTTCT.C T........T ..........
CMW 1652     C---------T .GAC-GAA.G TCTTG....G GCTGTTTGGC TAACCCTGTC TTTCTCTCTT CCCCTTCT.C A........T ..........
CMW 10455    .---------T .GAT-AAAGT CGTCTCT..G GCTTGTTTGC TAACC.TGTT TCTCTCCCCC CCCCCCCAAC .......... ..........
CMW 10477    .---------T .GAT-AAAGT CGTCTCT..G GCTTGTTTGC TAACC.TGTT TCTCTCCCCC CCCCCCCAAC .......... ..........
CMW 10463    C---------T .GAT-GAA.A TCTCG....G GCTTCTTGGC TAACCCCACG TTTCTCTCTT TC...CT..C T.......... ..........
CMW 10518    C---------T .CAT-GAA.A TCTTG....G GCTTTTTGGC TAACCCCATG TTTCTCTCTT TCCCCCTT.C T.......... .G........
CMW 10435    ..A.GGCTTG T.TT.T.CTG ACCCC..... .......... TATCCCTC.. .......... .........C -.......... ..........
CMW 10442    ..A.GGCTTG T.TT.T.CTG ACCCC..... .......... TATCCCTC.. .......... .........C -.......... ..........
CMW 5288     ---------- --GT.T.CGC GCTGACACTG TCTT...... .......... .......... .........C T........A ..........
CMW 5587     ---------- --GT.T.CGC GCTGACACTG TCTT...... .......... .......... .........C T........A ..........
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[                280       290       300       310       320       330       340       350       360]
[                .         .         .         .         .         .         .         .         .]
CMW 2113    TGAAGGAGGT CGAGGACCAG ATGCGCAACG TCCAGAGCAA GAACTCGTCC TACTTCGTCG AGTGGATCCC CAACAACGTC CAGACCGCCC
CMW 62      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756    .......... T......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632    .......... T......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839    .......... T......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758    .......... T......... ........T. .......... .......... ........A. .......... .......... ..........
CMW 8757    .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 9970    .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 9996    .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10025   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10026   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10028   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 9980    .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 9993    .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10626   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10022   .......... T......... ........T. .......... .......... .......... .......... ........A.. ..........
CMW 10024   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10639   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10775   .......... T......... ........T. .........G .......... .......... ..G....... .......... ..........
CMW 10776   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 10777   .......... T......... ........T. .......... .......... .......... .......... .......... ..........
CMW 1651    .......A.. .......... .......... .......... .......... .......... .......... .......T.. ..........
CMW 1652    .......A.. .......... .......... .......... .......... .......... .......... .......T.. ..........
CMW 10455   .......... A......... .......... .......... .......... .......... .......... .......... ..........
CMW 10477   .......... A......... .......... .......... .......... .......... .......... .......... ..........
CMW 10463   .......... .......... .......... .......... .......... .......... .A........ .......... ..........
CMW 10518   .......... .......... ..........T .......... .......... .......... .A........ .......... ..A.......
CMW 10435   .......... .........A .......... .......... .......... .......... .......... .......... ..........
CMW 10442   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 5288    .......... .......... .......... .......... .......... .......... .A.....T.. .......... ..........
CMW 5587    .......... .......... .......... .......... .......... .......... .A.....T.. .......... ..........
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[                370        380        390        400        410        420        430        440        450]
[                .          .          .          .          .          .          .          .          .]
CMW 2113    TCTGCTCCAT CCCCCCCAAG GGTCTCAAGA TGTCCTCCAC CTTTGTTGGC AACTCCACTG CCATCCAGGA GCTCTTCAAG CGTATCGGCG
CMW 62      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025   .G........ .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022   .......... .......... .......... ..A....... .......... .......... .......... .......... ..........
CMW 10024   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651    .......... T......... ..C....... .......G.. ......C... .........C. .......... .......... ...G......
CMW 1652    .......... T......... ..C....... .......G.. ......C... .........C. .......... .......... ...G......
CMW 10455   .......... .......... ..C....... .......... ......C... .........C. .......... .......... ...G......
CMW 10477   .......... .......... ..C....... .......... ......C... .........C. .......... .......... ...G......
CMW 10463   .......... .......... ..C....... .......T.. ......C... .........C. .......... .......... ...G......
CMW 10518   .......... ......T... ..C....... .......T.. ......C... .........C. .......... .......... ...G......
CMW 10435   .......... .....:.G.. .......... .......... .......... .....T..C. .......... ...T...... ..CG.T....
CMW 10442   .......... ........G. .......... .......... .......... .....T..C. .......... ...T...... ..CG.T....
CMW 5288    .G.....G.. ...T...... .......... .......T.. ...C.....T .....G.... .T......... ...G...... ...G......
CMW 5587    .G.....G.. ...T...... .......... .......T.. ...C.....T .....G.... .T......... ...G...... ...G......
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[                460       470       480       490       500       510       520       530       540]
[                .         .         .         .         .  β-tub 2a/2b      .         .          .]
CMW 2113     AGCAGTTCAC TGCTATGTTC CGTCGCAAGG CTTTCTTGCA TTGGTACACT GGCAAACCAT CTCTGGCGAG CACGGCCTCG ACAGCAA-TG
CMW 62       .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775    .......... .......... .......... .......... C......... .......... .......... .......... ..........
CMW 10776    .......... .......... .......... .......... C......... .......... .......... .......... ..........
CMW 10777    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651     .......T.. C..C...... ..G....... .......... .......... .......... ...C...... .......... ..........
CMW 1652     .......T.. C..C...... ..G....... .......... .......... .......... ...C...... .......... ..........
CMW 10455    .......... C..C...... .......... .......... ......T... .......... ...C...... .......... ......G...
CMW 10477    .......... C..C...... .......... .......... ......T... .......... ...C...... .......... ......G...
CMW 10463    .......... C..C...... .......... .......... .......... .......... ...C...... ..T..T.... ..........
CMW 10518    .......... C..C...... .......... .......... .......... .......... ...C...... ..T....... ..........
CMW 10435    .......... C..C...... .......... .C........ .........C .......... ...C...... .......... .....G....
CMW 10442    .......... C..C...... .......... .C........ .........C .......... ...C...... .......... .....G....
CMW 5288     .......... ...C...... A.G....... .......... .......... .......... .......... .....T.... ..........
CMW 5587     .......... ...C...... A.G....... .......... .......... .......... .......... .....T.... ..........
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[                 550       560          570         580         590         600         610         620       630]
[                  .         .            .           .           .           .           .           .        .]
CMW 2113     GCGTGTACGT ---ACCCTCC TGTTGCACCA GGCGG----- ---------- -CGCGCCTC- --GAGCTT-C CC-GCTGACC A-CTGCACAG
CMW 62       .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756     .......... .......... ..C....... .......... .......... .......... .......... .......... ...C......
CMW 2632     .......... .......... ..C....... .......... .......... .......... .......... .......... ...C......
CMW 3839     .......... .......... ..C....... .......... .......... .......... .......... .......... ...C......
CMW 8758     .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 8757     .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 9970     .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 9996     .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10025    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10026    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10028    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 9980     .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 9993     .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10626    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10022    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10024    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10639    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10775    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10776    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 10777    .......... .......... ..C....... .......... .......... .......... .......... .......... ..........
CMW 1651     .......... ........AT CT------.GG CTT--CCCAA G.CAAGACAG A....A..T. ........T. .T........ ...CA..T..
CMW 1652     .......... ........AT CT------.GG CTT--CCCAA G.CAAGACAG A....A..T. ........T. .T........ ...CA..T..
CMW 10455    .......T.. ......G.G. CT.ACAC.GG CTTT.CCCAG A.CAAGACAG A.......T. .......CT. .TT....... .A.CA.....
CMW 10477    .......T.. ......G.G. CT.A-....G .CTTTGCCCA GACAAGACAG A.......T. .......CT. .TT....... .A.CA.....
CMW 10463    .......T.. ........A. CT-------G .CTT-CCCCA G.CAAGATAG A....A..T. GT......T. .T.......G ...CA..T..
CMW 10518    .......T.. .....T..A. CT-------G .CTT-CCCCA G.CAAGATAG A....A..T. GT...AC.T. .T......T. ...CA..T..
CMW 10435    .......T.. TGT.....A. ..C..----- -----CCCGG CC.......G A......... .G.GC.C.T. ..C....... ...C......
CMW 10442    .......T.. TGT.....A. ..C..----- -----CCCGG CC.......G A......... .G.GC.C.T. ..C....... ...C......
CMW 5288     .......... ......TCGT ATC------- -----CCCTG CCCACTGGTC T..TC.TCTC CCTC.GC.TG G..A.....A ..........
CMW 5587     .......T.. ......TCGT ATC------- -----CCCTG CCCACTGGTC T..TC.TCTC CCTC.GC.TG G..A.....A ..T.......
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[                640       650       660       670       680       690       700       710       720]
[                .         .         .         .         .         .         .         .         .]
CMW 2113    CTACAACGGC ACCTCCGAGC TCCAGCTCGA GCGCATGAAC GTCTACTTCA ACGAGGTATG TC-TGT---- ----CGG--G AC-CA-GGCT
CMW 62      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756    .......... .......... .T........ .......... .......... .......... .......... .......... .......T..
CMW 2632    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651    .......... .......... .......... .......... .........T .......... ....TAT... ........GT. .T...A.-..
CMW 1652    .......... .......... .......... .......... .........T .......... ....TAT... ........GT. .TA..A.-..
CMW 10455   ......T... .......... .......... .......... .......... .......... ..C.TATCAT CCAT...GT. .....A....
CMW 10477   ......T... .......... .......... .......... .......... .......... ..C.TATCAT CCAT...GT. .....A....
CMW 10463   .......... .......... .......... .......... .......... .......... ....TAT... ........GT. ..........
CMW 10518   .......... .......... .......... .......... .......... .......... ....TAT... ........GT. ..........
CMW 10435   .......... .......... .......... .......... .......... .......... ...-TAT... ....G..G.. .........C
CMW 10442   .......... .......... .......... .......... .......... .......... ...-TAT... ....G..G.. .........C
CMW 5288    T......... ..T....... .......... .......... .......... ........A. ..AACAGCCA CGTCGTCAAT T.AA.TTTGA
CMW 5587    T......... ..T....... .......... .......... .......... ........A. ..AACAGCCA CGTCGTCAAT C.AA.TTTGA
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[                730       740       750       760       770       780       790       800       810]
[                .         .         .         .         .         .         .         .         .]
CMW 2113     GGCGCGTCA- --------TC CCGCCCGCGA ACCCCCTGTG CGT-----GA CCGAGCTCCC G--------- --------CT GACGCGCTCC
CMW 62       ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993     ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777    ..G..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651     CAA.-C.T-C ACCTCGGC-A A.C...C.CC C..TTTCCG. G.CCTT.... .TTCTGGTAT AGGCGAGCTT CC.TCTT... .........T.
CMW 1652     ACAAGC.TCC ACCTGGGCCA A.C...C.CC C..TTTCCG. G.CCTTCT.. .TTCTGGTAT AGGCGAGCAT CC.TCTT... .........T.
CMW 10455    CAA..A..CA TCTCGACC.T GG....C.C. .......C.C .C-....... .TTCTGG.AT AGGCGAAGTT CCCTCTTT.. .........TT
CMW 10477    CAA..A..CA TCTCGACC.T GG....C.C. .......C.C .C-....... .TTCTGG.AT AGGCGAAGTT CCCTCTTT.. .........TT
CMW 10463    C.A..A..CA TCTCAACCC. ..C...CTCC CAAAT.CCG. GCCCCTC... .TTCTGG.AT AGGCGAGCTT CC.TCTT... .........T.
CMW 10518    C.A..A..CA TCTCAGCCCA ..C.TGTTCC ---------- ---.CTC.C. .TTCTGGTA. AGGCGAGCTT CC.TCTT... .........T.
CMW 10435    CTG------. .....GCG.G G.C..GC.CG CGG...CTG. ---..CGT.. .......... A......... .......... ..........
CMW 10442    CTG------. .....GCG.G G.C..GC.CG CGG...CTG. ---..CGT.. .......... A......... .......... ..........
CMW 5288     CAAC.TA.GG CA.....-- -T.GTTT--- ---------- .CCGCCGTCG .---------- -CAAGGCCTT G......... A....AT.TA
CMW 5587     CAAC.TA.GG CA.....-- -T.GTTT--- ---------- ..CGCCGTCG .---------- -CAAGGCCTT G......... A....AT.TA
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[                820       830       840       850       860       870       880       890       900]
[                .         .         .         .         .         .         .         .         .]
CMW 2113    -TGTCACAGG CCTCCGGCAA CAAGTATGTC CCCCGCGCCG TCCTCGTCGA TCTCGAGCCC GGCACCATGG ACGCCGTCCG TGCCGGCCCC
CMW 62      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756    .......... .......... .........T .......... .......... .........T .......... .......... ..........
CMW 2632    .......... .......... .........T .......... .......... .........T .......... .......... ..........
CMW 3839    .......... .......... .........T .......... .......... .........T .......... .......... ..........
CMW 8758    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651    T....-.... .......... .........T .......... .......... .......... ..T....... .T....... C..T......
CMW 1652    T....-.... .......... .........T .......... .......... .......... ..T....... .T....... C..T......
CMW 10455   T....-.... .T....C... .........T .......... .......... .........G ..T....... .T....... C..........
CMW 10477   T....-.... .T....C... .........T .......... .......... .........G ..T....... .T....... C..........
CMW 10463   T.A..-.... .....A.... .........T ........A. .......... .......... ..T....... .T....... C..........
CMW 10518   T.A..-.... .....A.... .........T ........T. .......... .......... ..T....... .......... C..........
CMW 10435   .....-.... .......... .......... .......... .......... .......... ..T....... .......... C..........
CMW 10442   .....-.... .......... .......... .......... .......... .......... ..T....... .......... C..........
CMW 5288    TC.C.-.... .......... .........G ..T....... .......... .......... ..T....... .......... ......T...
CMW 5587    TC.C.-.... .......... .........G ..T....... .......... .......... ..T....... .......... ......T...
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[                910       920       930       940       950       960       970       980       990]
[                .         .         .         .      ITS 1 →      .         .         .         .]
CMW 2113    TTCGGCCAGC TGTTCCGCCC CGACAACTTC GTCTTCGGCC AGTCCCCCAG ATACCCTTTG TGAACTTATA -CCTTTTTAT CGTTGCCTCG
CMW 62      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022   .......... .......... .......... .......... .......... ...G...... .......... ......G...
CMW 10024   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639   .......... .......... .......... .......... .......... ........G. .......... .......... ..........
CMW 10775   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777   .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651    ..T..T.... .......T.. .......... .....T.... .......... .......... .......... ...A..A..... ..........
CMW 1652    ..T..T.... .......T.. .......... .....T.... .......... .......... .......... ...A..A..... ..........
CMW 10455   ..T..T.... .......T.. .......... .....T.... .......... .......... .......... ....A..... ..........
CMW 10477   ..T..T.... .......T.. .......... .....T.... .......... .......... .......... ....A..... ..........
CMW 10463   ..T..T.... .......T.. .......... .....T.... .......... .......... ......C... ....A..... ..........
CMW 10518   ..T..... .......T.. .......... .....T.... .......... .......... ......C... ....A..... ..........
CMW 10435   .......... .......... .......... .......... .......... .......... .......... ....A..... ..........
CMW 10442   .......... .......... .......... .......... .......... .......... .......... ....A..... ..........
CMW 5288    .......... .......... .......... .......... .......... .A........ .......... ....----.. ..........
CMW 5587    .......... .......... .......... .......... .......... .A........ .......... ....----.. ..........
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[                    1000       1010       1020       1030       1040       1050       1060       1070     1080]
[                    .          .          .          .          .          .          .          .          .]
CMW 2113      GCGCCGAGCC --GGGAGTGC TCTTCTGTGC ---------- ---------- --------TC CCC------- ---------- ------CACC
CMW 62        .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993      .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775     .........T .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651      ....T..... TCT..G.G-- ---------.G GGTTGGCGAA GGCAGATTTT CTTCCTTC.. ....TCCCTC CCCCCCCT.. ..CTTC....
CMW 1652      ....T..... TCT..G.G-- ---------.G GGTTGGCGAA GGCAGATTTT CTTCCTTC.. ....TCCCTC CCCCCCCT.. ..CTTC....
CMW 10455     ....T..... CG...G--AG GGAAAAAAAA AAAAAAAAGG GGGGAAATTC TGTTTCCCCT TTTC.TTTTT CCCCCCCTTC CCCTTCAT..
CMW 10477     ....T..... C....G.GAG GGAAAAAAAA AAAAAAAAGG GGGGAAATTT GTTTCCCCCT TTTTTTTTTT CCCCCCCTTC CCCTTTAT..
CMW 10463     ....T..... CC...G.G.A .T..T--GAG AGAGTC..TC TCTCTCCTTC CTTC.....-- -T.GC..... .....CTTCT ACC.------
CMW 10518     ....T..... CC...G.G.A .T..T--GAG AGAGTC..TC TCTCTCCTTC CTTC.....-- -T.GC..... .....CTTCT C.---------
CMW 10435     ....T.....- ..T..G.GC- ---------- .......... ....ACTCTC CTGTG..CC. ...C...... .....ACCGT GCAAGCG---
CMW 10442     ....T.....- ..T..G.GC- ---------- .......... ....ACTCTC CTGTG..CC. ...C...... .....ACCGT GCAAGCG---
CMW 5288      ...AA.GC.G GCC------- ---------- .......... .......... .......... .......... .....ACCGA GGCCCCTTGG
CMW 5587      ...AA.GC.G GCC------- ---------- .......... .......... .......... .......... .....ACCGA GGCCCCTTGG
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[                  1090       1100       1110       1120       1130       1140       1150       1160     1170]
[                  .          .          .          .          .          .          .          .          .]
CMW 2113     GCGCAAGCAG TG------GA GCAGGCCCGC CGGCGGCCCA CCAAACTCTT TGTTTTTAGA A-CGTATCTC TTCTGAGTGT TTATAACAAA
CMW 62       .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776    .......... .......... .......... .......... .T........ .......... .......... .......... ..........
CMW 10777    .......... .......... .......... .......... .T........ .......... .......... .......... ..........
CMW 1651     .T....A.G. .TGTTGGG.. .......... .......... .T........ ........T. ...C...... ........AC A..A.-....
CMW 1652     .T....A.G. .TGTTGGG.. .......... .......... .T........ ........T. ...C...... ........AC A..A.-....
CMW 10455    .T.T..AATC G.GTGCTG.. .....G.... .......... TT........ ..G.....A. .A.C...... ..T.....T- -..A..A...
CMW 10477    .G.A..AATC G.GGGCTG.. A.......C. .......... TT.....T.. ........A. ...C.....T ..T.....T- -..A..A...
CMW 10463    .T....A.G. .TGTT..G.. .......... .......... .......... ........T. ...C...... ........AC A.T...A...
CMW 10518    .T....A.G. .TGTT..G.. .......... .......... .......... ........T. ...C...... ........AC A.T...A...
CMW 10435    .T.------- --........ .......... .......... .......... .......... C......... C......... ...C..A..-
CMW 10442    .T.------- --........ .......... .......... .......... .......... C......... C......... ...C..A..-
CMW 5288     .AA...--- --........ ....-..... ........A. .......... -....C.TAG T.-.A..--- C.....--- -..A..A...
CMW 5587     .AA...--- --........ ....-..... ........A. .......... -....C.TAG T.-.A..--- C.....--- -..A..A...
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[                1180       1190       1200       1210       1220       1230       1240       1250     1260]
[                .    5.8S →          .          .          .          .          .          .          .]
CMW 2113     CAAA--TGAA TCAAAACTTT CAACAACGGA TCTCTTGGTT CTGGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAAT GTGAATTGCA
CMW 62       .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 2632     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651     A...AA.... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1652     A...AA.... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10455    A...A..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10477    A...A..... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10463    A...A..... .......... .......... .......... .......... ....A..... .......... .......... ..........
CMW 10518    A...A..... .......... .......... .......... .......... ....A..... .......... .......... ..........
CMW 10435    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10442    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 5288     ..T.AA.... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 5587     ..T.AA.... .......... .......... .......... .......... .......... .......... .......... ..........
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[                  1270        1280        1290        1300        1310        1320        1330        1340     1350]
[                  .           .           .           .           .           .           .           ITS 2 →    .]
CMW 2113     GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC CCGCTGGAAT TCCAGCGGGC ATGCCTGTTC GAGCGTCATT TCAACCCTCA
CMW 62       .......... .......... .......... .......... ..CG...... .......... .......... .......... ..........
CMW 8755     .......... .......... .......... .......... ..CG...... .......... .......... .......... ..........
CMW 8756     .......... .......... .......... .......... ..CG...... .......... .......... .......... ..........
CMW 2632     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 3839     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8758     .......... .......... .......... .......... ..CG...... .......... .......... .......... ..........
CMW 8757     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9996     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10024    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10776    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651     .......... .....G.... .......... .......... .......... .......... ....-..... .......... ..........
CMW 1652     .......... .....G.... .......... .......... .......... .......... ....-..... .......... ..........
CMW 10455    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10477    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10463    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10518    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10435    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10442    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 5288     .......... .......... .......... .......... ..T....T.. ...G.A.... .......... .......... ..........
CMW 5587     .......... .......... .......... .......... ..T....T.. ...G.A.... .......... .......... ..........
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[                  1360       1370       1380       1390       1400       1410       1420       1430     1440]
[                  .          .          .          .          .          .          .          .          .]
CMW 2113     AGCCTGGCTT GGTGTTGGGG CACTACCTGT TC-ACAGCGG GTAGGCCCTG AAATTTAATG GCGGGCTCGC TAAGACTCTG AGCGTAGTAG
CMW 62       .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8755     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8756     .......... .......... ....G..... .TT....... .......... .......G.. .......... .......... .A........
CMW 2632     .......... .......... ....G..... .TT....... .......... .......G.. .......... .......... ..........
CMW 3839     .......... .........N ....G..... .TT....... .......... .......G.. .......... .......... ..........
CMW 8758     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 8757     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9970     .......... .......... .......... .......... .......... .......... .......... .N........ ..........
CMW 9996     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10025    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10026    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10028    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9980     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 9993     .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10626    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10022    .......... .......... .......CT. .......... .......... .......... .......... .......... ..........
CMW 10024    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10639    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10775    .......... .......... .......... .T........ ..G....... .......... .......... .......... ..........
CMW 10776    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 10777    .......... .......... .......... .......... .......... .......... .......... .......... ..........
CMW 1651     ...T...... .......... T......C.. AA.--.A... -......... .......G.. .......... .......... ...C......
CMW 1652     ...T...... .......... T......C.. AA.--.A... -......... .......G.. .......... .......... ..........
CMW 10455    ...T.A.... .......... ......TC.. AA.--.A... -......... ....C..G.. .......... .......... ..........
CMW 10477    ...T.A.... .......... ......TC.. AA.--.A... -......... ....C..G.. .......... .......... ..........
CMW 10463    .......... .......... T......C.. CA.--.A... -......... .......G.. .......... .......... ..........
CMW 10518    .......... .......... T......C.. CA.--.A... -......... .......G.. .......... .......... ..........
CMW 10435    .......... .......... .......... A..--.A... -......... .......G.. .......... .......... ..........
CMW 10442    .......... .......... .......... A..--.A... -......... .......G.. .......... .......... ..........
CMW 5288     .......... ....A..... ....G.T.CC GAG.GG.A.- -C........ ....C..G.. ...A...... C.G...C.C. ..........
CMW 5587     .......... ....A..... ....G.T.CC GAG.GG.A.- -C........ ....C..G.. ...A...... C.G...C.C. ..........
```

```
[                    1450        1460        1470        1480        1490  1500]
[                    .           .           .           .           .        .]
CMW 2113     TTTTTAT--- ---CACCTCG CTTTGGAA-G GATTAGCGG- TGCTCTTGCC GTAAAACC
CMW 62       .......... .......... .......... .......... .......... ........
CMW 8755     .......... .......... .......... .......... .......... ........
CMW 8756     .......... .......... .......... .....CGA.. ...C...... ........
CMW 2632     .......... .......... .......... .......... .......... ........
CMW 3839     .......... .......... .......... .......A.. .......... ........
CMW 8758     .......... .......... .......... .......... .......... ........
CMW 8757     .......... .......... .......... .......... .......... ........
CMW 9970     .......... .......... .......... .......... .......... .....C..
CMW 9996     .......... .......... .......... .......... .......... ........
CMW 10025    .......... .......... .......... .......... .......... ........
CMW 10026    .......... .......... .......... .......... .......... ........
CMW 10028    .......... .......... .......... .......... .......... ........
CMW 9980     .......... .......... .......... .......... .......... ........
CMW 9993     .......... .......... .......... .......... .......... ........
CMW 10626    .......... .......... .......... .....T.... .......... .......T
CMW 10022    .......... .......... .......... ......NNN. NNNNNNNNNN NNNNNNNN
CMW 10024    .......... .......... .......... .......... .......... .......T
CMW 10639    .......... .......... .......... .......... .......... ........
CMW 10775    .......... .......... .......... .......... .......... ........
CMW 10776    .......... .......... .......... .......... .......... ........
CMW 10777    .......... .......... .......... .......... .........G ........
CMW 1651     .....T.TTC TTCA...... .......... .........T .......... ........
CMW 1652     .....T.TTC TTCA...... .......... .........T .......... ........
CMW 10455    .....T.TTC TTC....... ........A. .........T ....T..... ........
CMW 10477    .....T.TTC TTC....... ........A. .........T ....T..... ........
CMW 10463    .....T-... ..CA...... .......... .........T .......... ........
CMW 10518    .....T-... ..CA...... .......... .........T .......... ........
CMW 10435    ..--...... .......... .......... .........T -......... ........
CMW 10442    ..--...... .......... .......... .........T -......... ........
CMW 5288     ..A-...... ...---.... ..CC....G. CCC.G....T -..C..-... ..T.....
CMW 5587     ..A-...... ...---.... ..CC....G. CCC.G....T -..C..-... ..T.....
```

Fig. 4. The phylogenetic tree (tree length = 1198 steps, consistency index/CI = 0.8, retention index/RI = 0.9) generated from a combined data set comprising ribosomal and β-tubulin gene sequences. Confidence levels of the tree branch nodes >50% are indicated and were determined by a 1000 replicate bootstrap analysis. Isolates sequenced in this study are bolded. Host species for *C. cubensis* are indicated in capital letters. The *Diaporthe ambigua* isolates were used as the outgroup taxa.

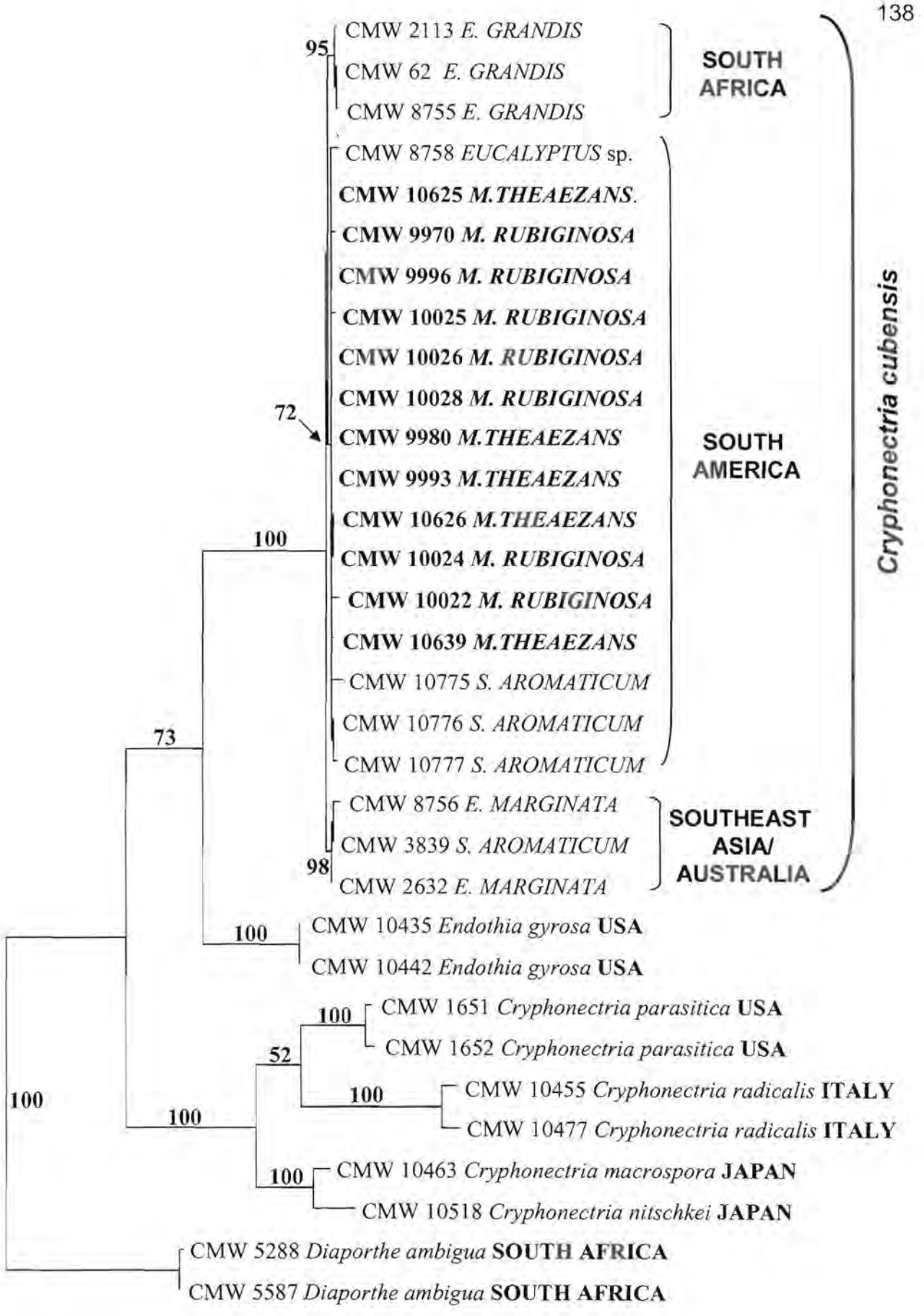


Fig. 5. Light micrographs of *Cryphonectria cubensis* on *Miconia rubiginosa* in Colombia. **(a)**. Perithecial neck and orange stromatic tissue (arrow) on bark. **(b)**. Vertical section through ascoma. **(c)**. Perithecial neck and surrounding tissue (arrow). **(d)**. Stromatic tissue of ascoma. **(e)**. Ascus. **(f)**. Ascospores. **(g)**. Conidioma on bark. **(h)**. Vertical section through conidioma. **(i)**. Tissue of the conidiomal base and neck (arrow). **(j)**. Conidiophores. **(k)**. Enteroblastic phialidic conidiogenous cell (arrow). **(l)**. Conidia. Bars a-b, g-h = 100 µm; c-d, i = 20 µm; e-f, j-l = 10 µm.

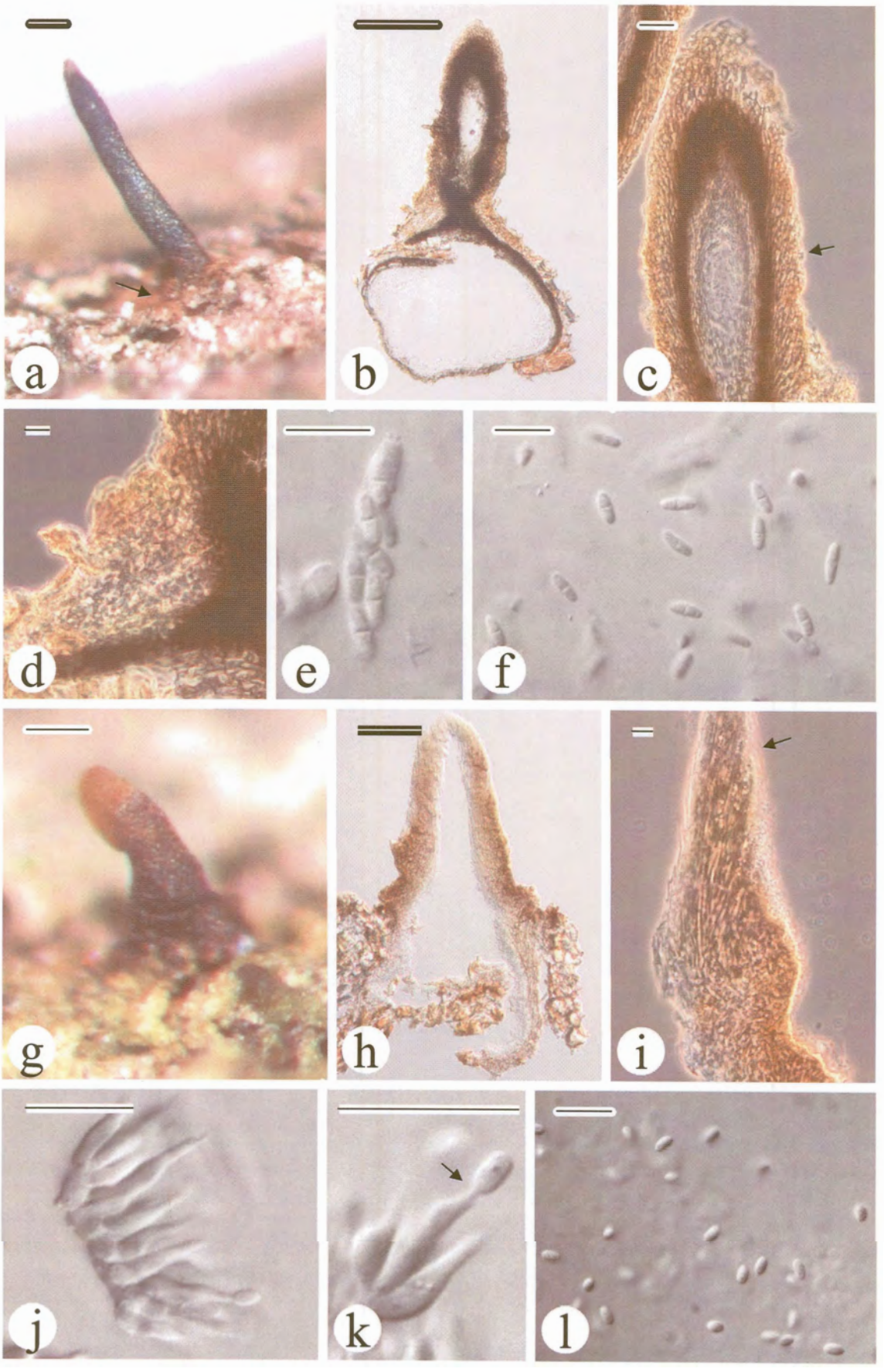


Fig. 6. Results of inoculation trial with isolates of *Cryphonectria cubensis* from *Miconia theaezans* (CMW 10625, CMW 10626) and *Eucalyptus grandis* (CMW 10640, CMW 10638, CMW 10639) from Colombia, and a negative control. Inoculations were done in a greenhouse on seven-month-old *Tibouchina urvilleana*. Mean length of lesions is shown with 95% confidence limits.

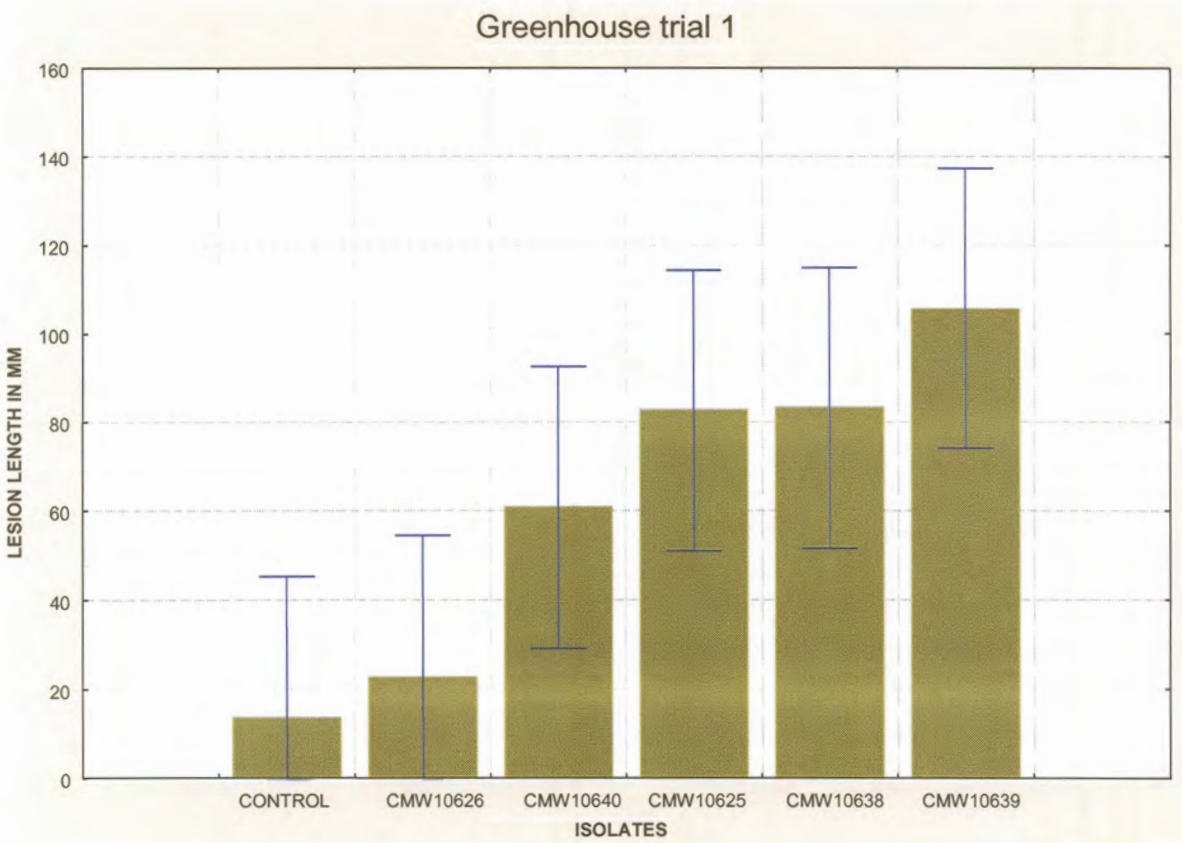

Greenhouse trial 1
LESION LENGTH IN MM
160
140
120
100
80
60
40
20
0
CONTROL
CMW10626
CMW10640
CMW10625
CMW10638
CMW10639
ISOLATES

Fig. 7. Results of inoculation trials in the greenhouse with isolates of *Cryphonectria cubensis* from *Miconia rubiginosa* (CMW 10022, CMW 10024) and a negative control. Inoculations were done on one-year-old *Tibouchina urvilleana* and a ZG14 clone of *Eucalyptus grandis*. A *C. cubensis* isolate from *E. grandis* in South Africa (CMW 2113) was also included. Mean length of lesions is shown with 95% confidence limits.

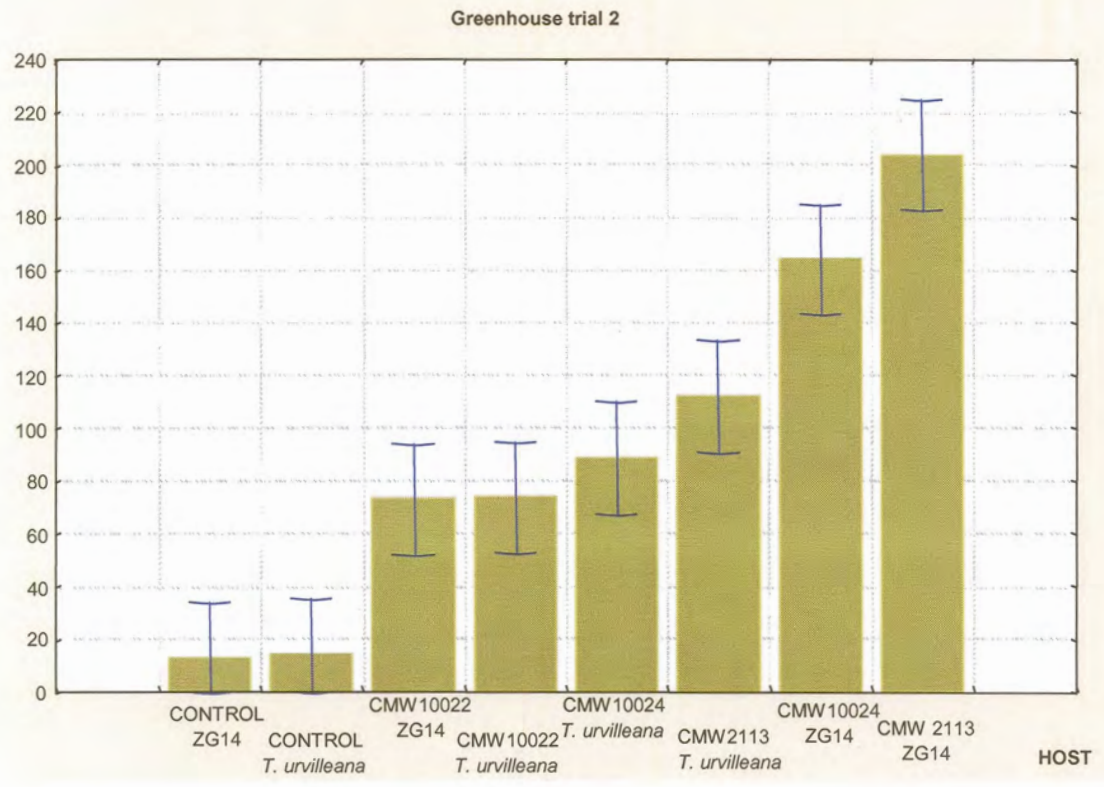

Greenhouse trial 2
240
220
200
180
160
140
120
100
80
60
40
20
0
CONTROL ZG14
CONTROL T. urvilleana
CMW10022 ZG14
CMW10022 T. urvilleana
CMW10024 T. urvilleana
CMW2113 T. urvilleana
CMW10024 ZG14
CMW 2113 ZG14
HOST

Fig. 8. Lesions produced by isolates of *Cryphonectria cubensis* from *Miconia theaezans* (CMW 10625) and *M. rubiginosa* (CMW 10022) on various hosts inoculated in field trials in Colombia. Control inoculations are indicated with a "c". **(a)**. Lesions on *Tibouchina urvilleana* inoculated with isolate CMW 10625. **(b)**. Lesions on *T. lepidota* inoculated with isolate CMW 10625. **(c)**. Lesions on *T. semidecandra* inoculated with CMW 10625. **(d)**. Lesions on *M. theaezans* inoculated with CMW 10625. **(e)**. Lesions on an *Eucalyptus grandis* clone (274) inoculated with CMW 10625. **(f)**. Lesions on *M. rubiginosa* inoculated with CMW 10022. **(g)**. Lesions on an *E. grandis* clone (275) inoculated with CMW 10022. **(h)**. Lesions on a cross between *E. grandis* and *E. urophylla* (*"E. urograndis"* 212) inoculated with isolate CMW 10022.

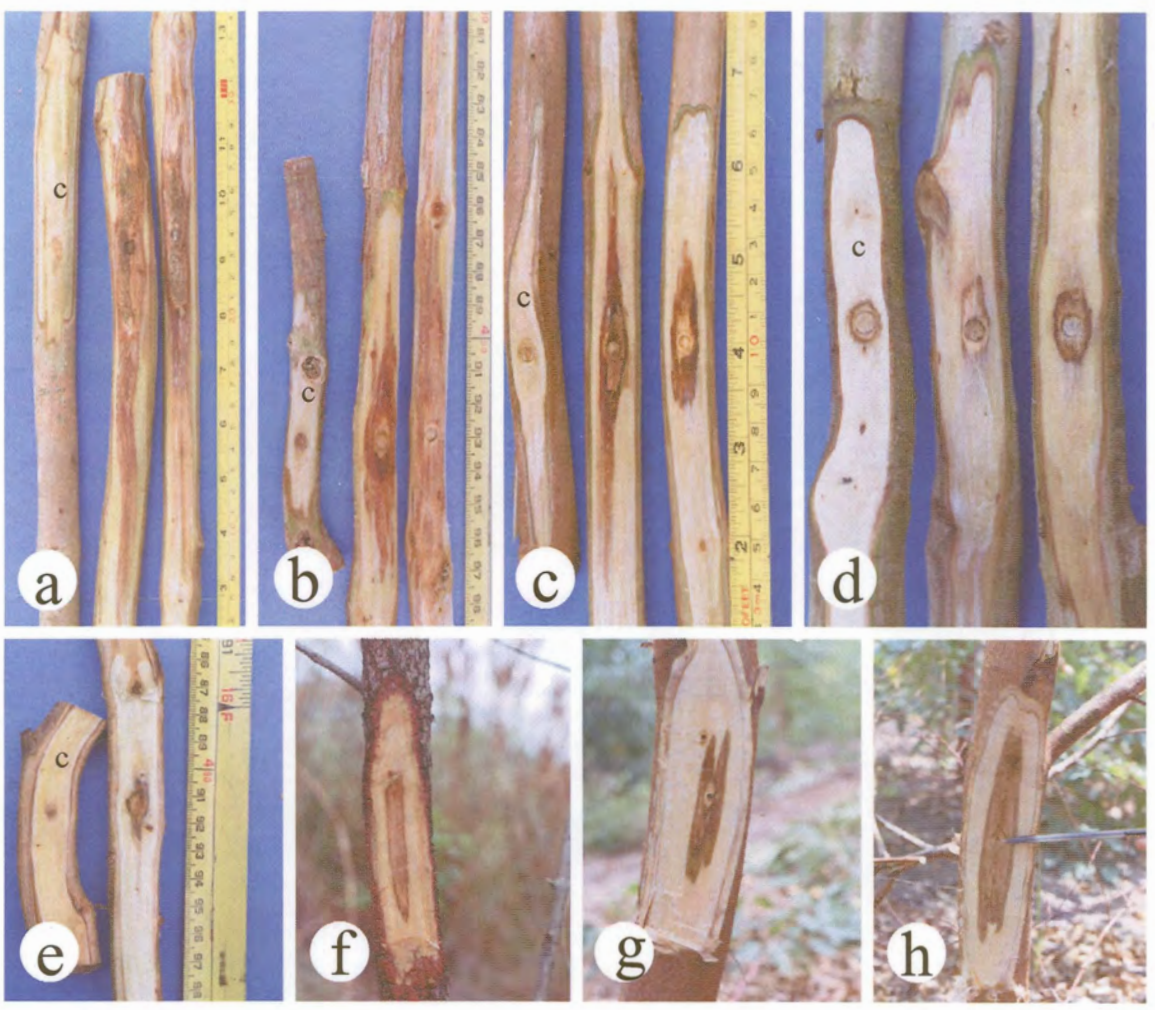


Fig. 9. Results of inoculation trials with isolates of *Cryphonectria cubensis* from *Miconia theaezans* (CMW 10625) and *Eucalyptus grandis* (CMW 10639) from Colombia, and a negative control. The field inoculations were done in Colombia on one-year-old *Tibouchina urvilleana*, *T. lepidota*, *T. semidecandra*, *M. theaezans* and an *E. grandis* clone (274). Mean lesion length is shown with 95% confidence limits.

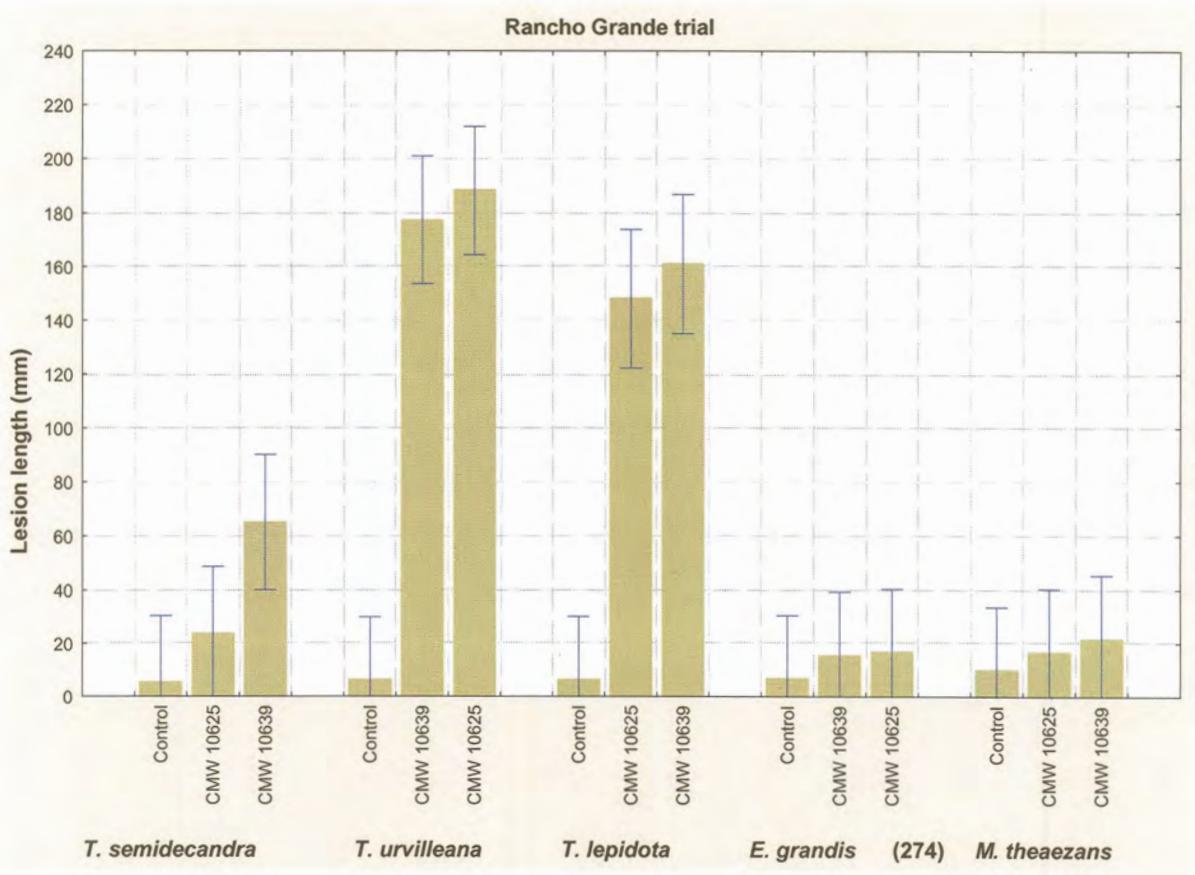
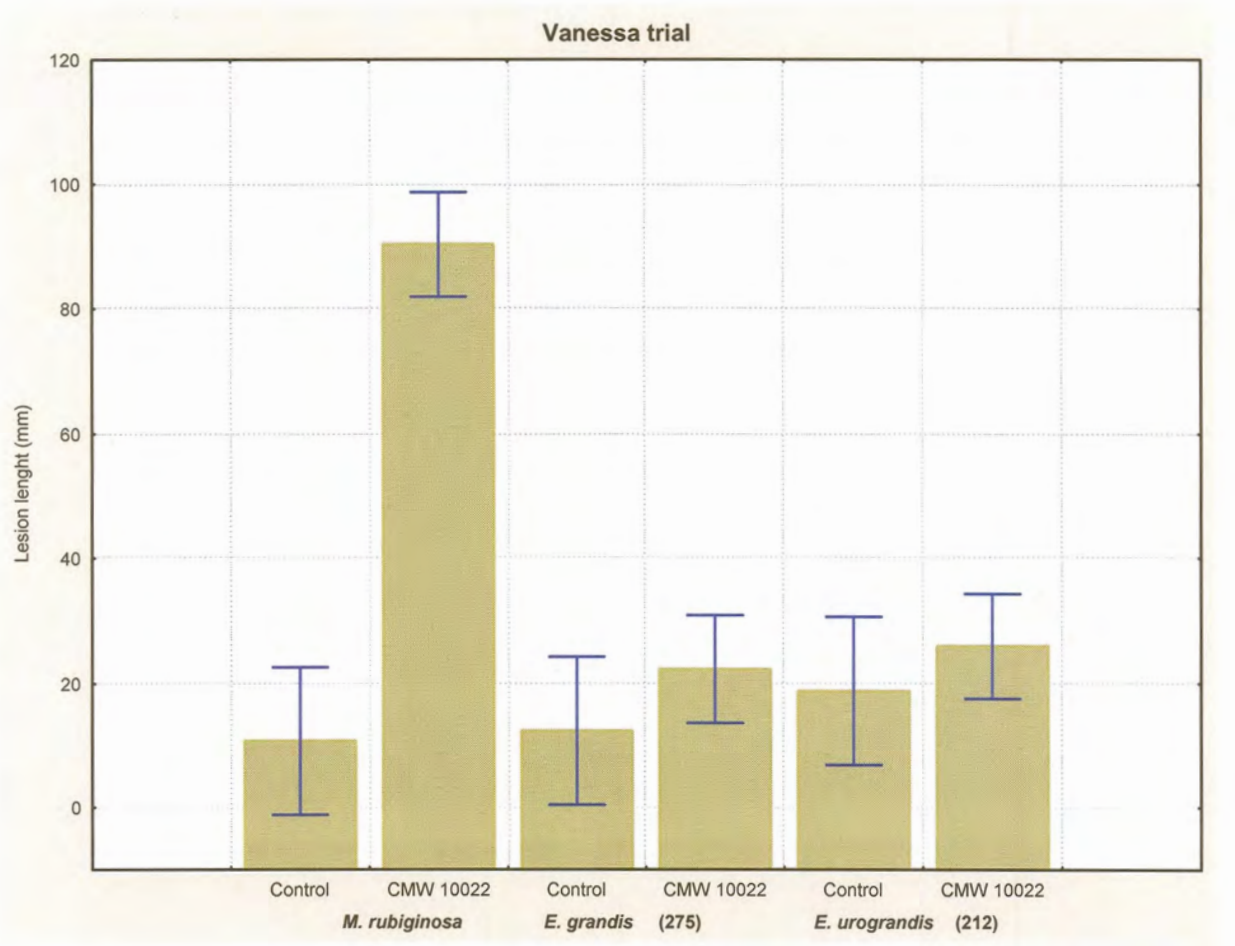


Fig. 10. Results of field inoculation trials with an isolate of *Cryphonectria cubensis* from *Miconia rubiginosa* (CMW 10022) and a negative control. Inoculations were done on six-year-old *M. rubiginosa,* a three-year-old *E. grandis* clone (275) and an *E. urograndis* cross (212). Mean length of lesions is shown with 95% confidence limits.



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.

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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.

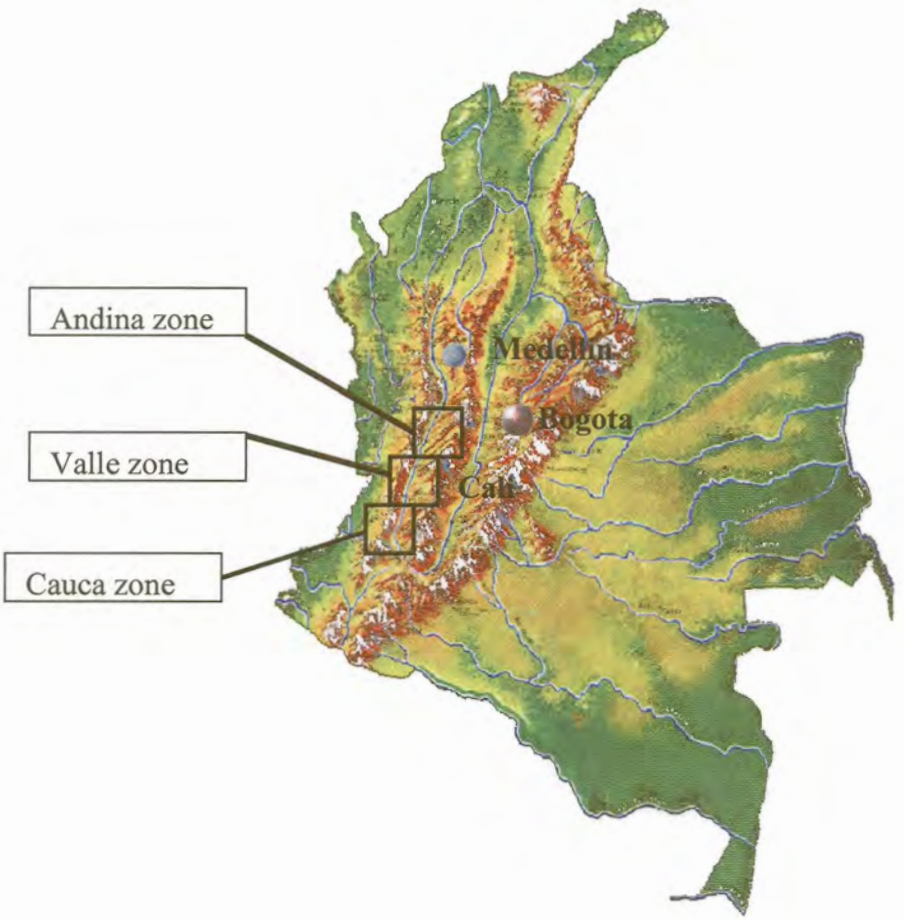

Fig. 3. Morphology of *Cylindrocladium spathulatum*. **(a)** Superficial sporulation on a *Eucalyptus grandis* leaf. **(b-d)** Colony morphology on MEA at (left to right) three days, five days and eight days. **(e)** Perithecia produced on carnation leaf in CLA. **(f)** Perithecia produced on MEA medium. **(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.

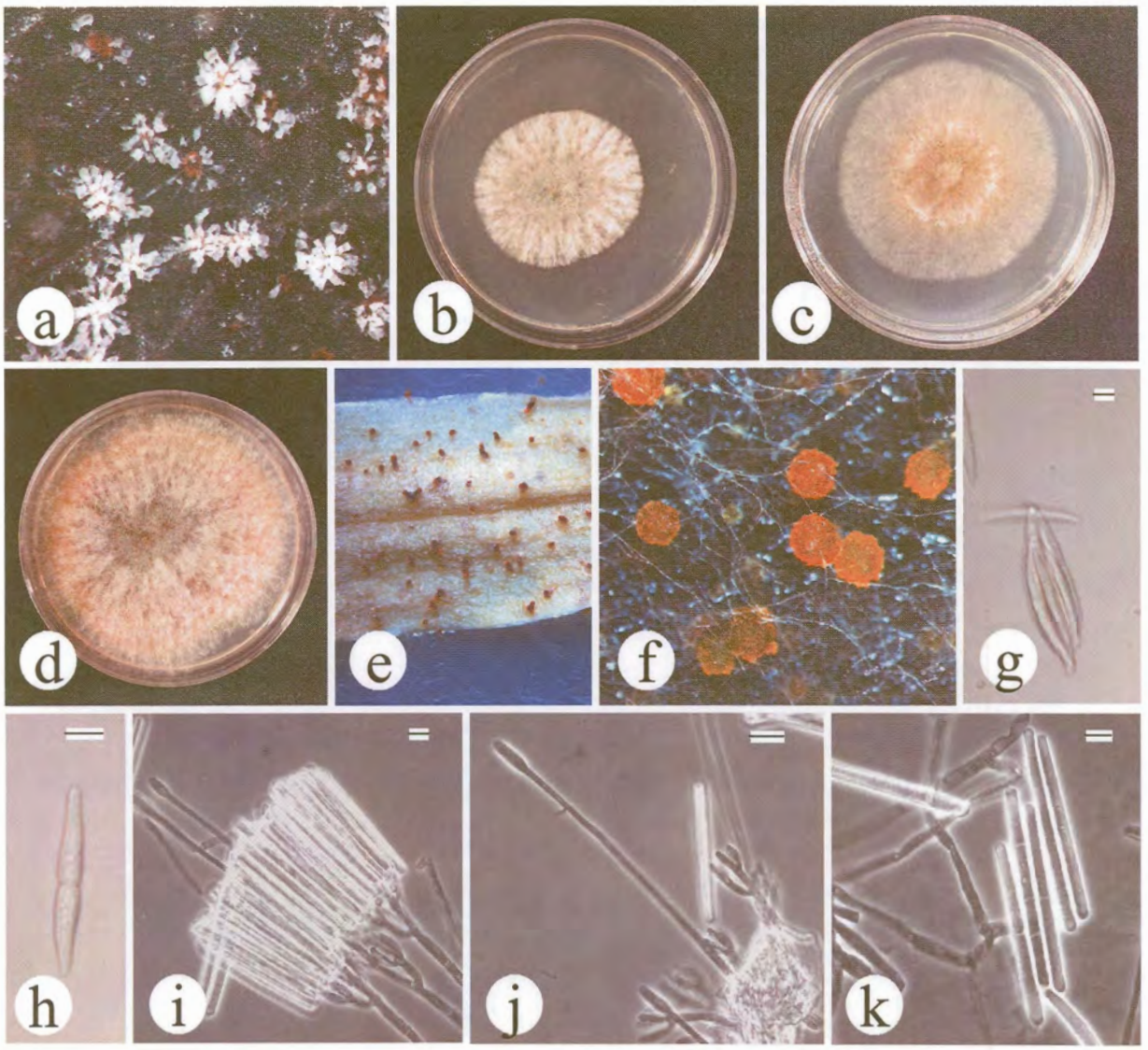

Fig. 4. Raw sequence data of the β-tubulin gene. Unknown sequence characters are indicated with a "**N**", while gaps inserted to achieve sequences alignment are indicated with "-". Bases matching those of STE-U 2712 are indicated with a ".".

```
[                    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
```

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[                    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.
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 [                    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.

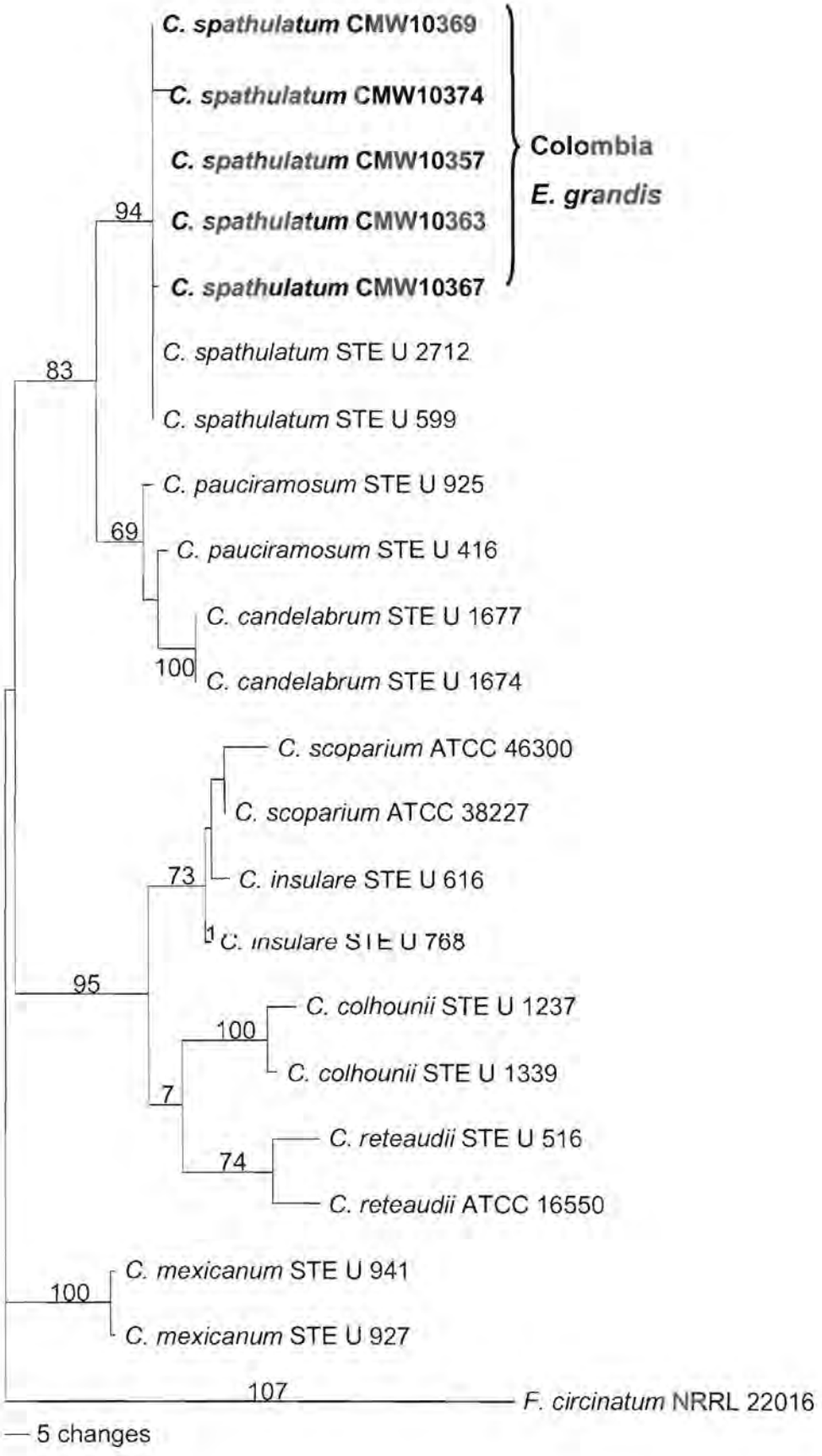
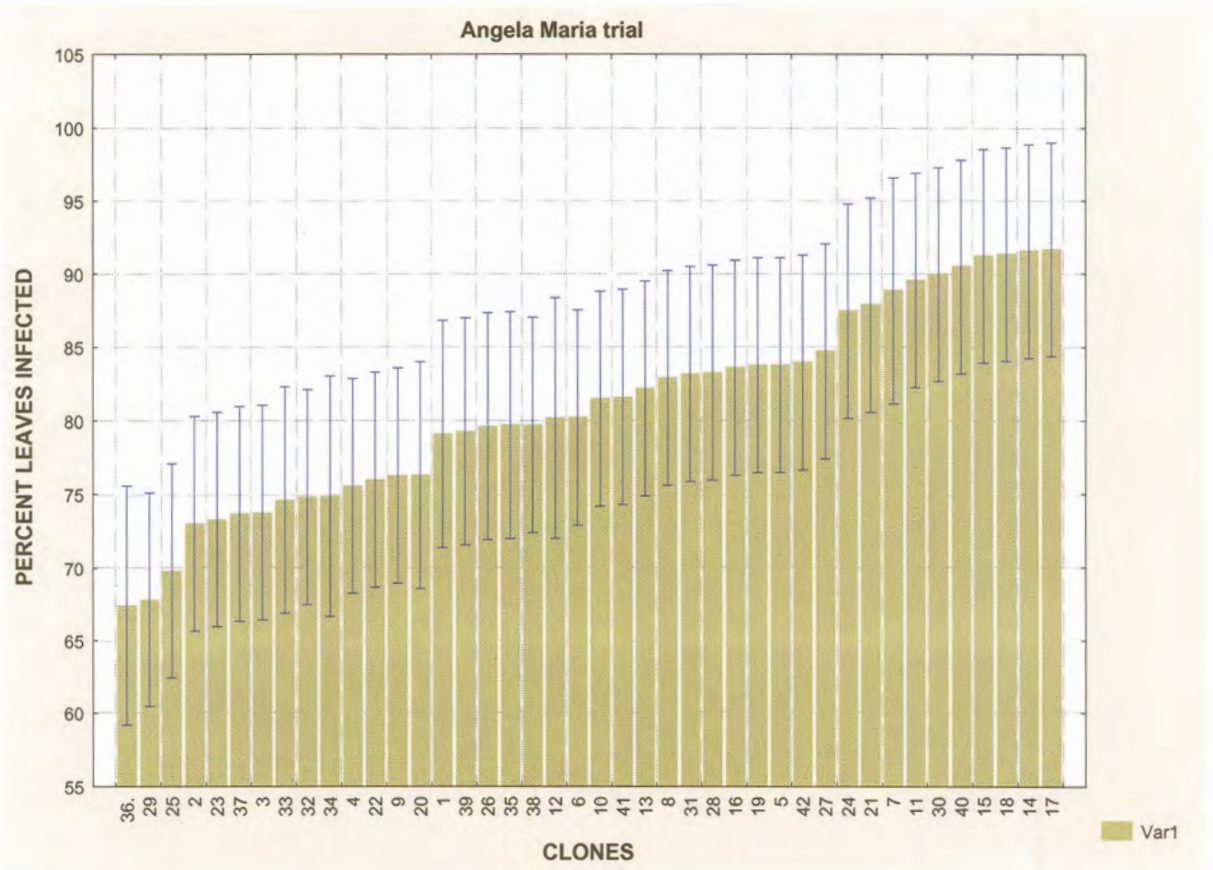


Fig. 6. Graphical presentation of leaves of 42 *Eucalyptus grandis* clones infected by *Cylindrocladium spathulatum* on the Angela Maria farm in Colombia. Data are presented as percentage leaves infected and 95% confidence limits are also shown.



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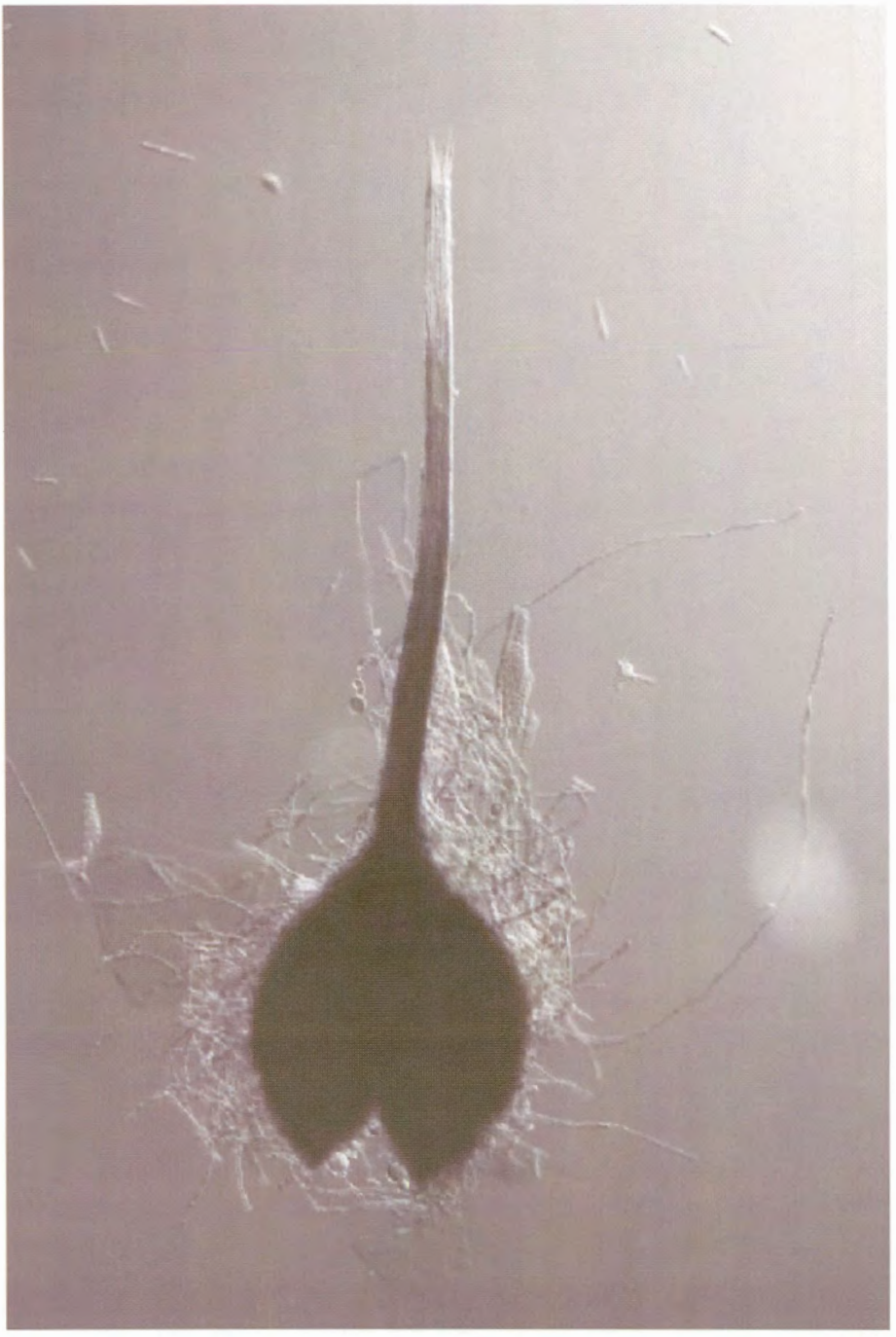

First report of *Ceratocystis fimbriata* on *Eucalyptus grandis* in Colombia

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.

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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.

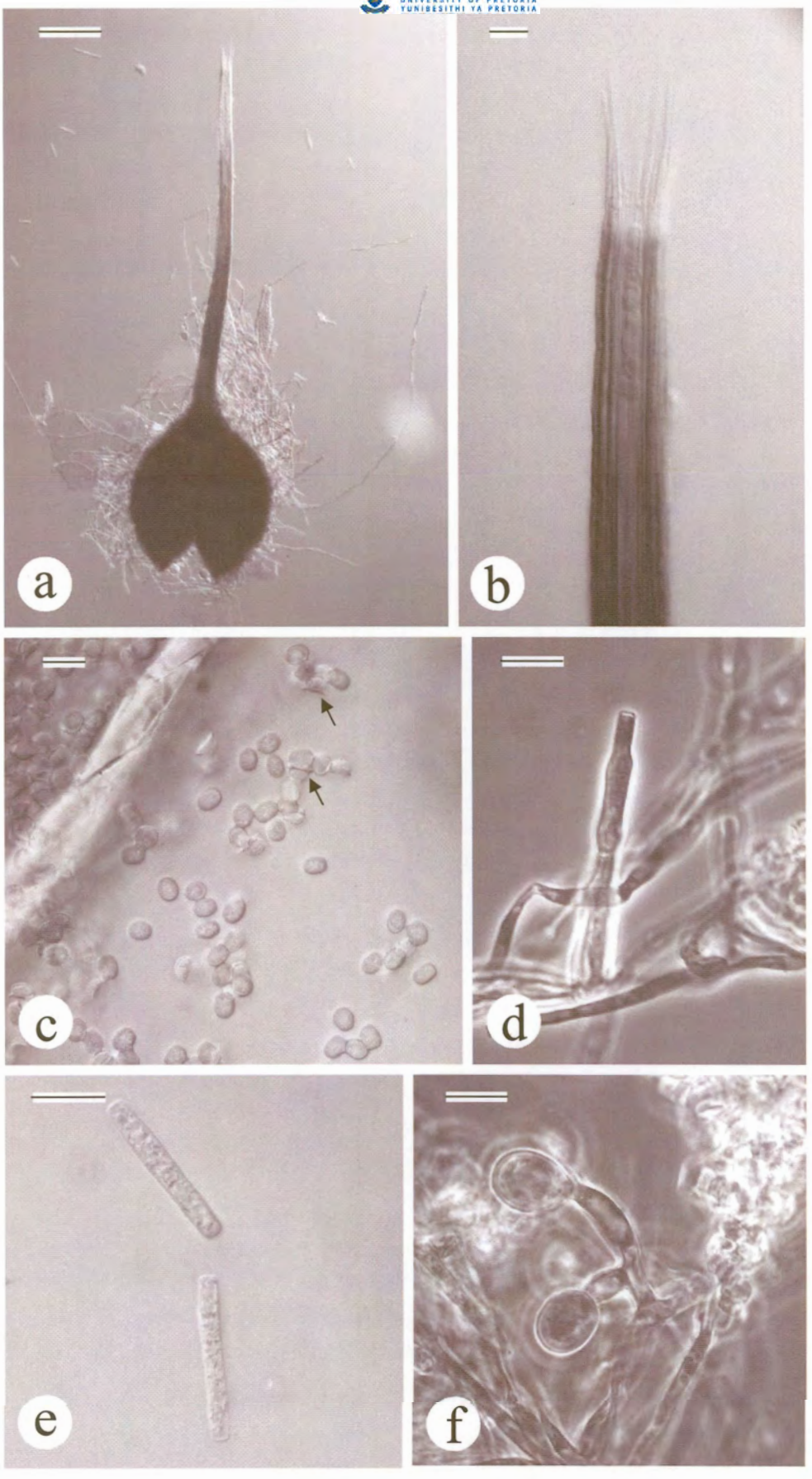

Fig. 2. Raw sequence data of the ITS1 and ITS2 regions and 5.8S gene of the ribosomal operon for various species of *Ceratocystis*. Unknown sequence characters are depicted with an "**N**", while gaps inserted to achieve sequence alignment are shown as "-". Bases matching those of CMW 2242 are indicated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

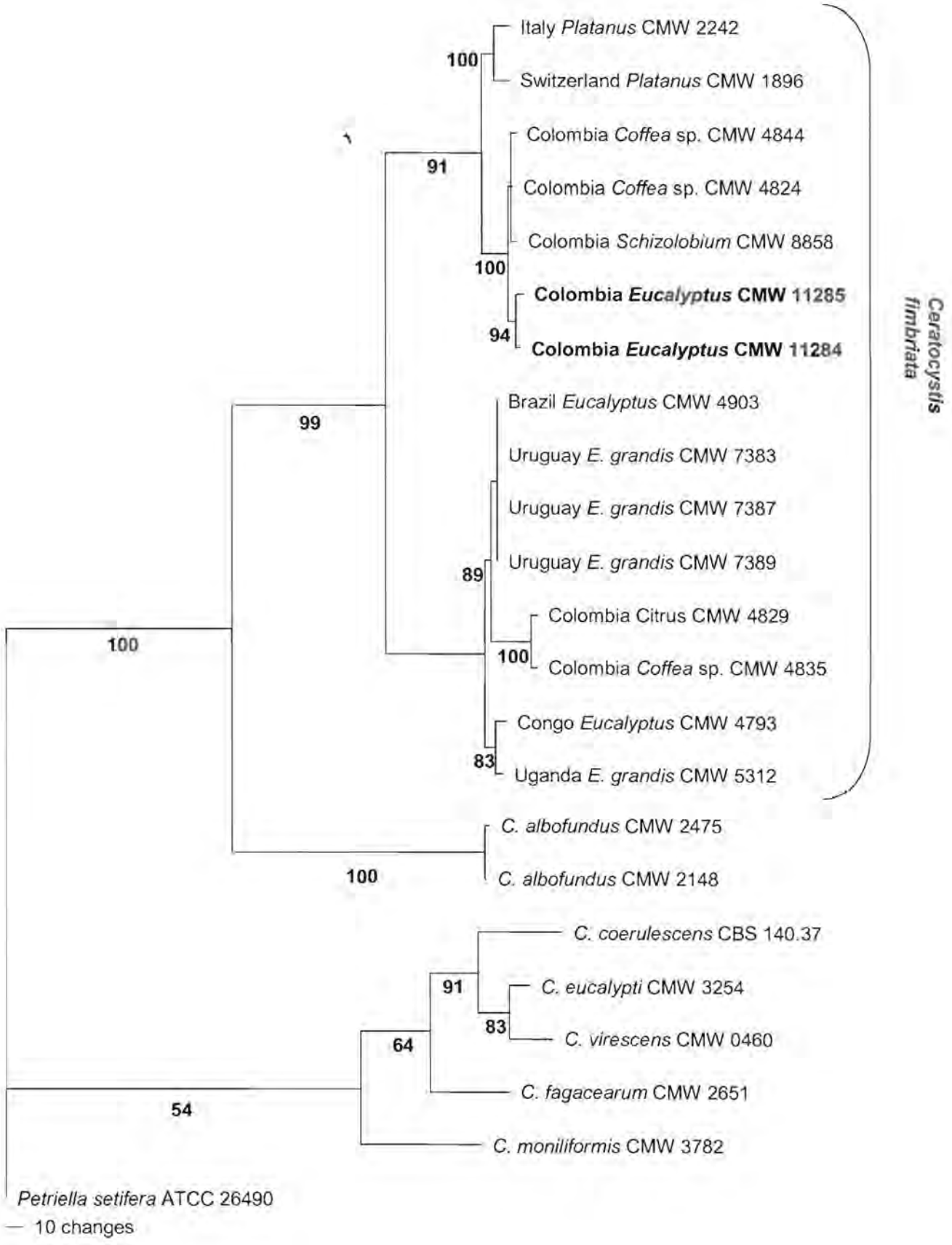


Fig. 4. Lesions produced by isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and an isolate of *Schizolobium parahybum* (CMW 8858) on various clones or seedling trees inoculated in field trials in Colombia.

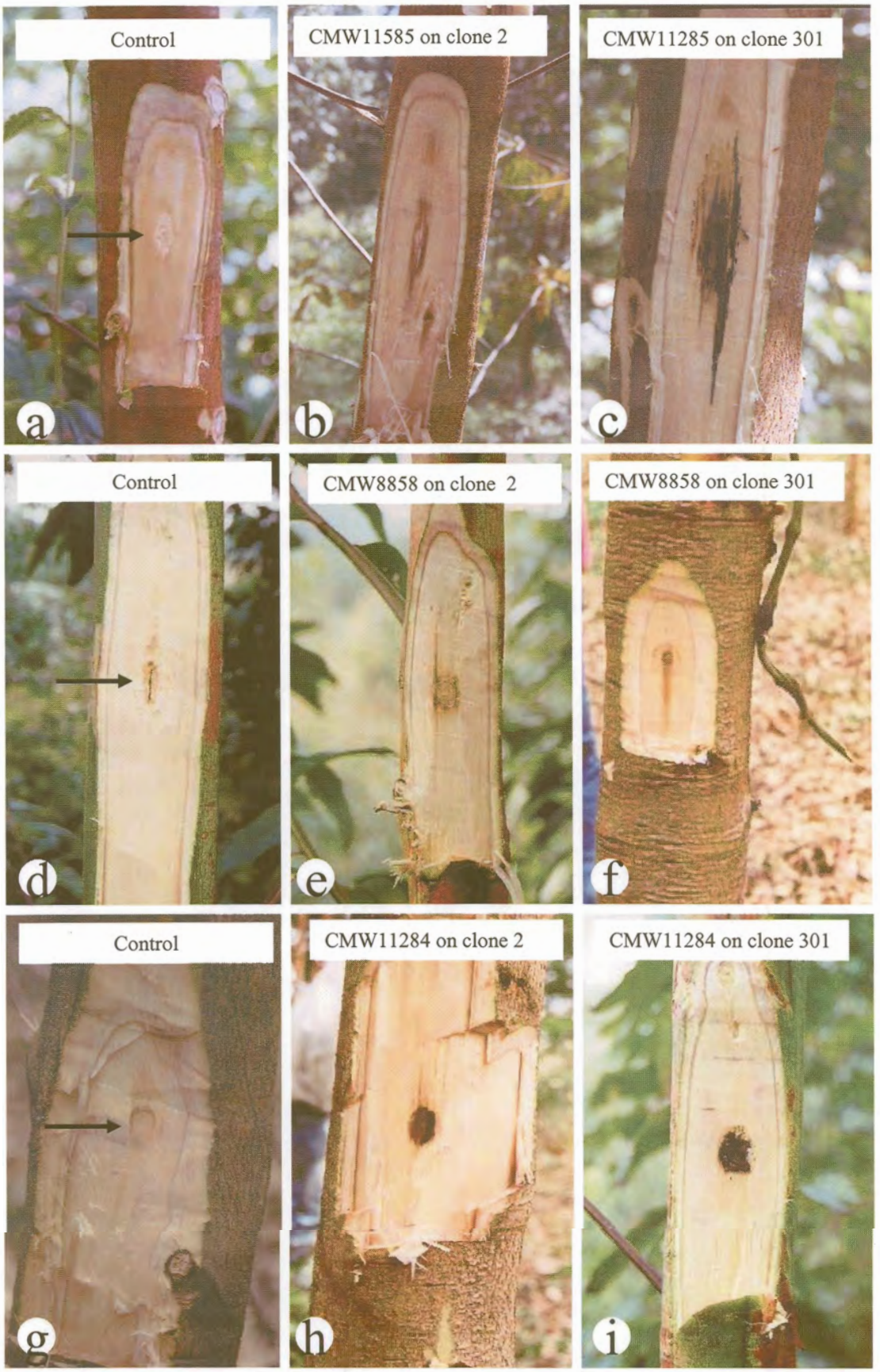


Fig. 5. Results of an inoculation trial with isolates of *Ceratocystis fimbriata* from *Eucalyptus grandis* (CMW 11285 and CMW 11284) and *Schizolobium parahybum* (CMW 8858) from Colombia and a negative control. Inoculations were done on *E. grandis* clones 301 and 2 at Buenos Aires farm, Trujillo Valle.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

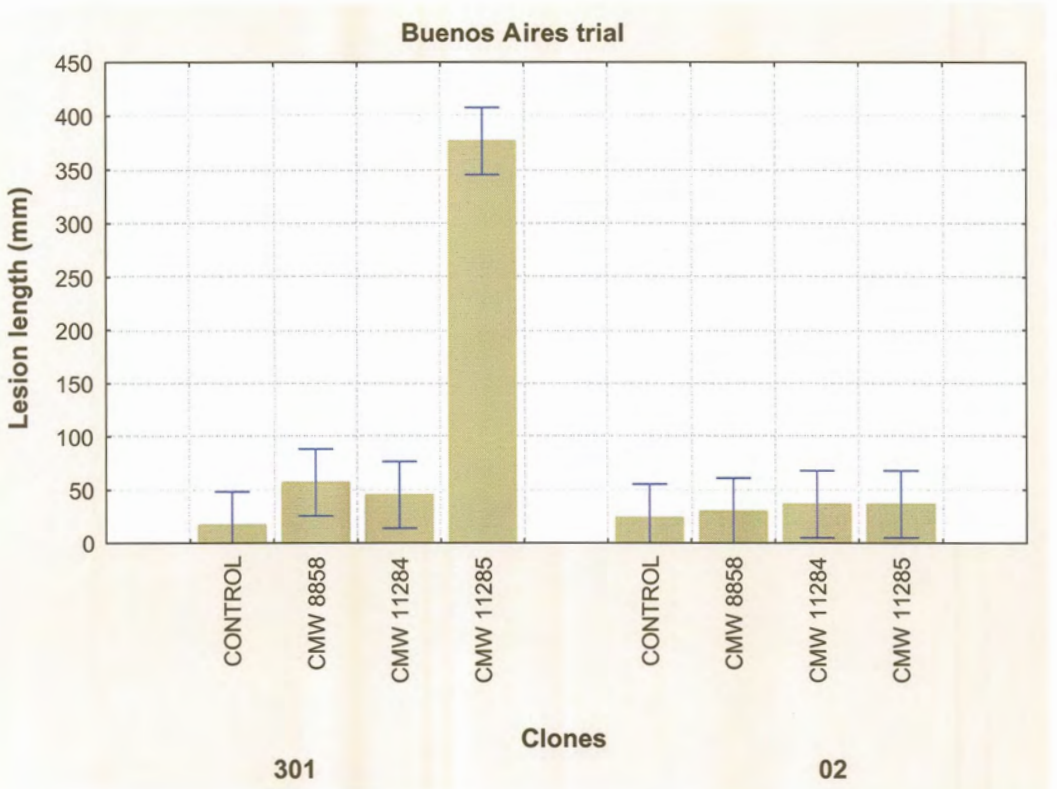

Buenos Aires trial
Lesion length (mm)
450
400
350
300
250
200
150
100
50
0
CONTROL
CMW 8858
CMW 11284
CMW 11285
CONTROL
CMW 8858
CMW 11284
CMW 11285
Clones
301
02

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.

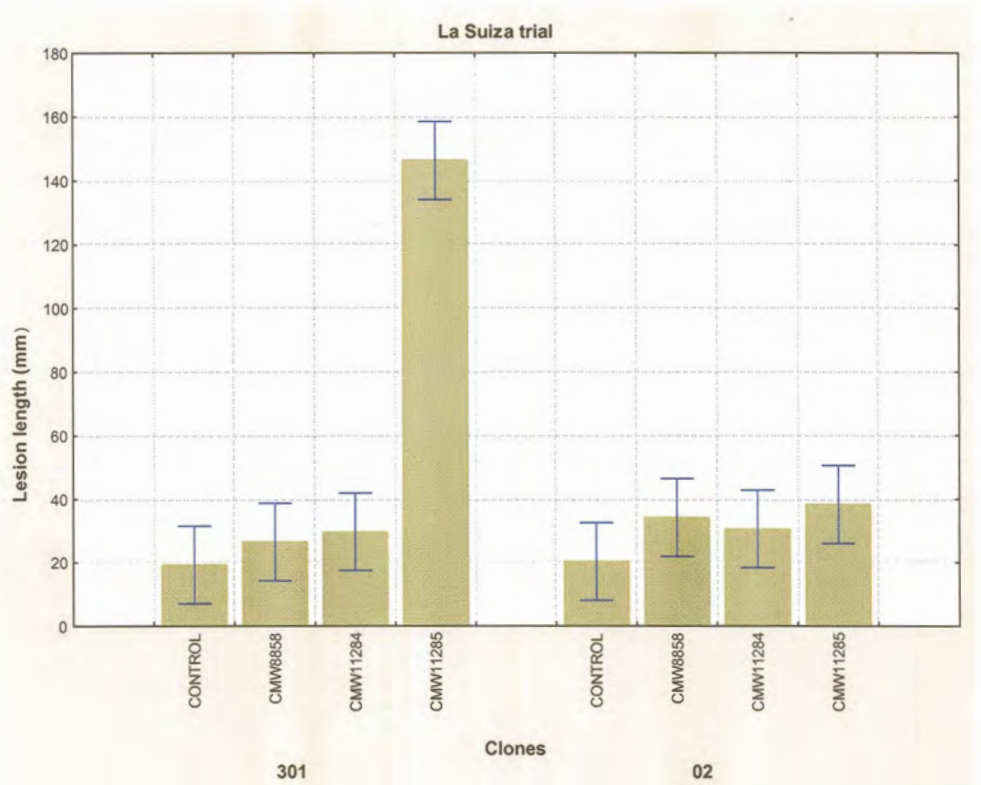
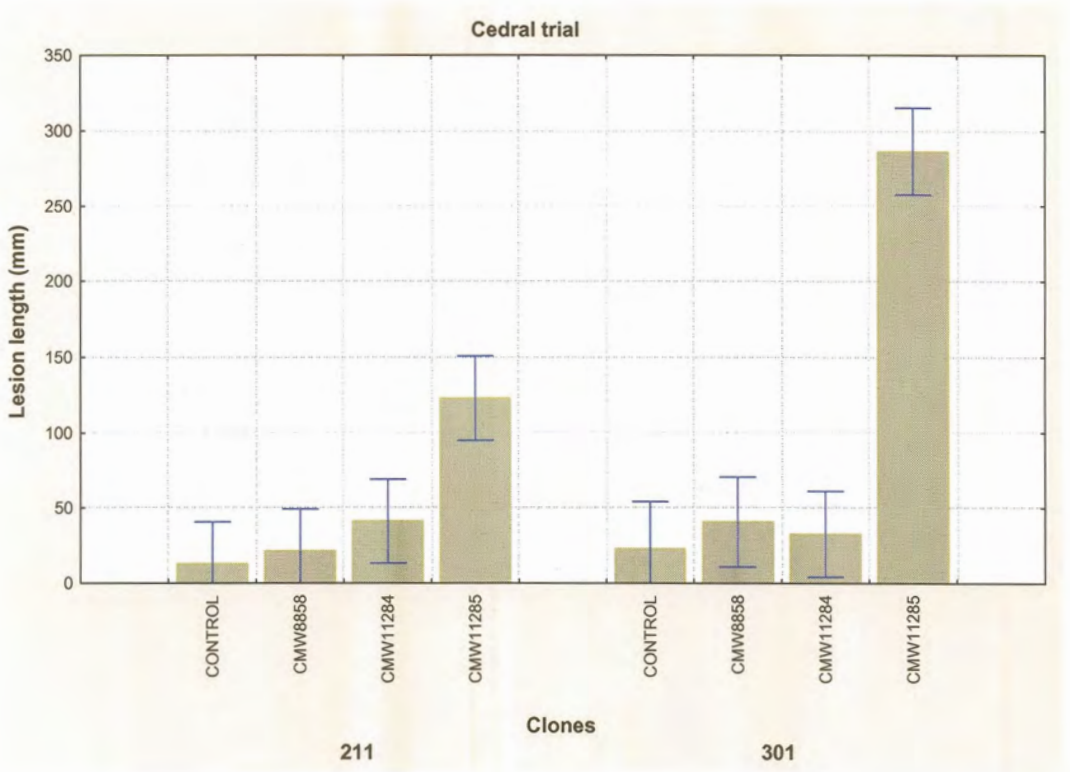

La Suiza trial
Lesion length (mm)
180
160
140
120
100
80
60
40
20
0
CONTROL
CMW8858
CMW11284
CMW11285
CONTROL
CMW8858
CMW11284
CMW11285
Clones
301
02

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