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DISEASES OF ACACIA MEARNSII IN SOUTH AFRICA WITH PARTICULAR REFERENCE TO CERATOCYSTIS WILT

DISEASES OF *ACACIA MEARNSII* IN SOUTH AFRICA, WITH PARTICULAR REFERENCE TO CERATOCYSTIS WILT

By

JOLANDA ROUX

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Promoter: Prof. M.J. Wingfield

Dedicated to my parents and friends

The only way of discovering the limits of the possible is to venture a little way past them into the impossible.

Clarke's Second Law

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PREFACE

Acacia mearnsii (black wattle) production has become one of the most profitable components of the South African forestry industry. It forms only the third largest portion of this industry, but it has been the only sector to be consistent in the prices being received for pulp and bark in recent years. Cultivation of *A. mearnsii* is especially popular amongst private and small holder farmers since, for every ton of bark, approximately five tons of utilizable timber can also be harvested.

Currently, black wattle wood is exported from South Africa to Japan and Norway for the production of high quality pulp, used in the production of paper and viscose. The timber is also used for charcoal production and building poles. The bark of *A. mearnsii* is chipped and the extracts are used for the production of tanning extracts as well as industrial adhesives for the manufacture of weather and boil proof particle board, plywood, medium density fibre board and corrugated cardboard. These bark extract products are exported to more than 50 countries world-wide.

Acacia mearnsii is a member of the family Leguminosae and is capable of nitrogen fixation. This makes it very attractive for planting in rotation with other forest species and agricultural crops. With the increased pressure on International Forestry from environmental agencies, this aspect of black wattle will be taken into consideration when planning future plantings. Farmers who have been growing sugarcane in rotation with *A. mearnsii* have, for example, reported considerable increase in yields.

Despite the fact that *A. mearnsii* has been grown commercially in South Africa for more than a century, very little research has been conducted on the diseases affecting these trees. Where *Eucalyptus* propagation in South Africa has benefited from intensive research into increased growth and disease resistance, very little work has been done with *A. mearnsii* in this regard. This situation is changing as the importance of *A. mearnsii* is realised by larger forestry companies, and as the demand for the higher quality wood

increases. Attention is now being focused on breeding trees with higher quality wood, shorter rotations and disease resistance.

Funding for research into *A. mearnsii* diseases was initiated in the 1990's, after a serious wilt disease, caused by *Ceratocystis albofundus*, was identified in the KwaZulu-Natal Midlands. The disease, known as wattle wilt (Ceratocystis wilt) is currently the most serious disease affecting *A. mearnsii*, with the pathogen capable of killing infected trees within a period of six weeks after inoculation. Discovery of Ceratocystis wilt also prompted a survey of diseased *A. mearnsii* in South Africa and *C. albofundus* is now known to occur throughout South Africa. A number of diseases have been reported to affect *A. mearnsii*, and, in a comprehensive survey of diseased trees conducted from 1994-1995, a number of new pathogens were also reported.

This thesis is a continuation of the research conducted in 1994/1995 and expands the available knowledge regarding the diseases affecting *A. mearnsii* in South Africa. It also aims to show the potential connection between pathogens occurring on different forestry species, illustrating the importance of taking other crops and their pathogens into consideration as possible sources of pathogens. The primary focus of this thesis is, however, on Ceratocystis wilt. Apart from Ceratocystis wilt it also investigates other diseases of *A. mearnsii* and the connection to pathogens on *Eucalyptus* spp. which are also grown extensively in South Africa. Each chapter has been written as an individual entity, although a close interaction is found between research represented in each of these units. Nevertheless, a degree of repetition between chapters has been unavoidable.

As an introduction, the thesis commences with a literature review on the diseases affecting commercially grown *Acacia* spp., focusing on *A. mearnsii* and the South African situation. A list of pathogens reported from *Acacia* spp. is provided at the end of Chapter one. Information gained from this literature review can be used in the identification and quarantine of plant material to prevent diseases of *A. mearnsii*, not yet occurring in South Africa, from entering the country.

In Chapter two, I investigated the population diversity of the wattle wilt pathogen, *C. albofundus*, in South Africa. The diversity of a pathogen population plays a role in the success of potential control measures against the disease. It also provides data pertaining to the mode of reproduction and the origin of the pathogen. The more diverse the population, the more likely it is to overcome disease tolerance in clones and the more likely it is that it is native to the country. *Ceratocystis albofundus* is known only from South Africa and the only other report of this fungus is from native *Protea* spp.

Very little is known about *Ceratocystis sensu stricto* in South Africa or the rest of Africa. Apart from *C. albofundus* and *Chalara elegans* (known to be an anamorph of *Ceratocystis*), only a few superficial reports of fungi in this genus have been made on this continent. Whether *C. fimbriata* occurs in South Africa has been questioned, since the reports of *C. fimbriata* from *Protea* spp. and *A. mearnsii* were shown to be incorrect and rather, to represent *C. albofundus*. During surveys of *A. mearnsii* diseases, two previously unrecorded fungi in the genus *Ceratocystis* were isolated. In Chapter three, I consider the identity of these two fungi and present the results of laboratory pathogenicity tests that show that they are capable of causing disease of *A. mearnsii* seedlings.

In Chapter four, *C. fimbriata* is reported as a pathogen of *Eucalyptus* in the Republic of the Congo for the first time. Isolates are compared with other *C. fimbriata* isolates, including some from *A. mearnsii* in South Africa. It is also the first report of a true *C. fimbriata* isolate, and not *C. albofundus*, from *A. mearnsii*. This chapter is the first report of *C. fimbriata* causing a wilt disease of *Eucalyptus* in Africa and considers the phylogenetic relationship between *C. fimbriata* from *A. mearnsii* and *C. fimbriata* from *Eucalyptus* spp. in West Africa and Brazil. The data provide knowledge of the possible origin of *C. fimbriata* in South Africa and the Congo and also of the taxonomy of *C. fimbriata*.

In a previous study, a species of *Seiridium* was reported from diseased *A. mearnsii* in South Africa. In Chapter five, the phylogenetic relationship of this *Seiridium* sp. from *A. mearnsii* is considered. This is done using morphological and molecular techniques, comparing *A. mearnsii* isolates to *Seiridium* isolates responsible for cankers on *Cupressus* spp. Molecular evidence is sought to support the identity of the *A. mearnsii* isolates and to show their similarity to the *Seiridium* spp. that cause cypress canker. Pathogenicity tests on *A. mearnsii* and *Cupressus lusitanica* are also conducted to support molecular and morphological data.

Many plant pathogens can live as symptomless endophytes in their hosts for part or all of their life cycle. These fungi are often activated to cause disease under unfavourable environmental conditions, such as drought and frost. Previous disease surveys of *A. mearnsii* have yielded a number of fungal species that may be capable of endophytic growth. In Chapter six, I investigate the endophytes of *A. mearnsii*, with the aim of identifying possible pathogens of this host. This would give an indication of the likelihood of the appearance of disease on *A. mearnsii* under unfavourable climatic conditions and provide the first list of endophytes of *A. mearnsii*.

The final chapter of this thesis deals with an unexpected and unusual report of *Fusarium graminearum* from *A. mearnsii*. This fungus is best known as a pathogen of wheat and maize but was shown to be capable of producing lesions on *A. mearnsii*. It was isolated infrequently from stem cankers and branches showing die-back. In Chapter seven, I consider the identity of this fungus, using molecular techniques, and pathogenicity tests on *A. mearnsii*.

This thesis represents a continuation of research previously undertaken on the fungal diseases of *A. mearnsii*. It also expands our knowledge on these pathogens, especially the economically important *C. albofundus*. It is my sincere hope that this research will contribute towards an increased knowledge pertaining to pathogens such as *Ceratocystis* spp. and also to the improvement of *A. mearnsii* propagation in South Africa.

CHAPTER 1

FUNGAL DISEASES OF PLANTATION ACACIA SPECIES, WITH SPECIAL REFERENCE TO ACACIA MEARNSII IN SOUTH AFRICA: A REVIEW

1

FUNGAL DISEASES OF PLANTATION *ACACIA* SPECIES, WITH SPECIAL REFERENCE TO *ACACIA MEARNSII* IN SOUTH AFRICA: A REVIEW

ABSTRACT

Plantations of fast growing exotic tree species have become the basis of an important industry in many developing countries of the world. Among the most common trees planted are a number of species in the genus *Acacia*. *Acacia* spp. possess excellent wood qualities for pulping and are also widely used for firewood and construction. In the past, detailed studies on diseases affecting these trees have been neglected in favour of the more widely planted *Eucalyptus* spp. Many diseases have, however, now been reported on *Acacia* spp., and research aimed at a better understanding of them is increasing. In South Africa, *A. mearnsii* is especially versatile in that both the wood and the bark are used commercially. The industry has, however, experienced a number of disease problems, of which the recently reported Ceratocystis wilt is the most serious. The aim of this chapter is to provide a review of the diseases of the most widely planted plantation *Acacia* spp. of the world, but with particular reference to *A. mearnsii* in South Africa.

1.0 INTRODUCTION

The genus *Acacia* resides within the family Leguminosae (= Fabaceae) and includes 1250 described species. *Acacia* spp. form an important component of the natural shrub and wood vegetation in many parts of the world (Carr, 1976; Ross, 1979; Davidson & Jeppe, 1981). In Africa, *Acacia* spp. are considered important for grazing and are unrivalled as pioneer species (Barnes, Filer & Milton, 1996). The genus is endemic to various countries, including countries in Asia (Barnes *et al.*, 1996), Australia (Larsen, Lombard & Hodges, 1985; Barnes *et al.*, 1996), Hawaii (Hodges & Gardner, 1984; Larsen *et al.*, 1985), New Guinea (Lee & Arentz, 1995), Indonesia (Lee & Arentz, 1995) and various countries in Africa (Barnes *et al.*, 1996).

Acacia spp. are extensively planted as exotics in plantations in many parts of the world. The Australian species are most widely planted as exotics, because of their outstanding wood properties, as well as for the high tannin contents of their bark (Sherry, 1971; Gibson, 1975; Bakshi, 1976; Turnbull, 1991). These species include *A. auriculiformis* A. Cunn. ex Benth., *A. decurrens* Wendl., *A. mangium* Willd. and *A. mearnsii* de Wild. All four species are economically important to countries such as Brazil, India, Malaysia and South Africa (Sherry, 1971; Bakshi, 1976; Florance & Balasundaran, 1991; Turnbull, 1991; Lee, 1993).

Where exotic plants are established as monocultures in plantations, they are more susceptible to infection by pathogens. There are thus many reports of diseases affecting plantation trees (Gibson, 1964; Ahmad, 1987; Roux & Wingfield, 1997). During the last century, there have also been many reports of disease problems on *Acacia* spp. Unfortunately, few of these diseases have been investigated in any detail with the result that most disease situations are still unresolved. In some instances, there are also contradictory reports regarding the cause of diseases. There is thus a need for detailed study of the diseases of commercially planted *Acacia* spp., especially considering their importance to forestry. This review is intended to provide a background on some of the more serious diseases known to affect *Acacia* spp. A list of possible pathogens reported on plantation *Acacia* spp. is also included.

2.0 *Acacia auriculiformis*

Acacia auriculiformis is widely planted in south east Asia, including Java and Madera, and Oceana, as well as in Africa for fuel wood. It is planted in urban forests and in aforestation (Suharti, 1980; Wiersum & Ramlan, 1982; Turnbull, 1991). The wood is used for furniture and farm tool manufacture, and gives high pulp yields. This tree is often preferred to other *Acacia* spp., since it is very fast growing and has proven to be adaptable to extremes in temperature and moisture availability (Wiersum & Ramlan, 1982; Supriana & Natawiria, 1987; Turnbull, 1991; Barari, 1993).

2.1 Root, butt and stem rots

Various root diseases of *A. auriculiformis* have been described from India. These pathogens include an unidentified *Ganoderma* sp. which was reported to cause trunk rot of mature trees. The disease is characterised by the defoliation of the trees and the eventual hollowing of the stems, due to decay. The primary inoculum source was reported to be older stumps (Barari, 1993). Two species of *Ganoderma* cause wood rot of *A. auriculiformis. Ganoderma applanatum* (Pers.: Wallr.) Pat. causes white mottled heart rot and *G. lucidum* (Leyss.: Fr.) Karst. causes white spongy rot (Browne, 1968; Lenné, 1992). *Ganoderma lucidum* was reported to cause root rot in an *Acacia* arboretum in the Seoni district in central India. *Ganoderma applanatum* has also been reported from India where it is considered to be the cause of mortalities of various *Acacia* spp. in an arboretum (Harsh, Soni & Tiwari, 1993). Two other pathogens causing root disease in India are *Macrophomina phaseolina* (Tassi.) G. Goid. and *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. (Synonyms: *Botryodiplodia theobromae* Pat.; *Diplodia natalensis* Pole Evans) (Lenné, 1992). Symptoms of the disease caused by *M. phaseolina* include die-back, gummosis and root death (Lenné, 1992). Another root disease of note is caused by an unidentified species of *Phellinus* in Papua New Guinea (Lenné, 1992). *Phellinus noxius* (Corner) G. H. Cunn. has been associated with rot and tree deaths in Malaysia (Lee & Arentz, 1995), but it is not known if this species is also the cause of the rot in Papua New Guinea.

In Kerala, India, *Corticium salmonicolor* Berk. & Br. (Syn.: *Phanerochaeta salmonicolor*) causes a severe disease, of which the first symptoms are wilting and die-back of the main stem. Other symptoms include girdling of the stem and splitting of the bark, due to canker formation. Affected

areas are covered in a pink encrustation (Florence & Balasundaran, 1991). This pathogen was reported to affect 2- to 3-year-old trees and is common in tropical areas with high rainfalls, with mortalities of 25 - 100 % (in *Eucalyptus* plantations) in Kerala (Florence & Balasundaran, 1991).

2.2 Foliar diseases

In India, *Cylindrocladium quinqueseptatum* Boedijn & Reitsma causes leaf spot and defoliation of trees, while *Rhizoctonia solani* Kühn causes web blight which leads to defoliation (Lenné, 1992). In the same country *Exserohilum rostratum* (Drechsler) Leonard & Suggs. causes lesions on foliage of young trees (Lenné, 1992). An unknown *Oidium* sp. has also been reported as the cause of seedling disease in China (Wang & Fang, 1991).

There have been three reports of rusts on *A. auriculiformis*. In all three instances the causal agent was described as a species of *Uromyces*. In India, *U. digitatus* Winter reduced growth of plants in nurseries and also of new transplants, while in Indonesia, an unidentified *Uromyces* sp. causes rust (Lenné, 1992). In nurseries throughout Java and Madera, a rust causing the formation of galls, chlorosis and stunted growth is commonly found. If left untreated the disease spreads into the field. The complete taxonomy of this rust fungus is, however, still unresolved (Suharti, 1980; Supriana & Natawiria, 1987). It was reported that *A. auriculiformis* is the primary host of this rust, since the pycnial, uredial and telial phases of the fungus were found on it (Suharti, 1980).

It is, however, believed that the rust of *Acacia* in Indonesia is in fact not a species of *Uromyces*, but a species of *Atelocauda*. Later reports of the rust from Java, reported by Suharti (1980) as a species of *Uromyces*, is in fact as *Atelocauda digitata* (Wint.) Cumm. & Y. Hiratsuka (Gardner, 1991). Teliospores of the Indonesian rust (demicyclic) are identical to *A. digitata* in Hawaii. It is speculated that either the Hawaiian or the Indonesian rust represents a new species (Hodges, personal communication).

3.0 *Acacia catechu* (L.f.) Willd. - Khair/Cutch tree

Khair has been classified as one of India's most important cash crops. Wood from these trees yields cutch, which is used for dyeing and tanning, and also katha. The wood is also useful for cabinet

building (Howard, 1920; Bakshi, 1957; Karnik *et al.*, 1971). The tree is considered very valuable by having a good growth rate and by performing well under poor soil conditions (Rout, Samantary & Das, 1995).

Most reports of diseases of *A. catechu* originate from India. *Ganoderma lucidum* has been reported to cause heavy mortalities due to root rot (Bakshi, 1957; Gibson, 1975; Lenné, 1992). The fungus forms white mycelium in the living roots and produces a white spongy rot (Gibson, 1975). Root rot leading to death of trees may also be caused by *Polyporus gilvus* Schwein [Synonym: *Phellinus gilvus* (Schw.) Pat.] (Bakshi, 1957). These pathogens infect trees when they are under stress and affect the sapwood, causing a soft spongy rot (Bakshi, 1957).

Wood rot of *A. catechu* has been reported to be caused by *Phellinus badius* (Cooke) G.H. Cunn. Losses of up to 50% have been reported in plantations after infection (Gibson, 1975). *Polyporus gilvus* was said to infect both sapwood and heartwood (Gibson, 1975). Also in India, *Fomes badius* [Syn.: *Phellinus badius* (Berkley) Cunningham] is reported as the cause of heart rot (Bakshi, 1957; Ito & Nanis, 1997). This fungus infects the trees through wounds, rendering the heartwood unusable. *Fomes badius* was described as a facultative wound parasite, only infecting heartwood and not sapwood (Bakshi, 1957). Root and wood rots are, however, not the only diseases that have been described on *A. catechu*. More recently, a wilt disease was reported to be caused by *Fusarium solani* (Mart.) Sacc. (Lenné, 1992). Witches broom, caused by *Ravenelia tandonii* Syd., has also been reported from India (Patil & Date, 1980).

4.0 *Acacia dealbata* Link - Silver Wattle

Silver wattle is grown for its timber and for the making of cask staves (Howard, 1920). Countries in which it is grown commercially include China and many others (Wang & Fang, 1991). It is a very frost hardy tree, but has, in many instances, been replaced by *A. mearnsii* because the latter has a higher tannin yield (Bakshi, 1976). It is also very useful in being able to colonise very poor sites (Wang & Fang, 1991).

4.1 Root, butt and stem rots

In Australia and New Zealand a number of root diseases caused by Basidiomycetes have been reported on *A. dealbata*. *Peniophora incarnata* (Fr.) Karst., in Australia, and *P. sacrata* G.H. Cunn, in New Zealand, for example, were reported as the cause of root diseases (Gibson, 1975; Bakshi, 1976). Other diseases are heart rot caused by *Fomes mastoporus* (Lev.) Cke in New Zealand, *G. applanatum* and *G. australe* (Fr.) Pat. in Australia and New Zealand (Ito & Nanis, 1997), as well as *Trametes tawa* G. H. Cunn. in Australia (Bakshi, 1976). Another root disease reported on *A. dealbata* is said to be caused by *Armillaria mellea* (Fr.) Kummer *sensu lato* (Bakshi, 1976).

4.2 Stem diseases

Hypoxylon hypomiltum Mont. and *H. rubiginosum* Fr. cause stem cankers on *A. dealbata* in Australia (Bakshi, 1976). In Japan, *Glomerella acaciae* (K. Ito & Shibukawa) K. Ito cause anthracnose and lesions on leaves, stems and petioles. During wet periods young shoots are girdled and die. This disease was reported to be seed-borne (Hodges, 1964).

4.3 Foliar diseases

Calonectria indusiata Seaver (Syn. *Calonectria theae* Loos) (Bakshi, 1976) and its anamorph *Cylindrocladium theae* (Petch) Subramanian (Syn. *Cercosporella theae* Petch) cause leaf spots and lesions on twigs in Sri Lanka (Gibson, 1975; Crous & Wingfield, 1994). In severe cases, *C. indusiata* can cause complete defoliation (Bakshi, 1976). In Australia, *Uromyces phyllodiorum* (Berk. & Br.) McAlpine and *Uromycladium alpinum* McAlpine cause phyllode and leaf rust, while *Uromycladium acaciae* (Cke.) Syd. (syn. *U. bisporum* McAlpine) causes powdery leaf spots and swellings on branches. This latter disease also occurs in New Zealand (Bakshi, 1976; Dick, 1985). *Uromycladium notabile* causes galls on branches, phyllodes and pods in Australia and New Zealand (Bakshi, 1976; Dick, 1985).

In Japan, *Glomerella cingulata* (Stonem.) Spauld. & Schrenk. (Syn: *Physalospora acaciae* K. Ito & Shibukawa) has been reported as a serious pathogen, affecting both the leaves and the stems. The disease first starts as spots on seedlings during moist weather, and these will lead to leaf drop and the eventual girdling of the stems as the disease worsens. *Cylindrocladium scoparium* Morgan and

Fusarium oxysporum Schlecht. were often associated with this disease as secondary pathogens (Gibson, 1975).

5.0 *Acacia decurrens* - Green wattle

This tree is grown for its timber and tannin in Indonesia, South Africa and Brazil (Ribeiro *et al.*, 1988; Turnbull, 1991; Evans, 1992). It is rated second only to black wattle (*A. mearnsii*) in the quality of its bark and it is also more frost hardy (Bakshi, 1976).

5.1 Root, butt and stem rots

Reports of root disease of *A. decurrens* include pathogens such as *A. mellea sensu lato* and *A. fuscipes* Petch. in India (Bakshi, 1976), *Fomes lamaoensis* (Murr.) Sacc. & Trott. in Indonesia and *Poria albobrunnea* Petch. in Sri Lanka (Bakshi, 1976). In the East Indies *Rosselinia acruata* Petch and *R. bunodes* (Berk. & Br.) Sacc. cause black root rot (Gibson, 1975).

Root rot, caused by *Ganoderma lucidum,* has resulted in severe losses to arboretum trees in India (Harsh *et al.*, 1993). Mortality of these trees was noticed within the first year after planting, with infection originating from previously colonised stumps. After infection of living trees from stumps, infection was also reported to spread through root contacts within plantation blocks (Harsh *et al.*, 1993).

5.2 Stem diseases

Stem diseases of *A. dealbata* are known to be caused by three pathogens that result in cankers on stems and twigs. *Corticium salmonicolor* in Mauritius, South Africa and Formosa causes pink disease (Gibson, 1975; Bakshi, 1976), while in South Africa *Physalospora abdita* (Berk. & Curt.) N.E. Stevens has been reported to cause stem cankers (Bakshi, 1976). A serious wilt and canker disease, accompanied by gummosis, of *A. dealbata* occurs in Brazil. The causal agent of this disease has been identified as *Ceratocystis fimbriata* Ell. & Halst. (Ribeiro *et al.*, 1988).

5.3 Foliar diseases

Calonectria indusiata causes dark brown to black spots on leaves in Sri Lanka, Indonesia and India. The disease was reported to be very serious in some of these areas, leading to complete defoliation of trees. *Calonectria indusiata* was also reported to be capable of causing cankers on young plants in Sri Lanka (Bakshi, 1976). Other leaf spot diseases have also been reported to be caused by *Camptomerris albizziae* (Petch) Mason and *C. verruculosa* (Syd.) Bessey in South Africa (Bakshi, 1976). In India and Sri Lanka *C. theae* causes leaf spots and lesions on twigs (Gibson, 1975).

Uromycladium notabile causes galls on branches, stems, seed pods, leaves and petioles. In addition it causes die-back of the branches beyond the galls and may lead to the death of young trees (Dick, 1985). The galls restrict water conduction within the branches, resulting in the die-back of the affected parts (Dick, 1985). This disease has been reported from Australia, where *A. decurrens* is native, as well as in New Zealand (Bakshi, 1976; Dick, 1985).

5.4 Nursery diseases/Damping-off

A number of nursery diseases, including damping-off, have been recorded on *A. dealbata*. The pathogens include fungi such as *C. scoparium* and *F. oxysporum*, associated with post-emergence damping-off (Bakshi, 1976). In Japan, *G. cingulata* has been described as the cause of brown to dark brown lesions on above ground parts of seedlings. In wet weather, these lesions develop rapidly, leading to girdling and death of the affected seedlings. The fungus is reported to be seed borne, with mycelium found on the seed surface, in the parenchyma and in the embryos (Bakshi, 1976).

6.0 *Acacia koa* Gray - Koa

The koa tree is a tropical timber tree, native to the Hawaiian islands and grown for the production of hardwood furniture on these islands (Gardner, 1978; Stein, 1983). Diseases affecting *A. koa* are mostly rusts, caused by a number of different genera and species. A number of other diseases have, however, also been documented.

The rusts reported on *A. koa* include several species previously placed in the genus *Uromyces*. All the pacific rusts of *Acacia* have, however, been transferred to the genus *Atelocauda* (Hodges & Gardner, 1984; Chen, Gardner & Webb, 1996). *Atelocauda koae* (Arthur) Cummins & Hiratsuka (=*Uromyces koae* Arthur) infects mainly young trees, leading to the distortion of leaves and small branches. In severe cases, entire stems are deformed (Gardner, 1978; Hodges & Gardner, 1984; Chen *et al.*, 1996). *Atelocauda digitata* (Wint.) Cumm. & Y. Hirat. (=*Uromyces digitatus* Winter) may produce witches brooms on affected trees and it also causes hypertrophy of leaves, shoots, flowers and seed pods. Both a macro- and microcyclic form of *A. digitata* has been found on the Hawaiian islands (Hodges & Gardner, 1984). Other rust genera reported from Koa include *Endoraecium acaciae* Hodges & Gardner, *E. hawaiiense* Hodges & Gardner (Hodges & Gardner, 1984) and *A. angustiphylloda* Gardner (Gardner, 1991).

Diseases reported from *A. koa* also include the reduction of seed production caused by *Colletotrichum gloeosporioides* Penz. (Stein, 1983). A number of heart and root rot fungi also occur on *A. koa*. These include *A. mellea sensu lato*, *Laetiporus sulphureus* (Bull.:Fr.) Bond. & Sing., *Phaeolus schweinitzii* (Fr.) Pat., *Pleurotus ostreatus* (Jacq.:Fr.) Quél, a species of *Ganoderma* and *Phellinus kawakamii* Larsen, Lombard & Hodges (Bega, 1979; Larsen *et al.*, 1985). *Phellinus kawakamii* causes a white pocket rot of *A. koa*, leading to wood decay in the basal part of the trees (Larsen *et al.*, 1985).

7.0 *Acacia mangium*

Malaysia and Indonesia are the main countries in which *A. mangium* is planted as a forest plantation tree (Nixon, 1995). It is, however, also planted widely throughout tropical Asia, the Pacific Islands, West Africa and the Americas (Turnbull, 1991; Barari, 1993; Ito & Nanis, 1997). The wood is used mainly for pulp, particle board and timber, although in Zaire it is also planted for fuel wood (Logan & Balodis, 1982; Zakaria, 1990; Clark *et al.*, 1991; Nixon, 1995; Ito & Nanis, 1997). *Acacia mangium* is used extensively in the reforestation of degraded grasslands and logged forests and grows well on poorer, acid soil types (Logan & Balodis, 1982; Lee & Arentz, 1995; Kapp, Beer & Lujan, 1997). It also readily forms hybrids with *A. auriculiformis*, producing progeny that are taller than either of the parents (Logan & Balodis, 1982).

7.1 Root, butt and stem rots

Phellinus noxius causes brown root disease and a *Macrophomina* sp., charcoal root disease of *A. mangium* in Malaysia (Ahmad, 1987). Characteristic symptoms of *P. noxius* is the formation of a continuous fungal "skin", covering the surface of the affected roots, and the presence of brown lines in the infected roots (Ahmad, 1987). This wood rotting fungus is known to cause a rot called honeycomb rot (pocket rot) (Lee & Arentz, 1995). *Macrophomina* spp. infect the root tips, killing the entire root system, which leads to the stunting and death of seedlings (Ahmad, 1987; Lenné, 1992). Other root pathogens reported to cause disease of *A. mangium,* are *L. theobromae* in India (Lenné, 1992) and an *Armillaria* sp. in Malaysia (M. J. Wingfield, unpublished).

There are a number of reports of heart rot caused by *Ganoderma* spp. In Bengal an unidentified *Ganoderma* sp. cause trunk rots accompanied by defoliation and the hollowing of trees. Fruiting bodies of a *Ganoderma* sp. were found at the base of affected trees, but the fungus species was not identified (Barari, 1993). In Malaysia, a species of *Ganoderma* causes red rot disease of *A. mangium*, while *P. noxius* causes brown root disease, killing seedlings (Lenné, 1992). Brown root disease is characterised by the decay of woody tissue and the yellowing and death of the foliage (Lenné, 1992).

Heart rot in the tropics, especially in Malaysia, Indonesia and Papua New Guinea, cause volume loss, reduction in the quality of wood and it leads to death of many trees (Lee, 1995; Ito & Nanis, 1997). Rot types reported include honeycomb rot caused by *P. noxius,* spongy rots, fibrous rots, brittle rot, pink pocket rot (Lee & Arentz, 1995) and white rot (Ito & Nanis, 1997). A number of possible wood rot fungi have been isolated from infected wood, but no single fungus has been identified as the primary, or sole cause of rot. Infections occur through wounds, especially branch stubs, and the severity of the disease increases with the age of trees. A direct correlation between the number and size of the side branches and the occurrence of heart rot has also been found. The more side branches and the thicker the side branches, the higher the incidence of disease. It is recommended that side branches be pruned at any early age, so as to produce only small wounds that can heal rapidly, thereby reducing the occurrence of heart rot (Lee, 1993; Ito & Nanis, 1997).

7.2 Stem diseases

In Malaysia, *Corticium salmonicolor* causes pink disease that results in serious damage to stems (Ahmad, 1987). *Corticium salmonicolor* predominantly infects young trees, causing death by girdling of the stems and branches (Ahmad, 1987). *Trametes corrugata* (Pers.) Bres. has not been shown to cause disease in Malaysia, but is found to be commonly associated with trees suffering from die-back. This disease is especially prevalent on soils that are low in nutrients (Ahmad, 1987).

A number of fungi are reported to cause twig die-back. In the Philippines *T. corrugata* and a *Diplodia* sp. cause die-back (Lenné, 1992). In the Solomon Islands, the same problem is thought to be caused by *Nectria pseudotricha* (Lenné, 1992). A *Nectria* sp. is also reported to cause extensive canker formation of up to 3 meters on *A. mangium* in Central America. This pathogen is capable of killing trees when it girdles the main stems (Kapp *et al.*, 1997)

7.3 Foliar diseases

Minor leaf spots of *A. mangium*, caused by *G. cingulata*, *Phyllostictina* sp., *Phomopsis* sp. and *Pestalotiopsis* sp. have been reported from Malaysia (Ahmad, 1987; Zakaria, 1990). A more serious problem occurs in India, where *C. quinqueseptatum* causes leaf spot and defoliation of trees (Lenné, 1992). In Malaysia, *Cylindrocladium theae* causes dark spots on leaves and sunken lesions on green twigs (Lenné, 1992), while in Malaysia, India and Thailand, sooty mold caused by a *Meliola* sp. is reported to be a serious problem on young trees (Lenné, 1992).

7.4 Nursery diseases/Damping-off

Damping-off diseases of nursery seedlings are very common, especially among seedlings that have been planted too densely, and where soils are damp. In Malaysia, the most common fungi associated with damping-off are species of *Fusarium*, *Pythium* and *Rhizoctonia* (Ahmad, 1987; Zakaria, 1990). In Malaysia, *F. solani* and in Sabah, *R. solani* are the cause of damping-off of seedlings (Zakaria, 1990). Apart from damping-off, a number of nursery diseases affecting the foliage of *A. mangium* also occur. Powdery mildew caused by an unidentified species of *Oidium* has led to mortalities as high as 75 % in Thailand nurseries and has also caused problems in Australia and China (Wang & Fang, 1991; Lenné, 1992). The problem also occurs in nurseries in Malaysia and may lead to

premature defoliation (Zakaria, 1990). In Indonesia and Papua New Guinea, *G. cingulata* causes seedling blights characterised by dark elliptical to irregular lesions on phyllodes as well as defoliation and death under humid conditions (Lenné, 1992).

8.0 *Acacia mearnsii* - Black wattle

In South Africa *A. mearnsii* is planted commercially for both its wood and bark. Tannins in the bark are used for the production of wood adhesives and flotation agents, as well as for leather tanning (Saayman & Oatley, 1976; Turnbull, 1991). The wood is used to produce paper, pulp and rayon and also for charcoal (Sherry, 1971; Turnbull, 1991; Evans, 1992; Anonymous, 1997). *Acacia mearnsii* is planted extensively in China, India, Japan, Kenya, Tanzania, Uganda, Brazil, Uruguay and Argentina (Boucher, 1978; Kihiyo & Kowero, 1986; Turnbull, 1991) and was also planted widely in Sri Lanka, Kenya and Zimbabwe (Sherry, 1971; Bakshi, 1976).

8.1 Root and butt rots

Various root and butt rots have been described on *A. mearnsii*. One of the most common root pathogens, *M. phaseolina*, has been reported as the cause of a root disease in Sri Lanka and South Africa (Gibson, 1975; Bakshi, 1976). *Armillaria mellea sensu lato* and *G. lucidum* are reported to cause root disease in South Africa (Bakshi, 1976; Gorter, 1977). *Ganoderma applanatum* is also reported from Sri Lanka and South Africa, where it causes heart rot (Bakshi, 1976). Collar rot in South Africa has also been ascribed to *G. rugosum* Blume & Nees, suggesting that three species of *Ganoderma* are responsible for root and collar rots of *A. mearnsii* in South Africa (Gibson, 1964; Lückhoff, 1964).

The best described disease of *A. mearnsii* in South Africa is black butt, caused by *Phytophthora parasitica* (Dastur) Waterhouse (= *P. nicotianiae*) (Zeijlemaker, 1971). It was originally believed that this pathogen causes two types of symptoms on trees, depending on the prevailing environmental conditions. Zeijlemaker (1971) described both mottled lesions (under cool conditions) and black to brown "tongues" of dead bark extending up the stem of the tree (warmer temperatures, ca. 30°C).

A number of reports of *L. theobromae* have originated from South Africa. Reports of collar rot from the Eastern Cape and throughout plantations in KwaZulu-Natal in the 1930's were ascribed to this pathogen. The rot was reported to start in the roots and lead to trees being blown over by the wind (Stephens & Goldschmidt, 1938). In KwaZulu-Natal and Mpumalanga whole root systems were affected and infection spread up stems to form black cankers. The affected roots were all stained a dark colour (Laughton, 1937). In the Eastern Cape Province, a *Rhizoctonia* sp. was also reported to cause infection of trees, leading to epidemic occurrences of root disease (Kotzé, 1935; Laughton, 1937).

8.2 Stem diseases

A number of stem diseases have been reported in South Africa. *Schizophyllum commune* Fries was reported as an opportunistic wound parasite, leading to the death of trees, and the rotting of the wood. Pruning wounds, especially seemed to be sites of infection for this opportunistic parasite (Ledeboer, 1940). Two other stem canker pathogens in South Africa include *Physalospora abdita* (Bakshi, 1976) and *Botryosphaeria dothidea* (Moug.) Ces. Et de Not. causing wood discolouration, die-back and canker of trees (Roux & Wingfield, 1997; Roux *et al.*, 1997).

In Malaysia and Mauritius *C. salmonicolor* was reported to cause stem and twig cankers (Gibson, 1975; Bakshi, 1976). Anthracnose, caused by *Glomerella acaciae,* has been problematic in Japan (Hodges, 1964), and in the Lower Pulneys, stem canker caused by *Dothiorella pithyophilla* Sacc. caused large scale losses (Panneerselvam *et al.*, 1975). Heart rot has been reported from a number of countries on various *Acacia* hosts, with *G. applanatum* causing white mottled heart rot and *G. lucidum* white spongy rot (Lenné, 1992).

8.3 Foliar diseases

Calonectria indusiata causes dark brown to black spots on leaves of *A. mearnsii* in Sri Lanka and India (Bakshi, 1976). This fungus can also cause sunken lesions on twigs and result in defoliation of trees (Gibson, 1975; Lenné, 1992). Another leaf disease occurring in India is caused by *R. solani*, which causes web blight, also resulting in defoliation (Lenné, 1992). In South Africa, leaf spots are caused by *Camptomerris albizziae* (Wingfield & Kemp, 1993) and *C. verruculosa* (Bakshi, 1976).

The disease is, however, not considered to be serious, usually being associated with leaf drop during fall (Wingfield & Kemp, 1993).

Various rusts have been reported from *A. mearnsii*. In Australia and New Zealand, *U. acaciae* causes powdery leaf spot and swellings on branches (Bakshi, 1976; Dick, 1985), while *U. tepperianum* (Sacc.) McAlpine causes galls on the phyllodes and branches (Bakshi, 1976). This report has been questioned, since *U. tepperianum* was not found in subsequent studies. The fungus deposited as *U. tepperianum* was later found to be *U. notabile* (Morris & Wingfield, 1988). The report of *U. acaciae* on *A. mearnsii* has also been questioned (Morris & Wingfield, 1988). *Uromycladium notabile* causes galls on branches, stems, seed pods, leaves and petioles (Sherry, 1971; Dick, 1985). The first and only rust described thus far from *A. mearnsii* in South Africa is caused by *U. alpinum* (Morris & Wingfield, 1988). The disease was described in areas ranging from the Western Cape Province to Swaziland in the East, causing severe leaf drop of the lower leaves (Morris & Wingfield, 1988).

8.4 Wilts

Wilt and die-back diseases of *A. mearnsii* have been reported regularly since the beginning of the century. In South Africa a serious disease was known as Albert Falls disease (Stephens & Goldschmidt, 1938). The causal agent was, however, never found although a range of fungi were isolated from diseased tissue (Stephens & Goldschmidt, 1938). Some authors reported *Rhizoctonia lamellifera* Small. to be the cause of Albert Falls disease (Gibson, 1964; Lückhoff, 1964), but this was never proven.

In 1989, a serious die-back and wilt disease of black wattle was ascribed to *Ceratocystis fimbriata* (Morris, Wingfield & de Beer, 1993). The disease is characterised by the rapid wilting and die-back of trees, gummosis, stem and wood lesions (Morris *et al.*, 1993). Since this report the disease has continued to be the focal point of disease research of black wattle in South Africa. The causal agent has more recently been described as a new species of *Ceratocystis*, known as *C. albofundus* de Beer, Wingfield & Morris (Wingfield *et al.*, 1996).

A die-back disease, caused by *Phoma herbarum* Westend. has been reported from Kenya. The fungus was described to be a wound associated pathogen only. Spores of *P. herbarum* could not

infect healthy bark, although it was found that mycelium of the fungus could infect both wounded and healthy bark (Olembo, 1972).

8.5 Nursery diseases

Cylindrocladium scoparium Morgan is reported to be the cause of post emergence damping-off (Bakshi, 1976). It is, however, suggested that all South African isolates of *C. scoparium* may in fact reside in another species. It is suggested that all previous reports are in fact of *C. candelabrum* Viégas and not of *C. scoparium* (Crous & Wingfield, 1994). An undetermined species of *Oidium* regularly causes powdery mildew of seedlings (Sherry, 1971).

9.0 HEALTH OF *A. MEARNSII* IN SOUTH AFRICA

In South Africa, *A. mearnsii* (black wattle) trees provide tannin for the production of Bondtite products such as water resistant glues, while the wood is used in the production of pulp (Anonymous, 1997). The tannins, extracted from the bark, are also used in the leather tanning industry (Anonymous, 1997). The pulp is used for the manufacture of high quality paper as well as rayon. Apart from this, *A. mearnsii* wood is used for chipboard, plywood and charcoal manufacture (Anonymous, 1997). The *A. mearnsii* industry is the third largest forestry industry in the country, and has shown itself to be invaluable to the success of the industry (Anonymous, 1992; Anonymous, 1996). Diseases of *A. mearnsii* are thus of great concern. Black wattle is fast growing, relatively drought tolerant and versatile. An added benefit is also their ability to fix nitrogen (Sherry, 1971).

Between the period 1994-1995 a comprehensive survey of diseases of *A. mearnsii* was conducted (Roux & Wingfield, 1997). This survey resulted in the identification of a number of fungi that had not previously been reported from *A. mearnsii* in South Africa. A number of new pathogens were also identified during this survey (Roux & Wingfield, 1997). Currently, diseases are common on *A. mearnsii* in South Africa. Black butt is found in many plantations. The typical black discolouration of the bark may either be restricted to the basal parts of the stems, but it often spreads and eventually covers the entire length of the trees (Roux & Wingfield, 1997). In severe cases the disease often leads to tree death. If trees survive, bark can be of a very low quality and is mostly unsaleable (Haigh, 1993).

Initially, the most serious disease of *A. mearnsii* in South Africa, was considered to be Ceratocystis wilt. This disease is of great concern to the industry, since it usually leads to tree death (Morris *et al.*, 1993). During the recent surveys, however, very few isolates of *C. albofundus* were collected from symptomatic trees (Roux & Wingfield, 1997). This was probably a result of the difficulty with which this pathogen is isolated, and not because it is uncommon in plantations. Symptoms of the disease are reportedly abundant (Roux & Wingfield, 1997), but many questions regarding the etiology of this disease and the origin of the pathogen remain unresolved.

A number of previously unreported fungal taxa were isolated from diseased *A. mearnsii* during these surveys. These included the probable pathogens, *Phytophthora boehmeriae* Sawada, *Botryosphaeria dothidea* [=*B. ribis* (Tode.:Fr.) Grosenb. & Drugger], a *Sphaeropsis* sp. and a *Fusarium* sp. (Roux & Wingfield, 1997; Roux *et al.*, 1997). A study of the role of these fungi, as well as a detailed study of Ceratocystis wilt, is now a priority.

Phytophthora boehmeriae has been shown to be capable of producing lesions similar in size to those produced by *P. parasitica* on *A. mearnsii* seedlings, both in glass house and in field inoculations (Roux & Wingfield, 1997). During the 1994-1995 surveys a number of oomycetous fungi were isolated from diseased material. These fungi included *P. meadii* McRae, which was shown to be capable of producing significant lesions on *A. mearnsii* seedlings in glass house and field trials (Roux, 1996; Roux & Wingfield, 1997). It is clear that more than one *Phytophthora* species might be involved in diseases of *A. mearnsii*. A study of these fungi, their distribution and etiology is needed.

Botryosphaeria dothidea and *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & B. Sutton are serious stress related pathogens of *Eucalyptus* and *Pinus* spp. in South Africa and other parts of the world (Swart, Wingfield & Knox-Davies, 1987; Shearer, Tippett & Bartle, 1987; Smith, Kemp & Wingfield, 1994). Both pathogens are known as endophytes on various plants (Fisher *et al.*, 1993; Smith *et al.*, 1996a; Smith, Wingfield & Petrini, 1996). These fungi can infect healthy trees through wounds or stomata and live asymptomatically within the host tissues until the host tree is stressed or weakened. These endophytic fungi can then become aggressive pathogens, capable of killing mature trees (Carroll, 1988; Stone & White, 1997). *Sphaeropsis sapinea* has been shown to be especially aggressive after hail damage to *Pinus* spp. Trees that would normally have recovered from the hail

damage are killed within a few weeks (Swart *et al.*, 1987; Zwolinski, Swart & Wingfield, 1990). The same has been found with *B. dothidea* and frost damage on *Eucalyptus* spp. (Smith *et al.*, 1994). The isolation of *B. dothidea* and a *Sphaeropsis* sp. from diseased *A. mearnsii* suggests that these fungi may be endophytes on this tree, and thus play the same role in disease development as they do on other trees.

10.0 CONCLUSIONS

10.1 It is clear that there are many diseases affecting the planting of *Acacia* spp. in plantations. Many of these diseases can be controlled with management practices and sound breeding programmes. The most common disease problems are infection by wood rot fungi. It is clear that there is great room for improvement, especially in the breeding aspects of *Acacia* forestry. Although expensive, it is possible to control nursery diseases with chemicals. Once the trees have been taken to the plantation this option becomes impractical and uneconomical. This is especially true when considering that many of the countries planting *Acacia* spp. are in fact developing countries with limited financial resources.

10.2 Although considerable progress have been made with the identification of disease problems on *A. mearnsii* in South Africa, a number of questions remain to be answered. The most pressing of these regards the etiology of *C. albofundus* and the development of disease tolerant clones for future planting. With profits from *A. mearnsii* increasing, the industry will continue to grow in importance. Disease problems should thus be clarified and controlled as early as possible, so as to ensure the success of the industry in South Africa and in other countries.

10.3 Many of the disease problems reported on plantation *Acacia* spp. are wound and stress related. Reducing the number of wounds to trees would thus greatly reduce disease problems. Basic silvicultural practices combined with improved genetic stock will ensure that the *Acacia* industry maintains a strong position in international forestry. *Acacia* spp. are fast growing and yield high quality products. Unlike *Eucalyptus* and *Pinus* spp., *Acacia* spp. also provide nitrogen to the soil and many may be a source of food and feed, an important consideration for developing countries. There is great potential for using *Acacia* forestry in rotations with other forestry

genera as a way of reducing soil depletion due to nutrient losses. These trees thus deserve a concerted research effort into maximising their performance and yield.

Table 1: List of pathogens reported from plantation *Acacia* species of the world.

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. auriculiformis	*Corticium salmonicolor*	Pink disease	Florence & Balasundaran, 1991
	Cylindrocladium quinqueseptatum	Leaf spot, defoliation	Lenné, 1992
	Exserohilum rostratum	Leaf spot	"
	Ganoderma applanatum	Root and butt rot	Browne, 1968; Lenné, 1992
	G. lucidum	Root rot & white spongy rot	Browne, 1968
	Ganoderma sp.	Wood rot	Barari, 1993
	Lasiodiplodia theobromae	Root rot	Lenné, 1992
	Macrophomina phaseolina	Root rot, wilt, gummosis	"
	Phellinus noxius	Wood rot	Lee & Arentz, 1995
	Phellinus spp.	Wood rot	Lenné, 1992
	Rhizoctonia solani	Web blight, defoliation	"
	Uromyces digitatus	Rust	"
A. catechu	*Colletogloeum acaciicola*		Sutton & Swart, 1986
	Erysiphe acaciae Blumer		Browne, 1968
	Fomes badius	Heart rot	Bakshi, 1957; Browne, 1968; Ito & Nanis, 1997
	F. fastuosus [Syn.: *Phellinus fastuosus* (Leveille) Cunningham]		Browne, 1968
	F. senex [Syn.: *Phellinus senex* (Nees ex. Montagne) Imazeki]		"
	Fusarium solani	Wilt	Lenné, 1992

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. catechu	*Glomerella cingulata*	Anthracnose	Gibson, 1975
	Ganoderma applanatum	Root rot	Browne, 1968
	G. lucidum	Root rot	Bakshi, 1957; Browne, 1968; Lenné, 1992
	Microstroma acaciae		Browne, 1968
	Phellinus badius	Wood rot	Gibson, 1975
	P. gilvus	Root rot	Bakshi, 1957
	Ravenelia tandonii		Browne, 1968; Patil & Date, 1980
A. dealbata	*Armillaria mellea*	Root rot	Bakshi, 1976
	Calonectria indusiata [Imperfect = *Cylindrocladium theae*]	Leaf spot	Browne, 1968; Bakshi, 1976; Crous & Wingfield, 1994
	Cylindrocladium scoparium	Leaf drop, stem disease, damping-off	Bakshi, 1976
	C. floridanum Sobers & Seymour	Leaf spot, root rot	Crous *et al.*, 1991
	Daldinia concentrica (Bolt. ex Fr.) Ces. & De Not.		Browne, 1968
	Fomes endapalus		"
	F. mastoporus (Lev.) Cke.	Heart rot	Browne, 1968; Bakshi, 1976
	Fusarium oxysporum	Leaf drop, stem disease, damping-off	Bakshi, 1976
	Ganoderma applanatum	Heart rot	Browne, 1968; Bakshi, 1976; Ito & Nanis, 1997
	G. australe	Heart rot	Browne, 1968; Bakshi, 1976
	Glomerella acaciae	Anthracnose	Hodges, 1964

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. dealbata	*Glomerella cingulata* (Imperfect: *Colletotrichum acaciae* K. Ito & Shibukawa)	Leaf drop, stem disease	Bakshi, 1976
	Hypoxylon hypomiltum	Stem cankers	Browne, 1968; Bakshi, 1976
	H. rubiginosum	Stem cankers	Browne, 1968; Bakshi, 1976
	Peniophora incarnata	Root rot	Browne, 1968; Bakshi, 1976
	P. sacrata	Root rot	Browne, 1968
	Polyporus laevigatus [Syn.: *Phellinus laevigatus* (Fries) Bourdot et Galzin]		Browne, 1968
	P. zonatus Fr.		Browne, 1968
	Trametes tawa	Heart rot	Browne, 1968; Bakshi, 1976
	Uromyces phyllodiorum	Phyllode and leaf rust	Browne, 1968; Bakshi, 1976
	Uromycladium acaciae	Leaf spot, branch and stem distortions	Browne, 1968; Bakshi, 1976; Dick, 1985
	U. alpinum	Phyllode and leaf rust	Browne, 1968; Bakshi, 1976
	U. notabile	Galls, die-back	Browne, 1968; Dick, 1985
A. decurrens	*Armillaria mellea*	Root rot	Bakshi, 1976
	A. fuscipes Petch.	Root rot	"
	Calonectria indusiata	Leaf spot, defoliation	Bakshi, 1976; Crous & Wingfield, 1994

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. decurrens	*Camptomeris albizziae*	Leaf spot	Bakshi, 1976
	C. verruculosa	Leaf spot	"
	Ceratocystis fimbriata	Wilt and die-back, stem cankers	Ribeiro *et al.*, 1988
	Corticium salmonicolor	Pink disease	Bakshi, 1976
	Cylindrocladium scoparium	Damping-off	"
	C. theae	Leaf spot	Gibson, 1975
	Fomes lamaoensis	Root rot	"
	Fusarium oxysporum	Damping-off	"
	Ganoderma lucidum	Root rot	Harsh *et al.*, 1993
	Glomerella cingulata	Stem canker	Bakshi, 1976
	Irpex subvinosus (B. & Br.) Petch.		Bertus, 1961
	Macrophomina phaseolina	Root rot	Gibson, 1975
	Physalospora abdita	Stem cankers	Bakshi, 1976
	Poria albobrunnea	Root rot	Bertus, 1961; Bakshi, 1976
	Rosellinia acruata	Black root rot	Gibson, 1975
	R. bunodes	Black root rot	"
	Trametes mollis Fr.		Bertus, 1961
	Uromycladium acaciae	Leaf spot, branch and stem distortions	Dick, 1985
	U. notabile	Galls, die-back	Bakshi, 1976; Dick, 1985
	U. tepperianum		Browne, 1968
A. koa	*Armillaria mellea*	Root rot	Larsen *et al.*, 1985
	Atelocauda angustiphylloda	Rust	Gardner, 1991
	A. digitata	Rust	Hodges & Gardner, 1984
	A. koae	Rust	Gardner, 1978

Acacia species	**Fungal taxon**	**Associated disease/symptoms**	**References**
A. koa	*Colletoctrichum gloeosporioides*	Seed rot	Stein, 1983
	Cylindrocladium parasiticum Crous, Wingf. & Alfenas (Syn: *Calonectria crotalariae* (Loos) Bell & Sobers)	Collar rot	Gibson, 1975 Crous & Wingfield, 1994
	Endoraecium acaciae	Rust	Hodges & Gardner, 1984
	E. hawaiiense	Rust	"
	Ganoderma sp.	Root rot	Bega, 1979
	Ganoderma lucidum	Root rot	Harsh *et al.*, 1993
	Laetiiporus sulphureus	Wood rot	Larsen *et al.*, 1985
	Phaeolus schweinitzii	Wood rot, brown cubical rot	"
	Phellinus kawakamii	White pocket rot	"
	Pleurotus ostreatus	Wood rot/ white rot	"
	Polyporus sulphureus Bull ex. Fr.	Brown cubical rot	Bega, 1979
A. mangium	*Armillaria* sp.	Root rot	Wingfield, unpublished
	Colletotrichum gloeosporioides	Leaf spot	Lee, 1993
	Corticium salmonicolor	Pink disease	Ahmad, 1987; Lee, 1993
	Corynespora sp.	Leaf spot	Lee, Lenné, 1993
	Cylindrocladium theae	Leaf spot, lesions	Lenné, 1992; Crous & Wingfield, 1994
	Cylindrocladium quinqueseptatum	Leaf spot, defoliation	Lenné, 1992
	Cylindrocladium sp.	Damping-off	Lee, 1993

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. koa	*Diplodia* sp.	Die-back	Lenné, 1992
	Fusarium solani	Damping-off	Zakaria, 1990; Lee, 1993
	Fusarium sp.	Damping-off	Ahmad, 1987; Lee, 1993
	Ganoderma sp.	Heart rot	Lee, 1993
	G. lucidum	Root rot	Harsh *et al.*, 1993
A. mangium	*G. weberianum* (Bresadola et Hennings) Steyaert	Root rot	Lee, 1993
	Gloeosporium sp.	Leaf spot	Lee, 1993
	Glomerella cingulata	Leaf spot, seedling blight	Ahmad, 1987; Lenné, 1992; Lee, 1993
	Lasiodiplodia theobromae	Root disease, leaf spot	Lenné, 1992; Lee, 1993
	Macrophomina sp.	Charcoal root disease	Ahmad, 1987; Lenné, 1992; Lee, 1993
	Meliola sp.	Sooty mold	Lenné, 1992
	Nectria pseudotricha	Die-back	Lenné, 1992
	Nectria sp.	Stem cankers	Kapp *et al.*, 1997
	Oidium sp.	Powdery mildew	Lenné, 1992; Lee, 1993
	Phellinus noxius	Brown root disease, honeycomb rot	Ahmad, 1987; Lenné, 1992; Lee, 1993
	Phialophora sp.	Heart rot	Ito & Nanis, 1997
	Phomopsis sp.	Leaf spot	Ahmad, 1987
	Phyllostictina sp.	Leaf spot	Ahmad, 1987
	Phytophthora sp.	Damping-off	Lee, 1993
	Pythium sp.	Damping-off	Ahmad, 1987; Lee, 1993
	Rhizoctonia solani	Damping-off	Zakaria, 1990; Lee, 1993
	Rhizoctonia sp.	Damping-off	Ahmad, 1987
	Rosellinia sp.	Root disease, die-back	Kapp *et al.*, 1997
	Trametes corrugata	Die-back	Lenné, 1992
A. mearnsii	*Armillaria mellea*	Root rot	Browne, 1968; Bakshi, 1976

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. mearnsii	*Amauroderma rude* (Berk.) G. H. Cunn.	Root rot	Doidge *et al.*, 1953; Roberts, 1957
	Botryosphaeria dothidea	Stem canker	Roux *et al.*, 1997a, b
	Calonectria indusiata	Leaf spot	Browne, 1968; Bakshi, 1976
	Camptomeris albizziae	Leaf spot	Bakshi, 1976
	C. verruculosa	Leaf spot	"
	Ceratocystis albofundus	Wilt, die-back, gummosis, stem cankers (Ceratocystis wilt)	Wingfield *et al.*, 1996
	Cylindrocladium theae	Leaf spot, cankers	Lenné, 1992; Crous & Wingfield, 1994
	Coniophora arida (Fr.) Karst.		Sherry, 1971
	Corticium salmonicolor	Pink disease	Roberts, 1957; Browne, 1968; Bakshi, 1976; Lenné, 1992
	Cylindrocladium candelabrum	Damping-off, stem cankers	Roux & Wingfield, 1997
	C. scoparium	Root disease	Doidge *et al.*, 1953; Browne, 1968; Bakshi, 1976; Crous *et al.*, 1991
	Dothiorella pithyophilla Sacc.	Stem canker	Panneerselvam *et al.*, 1975
	Ganoderma applanatum	White mottled heart rot	Browne, 1968; Bakshi, 1976
	C. scoparium	Root disease	Doidge *et al.*, 1953; Browne, 1968; Bakshi, 1976; Crous *et al.*, 1991

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. mearnsii	*Dothiorella pithyophilla* Sacc.	Stem canker	Panneerselvam *et al.*, 1975
	Ganoderma applanatum	White mottled heart rot	Browne, 1968; Bakshi, 1976
	G. lucidum	Root rot, white spongy rot	Browne, 1968; Sherry, 1971; Bakshi, 1976; Gorter, 1977
	G. rugosum	Collar rot	Lückhoff, 1964; Gibson, 1964
	Glomerella acaciae	Anthracnose	Hodges, 1964
	Hydnum henningsii Bres.	Wood rot	Roberts, 1957
	Irpex subvinosus		Browne, 1968
	Lasiodiplodia theobromae	Collar rot	Stephens & Goldschmidt, 1938; Lenné, 1992
	Macrophomina phaseolina	Root rot	Browne, 1968; Bakshi, 1976
	Oidium sp.	Powdery mildew	Sherry, 1971
	Phoma herbarum	Wilt and die-back	Olembo, 1972
	Phytophthora nicotianiae	Black butt/root rot	Zeijlemaker, 1971
	Physalospora abdita	Die-back, stem canker	Browne, 1968; Bakshi, 1976
	Polystictus hirsutus Fr.		Roberts, 1957
	Poria albobrunnea		Browne, 1968
	Rhizoctonia lamellifera	Wilt & die-back (Albert Falls Disease), root rot	Gibson, 1964; Lückhoff, 1964
	R. solani	Web blight, defoliation	Lenné, 1992

Acacia species	Fungal taxon	Associated disease/symptoms	References
A. mearnsii	*Schizophyllum commune*	Wood rot	Ledeboer, 1946
	Stereum ostrea (Fr.) Fr.	Heart rot	Browne, 1968; Bakshi, 1976
	Stigmina verruculosa Syd.	Leaf spot	Doidge *et al*, 1953
	Uromycladium acaciae	Leaf spot, branch and stem distortions	Browne, 1968; Bakshi, 1976; Dick, 1985
	U. alpinum	Rust	Morris & Wingfield, 1988
	U. notabile	Galls, leaf drop, die-back	Browne, 1968; Dick, 1985
	U. tepperianum	Galls	Browne, 1968; Bakshi, 1976

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CHAPTER 2

GENETIC VARIATION IN THE WILT PATHOGEN, CERATOCYSTIS ALBOFUNDUS, IN SOUTH AFRICA

2

GENETIC VARIATION IN THE WILT PATHOGEN, *CERATOCYSTIS ALBOFUNDUS*, IN SOUTH AFRICA

ABSTRACT

Ceratocystis albofundus is a serious wilt pathogen of *Acacia mearnsii* in South Africa where it kills large numbers of trees each year. Currently, no effective control measures are available for reducing losses due to this pathogen. Recent success with clonal propagation of *A. mearnsii* has raised hopes of being able to select disease tolerant clones for future planting. The durability of disease tolerance in these clones will depend strongly on the genetic diversity of *C. albofundus* in South Africa. The aim of this study was to determine the genetic diversity of the *C. albofundus* population in South Africa. Isolates were collected from a number of geographic regions, focusing on the commercial *A. mearnsii* growing areas. Total genomic DNA was extracted for each isolate and restricted with *Pst*I for determination of nuclear DNA diversity and *Hae*III for mitochondrial diversity. The resultant *Pst*I fragments were probed with a radioactively labeled 15bp oligonucleotide marker $(CAT)_5$. For the mitochondrial DNA the RFLP's were scored directly without probing. Nei's gene diversity (*H*) was determined for both methods and compared with published values for other *Ceratocystis* species. A distance matrix was developed for each technique using UPGMA and Neighbor-joining. The *C. albofundus* population was found to have a high level of both nuclear and mitochondrial gene diversity when compared with other *Ceratocystis* spp. These results support the hypothesis that *C. albofundus* is native to South Africa. Data also suggest that selection and breeding for disease tolerance will be complicated by genetic variation in the pathogen.

INTRODUCTION

The genus *Ceratocystis sensu stricto* Ell. & Halst. includes some of the most serious plant pathogens known (Wingfield, Seifert & Webber, 1993). Fungi in this genus range from aggressive primary pathogens to opportunistic secondary invaders of stressed plants (Kile, 1993). Some of the more serious diseases caused by *Ceratocystis* spp. or their *Chalara* (Corda) Rabenh. anamorphs include black rot of sweet potato (Halsted, 1890), oak wilt (French & Stienstra, 1978), canker and wilt of stone fruits (De Vay, Davidson & Moller, 1968), canker and rot of coffee and rubber (Upadhyay, 1981) and canker and wilt of *Nothofagus* sp. (Kile & Walker, 1987).

Ceratocystis albofundus De Beer, Wingfield & Morris causes Ceratocystis wilt (wattle wilt) of *Acacia mearnsii* de Wild. in South Africa (Morris, Wingfield & De Beer, 1993; Wingfield *et al.*, 1996b). The fungus results in rapid wilting and die-back of trees, leading to death within a few weeks (Morris *et al.*, 1993; Roux, Wingfield & Dunlop, 1998). Ceratocystis wilt was first recorded in 1989 from the KwaZulu-Natal Midlands where *A. mearnsii* trees were found dying of an unknown cause. Since then, regular outbreaks of the disease have been reported (Roux & Wingfield, 1997).

Ceratocystis albofundus has been described only from South Africa and is suspected to be endemic to the country. The only other records of this fungus are from *Protea gigantea* L., from the Mpumalanga Province in 1977 (Gorter, 1977) and a collection from *P. cynaroides* near Pretoria (PREM44932). These two records were of *C. fimbriata* Ell. & Halst., but a re-examination has shown that the specimens resemble *C. albofundus.* Thus, both specimens have perithecia with the light coloured bases with dark necks that distinguish *C. albofundus* from *C. fimbriata* (Wingfield *et al.* 1996b).

No control measures are currently available for the management of wattle wilt caused by *C. albofundus*. In an effort to reduce losses caused by diseases, a breeding and selection programme has been initiated. This is focused strongly on selection of trees that have

outstanding growth and other quality characteristics, including tolerance to Ceratocystis wilt. To ensure durability in crop resistance, a knowledge of the pathogen population is needed (Wolfe & Caten, 1987; McDonald, 1997; Milgroom & Fry, 1997). Thus, pathogens with more variable populations will be most likely to adapt to disease tolerant planting stock (McDonald & McDermott, 1993). This is especially true in situations where the host plants have very little genetic variation (McDonald & McDermott, 1993).

A recent technique described for determining the genetic variation within and between populations of ascomycetous fungi, is the use of synthetic oligonucleotides as probes. These probes are used to hybridize to variable number tandem repeat (VNTR) loci found in the microsatellite DNA regions of the genome (Jeffreys, Wilson & Swee, 1985; Kistler, Momol & Benny, 1991; DeScenzo & Harrington, 1994; Haymer, 1994). These microsatellite regions are useful for studying population diversity since they have a higher level of variation than coding regions of the genome and are not influenced by codon bias or selection (Haymer, 1994; Akagi *et al.*, 1996). One such mini-sattelite probe is $(CAT)_5$ which has been found to detect restriction fragment length polymorphisms (RFLP's) in a wide variety of organisms, including Basidiomycetes and Ascomycetes (DeScenzo & Harrington, 1994). This probe is also effective in quantifying gene diversity across different species (Harrington, Steimel & Kile, 1998). $(CAT)_5$ is a 15 base pair oligonucleotide which enables its use with in-gel hybridization techniques. It was shown to reproducibly detect large numbers of hypervariable loci in *Heterobasidion annosum* (Fr.:Fr.) Bref., *Ophiostoma piliferum* (Fr.:Fr.) Syd. and *Leptographium wagneri* (Kendrick) M.J. Wingfield (DeScenzo & Harrington, 1994). It has also been used with success to show the differences in genetic variation between outcrossing, selfing and asexual species of *Ceratocystis* (Harrington *et al.*, 1998).

The detection of mt DNA polymorphisms has been refined greatly by the development of rapid methods to obtain restriction patterns. It is possible to isolate total genomic DNA to use restriction enzymes that specifically digests the GC-rich nuclear DNA. Restriction enzymes *Hae*III, *Cfo*I and *Msp*I have proven very successful for a number of fungal

genera, recognizing the sites GGCC and GCGC respectively (Lacourt *et al.*, 1994; Wingfield, Harrington & Steimel, 1996a; Harrington *et al.*, 1998) This leaves AT-rich fragments of mtDNA that can be visualized directly with ethidium bromide staining of agarose gels (Freeman, Pham & Rodriques, 1993; Wingfield *et al.*, 1996a). These methods have been useful in studies of intraspecific polymorphisms in a range of fungi, providing valuable knowledge about plant pathogenic populations (Milgroom & Lipari, 1993; Lacourt *et al.*, 1994; Harrington *et al.*, 1998).

The objective of this study was to determine the genetic diversity of a population of *C. albofundus* isolates. This would enable us to re-consider the hypothesis that the fungus might be native to South Africa. Furthermore, it would reflect the durability in tolerance that might be expected from clones of *A. mearnsii*. This goal was achieved by considering nuclear and mitochondrial DNA diversity of isolates of *C. albofundus.*

MATERIALS & METHODS

Isolates

The 49 isolates used in this study were obtained from dying *A. mearnsii* trees throughout South Africa (Fig. 1; Table 1). Isolations were made from diseased trees using the carrot slice technique described by Moller & De Vay (1968). Each isolate originated from a different tree and was transferred from a single drop of ascospores on one perithecium. All isolates are maintained in the culture collection (CMW numbers) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, and in the culture collection of T.C. Harrington, Iowa State University. Results for *C. albofundus* were compared with published data for three other *Ceratocystis* spp. (Harrington *et al.*, 1998), since no other populations of *C. albofundus* exist.

The three *Ceratocystis* spp. chosen for comparison with *C. albofundus* were selected on the grounds of their reproductive strategies. *Ceratocystis eucalypti* Yuan & Kile is an

obligate outcrossing fungus, producing perithecia only when two strains of opposite mating types are crossed. It is a weak, wound colonizing pathogen of *Eucalyptus* spp. in Australia and it is also reported to be native to that country (Kile *et al.*, 1996). The second species, *Chalara australis* Walker & Kile, is an asexually reproducing fungus also native to Australia. It has only one mating type (*MAT*-2) and causes a serious wilt disease of *Nothofagus cunninghamii* (Hook.) Oerst. (Kile & Walker, 1987; Harrington *et al.*, 1998). *Ceratocystis virescens* (Davids) C. Moreau causes sap streak disease of maple (*Acer* spp.), tulip poplar (*Liriodendron tulipifera*) and other hardwoods in the U.S.A. It has two mating types, with one of the mating types capable of unidirectional mating type switching and thus selfing (Harrington & McNew, 1997).

DNA Extraction

Total genomic, high molecular weight DNA was extracted from all isolates by culturing them in 20 ml of liquid media (2 % malt extract, 1 % yeast extract) in 250 ml Erlenmeyer flasks. Flasks were kept at room temperature for 10 days. Cells were collected using vacuum filtration through 1mm Whatmann filter paper and care was taken to remove all the agar. The harvested cells were ground to a fine powder in liquid nitrogen with a mortar and pestle. Ten ml of extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl, 1.25% SDS; 10 mM β-mercaptoethanol; 4 mM spermidine; 1 mM spermine; 1 mM PMSF), maintained at 65°C, was added to each isolate. The resultant slurry was transferred to a sterile centrifuge tube and stored at -20°C until all samples were ready for further processing.

Samples were incubated in a water bath at 65°C for 60 min with frequent mixing. Potassium acetate (0.4 volumes of 5 M stock) was added to each sample and the samples incubated on ice for 20 min. The supernatant was collected by centrifugation for 15 mi. at 17 000 RPM at 4°C. Ice cold isopropanol (0.58 volumes) was added to the supernatant of each sample in a 30 ml glass Corex tube. Tubes were placed at -20°C overnight. The

resultant pellets were collected by centrifugation for 10 min at 10 000 rpm's and 4°C, after which they were washed with 10 ml of 70% ethanol for 10 min on ice.

Pellets were collected by centrifugation at 10 000 RPM for 10 min and air dried in a fume hood at room temperature after which they were resuspended in 1000 µl of sterile distilled water for 60 min at 37°C. Samples were transferred to 1.5 ml Eppendorf tubes and centrifuged at 10 000 RPM for 10 min. The supernatant, containing the DNA, was collected in sterile 1.5 ml Eppendorf tubes and the DNA concentrations were determined using a TKO fluorometer and then stored at -20°C.

DNA restrictions

Restriction digests of the total genomic DNA was done using 25 µg of DNA at 37°C for 15-20 hours, or overnight. Restrictions were carried out in a total volume of 500 µl containing DNA, 10X buffer, spermidine (1 mM), water and the specific enzyme, *Hae* III or *Pst* I (5 U/µg genomic DNA) (GIBCO BRL). Rnase (A 1.35 un/µl + T 37 un/µl) was added after 2 hours to remove the RNA.

Samples were precipitated with NaCl (0.2 M) and 2 volumes of 100 % cold ethanol for 45 min at -20°C. Pellets were collected by centrifugation at 12 000 rpm's for 3 min and then washed in 800 µl of 70% ethanol for 20 min on a rotary shaker. The samples were again centrifuged at 12 000 rpm's for 3 min and the pellets dried in a speed vac for ~30 min or until dry. Pellets were resuspended in sterile water at 37°C to a concentration of 0.2 µg/µl and stored at -20°C.

Separation of fragments

For the separation of fragments, 2 µg of restricted DNA was loaded onto 1% Agarose (Biorad analytical grade) gels in 1X TBE. Gels were run at 88 volts for 17.5 hours with constant stirring for *Pst*I gels and at 80V for 17 hours for *Hae*III gels. Lanes 1 and 20

contained λ *Hind*III DNA (1μg) (GIBCO BRL) as molecular marker and samples were loaded into lanes 2-19. Gels were stained for ~15 min in ethidium bromide on a rotary shaker, washed in water for ~30 min and visualized under UV light. Successful genomic DNA gels were dried using a gel drier for 60 min at 50°C. They were then sealed between sheets of plastic, enclosed in tin foil and stored at 4°C until further use. Mitochondrial DNA gels were photographed and analyzed from these images.

$(CAT)_5$ hybridization

In-gel hybridization was performed by rinsing gels for 45 min in 250 ml denaturing solution (0.5 M NaOH; 1.5 M NaCl) on a rotary shaker. Denaturation was followed by rinsing for 45 min in 250 ml neutralizing solution (1.0 M Tris, pH 8.8; 1.5 M NaCl). Gels were washed for 20 min in 250 ml water and then placed in Church's solution (250 mM Na_2HPO_4 - NaH_2PO_4, pH 7.4; 7% SDA; 1 mM EDTA; 1% BSA) for 2 hours at 42°C on a shaker. The P^{32} labeled $(CAT)_5$ probe was added to the hybridization mix and incubated for 16-18 hours or overnight. Probes were prepared by end labeling 50 ng of $(CAT)_5$ with 50 μCi of ^{32}P-dCTP using terminal deoxynucleotidyl transferase (GIPCO BRL). The reaction was done at 37°C for 1 hour and stopped by adding 50 μl of Buffer EB (QIAGEN). Unincorporated nucleotides were removed by spin column chromatography with Sephadex G25/80 (Sigma). Purified probe was added directly to the Church's hybridization solution and hybridization was carried out for 16-18 hours at 42°C with continuous shaking.

For the labeling of the *Hind*III marker the Prime-a-Gene Labeling System (PROMEGA) was used. Labeled marker was prepared in a total volume of 50 μl containing 5 Units of Klenow DNA Polymerase I (5U/ μl), 50 μCi P^{32}, 5X labeling buffer, unlabelled DNTP's (dGTP, dTTP, dATP), Nuclease free BSA and λ *Hind*III marker. The marker was denatured before adding it to the reaction mixture. The reaction mixture was incubated at 37°C for 1 hour before the volume was adjusted to 100 μl with QIAGEN elution buffer

and spun through a Sephadex G25/80 column. Labeled marker was then denatured again and added to the gels with the labeled $(CAT)_5$ probe.

The probe solution was removed and gels were washed for 45 min with 6X SSC (Prepared from 20X SSC stock: 3 M NaCl; 0.3 M $Na_2citrate{\cdot}2H_20$; pH 7) on a rotary shaker. This SSC was replaced with fresh SSC and again washed for 45 min. Gels were then washed in 5X SSC for 45 min and wrapped between two layers of plastic. Gels were visualized in two ways. They were either exposed to Kodak X-ray film for one week or to a Phosho Imager Screen for 1-2 days, depending on the strength of the probe.

Gel analysis

Nuclear DNA

Each unique band was scored as either present (1) or absent (0) for each isolate tested. Band sizes were determined using the programme GelReader 2.0.5 (NCSA, University of Illinois, Champagne, Urbana, IL). The procedure was repeated for all isolates and only bands that were clearly visible in all runs were scored. Nei's (1973) gene diversity (*H*) was calculated and a distance matrix and dendogram compiled using Neighbor-joining and the Unweighted Pair-Group Mean Arithmetic Analysis (UPGMA) (Felsenstein, 1993). Data obtained for *C. albofundus* were compared with published data for *C. virescens*, *C. eucalypti* and *Chalara australis* (Harrington *et al.* 1998). Although only a small number of isolates were available per geographic area, gene diversity values were also calulated for each area separately.

Mitochondrial DNA

For the analysis of bands generated from the RFLP's using *Hae*III, no probing was necessary. All bright bands, larger than 2kb in size, were scored. Band sizes were determined using GelReader. The procedure was repeated for each isolate. The gene

diversity (*H*) was determined and the data obtained for *C. albofundus* were compared with those published by Harrington *et al.* (1998) for *C. virescens*, *C. eucalypti* and *Chalara australis*. Gene diversity values were also calculated for each geographic area to determine the influence of geographic distribution on the population diversity.

RESULTS

Nuclear DNA diversity

The $(CAT)_5$ markers were highly variable for *C. albofundus* (Fig. 2) when compared to published results for the other *Ceratocystis* spp. (Table 2). For *C. albofundus,* 47 of the 50 loci scored were found to be polymorphic. Harrington *et al.* (1998) found only 2 of 22 loci polymorphic for the asexual *Ch. australis;* 17 of 19 loci polymorphic for the obligately outcrossing *C. eucalypti* and an intermediate level of variation, 1 of 4 loci, for the homothallic *C. virescens*. Values for *C. albofundus* are thus most similar to those of *C. eucalypti*.

A total of 37 phenotypes were found for the 37 isolates of *C. albofundus* tested for nuclear diversity. This is higher than that published for any of the *Ceratocystis* spp. for which similar values are available. The closest similarity was again with *C. eucalypti*, showing 9 phenotypes from 10 isolates. For *C. virescens* 2 phenotypes were found in 16 isolates and for *Ch. australis*, 3 phenotypes were seen in 30 isolates (Harrington *et al.*, 1998). The average gene diversity (*H*) of the $(CAT)_5$ markers for *C. albofundus* was 0.2137. This is higher than values for *Ch. australis* (0.0111) or *C. virescens* (0.0935), but lower than those of *C. eucalypti* (0.3747) (Harrington *et al.*, 1998).

Gene diversity values for individual plantations were similar to those for the country as a whole (Table 2). When considering gene diversity values for individual plantations, the results suggest that the total diversity is based not only on diversity between plantations, but also on diversity within plantations. Values for individual plantations were similar to

those of the entire South African population, with the highest value found for the Vryheid area (0.282).

Phylograms obtained from the UPGMA and Neigbor-joining analysis of the distance matrix produced after scorring of the bands, show a tendency for isolates from different plantations to group together in clusters (Fig. 3). Most clusters consisted of isolates from more than one geographic area. Results suggests a migration of isolates between different geographic areas in South Africa. Data obtained from UPGMA and Neighbor-joining supported each other.

Mitochondrial DNA diversity

Variation in the mitochondrial DNA of *C. albofundus* (Fig. 4) was much higher than that of any of the three *Ceratocystis* spp. with which it was compared (Table 3). Forty-one of the 46 scored bands were polymorphic for *C. albofundus*. For the obligate outcrossing fungus, *C. eucalypti*, only 9 of 33 bands were polymorphic, while for *Ch. australis* 1 of 28 and for *C. virescens* only 13 of 40 were polymorphic (Harrington *et al.*, 1998).

For *C. albofundus* 30 different phenotypes were found for the 31 isolates tested. As with the nuclear DNA, *C. eucalypti* showed the most similar values, with 6 different phenotypes from 10 isolates. *Ceratocystis virescens*, which has a similar mating strategy to that of *C. albofundus*, had only 10 different phenotypes in 16 different isolates. For the asexual *Ch. australis*, only 2 different phenotypes were found from 30 isolates.

The average gene diversity value for *C. albofundus*, using the *Hae* III marker was 0.249. In contrast, the average diversity value for *C. eucalypti*, which is reported to be native to Australia, was only 0.1115, while for *C. virescens* a value of 0.0928 was reported. The lowest value (0.0023) was again found for *Ch. australis* (Harrington *et al.*, 1998).

Phylograms showed a grouping of isolates from different geographic areas in most clusters (Fig. 5). Genetic diversity for individual plantations was also similar to that for the country as a whole (Table 3). Mitochondrial data thus also show a mixing of genes between different areas in South Africa.

For the *Ha* III digests, isolate CMW4084 from Dalton grouped with isolate CMW4758 from Umtata. These two areas are approximately 400 kilometers apart, the one occurring in a plantation of a commercial *A. mearnsii* growing area and the other originating from an area with only "jungle" stands of *A. mearnsii* (Fig. 1). Isolates CMW4093 and CMW4094 from East London in the Eastern Cape province and isolate CMW4105 from Piet Retief in the South Eastern Mpumalanga Province group together (Fig. 1). Isolate CMW4102 from Piet Retief originated from a commercial plantation, approximately 700 km distant from East London, where isolates originated from "jungle" stands.

DISCUSSION

Recently established populations are expected to have small effective population sizes and low levels of mtDNA diversity (Ellstrand & Elam, 1993; Milgroom & Lipari, 1993). The size of the founder population of *C. albofundus* is unknown and it is also not known if this fungus was introduced or is endemic to South Africa. Results obtained in this study show a level of nuclear and mtDNA diversity higher than those of any of the other three species to which *C. albofundus* was compared. This includes *C. eucalypti* and *Ch. australis,* which are thought to be native fungi in Australia (Kile *et al.*, 1996) and are thus expected to have relatively high levels of genetic diversity. The high gene diversity, together with the reports of *C. albofundus* from indigenous *Protea.* spp. (Gorter, 1977; Wingfield *et al.*, 1996b), supports the hypothesis that *C. albofundus* is native to South Africa.

Genetic diversity is influenced by the mode of reproduction of the organism, mutation, gene flow, genetic drift and selection (Kohn *et al.*, 1988; McDonald & McDermott,

1993; Milgroom & Fry 1997). High levels of genetic diversity in *C. albofundus* could thus be attributed to a number of factors, including its capacity for sexual reproduction. Organisms capable of sexual reproduction have been found to have a higher degree of genetic diversity than organisms that reproduce asexually (McDonald & McDermott, 1993; Wolf & McDermott, 1994; Milgroom, 1996). *Ceratocystis albofundus* has two mating types, a *MAT*-1 (self-sterile) requiring outcrossing and a MAT-2 (self-fertile) which is capable of selfing (De Beer, 1994; Harrington & McNew, 1997). This phenomenon has been shown for *C. virescens*, which has a similar reproduction system to *C. albofundus*. *Ceratocystis virescens* has an intermediate level of genetic diversity when compared to strictly outcrossing and strictly asexual species (Harrington *et al.*, 1998). The same situation could thus also have been expected for *C. albofundus*. The fact that *C. albofundus* has nuclear DNA diversity values higher than those for *C. virescens*, despite its similar reproductive strategy, strongly suggests that it is either native to South Africa, or has been in the country for an extended period of time.

Nuclear DNA diversity data for *C. albofundus* is supported by high levels of mitochondrial diversity in the fungus population. Sexual reproduction and outcrossing does not influence mitochondrial diversity (Taylor, 1986; Milgroom & Lipari, 1993; Harrington *et al.*, 1998). Mitochondrial diversity suggests that a population has been in existence for many years, or that it has not gone through a genetic bottle neck, such as an introduction into a new environment (Harrington *et al.*, 1998). The maternal and haploid inheritance of mtDNA makes this more sensitive than nuclear DNA to severe reductions in the number of individuals in a population of organisms, such as those caused by introductions to new areas (Cann, Stoneking & Wilson, 1987).

High levels of mitochondrial diversity could be attributable to a high mutation rate and large effective population sizes (Taylor, 1986). Most mutations in animal mtDNA take place through point mutations or nucleotide substitutions or deletions (Taylor, 1986). In fungi, it has been shown that a high number of length mutations (due to insertions and deletions) occur in the mitochondria (Taylor, 1986). In the U.S.A., *Cryphonectria*

parasitica (Murrill) Barr, is an introduced fungus with an extremely high mtDNA diversity and it is hypothesized that the high level of diversity is due to high mutation rates (Milgroom & Lipari, 1993). Observations on the occurrence of *C. albofundus* in the colder areas of South Africa (winter temperatures below 0 °C), however, strongly suggest that this fungus is a temperate species, unlike most other *Ceratocystis* spp., and that it is native to South Africa.

Although gene diversity values for the nuclear and mitochondrial DNA of *C. albofundus* are low when compared to the values provided by Nei (1973) for a diverse population (0 = clonal; 1 = diverse), results obtained in this study provide further support for the hypothesis that this pathogen may be native to South Africa. It has higher diversity values than those of other endemic *Ceratocystis* spp. (Harrington *et al.*, 1998) and its only known hosts include species of the native genus *Protea*.

As with any breeding programme, care should be taken in the clonal propagation of *A. mearnsii* in South Africa. Ceratocystis wilt tolerant trees and clones should be monitored continuously and new disease tolerant clones should be produced on a regular basis. Surveys to find more alternative hosts for *C. albofundus*, both native or introduced, will also continue in the future with the aim of unequivocaly determining the origin of this unique fungus in South Africa.

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Table 1. List of *Ceratocystis albofundus* isolates from wilted *Acacia mearnsii*, used to determine genetic diversity in South Africa.

Culture number [a]	Origin [b]	Collector
CMW4059 - CMW4068	Bloemendal, KZN	J. Roux & T.C. Harrington
CMW4069 - CMW4078	Vryheid, KZN	J. Roux & T.C. Harrington
CMW4079 - CMW4085	Dalton, KZN	J. Roux & T.C. Harrington
CMW4087 - CMW4090	"	"
CMW4092 - CMW4096	East London, EC	M.J. Wingfield & T.C. Harrington
CMW4097	Cintsa, EC	"
CMW4102	Bloemendal, KZN	J. Roux
CMW4103	Dalton, KZN	"
CMW4104	"	"
CMW4105	Piet Retief, MP	"
CMW4106	"	"
CMW4107	Vryheid, KZN	J. Roux & T.C. Harrington
CMW4109	Bloemendal, KZN	J. Roux
CMW4110	Bloemendal, KZN	"
CMW4757	Umtata, EC	J. Roux & M.J. Wingfield
CMW4758	"	"
CMW4905	Kataza, KZN	M.J. Wingfield
CMW4906	"	"

[a] CMW numbers represent cultures maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] All isolates were collected from diseased *Acacia mearnsii* in South Africa (Fig. 1). KZN refers to the KwaZulu-Natal Province, EC to the Eastern Cape Province and MP to the Mpumalanga Province.

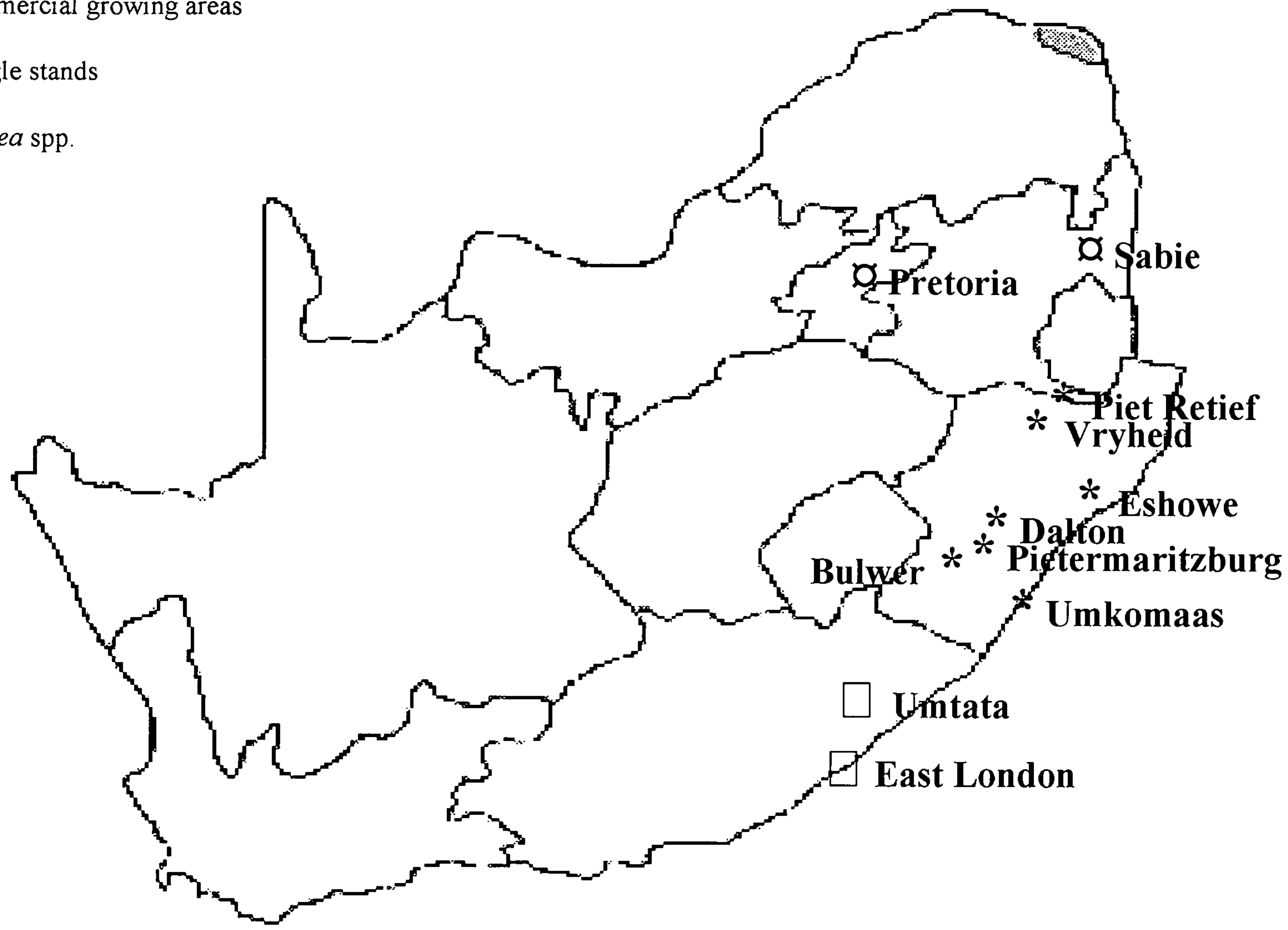
Table 2: Number of phenotypes, polymorphic loci and average gene diversity in *Ceratocystis albofundus* based on DNA fingerprinting with *Pst*I restrictions and the 15bp oligonucleotide probe, $(CAT)_5$. Results for *C. albofundus* are compared to the published data of Harrington *et al.* (1998).

Species	Number of Isolates	Number of Phenotypes	Number of Loci	Number of Polymorphic Loci	Genetic diversity (*H*)
C. albofundus	38	38	50	47	0.2137
C. eucalypti	10	9	19	17	0.3747
C. virescens	16	2	4	1	0.0935
Chalara australis	30	3	22	2	0.0111
C. albofundus					
Bloemendal	12	12	36	27	0.202
Dalton	12	12	40	35	0.258
Vryheid	8	8	37	30	0.282
Piet Retief	2	2	22	7	0.159
East London	4	4	27	20	0.278

Table 3: Number of phenotypes, polymorphic loci and average gene diversity in *Ceratocystis albofundus* based on RFLP's after restrictions with *Hae*III. Results for *C. albofundus* are compared to the published data of Harrington *et al.* (1998).

Species	Number of Isolates	Number of Phenotypes	Number of Loci	Number of Polymorphic Loci	Genetic diversity (*H*)
C. albofundus	31	30	46	41	0.249
C. eucalypti	10	6	33	9	0.1115
C. virescens	16	10	40	13	0.0928
Chalara australis	30	2	28	1	0.0023
C. albofundus					
Bloemendal	9	9	36	26	0.251
Dalton	9	9	33	22	0.207
Vryheid	7	7	40	33	0.296
Piet Retief	2	2	26	13	0.125
East London	2	1	18	0	0

Figure 1: Map of South Africa showing areas from which *C. albofundus* has been reported. Reports from the commercial *A. mearnsii* areas are indicated with (*), reports from jungle stands are indicated with (□) and reports made from *Protea* spp. are indicated with (¤).



Commercial growing areas
Jungle stands
Protea spp.
Sabie
Pretoria
Piet Retief
Vryheid
Eshowe
Dalton
Pietermaritzburg
Bulwer
Umkomaas
Umtata
East London

Figure 2: Nuclear fingerprint of *C. albofundus*, generated by probing *Pst*I restrictions with CAT_5. Lanes 1 and 20 are *Hin*dIII digested Lambda marker.

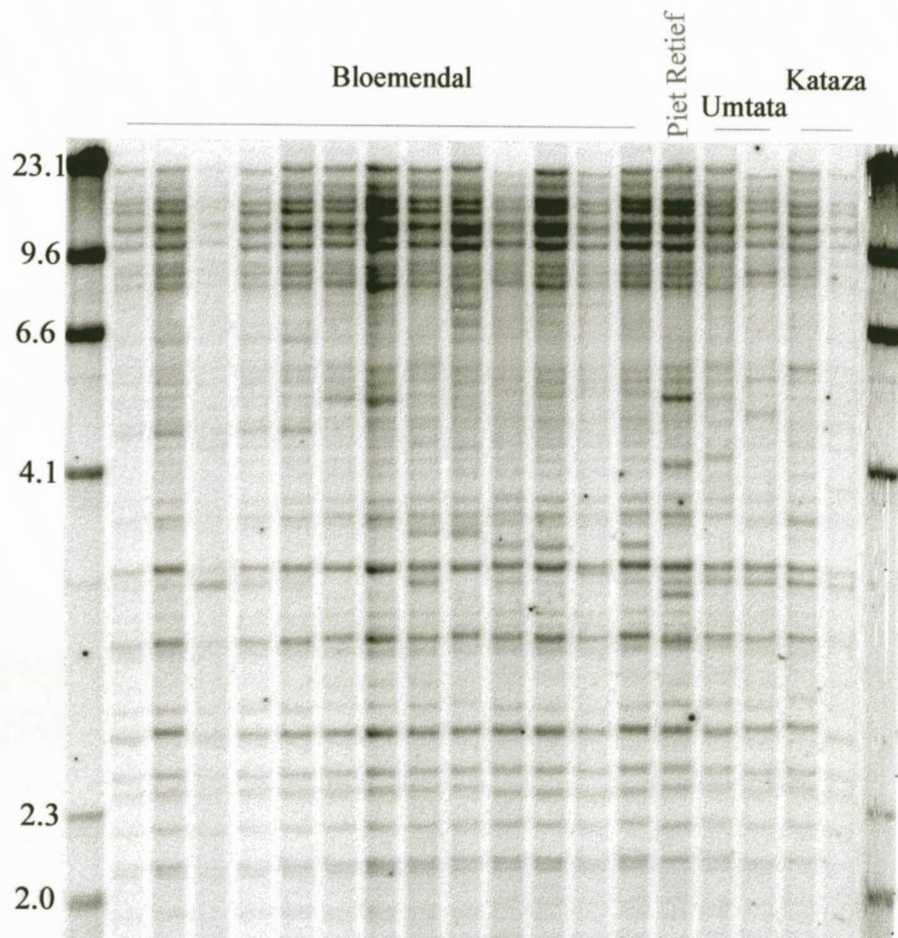

Bloemendal
Piet Retief
Umtata
Kataza
23.1
9.6
6.6
4.1
2.3
2.0

Figure 3: Phylogram of UPGMA cluster analysis of genetic distance matrixes of *C. albofundus* isolates after probing nuclear DNA with $(CAT)_5$. In the legends the letters refer to the area from which the isolate was obtained, while the numbers refer to the CMW number of the isolate. BLOEM refers to isolates obtained from Bloemendal, DALTO are isolates from Dalton, VRYHE represents isolates from Vryheid, EASTL represents isolates from East London and POTG represents isolates from Piet Retief.

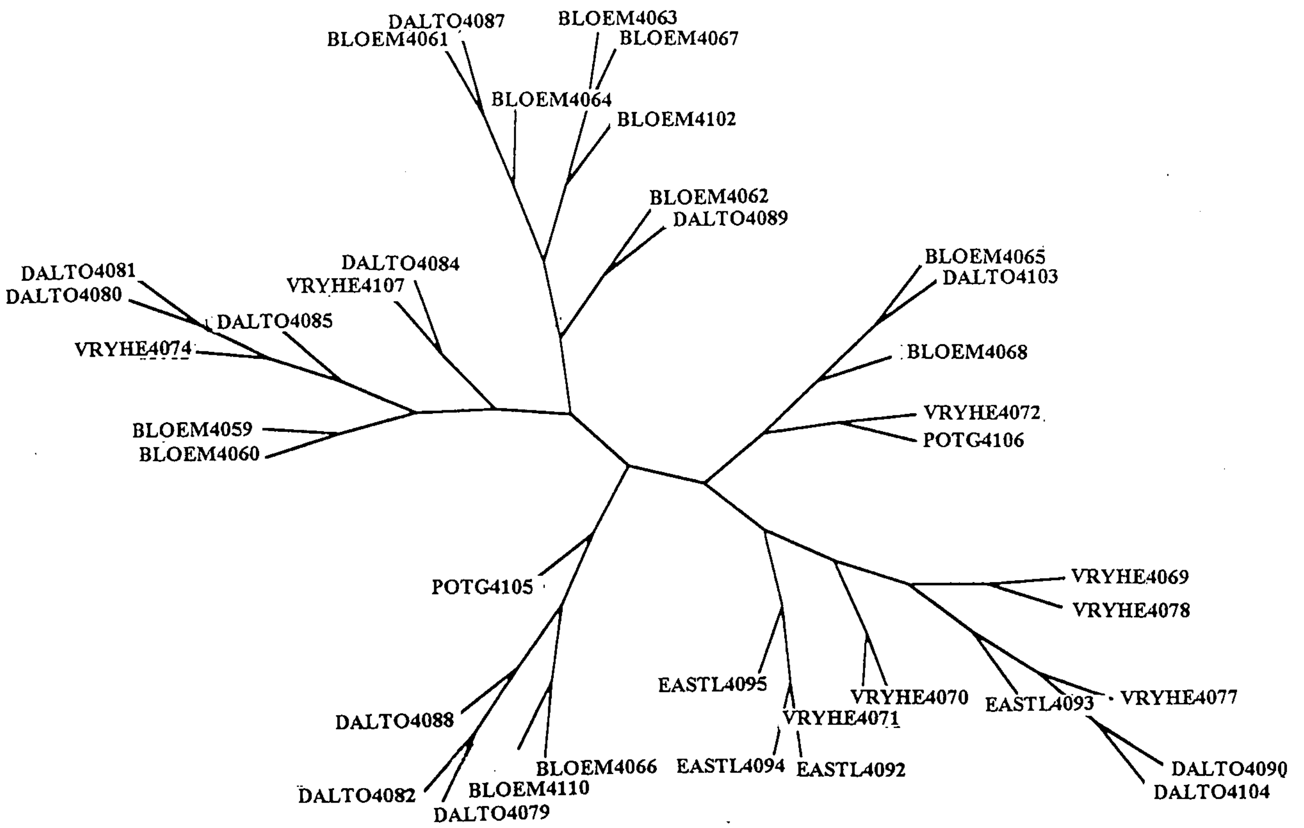

DALTO4087
BLOEM4061
BLOEM4063
BLOEM4067
BLOEM4064
BLOEM4102
BLOEM4062
DALTO4089
DALTO4081
DALTO4080
DALTO4084
VRYHE4107
DALTO4085
VRYHE4074
BLOEM4059
BLOEM4060
BLOEM4065
DALTO4103
BLOEM4068
VRYHE4072
POTG4106
POTG4105
VRYHE4069
VRYHE4078
EASTL4095
VRYHE4070
EASTL4093
VRYHE4077
VRYHE4071
DALTO4088
BLOEM4066
EASTL4094
EASTL4092
DALTO4090
DALTO4104
DALTO4082
BLOEM4110
DALTO4079

Figure 4: Mitochondrial fingerprints of *C. albofundus*, generated by *Hae*III restrictions of total genomic DNA. Lanes 1 and 20 are *Hind*III digested Lambda marker.

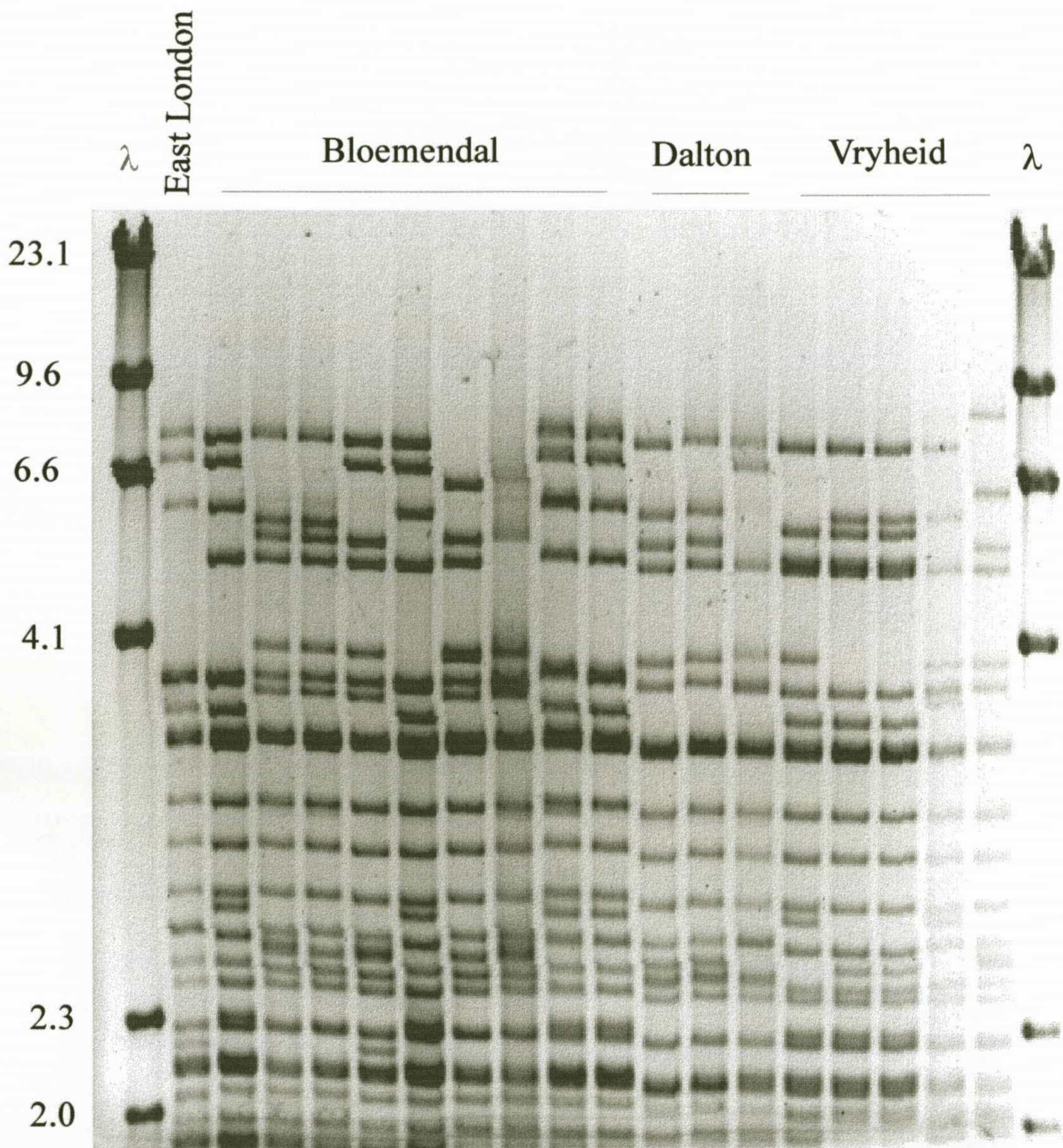
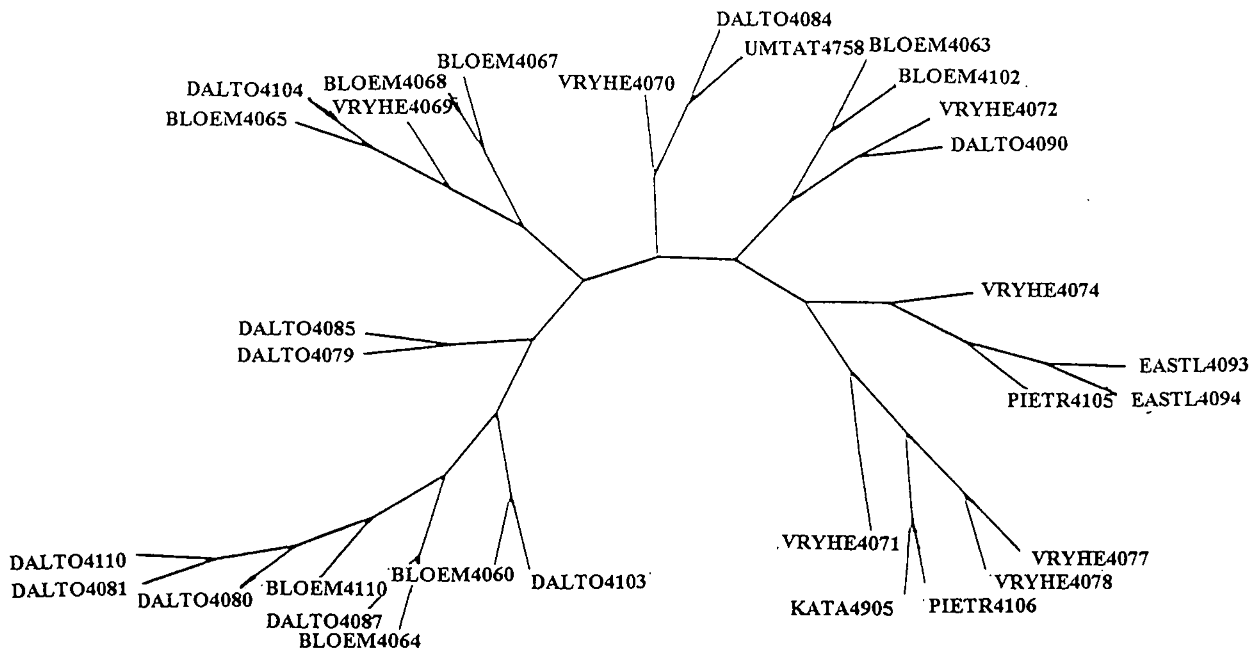

λ
East London
Bloemendal
Dalton
Vryheid
λ
23.1
9.6
6.6
4.1
2.3
2.0

Figure 5: Phylogram of UPGMA cluster analysis of genetic distance matrixes of *C. albofundus* isolates after restriction with *Hae*III. In the legends the letters refers to the area from which the isolate was obtained, while the numbers refer to the CMW number of the isolate. BLOEM refers to isolates obtained from Bloemendal, DALTO are isolates from Dalton, VRYHE represents isolates from Vryheid, EASTL represents isolates from East London, KATA isolates are from Kataza, UMTAT isolates from Umtata and POTG represents isolates from Piet Retief.


DALTO4084
UMTAT4758
BLOEM4063
BLOEM4067
BLOEM4102
VRYHE4070
DALTO4104
BLOEM4068
VRYHE4069
VRYHE4072
BLOEM4065
DALTO4090
VRYHE4074
DALTO4085
DALTO4079
EASTL4093
PIETR4105
EASTL4094
VRYHE4071
VRYHE4077
DALTO4110
BLOEM4060
DALTO4103
VRYHE4078
DALTO4081
DALTO4080
BLOEM4110
KATA4905
PIETR4106
DALTO4087
BLOEM4064

CHAPTER 3

CERATOCYSTIS FIMBRIATA AND CHALARA ELEGANS, PATHOGENS ON ACACIA MEARNSII IN SOUTH AFRICA

3

CERATOCYSTIS FIMBRIATA AND *CHALARA ELEGANS*, PATHOGENIC ON *ACACIA MEARNSII* IN SOUTH AFRICA

ABSTRACT

Ceratocystis albofundus causes a serious wilt disease of exotic *Acacia mearnsii* in South Africa. During the course of a recent country-wide survey of *A. mearnsii* showing symptoms of wattle wilt, two unusual fungal isolates were collected. Both isolates were associated with typical symptoms of wattle wilt, such as a streaked appearance of the infected timber. These fungi also had *Chalara* anamorphs, typical of *Ceratocystis* species. They were, however, clearly different from *C. albofundus* and from each other. The aim of this study was to identify the two fungi, based on morphology and rDNA sequence comparisons. Their relative pathogenicity to *A. mearnsii* seedlings was also tested in greenhouse studies. The one fungus, which produced a *Ceratocystis* teleomorph, was identified as a typical isolate of *C. fimbriata*, which is a well-known pathogen of woody plants, but has not previously been reported from South Africa. The second fungus was identified as *Chalara elegans*, which is a well-known pathogen of root crops in South Africa and elsewhere. Both fungi cause rapid death of susceptible seedlings after inoculation and I believe that they played a major role in the demise of the trees from which they were isolated.

INTRODUCTION

The genus *Ceratocystis sensu stricto* Ell. & Halst. includes many important plant pathogens that cause disease on a wide range of plant species. Species of *Ceratocystis* have been described as pathogens of sugarcane (Abbot, 1964), oak (French & Stienstra, 1978), maple (Beil & Kenneth, 1979), *Gmelina arborea* Roxb. (Muchovej, Albuquerque & Ribeiro, 1978), poplar (Gremmen & de Kam, 1978), sweat potato (Halsted, 1890), pimento (Leather, 1966), fruit trees (DeVay, Davidson & Moller, 1968), *Acacia mearnsii* de Wild. (Wingfield *et al.*, 1996), coffee and rubber (Pontis, 1951; Upadhyay, 1981) and many other plant species (Hunt, 1956; McDonald & Hindal, 1981; Wingfield, Seifert & Webber, 1993). In the past, *Ceratocystis* was collectively treated with *Ophiostoma,* which also represents an important genus of tree pathogens (Wingfield *et al.*, 1993). However, it is now generally accepted that *Ceratocystis* forms a discrete group of fungi that are phylogenetically distinct from *Ophiostoma* (Huasner & Reid, 1993; Samuels, 1993; Spatafora & Blackwell, 1994).

Ceratocystis species typically have *Chalara* (Corda) Rabenh. asexual states (Crone & Bachelder, 1961; Hinds, 1972; Perry, 1991; Rosseto & Ribeiro, 1991; Nag Raj & Kendrick, 1993; Wingfield *et al.*, 1993). There are also *Chalara* species that cause diseases of plants and for which sexual states are not known. A contemporary view, primarily based on sequence data, is that the latter fungi belong in *Ceratocystis* (Wingfield *et al.*, 1993; Witthuhn *et al.*, 1998a). *Chalara* spp. are pathogens of both hardwood trees and agricultural crops and cause diseases ranging from root rots to vascular stains (Nag Raj & Kendrick, 1975; Wills & Lambe, 1978; Yarwood, 1981; Specht & Griffon, 1985; Kile, 1993).

One of the best known pathogenic *Chalara* spp. is *Chalara elegans* Nag Raj & Kendrick [Syn. *Thielaviopsis basicola* (Berk. & Br.) Ferr.] which is a serious pathogen of tobacco (Gayed, 1972), carrot (Chittaranjan, 1994), cotton (Mauk & Hine, 1988) and many other, mostly herbaceous, plants (Nag Raj & Kendrick, 1975; Wingfield *et al.*, 1993). *Chalara*

elegans can also cause root rot and death of Japanese holly (*Ilex* sp.) and affects woody hosts such as citrus and black locust (Lambe & Wills, 1978a, 1978b; Yarwood, 1981; Sinclair, Lyon & Johnson, 1996). Other, highly pathogenic *Chalara* spp. include *Ch. neocaledoniae* Kiffer & Delon that causes important wilt diseases of *Coffea robusta* Linden and *Psidium guajava* L. (Kile, 1993) and *Ch. australis* Walker & Kile which causes a severe wilt disease of *Nothofagus cunninghamii* (Hook.) Oerst in Tasmania (Kile & Walker, 1987).

Ceratocystis spp. and their *Chalara* anamorphs are well adapted to dispersal by insects. These adaptations include sticky spores borne on long necked perithecia or conidiophores and the production of aromatics, which attract insects. These insects then transmit the spores to freshly wounded plant tissue (Lanza, Ko & Palmer, 1976; French & Stienstra, 1978; Juzwik & French, 1983; Kile, 1993; Christen, Meza & Revah, 1997). Many possible insect vectors have been described for *Ceratocystis* spp. *Ceratocystis fagacearum* (Bretz) Hunt, the oak wilt pathogen, is vectored by sap-feeding beetles in the family Nitidulidae (French & Stienstra, 1978; Juzwik & French, 1983). Nitidulid beetles have also been reported as the vectors of *C. fimbriata f. platani* Walter (Crone & Bachelder, 1961). A number of other species of *Ceratocystis* have been found to be vectored by insects in the families Rhizophagidae, Staphylinidae and Drosophilidae (Hinds, 1972). The exceptions are a number of *Ceratocystis* spp. on conifers that are vectored by bark beetles (Mathre, 1964; Upadhyay, 1981; Redfurn, Stoakley & Steele, 1987; Wingfield, Harrington & Solheim, 1997). The role of insects in *Ceratocystis* dissemination may be direct, through feeding on and crawling over fungal mats, or indirect by kicking frass, contaminated with fungal spores, from galleries (Kile, 1993).

Pathogenic *Ceratocystis* spp. can act in several ways to cause disease and death of plants. Vascular wilt pathogens, such as *C. fagacearum*, may cause physical blockage of the vascular tissue or may act by the production of toxins and hydrolytic enzymes, as well as the disruption of hormonal regulation (Kile, 1993). Blockage of vascular tissues take place by stimulating the production of tyloses that occlude the vessels, thus reducing water

movement within the plant (MacDonald & Hindal, 1981). Many of the phloem, pith and primary xylem cells are also plugged with hyphae (Zalasky, 1965). *Chalara elegans* produces methyl acetate, which acts as a phytotoxin and causes the swelling of cell walls and the discolouration of plant tissue (Kile, 1993).

Very few *Ceratocystis* and *Chalara* spp. have been reported from Africa. Reports include *C. paradoxa* (Dade) C. Moreau from pineapple, sugarcane and banana (Doidge *et al.*, 1953; Gorter, 1977), *Ch. elegans* from chicory, peanuts and tobacco (Doidge *et al.*, 1953; Gorter, 1977) and *C. albofundus* Wingfield, De Beer & Morris from *Protea gigantea* and an unidentified *Protea* sp. (Hunt, 1956; Gorter, 1977; Upadhyay, 1981). *Ceratocystis albofundus* has also been described as a serious pathogen of *Acacia mearnsii* de Wild. (Wingfield *et al.*, 1996) and is known only from South Africa.

Acacia mearnsii (black wattle) is the third most commonly planted forestry tree in South Africa and forms an important component of a major industry in the country. *Acacia mearnsii*, however, suffers from a serious wilt disease caused by *C. albofundus* (Morris, Wingfield & De Beer, 1993). Symptoms include rapid wilt and die-back (Fig.1), stem cankers (Fig. 2) and xylem discolouration (Fig. 3) of affected trees (Morris *et al.*, 1993). Initial reports of this disease described the causal agent as *C. fimbriata*, but this was later found to be incorrect (Wingfield *et al.*, 1996).

During recent surveys of diseased *A. mearnsii* in Cape Town (Western Cape Province) and Vryheid (KwaZulu-Natal Province), isolates of two unusual fungi with *Chalara* anamorphs were found associated with vascular discolouration. One fungus produced a typical *Ceratocystis* teleomorph, while the other produced only anamorphic structures. The aim of this study was to identify these fungi based on morphology and DNA sequence data. Furthermore, with pathogenicity tests we considered the role of these two fungi in diseases of *A. mearnsii*.

MATERIALS AND METHODS

Isolates

Isolates from *A. mearnsii* were collected from two mature trees in South Africa. The isolate from Vryheid (CMW4101) was collected from the stump of a recently harvested tree. This stump showed extensive discolouration of the xylem, with the streaked and flared discolouration commonly associated with *Ceratocystis* diseases (Fig. 4). Isolate CMW4690 from the Company Gardens in Cape Town was collected from a mature *A. mearnsii* tree showing wilt and die-back symptoms and extensive cracking and gummosis on the main stem. The sample was obtained from discoloured xylem, also with a streaked appearance. All isolates were grown on 2% Malt extract agar (MEA) (20 g/L Biolab malt and 15 g/L Biolab agar) amended with 0.1% streptomycin sulfate (Sigma) in Petri dishes at room temperature for morphological studies, DNA isolation and pathogenicity trials.

Morphological comparisons

Isolates collected from *A. mearnsii* were examined morphologically using a Zeis Axioskop light microscope. For the sexual structures, perithecial characteristics such as morphology, colour, shape, ornamentation, arrangement of ostiolar hyphae, ascospore shape and ascus size were noted. The morphology of chlamydospores in culture was also recorded. For asexual structures, conidial shape, shape and size of conidiogenous cells and the presence or absence of chlamydospores was recorded.

DNA Sequence comparisons

Polymerase chain reactions (PCR) were performed directly from mycelial scrapes from Petri dishes without the extraction of DNA (Harrington & Wingfield, 1995). Primers ITS1 and ITS4 were used to amplify the ITS region of the ribosomal RNA operon. The sequence for ITS1 is 5'TCCGTAGGTGAACCTGCGG3' and for ITS4 is

5'TCCTCCGCTTATTGATATGC3' (White *et al.*, 1990). Initial denaturation was performed at 96°C for 5 min, after which the temperature was lowered to 90°C until the PCR polymerase (Expand™, Boehringer Mannheim, South Africa) had been added. Primer annealing took place at 55°C for 30 sec, chain elongation at 72°C for 1 min and denaturation at 92°C for 1 min. These steps were repeated for 35 cycles. Final chain elongation was at 72°C for 5 min, followed by 2 min at 37°C. The PCR products were visualized under UV light on 1% agarose gels containing ethidium bromide.

DNA sequencing

The PCR fragments were purified using the Nucleon™ Qc kit for PCR/oligo clean up (Amersham Life Sciences) and a QIAquick PCR purification Kit (Quiagen, Germany). Sequence reactions were carried out with an ABI PRISM™ Dye Terminator Cycle Sequencing Kit with Amplitaq® DNA polymerase, FS (Perkin-Elmer, Warrington, UK). An ABI PRISM™ 377 DNA autosequencer (Perkin-Elmer) was used for the sequencing. The sequences were aligned manually by the insertion of gaps. All phylogenetic relationships among species were determined using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1985) and bootstrap analysis (bootstrap confidence intervals on DNA parsimony) (Felsenstein, 1988). Data obtained from the sequencing of the two *A. mearnsii* isolates were compared with data obtained from Genbank as well as from Wingfield *et al.* (1996) and Witthuhn *et al.* (1998a, b) (Table 1). The two unknown isolates were also compared to *C. albofundus* from *A. mearnsii*. *Petriella setifera* (Schmidt) Curzi. was used as an outgroup.

Pathogenicity trials

Isolates CMW4690 (*Chalara*), CMW4101 (*Ceratocystis*) and CMW4908 (*C. albofundus*) were used in glasshouse inoculation trials on *A. mearnsii*. Isolates were grown on 2 % MEA amended with 0.1% streptomycin sulfate for 2 weeks prior to inoculation.

Glasshouse conditions were set with day/night lighting, with the average daily temperature at 24/25°C and night temperature at approximately 20°C.

Ten *Acacia mearnsii* seedlings were inoculated for each isolate tested. A small wound (5 mm diam.) was made in the stem of each seedling by removing the bark and exposing the cambium. Mycelial plugs, of similar size, overgrown with the test fungi, were placed into each wound, mycelium towards the cambium. All wounds were closed with parafilm to prevent desiccation of the inoculum and wounds. For control inoculations, sterile MEA plugs were used as inoculum and inserted into the stems of five wounded trees. Lesion lengths were assessed after four weeks and statistical differences in lesion length for each isolate were determined using Tukey's studentised range test ($P=0.05$). Re-isolations were made from the lesions produced, to confirm that the lesions were caused by the inoculated fungi.

RESULTS

Morphological comparisons

Isolate CMW4101 produced both sexual and asexual structures in culture. Perithecia were characterized by black, un-ornamented globose bases and black necks (Fig. 5) with convergent ostiolar hyphae (Fig. 6). Ascospores were hat-shaped and accumulated in slimy drops at the tips of the perithecial necks. Chlamydospores, globose to sub-globose in shape, were produced either singly or in short chains. These characteristics are typical of *C. fimbriata*. Isolate CMW4690 did not produce a sexual state in culture and was characterized by thallic chlamydospores (Fig. 7), usually with 4 or more transverse septa. Phialides had typical cylindrical collarettes that gave rise to hyaline conidia that were cylindrical in shape and extruded in chains (Fig. 8). Morphological characteristics were typical of *Ch. elegans*.

Analysis of DNA sequence data

Both strands of the ITS region of the rRNA operon of the isolates used in this study were sequenced. Sequences for isolates from *A. mearnsii* were aligned manually with Genbank data for *Ceratocystis* spp. A total of 559 characters were aligned for the analysis (Fig. 9), after the insertion of gaps. A heuristic search using the no branch swapping option in PAUP generated a single tree (Figure 10). Values for the consistency index (CI), homoplasy index (HI) and retention index (RI) were 0.817, 0.183 and 0.867 respectively. The *Ceratocystis* isolate from *A. mearnsii* grouped with *C. fimbriata* isolates from sweet potato and plane trees. The *Chalara* isolate from *A. mearnsii* grouped with *Ch. elegans*, separate from other *Chalara* species. Sequence data comparisons, therefore, confirmed morphological studies that showed that one of the *A. mearnsii* isolates represented *C. fimbriata* and the other *Ch. elegans*.

Pathogenicity trials

Ceratocystis albofundus (CMW4908) produced the largest lesions on *A. mearnsii* seedlings (Ave. 77.4 mm). The next most virulent isolate was *Ch. elegans* (Ave. 52.4 mm) and the least virulent isolate was *C. fimbriata* (Ave. 42.6 mm) (Fig. 11). All isolates produced lesions significantly larger than those of the control inoculation (Ave. 14.6 mm) (Fig. 12) ($P=0.05$). Re-isolation from diseased material consistently yielded the inoculated fungi. Lesions were characterized by death of the bark and streaking of the xylem. Susceptible seedlings showed signs of wilt and die-back before the experiment was terminated.

DISCUSSION

This study investigated two unusual fungal isolates associated with wilt of *A. mearnsii* in South Africa. The one isolate was shown to be typical of *C. fimbriata* and represents the first unequivocal record of *C. fimbriata* from the country. Previous reports of this fungus on *Protea* spp. (Gorter, 1977) and *A. mearnsii* (Morris *et al.*, 1993) were later shown to represent the related, but distinct *C. albofundus* (Wingfield *et al.*, 1996). There is precedence for finding *C. fimbriata* on *Acacia* spp., since a report of this fungus from *A. decurrens* Wendl. in Brazil was made in the late 1980's (Ribeiro *et al.*, 1988).

The discovery of *Ch. elegans* associated with vascular discolouration and wilting of *A. mearnsii* is intriguing. This fungus is mostly known as an important pathogen of root crops (Gayed 1972; Yarwood, 1981; Kile, 1993) and graft failures (Longrée, 1940). Surveys of diseased *A. mearnsii* in South Africa during the course of the past 10 years have consistently yielded *C. albofundus.* The appearance of *C. fimbriata* and *Ch. elegans* was unusual. Although these fungi have each appeared only once on single wilting trees, the fact that they were both able to cause disease in pathogenicity tests suggests that their discovery is significant, and that they are both worthy of further study.

The best known *Ceratocystis* sp. in South Africa, associated with disease of a woody host is *C. albofundus*, the cause of wattle wilt of *A. mearnsii* (Morris *et al.*, 1993; Wingfield *et al.*, 1996). This fungus can lead to the death of susceptible trees within six weeks after inoculation. It also has a wide geographic distribution in South Africa (Roux *et al.*, 1998; Chapter 2). Although *C. albofundus* was initially confused with *C. fimbriata*, it has very distinctive morphological characteristics, distinguishing it from this closely related species. Typical *C. albofundus* isolates have light coloured perithecial basis and dark necks (Fig. 13). Perithecia are also characterized by having divergent ostiolar hyphae (Fig. 14), compared to the typically convergent ostiolar hyphae and dark perithecial basis of *C. fimbriata* (Fig. 5, 6). Morphological characteristics of the species collected from *A. mearnsii* in this study, place this fungus firmly in *C. fimbriata.*

Chalara elegans has been reported from a wide range of hosts and has a cosmopolitan distribution (Wills & Lambe, 1978; Yarwood, 1981; Kile, 1993). The host range of this fungus includes, among others, birch, citrus, poinsettia, American elm, Japanese holly and black locust (Lambe & Wills, 1978a, 1978b; Yarwood, 1981; Sinclair *et al.*, 1996). *Chalara elegans* is reported to be most infectious in non-lignified or slightly lignified tissue, especially in the Solanaceae, Leguminosae and Cucurbitaceae (Kile, 1993). It can saprophytically colonize the roots of various plant hosts, either directly or through wounds, and is known to survive in the soil by the formation of chlamydospores (Gayed, 1972; Wick & Moore, 1983). Symptoms on *A. mearnsii* from which *Ch. elegans* was isolated resemble those described for other pathogenic *Chalara* and *Ceratocystis* spp. from woody hosts. These include wilt, stem cankers and streaking of the xylem (Kile & Walker, 1987). The fact that the *Ch. elegans* is most likely a *Ceratocystis* anamorph might suggest that this report from *A. mearnsii* is not unusual. Additional isolations from diseased trees in future might lead to the appearance of the fungus on other trees.

Very few *Chalara* spp. are known to cause diseases of woody plants. Those that have been reported include *Chalara australis*, *Ch. neocaledoniae*, *Ch. thielaviodes* Peyr. and *Ch. populi* Veldeman ex. Kiffer & Delon (Kile, 1993). *Chalara australis* causes a serious wilt of *Nothofagus cunninghamii* in Australia (Kile & Walker, 1987), while *Ch. neocaledoniae* causes vascular stain of coffee and guava in New Caledonia (Kile, 1993). *Chalara populi* from Europe causes small cankers on *Populus* and *Salix* spp., known as trunk scab or brown patch disease, while *Ch. thielaviodes* causes root and stem rots of *Ulmus* spp., walnut and peach in Europe, North America and Australia (Lamb, Wright & Davidson, 1935; Baker & Thomas, 1946; Kile, 1993). *Chalara thielaviodes* has also been reported as the cause of graft union failure in several hosts (Longrée, 1940; Baker & Thomas, 1946; Kile, 1993).

Sequence data from the ITS region of the ribosomal DNA has proven to be most useful for distinguishing *Ceratocystis* spp. (Hausner & Reid, 1993; Visser *et al.*, 1995; Wingfield *et al.*, 1996; Witthuhn *et al.*, 1998a; 1998b). Analysis of sequence data from

this region has made it possible to confirm the morphological identification of *Ch. elegans* and *C. fimbriata* from *A. mearnsii*. It has been suggested that *C. fimbriata* may represent a complex of many different species (Wingfield *et al.*, 1996). Given the fact that this is the first time that *C. fimbriata* has been collected from South Africa it would thus be of considerable interest to compare the *A. mearnsii* isolate with isolates from additional hosts and origins.

Ceratocystis fimbriata and *C. albofundus* form a sub-clade within *Ceratocystis*, showing a close relationship between these two species (Witthuhn *et al.*, 1998b). In a search for the possible origin of *C. albofundus*, we have hypothesized that it might be derived from *C. fimbriata*. Apart from *C. moniliformis* (Hedgecock) Moreau, *C. fimbriata* and *C. albofundus* are also the only species of *Ceratocystis* with hat shaped ascospores. The fact that both fungi have now been found on the same tree species in South Africa, supports the view that they are closely related. The isolation of *C. albofundus* from the temperate and colder (below 0°C winter temp.) areas in South Africa suggests that it could be a temperate species that has developed from *C. fimbriata*.

The pathogenicity of *C. fimbriata* and *Ch. elegans* to *A. mearnsii* has been shown in this study for seedlings under laboratory conditions. Although a close correlation between greenhouse and field inoculation studies has been shown with *C. albofundus* (De Beer, 1994), tests in this study should be repeated under field conditions on older trees. Under laboratory conditions, *C. albofundus* appears to be the most pathogenic of the three species, but more isolates of each of them would need to be considered before clear conclusions can be made regarding the role that each fungus may play in disease. Although thorough surveys have already been conducted, we believe that it is likely that more isolates of *C. fimbriata* and *Ch. elegans* will be obtained in future surveys. Comparison of *C. fimbriata* and *Ch. elegans* with isolates from other hosts in Africa and the rest of the world will also be a future priority and will provide knowledge as to the possible origin of these two fungi from *A. mearnsii*.

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Table 1: *Ceratocystis* and *Chalara* species used in molecular comparisons and in pathogenicity studies.

SPECIES	**ISOLATE NUMBER** [a]	**ORIGIN**	**HOST**	**GENBANK ACCESSION NUMBER**
Ceratocystis adiposa	CMW1622	Japan		AF043606
C. albofundus	CMW4908	South Africa	*Acacia mearnsii*	
"	CMW2475	East London	"	AFO43605
"	PREM51639	KwaZulu-Natal	"	
"	PREM51645	"	"	
"	PREM51829	"	"	
C. coerulescens	C666	Norway	*Picea abies*	U75618
C. eucalypti	C639	Australia	*Eucalyptus sieberi*	U75627
C. fagacearum	CMW2651	Iowa, USA	*Quercus palustris*	AFO43598
C. fimbriata	**CMW4101**	**KwaZulu-Natal**	***Acacia mearnsii***	
"	CMW2220	France	*F. platani*	AFO43604
"	PREM51830	Italy	*P. orientalis*	
"	PREM51831	"	"	
"	PREM51644	France	*P. hybrida*	
"	C854	USA	*Ipomoea batatas*	AF007749
C. moniliformis	CMW3782	South Africa	*Erythrina* sp.	AF043579
C. paradoxa	CMW1546	New Zealand	*Musa* sp.	AF043607
C. pinicola	CMW1323	England	*Pinus* sp.	AF043602
C. virescens	CMW0460	USA	*Quercus* sp.	U75624
Chalara australis	C619	Australia	*Nothofagus cunninghamii*	U75629
Ch. elegans	**CMW4690**	**Cape Town**	***Acacia mearnsii***	
"	C185	USA	*Pelargonium* sp.	
Ch. neocaledoniae	C694	New Caledonia	*Coffea robusta*	U75628

[a] CMW numbers represent cultures maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. The two isolates, *C. fimbriata* (CMW4101) and *Ch. elegans* (CMW4690), presented in bold were used in sequence comparisons and pathogenicity tests. Data pertaining to the remaining isolates were derived from Genbank.

C numbers - Culture collection of T.C. Harrington, Department of Plant Pathology, Iowa State University

PREM - Official designation of the National Collection of fungi, Pretoria, South Africa

Figures 1-4: Disease symptoms caused by *Ceratocystis albofundus* on *Acacia mearnsii*.

Fig. 1: Wilt and die-back of *A. mearnsii* after inoculation with *C. albofundus*.

Fig. 2: Stem cankers and gummosis caused by a natural infection of *C. albofundus*. Note small cracks exuding gum higher up middle stem.

Fig. 3: Xylem discolouration caused by *C. albofundus* infection.

Fig. 4: Streaked appearance of xylem, caused by *C. albofundus* infection.

1

2

3

4

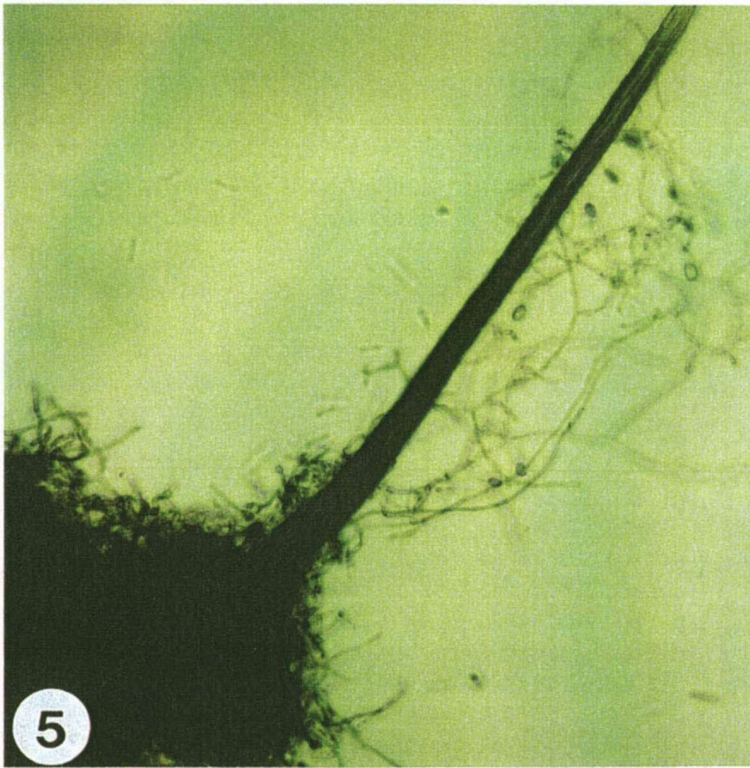
Figures 5-8: Morphological characteristics of *C. fimbriata* from *A. mearnsii*.

Fig. 5: Perithecium with black base and neck, typical of *C. fimbriata*.

Fig. 6: Convergent ostiolar hyphae of *C. fimbriata*.

Fig. 7: Thallic chlamydospores, typical of *Ch. elegans*.

Fig. 8: Cylindrical conidia produced in chains from phialide.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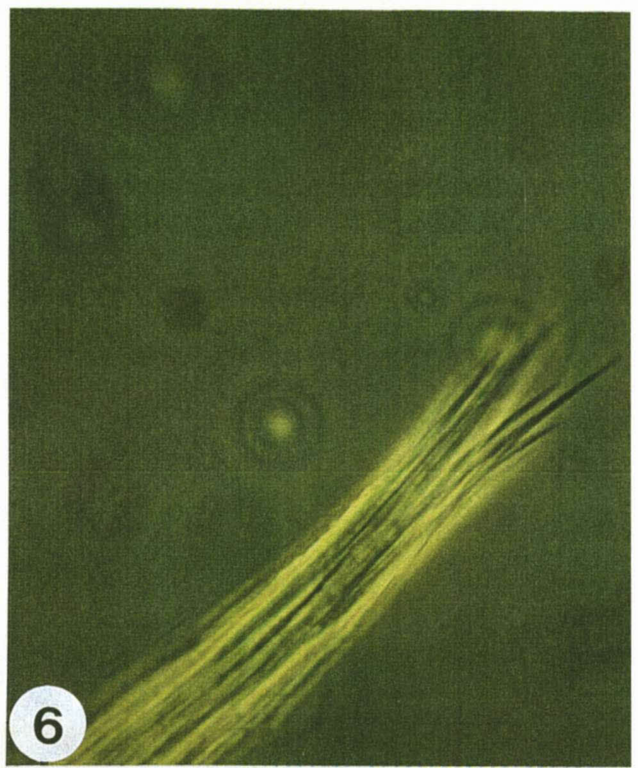
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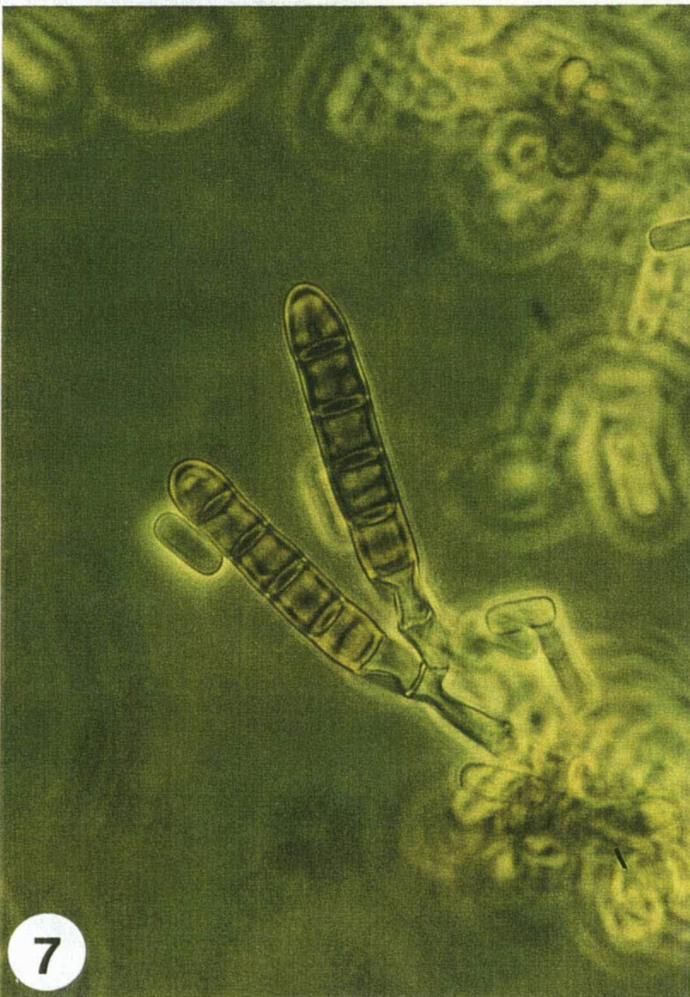
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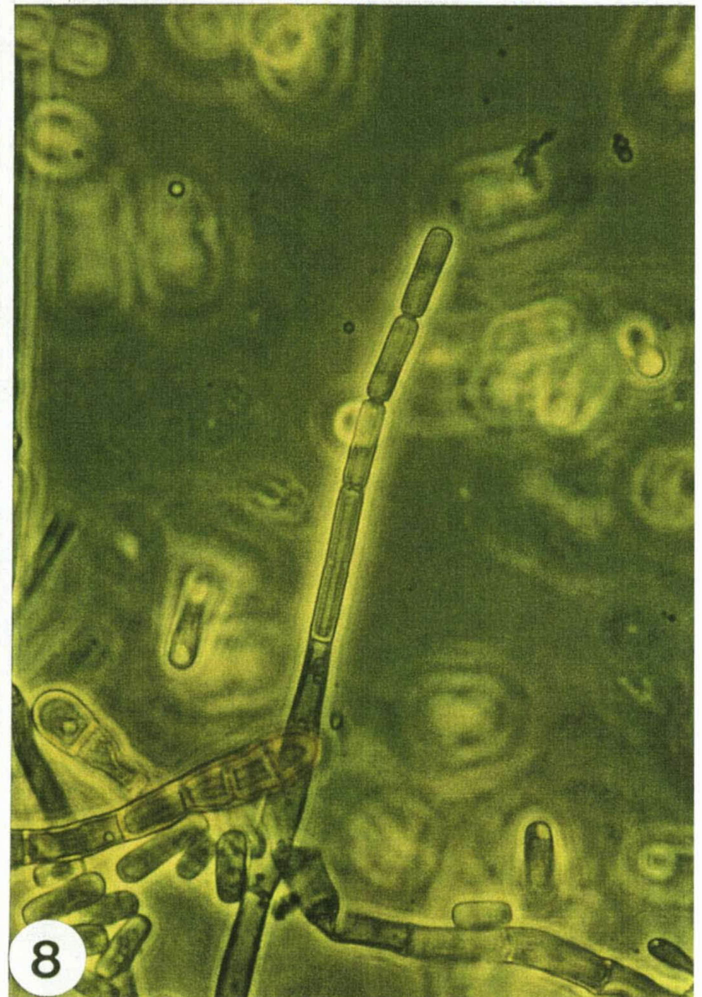
8

Figure 9: Aligned nucleotide sequences for the ITS region for different species of *Ceratocystis* and *Chalara*. N indicates unknown bases, a dash (-) indicates a gap in the sequence alignment and (.) indicates bases identical to those of isolate ALBOFUNDUS2475.

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                           10         20         30         40         50         60         70
ALBOFUNDUS2475      GCTGCCTTG- --GTGGGTG- TCT-G-TAGT GGTGTTAA-C CTCTTTTTAT ----AAGGGG GCAGCCC-AC
ALBOFUNDUS51829     .........- --.......- ...-.-.... ........-. .......... ----...... .......-..
ALBOFUNDUS51645     .........- --.......- ...-.-.... ........-. .......... ----...... .......-..
ALBOFUNDUS51639     .........- --.......- .N.-.-.... ........-. .......... ----...... .......-..
ACACIA4101          C.ATGTG..A AC..ACCCTA ...T.-.... .AGA.G..TG ..G..-..GG TGGT-....- ----...TT.
FIMBRIATA854        -.ATGTG..A AC..ACC-TA ...T.-.... .AGA.G..TG ..G..-..GG TGGT-....- ----...TT.
FIMBRIATA2220       C.ATGTG..A AC..ACC-TA ...T.-.... .AGA.G..TG ..G..-..GG TGGT-....- ----...TT.
FIMBRIATA51831      ---T---..A ---.------ ...T.-.... .AGA.G..TG G.G..G.-GG TGGT-..-T. .---...TT.
FIMBRIATA51830      ---T---..A ---.------ ...T.-.... .AGA.G..TG G.G..G.-GG TGGT-..-T. .---...TT.
FIMBRIATA51644      C------..A ---.------ ...T.-.... .AGA.G..TG G.G..G.-GG TGGT-..-T. .---...TT.
VIRESCENS0460       C.ATATG..A ACA.ACC-TA .-.------- -------.-G ..GC-...GG C----...-- ------.TTG
EUCALYPTI639        C.ATATG..A ACA.ACC-T- ...------- -------.-G ..GC-...GG C----...-- ------.TTG
MONILIFORMIS3782    C.ATTTG..A ATT.-CCACA AACA--.C-- -----G..-- ..GCGA..GG C----G..T- ------.T-.
PARADOXA1546        C.ATTTG..A ACT.ACC--T ...-.----- ---------G ..GC-...GG C----...T- ----...TTG
ADIPOSA1622         C.ATTTG..A ACA.ACC-TA ...T--..-- ---------- ..GC-...GG C-GT--..T- -----..TT.
PINICOLA1323        C.ATATG..A ACA.ACC-T- .-.T--..-- ---------G ..GC-...GG C----...-- ------.TTG
FAGACEARUM2651      C.ATTTG..A ACA.ACC-.A .-.TTT.TT. CTCTAAT.-- ..GC-...GG C----....A ------.TT.
COERULESCENS666     C.ATATG..A ACA.ACC--T ...------- -------.-G ..GC-...GG C----...-- ------.TTG
NEOCALEDONIA694     C.ATATG..A ACA.ACC--T ..----..-- ---------G ..GC-...GG C----...-- ------.TTG
AUSTRALIS619        C.ATATG..A ACA.ACC--T ..----..-- ---------G ..GC-...GG C----...-- ------.TTG
ELEGANS185          C.ATATG..A AC..ACC.TT ...------- -------.-G ..GC-...GG C----...T- ---...--T.
ACACIA4690          C.ATATG..A -CA.ACCCTT .-.C--..-- ---------G ..GC-...GG C----...T- ---...--T.
PETRIELLA           C.CTTTG..A ACC.TACC-A --.T.T..-- --....---G .CTCGGC-GG -GGTT..--- --C--..CCA
```

		80	90	100	110	120	130	140
ALBOFUNDUS2475	TACCGC-TAG	-CCACC----	----AGCAGC	ATACA--AGT	CTTTTACCAC	TAT---AAA-	C-CTTCTGT-
ALBOFUNDUS51829	......-...	-....-----	----......	....--..	....	..----..-	.........-
ALBOFUNDUS51645	......-...	-....-----	----......	....--..	....	..----..-	-........-
ALBOFUNDUS51639	......-...	-....-----	----......	....--..	....	..----..-	.........-
ACACIA4101	.-GAAGAG..	GG....G--C	TGCC.....-	-..--TT...	...-CG....	.G.----..-	.T...TAT.-
FIMBRIATA854	.-GAAGGG..	GG....G--C	TGCC.....-	-..--TT...	..--C.....	..-----..-	.T...T.---
FIMBRIATA2220	.-GAAGGG--	--....G--C	TGCC.....-	-..---T...	..--CG....	.G.----..-	.T..------
FIMBRIATA51831	.-GAAG----	--....G--C	TGCC.....-	-..---T...	..--CG....	.G.----..-	.T..------
FIMBRIATA51830	.-GAAG----	--....G--C	TGCC.....-	-..---T...	..--CG....	.G.----..-	.T..------
FIMBRIATA51644	.-GAAGGG--	--....G--C	TGCC.....-	-..---T...	..--CG....	.G.----..-	.T..------
VIRESCENS0460	GTAACA----	--..AGTC--	TGCCG.T..-	-.--------	-...------	-------..A	.T...T.T.T
EUCALYPTI639	GTAACA----	--..AGTC-T	TGCCG.T..-	-.--------	-...-----.	--------.-	.T...T.T--
MONILIFORMIS3782	----------	--.-..----	GCCCG....-	-...-----.	...-------	-----G..--	.T.G.T.T.-
PARADOXA1546	G------G.T	T---------	TGCCG.T..-	----------	--------..	-.AAC-...-	.T...T----
ADIPOSA1622	GG-----G.T	TG--------	TGCCG.T..-	-.--------	-...-----.	--------.-	.T...T.---
PINICOLA1323	GTAAAA--.-	---..AGTC--	TGCCG.T..-	-..TTT-.-A	AA--------	-------..A	.T...T----
FAGACEARUM2651	.TT-CTTC..	GGG.TGTTTC	TGCC..T..-	-..TTT-.--	--------.-	--------.A	.T...T.T--
COERULESCENS666	GTAAAA----	--..AGTC--	TGCCG.T...	--.TTT-.--	----------	-------..A	.T...T----
NEOCALEDONIA694	GTAACA----	---..AGTCT-	TGCCG.T..-	-..TTT-.C-	----------	-------..A	.T...T.T--
AUSTRALIS619	GTAACA----	--..AGTCT-	TGCCG.T..-	-..TTT-.C-	----------	-------..A	.T...T.T--
ELEGANS185	.--..-----	GGGC-TTCT-	-GCCG.T...	--.TTT-.T-	----------	-------..A	.T...T----
ACACIA4690	.--..-----	GGGC-TTCT-	-GCCG.TT..	--.TTT-.T-	----------	-------..A	.T...T----
PETRIELLA	A.--..T.CT	C..-G.CGG-	---C....--	C.--------	----------	------..-	---.....-.-

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                    150        160        170        180        190        200        210

ALBOFUNDUS2475     -AT-ATT-TT TTAAAA--TT TTT-AAAA-- -ATTGCTGAG TGGCAT--AA -CTATAAAAA -AAGTTAAAA
ALBOFUNDUS51829    -..-...-.. ......--.. ...-....-- -......... ......--.. -......... -.........
ALBOFUNDUS51645    -..-...-.. ..N...--.. ...-..N.-- -......... ......--.. -...NNNNN. -.........
ALBOFUNDUS51639    -..-...-.. ......--.. ...-....-- -......... ......--.. -......... -.........
ACACIA4101         -..T...T.- C..--GA-.. ...------- C......... ......--.. -......... -.........
FIMBRIATA854       -..T...T.- C..--GA-.. ...------- C......... ......--.. -......... -.........
FIMBRIATA2220      -..-...T.- CC.--GA-.. ...T------ C......... ......--.. -........- -.........
FIMBRIATA51831     -..-...T.. CC.--GA-.. ...T------ C......... ......--.. -........- -N........
FIMBRIATA51830     -..-...T.. CC.--GA-.. ...T------ C......... ......--.. -........- -.........
FIMBRIATA51644     -..-...T.. CC.--GA-.. ...T------ C......... ......--.. -........- -.........
VIRESCENS0460      T---...--- C....GAA.. ----.---TT C......... ......T-.. -.-....--- T.........
EUCALYPTI639       ----...T-- C..G.GAA.. ----.---TT C......... ......T-.. -.-....--- T.........
MONILIFORMIS3782   -..-.----- --...GAA.. .---.---TT C......... .A....TTT. ----....-- TGTA......
PARADOXA1546       -..-...T-- C..G.GAA.. ----.---TT C......... ......T-.. -.--....-- T.........
ADIPOSA1622        -.--...T-- C..G.GAA.. ----.---TT C......... .T....TT.. -.---...-- T.-.......
PINICOLA1323       A..-...T-- C..G.GAA.. .---.TT-TT C......... ......T-.. A.-....--- T.........
FAGACEARUM2651     -.--...T-- C..G.GAA.. ----.---TT C......... .T....TT.. -.-.-...-- T.-.......
COERULESCENS666    -..-...T-- C..G.GAA.. ----.---TT C......... ......T-.. -.-....--- T.........
NEOCALEDONIA694    -..--.---- C..G.GAA.. ----.---TT C......... ......T-.. -.-....--- T.........
AUSTRALIS619       -..--.---- C..G.GAA.. ----.T--TT C......... ......T-.. -.-....--- T.........
ELEGANS185         -..-...T-- C..T.GAA.. ----.T---T C......... ......T-.. -.--....-- T.........
ACACIA4690         -..-...T-- C..T.GAA.. ----.T---T C......... ......T-.. -.--....-- T.........
PETRIELLA          -.--...T.A .AGCG--GA. .---.T.--- C-..-....A .ACA..--.- -.---....- C..A.A....
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	220	230	240	250	260	270	280
ALBOFUNDUS2475	CTTTCAACAA	CGGATCTCTT	GGCTCTAGCA	TCGATGAAGA	ACGCAGCGAA	ATGCGATAAG	TAATGTGAAT
ALBOFUNDUS51829	....	....	....	....	....	....	....
ALBOFUNDUS51645	....	....	....	....	.......N..	....	....
ALBOFUNDUS51639	....	....	....	....	....	....	....
ACACIA4101	....	....	....	....	....	....	....
FIMBRIATA854	....	....	....	....	....	....	....
FIMBRIATA2220	....	....	....	....	....	....	....
FIMBRIATA51831	....	....	....	....	....	....	....
FIMBRIATA51830	....	....	....	N.........	....	....	....
FIMBRIATA51644	....	....	....	....	....	....	....
VIRESCENS0460	....	....	....	....	....	........C.	....
EUCALYPTI639	....	....	....	....	....	........C.	....
MONILIFORMIS3782	....	....	....	....	....	....	....
PARADOXA1546	....	....	....	....	....	........C.	....
ADIPOSA1622	....	....	....	....	....	....	....
PINICOLA1323	....	....	....	....	....	........C.	....
FAGACEARUM2651	....	....	....	....	....	....	....
COERULESCENS666	....	....	....	....	....	........C.	....
NEOCALEDONIA694	....	....	....	....	....	........C.	....
AUSTRALIS619	....	....	....	....	....	........C.	....
ELEGANS185	....	....	....	....	....	....	....
ACACIA4690	....	....	....	....	....	....	....
PETRIELLA	....	....	..T...G...	....	....	....	....

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                              290        300        310        320        330        340        350

ALBOFUNDUS2475        TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT GCCCCTGG-T AGTATTCTGC CAGGCATGCC
ALBOFUNDUS51829       .......... .......... .......... .......... .NN.....-N .......... ..........
ALBOFUNDUS51645       .......... .......... .......... .......... ..N.....-N .......... ..........
ALBOFUNDUS51639       .......... .......N.. .....N.... .......... .NN.....-. .......... ..........
ACACIA4101            .......... .......... .......... .......... ..G.....GC .......... ..........
FIMBRIATA854          .......... .......... .......... .......... ..G.....-C .......... ..........
FIMBRIATA2220         .......... .......... .......... .......... ........-. .......... ..........
FIMBRIATA51831        ...N...... .......... .......... .......... ..N.....N- .......... ..........
FIMBRIATA51830        .......... .......... ..NN...N.. .......... ..G.....-C .......... ..........
FIMBRIATA51644        .......... .......... .......N.. .......... ........N- .......... ..........
VIRESCENS0460         .......... .......... .......... .......... ..G.....-C .......... ..........
EUCALYPTI639          .......... .......... .......... .......... ..G.....-C .......... ..........
MONILIFORMIS3782      .......... .......... .......... .......... ..G..CA.-C ....C..... TG........
PARADOXA1546          .......... .......... .......... .......... ..G.....-C .......... ....T.....
ADIPOSA1622           .......... .......... .......... .......... ..G.....-C .......... ..........
PINICOLA1323          .......... .......... .......... .......... ..G.....-C .......... ..........
FAGACEARUM2651        .......... .......... .......... .......... ..G...A.-C .......... T.........
COERULESCENS666       .......... .......... .......... .......... ..G.....-C .......... ..........
NEOCALEDONIA694       .......... .......... .......... .......... ..G.....-C .......... ..........
AUSTRALIS619          .......... .......... .......... .......... ..G.....-C .......... ..........
ELEGANS185            .......... .......... .......... .......... ..G.....-C .......... ..........
ACACIA4690            .......... .......... .......... .......... ..G.....-C .......... ..........
PETRIELLA             .......... .......... .......... .......... ..G..C..-C ....A..... .G........
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                         360        370        380        390        400        410        420
ALBOFUNDUS2475      TGTCCGAGCG TCATTTCACC ACTCAA-GAC TT-GCTTT-- AGTT-TTGGT -GTT-GG-AG GTCCTGTTC-
ALBOFUNDUS51829     ...N...... .......... ......-... ..-.....-- ....-..... -...-..-.. .........-
ALBOFUNDUS51645     ...N...... .......... ......-... ..-.....-- ....-..... -...-..-.. .........-
ALBOFUNDUS51639     ...N...... .......... ......-... ..-.....-- ....-..... -...-..-.. .........-
ACACIA4101          .......... .......... ......-... .---....T- -...C....G C...G..-.. .........-
FIMBRIATA854        .......... .......... ......-..- ..--A..CT- --..C....- C...-..-.. .........-
FIMBRIATA2220       .......... .......... ......G... .CC--...-- -...C....- C...-..-.. .........-
FIMBRIATA51831      ...N...... .......... ......G... .CC--...-- -...C....- C...-..-.. .........-
FIMBRIATA51830      .......... .......... ......G... .CC--...-- -...C....- C...-..-.. .........-
FIMBRIATA51644      .......... .......... ......G... .CC--...-- -.NNC....- C...-..-.. .........-
VIRESCENS0460       .......... .......... ......-.-. .CC...---- -.G.G.---- ------.-.. .A......-G
EUCALYPTI639        .......... .......... ......-.-. .CC...---- -.G.G.---- ------.-.. .A.-.CG-.A
MONILIFORMIS3782    .......... .......... ......-.-. .CT...---- -...G.---- ------.-.. AG.....---
PARADOXA1546        .......... .......... ......-.-. .CT...---- -.GCG.---- ------.-.. .A.-------
ADIPOSA1622         .......... .......... ......-.-. .CT...---- -.G.G.---- ------.-.. .A.-------
PINICOLA1323        .......... .......... ......-.-. .CT...---- -.G.G.---- ------.-.. .A.-------
FAGACEARUM2651      .......... .......... ......-.-. C.T...---- -.G.G.---- ------.-.. .A.-------
COERULESCENS666     .......... .......... ......-.-. .CT...---- -.G.G.---- ------.-.. .A.-------
NEOCALEDONIA694     .......... .......... ......-.-. .CC...---- -.G.G.---- ------.-.. .A.-------
AUSTRALIS619        .......... .......... ......-.-. .CC...---- -.G.G.---- ------.-.. .A.-------
ELEGANS185          .......... .......... ......-.-. .CT...---- -.G.G.---- ------.-.. .A.-------
ACACIA4690          .......... .......... ......-.-. .CT...---- -.G.G.---- ------.G.. .A.-------
PETRIELLA           .......... ........A. C...G.G-C. .AA.T...TT .AA-C.-.-- ---AA..ATC --GG....-G
```
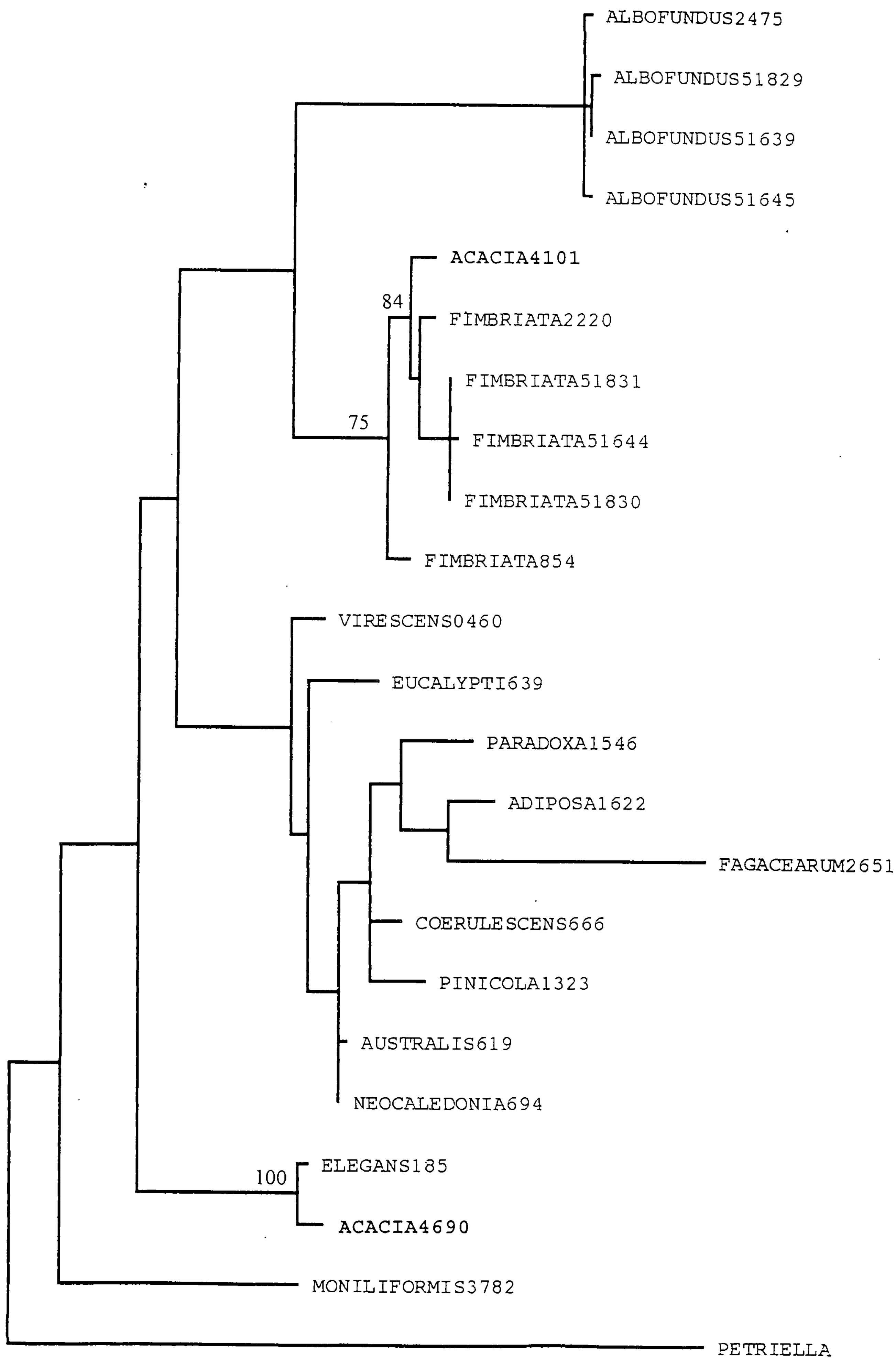

```
                          430        440        450        460        470        480        490
ALBOFUNDUS2475      TT----ACCC TTC-----TG AA-CAGGCC- GCCGAAATGC ATCGGCTGTT ATTTTTACTT GCCAACTCCC
ALBOFUNDUS51829     ..----.... ...-----C. ..-......- .......... .......... .......... ..........
ALBOFUNDUS51645     ..----.... ...-----.. ..-......- .......... .......... .......... ..........
ALBOFUNDUS51639     ..----.... ...-----C. ..-......- .......... .......... .......... ..........
ACACIA4101          .------... --.-----.. ..-......- .........T .......... ..----.... ..........
FIMBRIATA854        .------... --.-----.. ..-......- .......... .......... ..----.... ..........
FIMBRIATA2220       .------... --.-----.. ..-......- .........T .......... ..----.... ..........
FIMBRIATA51831      .------... --.-----.. ..-......- .........T .......... ..----.... ..........
FIMBRIATA51830      .------... --.-----.. ..-......- .........T .......... ..----.... ..........
FIMBRIATA51644      .------... --.-----.. ..-......- .........T .......... ..----.... ..........
VIRESCENS0460       ..TTC----- ---------- ..-......- A......... .......... ..----.... ..-.G..T..
EUCALYPTI639        .--------. ..-A----.. .TG.G....G -......... .......... ..----.... ..-.G..T..
MONILIFORMIS3782    -----GC--- -.ATGC---- ---GC..G.C T.T....... .........- G.---...A. ..-.GT.T..
PARADOXA1546        ----CGC--G ..-TGC---- -----..G.C .........A .......... .----..... ..-.G..T..
ADIPOSA1622         ----CGC--- ---TG----T C.AGC..G.C .......... .........A G----..T.. ..-.G..T..
PINICOLA1323        ----CGCAT. ..-T-TT--- ---GC..G.C .......... .......... .----..T.. ..-.G..T..
FAGACEARUM2651      ----C-CA-. ..GT-CA--- C.AGC-.G.C A......... .........- .G---..T.. ..-.G..T..
COERULESCENS666     ----CGCAT. ..-------- ---GC..G.C A......... .......... .----..T.. ..-.G..T..
NEOCALEDONIA694     ----CGCGT. ..AT------ ---GC..G.C .......... .......... .----..... ..-.G..T..
AUSTRALIS619        ----CGCGT. ..-T-AT--- ---GC..G.C .......... .......... .----..... ..-.G..T..
ELEGANS185          ----CGCGT- ..A-G-T--- C--GC..G.C .......... .......... G.A.A...-- ---.G..T..
ACACIA4690          ----CGCGT- ..A-G-T--- C--GC..G.C .......... .......... GNA.A...-- ---.G..T..
PETRIELLA           -----G-G-- ----GCGC.A ----CA..-- .GTTCTTC.G -AGCAGCTG. .GG---C.C. .--..A.A.-
```

```
                          500        510        520        530        540        550        559

ALBOFUNDUS2475      C-TGTGTAGT ACAAGATTTT -TTAAATTTT TACGCTTT-G GAGTGCTTGT GTAACAT-GC CGT--TAAA
ALBOFUNDUS51829     .-........ .......... -......... ........-. ...-...... .......-.G ..--.....
ALBOFUNDUS51645     .-........ .......... -......... ........-. .......... .......-.G :..--....
ALBOFUNDUS51639     .-........ .......... -......... ........-. .......... .......-.. ...--....
ACACIA4101          .-........ .T..A.-... C.--...... -..A....-. A...T..... ......C-.. ..C--....
FIMBRIATA854        .-........ .T..A.-... C.--...... ...A....-. A...T..... ......C-.. ..C--....
FIMBRIATA2220       .-........ .T..A.-... C.--...... ...A....-. A...T..... ......C-.. ..C--....
FIMBRIATA51831      .-........ .T..A.-... C.--...... ...A....-. A...T..... ......C-.G ..C--....
FIMBRIATA51830      .-........ .T..A.-... C.--...... ...A....-. A...T..... ......C-.G ..C--....
FIMBRIATA51644      .-........ .T..A.-... C.--...... ...A....-. A...T..... ......C-.G ..C--....
VIRESCENS0460       .-.......- -T..T.---- C.---.--.. ...A....-. A.ACT..... ACT....-.. .---.....
EUCALYPTI639        .-.......- -T..T....- -----.--.. ........-. A.ACT..... ACT....-.. .---.....
MONILIFORMIS3782    .-.......- -T..A.C... ------...G. .G.A....-. A.ACT..... ACT.....-.. ..--T....
PARADOXA1546        .-..C....- -T.----... ------..-G. ........-. A.ACT..... ACT....-.. .----.T..
ADIPOSA1622         .-..C....- -T..A.C... G.------G. ........-. A.CCT..... AC.....-.. .----....
PINICOLA1323        .-.......- -T..T....- -------...- ........-. A.ACT...T. AC......-.. .----....
FAGACEARUM2651      .-..C..... .-..AC.... G.------G. .......-C. A.ACT..... ACG....T.. .----....
COERULESCENS666     .-.......- -T..T....- -----.--.. ........-. A.ACT...A. AC......-.. .----.T..
NEOCALEDONIA694     .-.......- -T..T....- -----.--.. ........-. A.ACT..... ACT....-.. .----....
AUSTRALIS619        .-.......- -T..T....- -----.-... ........-. A.ACT..... ACT....-.. .----....
ELEGANS185          .C.......- -T..ATGC.. AGC------.. ...A....-. A.ACTT..A. A.......-.. .---A...
ACACIA4690          .-.......- -T..ATGC.. AGC------.. ...A....-. A.ACTT..A. A.......-.. ..--GA...
PETRIELLA           -------... GGCG.-.CCC GCCGC----- GG..------ ------..-- -CT-----.. -..AG....
```

Figure 9: Aligned nucleotide sequences for the ITS region for different species of *Ceratocystis* and *Chalara*. N indicates unknown bases, a dash (-) indicates a gap in the sequence alignment and (.) indicates bases identical to those of isolate ALBOFUNDUS2475.



ALBOFUNDUS2475
ALBOFUNDUS51829
ALBOFUNDUS51639
ALBOFUNDUS51645
ACACIA4101
84
FIMBRIATA2220
FIMBRIATA51831
75
FIMBRIATA51644
FIMBRIATA51830
FIMBRIATA854
VIRESCENS0460
EUCALYPTI639
PARADOXA1546
ADIPOSA1622
FAGACEARUM2651
COERULESCENS666
PINICOLA1323
AUSTRALIS619
NEOCALEDONIA694
100
ELEGANS185
ACACIA4690
MONILIFORMIS3782
PETRIELLA

Figures 11, 12: Symptoms produced on *A. mearnsii* seedlings during glasshouse inoculation trials with *C. fimbriata.*

Fig. 11: Black cankers developing on *A. mearnsii* seedling after inoculation with *C. fimbriata.*

Fig. 12: Comparison between control inoculation and *C. fimbriata* inoculation after four weeks.

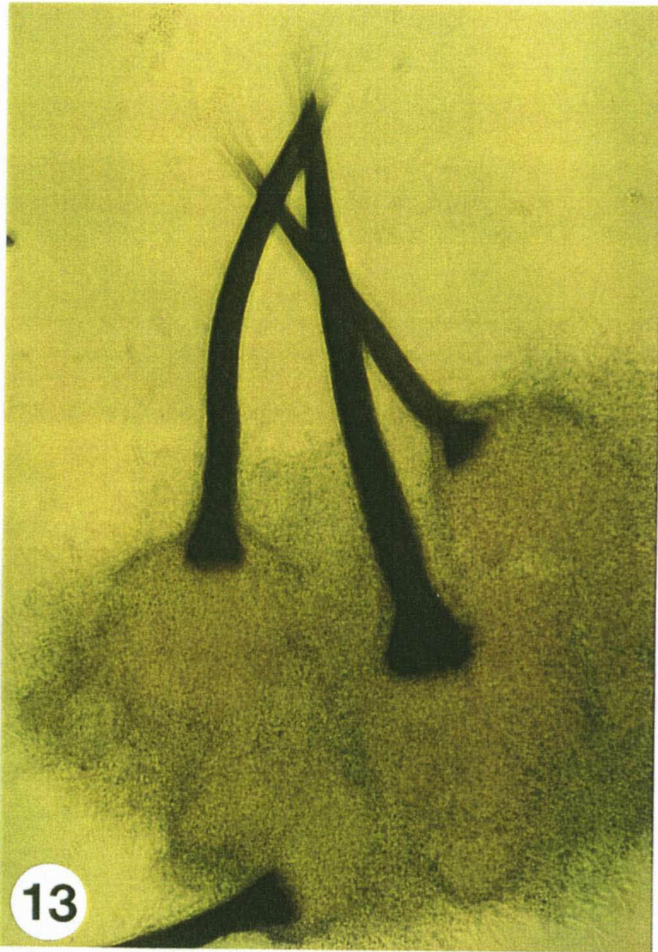
CMW 4101
11

CONTROL
CMW 4101
12

Figures 13, 14: Morphological characteristics of *C. albofundus*.

Fig. 13: Light coloured perithecial base with dark necks, typical of *C. albofundus*.

Fig. 14: Divergent ostiolar hyphae of *C. albofundus*.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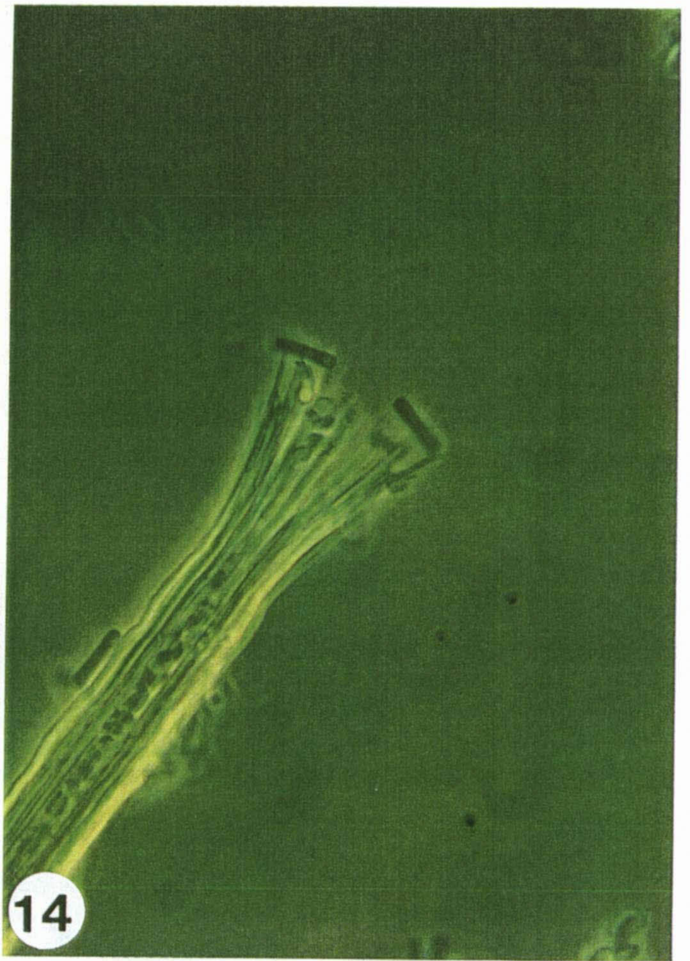
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CHAPTER 4

A SERIOUS NEW WILT DISEASE OF EUCALYPTUS CAUSED BY CERATOCYSTIS FIMBRIATA IN WEST AFRICA

4

A SERIOUS NEW WILT DISEASE OF *EUCALYPTUS* CAUSED BY *CERATOCYSTIS FIMBRIATA* IN WEST AFRICA

ABSTRACT

In a recent survey of *Eucalyptus* clones in the Republic of Congo, West Africa, a serious wilt and die-back disease of two different hybrid clones was observed. Affected trees ranged in age from approximately 6 months to 4 years. Isolations from symptomatic plant material consistently yielded a *Ceratocystis* species. Based on morphology, this fungus was identified as *C. fimbriata*, which is a well-known wilt and canker pathogen of many economically important plants. The *Eucalyptus* isolates were compared with other *Ceratocystis* spp. based on sequence data generated from the ITS region of the rRNA operon. The results confirmed the identity of the *Ceratocystis* species from *Eucalyptus* and also showed that it groups with South American *C. fimbriata* isolates, as well as with *C. fimbriata* from *Acacia mearnsii* in South Africa. Inoculations of young *Eucalyptus* plants were conducted in the greenhouse and all three of the isolates tested, produced lesions in the bark and xylem. This study represents the first report of *C. fimbriata* as a pathogen of *Eucalyptus* in Africa. This is a serious new disease which will require considerable study in order to ensure that losses caused by *C. fimbriata* do not continue.

INTRODUCTION

Eucalyptus spp. are native to Australia, but approximately 8 million hectares of plantations have been established, mostly in tropical and sub-tropical countries of the world (Mohanan & Sharma, 1986; Wingfield, Crous & Peredo, 1995; Wingfield, Crous & Boden, 1996; Wingfield & Wingfield, 1998). The wood from these trees are used for timber, paper and pulp, the production of rayon and viscose and for firewood (Turnbull, 1991). *Eucalyptus* spp. have been grown in the Republic of the Congo since 1953 and from 1978, clonal plantations, established from vegetatively propagated trees, have been utilized (Leaky, 1987).

Serious disease problems have emerged on exotic *Eucalyptus* spp. in most countries where they have been planted, despite the fact that they have been isolated from their natural enemies. These diseases include both stem, root and leaf diseases. Diseases such as Cryphonectria canker, caused by *C. parasitica* (Bruner) Hodges, have necessitated extensive clonal programmes to reduce losses in countries such as South Africa and Brazil (Florence, Sharma & Mohanan, 1986; Hodges, Alfenas & Ferreira, 1986; Conradie, Swart & Wingfield, 1990). Other stem and root diseases include Eucalyptus rust caused by *Puccinia psidii* G. Winter (Ferreira, 1989), Coniothyrium canker caused by *C. zuluense* (Wingfield, Crous & Coutinho, 1997) and Pythium and Phytophthora root rot (Linde, Kemp & Wingfield, 1994). Leaf diseases caused by species of *Mycosphaerella* Johanson and *Cylindrocladium* Morgan also cause serious problems, especially in tropical areas (Park & Keane, 1984; Crous & Wingfield, 1994; Crous & Wingfield, 1996). These are but a few of the diseases that have already lead to considerable economic losses to the *Eucalyptus* industry.

Until recently, no thorough survey of the diseases affecting plantation *Eucalyptus* in the Republic of the Congo had been conducted. Sporadic reports of tree deaths were made occasionally, but no detailed investigations into the causal agents were initiated, since the mortalities were not considered to be serious. One such report was of a wilt and die-back

disease, thought to be caused by an undetermined species of *Botryodiplodia* (Declert, 1996).

During 1998, a survey of diseases of eucalypt plantations in the Pointe-Noire area of the Republic of Congo was undertaken. This lead to the discovery of a serious wilt and die-back disease of *E. urophylla* S. T. Blake X *E. pellita* F. Muell. (UP) and *E. grandis* Hill ex. Maid. X *E. territicornis* Sm. (ET). Affected trees ranged from 6 months to more than 4 years old. The most common symptoms were the rapid wilting and death of trees and dark brown "streaking" of the xylem. The aim of this study was, therefore, to determine the cause of the *Eucalyptus* wilt disease in the Republic of Congo and to prove pathogenicity of the isolated fungus using greenhouse inoculation trials.

MATERIALS AND METHODS

Disease and symptoms

Disease was observed on 2-year-old trees and 6-month-old coppice stems of *E. urophylla* X *E. pellita* (UP) hybrids from Kissoko plantation and from 4-year-old coppice stems of *E. grandis* X *E. territicornis* (GT) growing at Tchittanga plantation, Republic of Congo. Approximately 50 % of these stands were affected. Hybrid *E. urophylla* X *E. pellita* (UP) showed symptoms of wilt, followed by death. Upon cutting into the xylem, extensive streaking was observed (Fig. 1). These streaks were more intense and concentrated towards the base of the tree. Adjacent to this UP stand was a stand of young UP coppice (less than 1-year-old), of which at least one of the coppice stems per stump was dead (Fig. 2) or dying. Again, distinct dark brown streaks were found in the xylem (Fig. 3). These symptoms were similar to those on the adjacent, approximately 2 year old, UP trees.

At Tchittanga plantation, trees showed signs of wilt and die-back of first rotation coppice of a 4-year-old GT clone. More than half of the trees in this stand were dead or dying. Many of the affected trees were exuding kino and the stems of many trees were cracked.

Extensive kino pockets were observed in the xylem. Epicormic shoots were also common on many of the trees. Some trees showed streaking of the xylem.

Isolations

Pieces of symptomatic tissue from the leading margin of the streaked and discoloured wood were plated directly onto 2% malt extract agar (MEA) (20 g/L Biolab malt and 15 g/L Biolab agar). Segments of symptomatic material were also placed in humidity chambers to induce the formation of fungal fruiting bodies. All plates were incubated at approximately 25°C to induce fungal growth. Perithecia formed in the stains within a few days and single ascospore drops were transferred to separate MEA plates. Direct isolation from plant material on to MEA resulted in the abundant formation of colonies of a fungus that produced long necked perithecia.

Greenhouse pathogenicity trials

Twenty trees, approximately 5 mm diameter, of a *E. grandis* X *E. camaldulensis* Denh. (GC) hybrid were artificially inoculated with 3 isolates of the suspected pathogen. The isolates were grown on MEA for 14 days before inoculation. Wounds were made into the xylem of the trees by removing the bark with a 4 mm diameter cork borer. Mycelial plugs of equal size, covered by the test fungus were placed into the wounds and the wounds sealed with parafilm to prevent desiccation of the inoculum and the wounds. Ten trees were inoculated with sterile agar plugs as controls. Lesions were measured after 5 weeks on the outer bark and in the xylem. Pieces of symptomatic material were placed in humidity chambers to confirm that the inoculated fungus were responsible for causing the observed lesions.

DNA amplification and sequencing

Isolates used in this study were grown on MEA plates and template DNA was obtained by scraping the mycelial surfaces with a pipette tip (Harrington & Wingfield, 1995). The polymerase chain reaction (PCR), using primers ITS 1 (5'TCCGTAGGTGAACCTGCGG3') and ITS 4 (5'TCCTCCGCTTATTGATATGC3') was used to amplify the Internal Transcribed Spacer (ITS) regions of the ribosomal RNA operon (White *et al.*, 1990). The PCR reaction mixture included Expand™ (Boehringer, High Fidelity PCR), 0.2 mM DNTP's, 10X Buffer (Boehringer), 1 mM MgCl (Boehringer) and 0.75 mM primer. Denaturation was performed at 96°C for 1 min, followed by 35 cycles of primer annealing at 55°C for 30 sec. Chain elongation took place at 72°C for 1 min and denaturation at 92°C for 1 min. Final chain elongation took place at 72°C for 5 min. PCR products were stained with ethidium bromide and visualized under UV light.

The PCR fragments obtained were purified using the QIAquick PCR purification kit (QIAGEN, Germany). PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit with Amplitaq® DNA polymerase, FS (Perkin-Elmer, Warrington, UK) on a ABI PRISM™ 377 DNA Autosequencer (Perkin-Elmer). Primers ITS 1 and ITS 4 was used in the sequence reaction. Sequences for the Congolese isolates were aligned against sequences obtained from Genbank, Witthuhn *et al.* (1998a, b) and Roux *et al.* (unpublished) (Table 1). Nucleotide sequences were manually aligned by inserting gaps and analyzed using the Heuristic search option in PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1985). Confidence intervals were determined using DNA BOOTSTRAP analysis (Bootstrap confidence intervals on DNA parsimony) (Felsenstein, 1988).

RESULTS

Isolations

Isolations made from the diseased 2-year-old UP and the 6-month-old coppice at Kissoko consistently yielded a species of *Ceratocystis*, both from humidity chambers and from isolations made on agar. The same fungus was also isolated from the UT coppice at Tchittanga. The *Ceratocystis* sp. was found to sporulate abundantly in the brown streaks. The fungus was identified as *Ceratocystis fimbriata* based on perithecial morphology and size, hat-shaped ascospores, chlamydospore morphology and the presence of a distinctive *Chalara* anamorph. Cultures of the fungus have been deposited in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), South Africa.

Greenhouse pathogenicity trials

All 3 isolates tested produced lesions on the outer bark as well as in the xylem of the inoculated trees (Fig. 4, Table 2). The typical streaking associated with *C. fimbriata* infection on naturally infected trees was also evident in many of the inoculated trees (Fig. 4). No symptoms developed on trees inoculated as controls and in all cases the inoculation wounds were covered with callus (Table 2). The inoculated pathogen was consistently re-isolated from the lesions on inoculated trees and never from control trees.

DNA amplification and sequencing

Sequences for the Congolese isolates were aligned against sequences obtained from Genbank, Witthuhn *et al.* (1998a, 1998b) and Roux *et al.* (unpublished) (Table 1). Sequences were manually aligned by the insertion of gaps, resulting in a total of 560 characters (Fig. 5). A Heuristic search using PAUP with the no branch swopping option, generated one tree (Fig. 6). Values for the consistency index (CI), homoplasy index (HI)

and retention index (RI) were 0.878, 0.122 and 0.852 respectively. Isolates from the Republic of Congo grouped with *C. fimbriata*, separately from other *Ceratocystis* spp. with which they were compared, with a confidence interval of 83 %. The Congolese isolates formed a clade with *C. fimbriata* isolates from *Eucalyptus* in Brazil and *A. mearnsii* in South Africa. This clade was separate from the clade containing *C. fimbriata* isolates from *Platanus* spp. in the Northern Hemisphere.

DISCUSSION

As far as we are aware, this report represents the first record of a *Ceratocystis* sp. as a pathogen of *Eucalyptus*. We are, however, aware of a similar report of a fungus representing *C. fimbriata* from a *Eucalyptus* sp. by colleagues in Brazil (Dr. A. Alfenas, University of Viçosa), although details of that disease are not known to us. This is also the first example of a serious wilt disease of *Eucalyptus* caused by a fungus. The appearance of the disease at a time when the propagation of these trees is increasing greatly is of concern and it deserves further study.

Ceratocystis spp. are well-known causal agents of wilt diseases and are amongst the most serious pathogens of woody plants in the world (Kile, 1993; Wingfield, Seifert & Webber, 1993). *Ceratocystis fimbriata* is perhaps the best known of these species and has a wide host range including sweet potato (Halsted & Fairchild, 1891), coffee (Pontis, 1951), cocoa (Kile, 1993), gmelina (Muchovej, Albuquerque & Ribeiro, 1978), fruit trees such as peach and almond (De Vay *et al.*, 1963; Teviotdale & Harper, 1991), poplar (Wood & French, 1962; Gremmen & de Kam, 1976), *Acacia decurrens* (Ribeiro *et al.*, 1988) and many others, on all of which it causes serious wilt and canker diseases. The fungus produces slimy droplets of spores from perithecia and also produces sweet smelling aromatics which are thought to play a role in insect dispersal (Hanssen, 1993; Christen, Meza & Revah, 1997). Trees usually require wounds for the initiation of infection (De Vay *et al.*, 1963; Teviotdale & Harper, 1991) and these wounds are usually visited by insects that transmit spores to them (Crone & Bachelder, 1961; Hinds, 1972; Rosetto &

Ribeiro, 1991). At this stage, we know very little concerning the factors associated with disease development on *Eucalyptus* in the Congo, but we must expect that it will be similar to the situation on other trees, elsewhere in the world.

There has been only one other report of a *Ceratocystis* sp. from *Eucalyptus*. This is *C. eucalypti* Yuan & Kile that was collected from wounds on the stems of *E. siberi* L. Johnson and *E. globoidea* Blakely in Australia (Kile *et al.*, 1996). There can, however, be no mistaking *C. fimbriata* for *C. eucalypti*. The latter has very large elongated, fusiform ascospores, whereas *C. fimbriata* has very characteristic hat-shaped ascospores. *Ceratocystis eucalypti* is reportedly not pathogenic to *Eucalyptus*.

Morphological characteristics of the fungus associated with *Eucalyptus* wilt in the Congo closely match the description of *C. fimbriata*. Recently, considerable data pertaining to the phylogenetic relationships between *Ceratocystis* spp. have become available (Hausner, Reid & Klassen, 1993; Visser *et al.*, 1995; Witthuhn *et al.*, 1998a; 1998b). For the present, *C. fimbriata* remains a discrete species. However, Webster and Butler (1967) have previously presented data that might suggest that this fungus represents a number of closely related, but different species. They, however, concluded, based on hybridisation studies, that *C. fimbriata* represents one species including several strains that differ in morphology and cultural characteristics. As additional sequence data become available, this situation might need reconsideration.

The recent discovery of a *Ceratocystis* sp. causing a serious wilt disease of black wattle (*A. mearnsii* de Wild.) in South Africa aptly illustrates the difficulty with morphological identification of species in the *C. fimbriata* group (Morris, Wingfield & DeBeer, 1993). The pathogen was first reported as *C. fimbriata* but, later, based largely on sequence data, was described as a new species that is now known as *C. albofundus* Wingfield, De Beer & Morris (Wingfield *et al.*, 1996). The grouping in the present study of *C. fimbriata* isolates from South America and Africa in a clade separate from European and North American

isolates, also supports the hypothesis that *C. fimbriata* represents a species aggregate. This matter requires further investigation.

Pathogenicity tests on young trees in the greenhouse confirmed the likely role of *C. fimbriata* as the causal agent of the *Eucalyptus* disease in the Congo. The results are also seen together with the symptoms on trees which are similar to those usually associated with *C. fimbriata* infection of woody crops (Leather, 1966; Muchovej *et al.*, 1978; Kile & Walker, 1987; Ribeiro *et al.*, 1988). In the future, I would, however, hope to conduct pathogenicity tests on established trees in the Republic of Congo. Such tests will expand our understanding of disease development, and perhaps more importantly, will allow us to compare the susceptibility of different species and hybrids. Ultimately, the aim must be to reduce the effects of this disease. This could potentially be achieved through selection of disease tolerant planting stock.

It is intriguing to consider what the possible origin of *C. fimbriata* on *Eucalyptus* in the Republic of Congo might be. At the present time, there are no reports of this fungus causing disease in this country or any other African countries. This might be due to the fact that intensive surveys for this pathogen, which can be inconspicuous and difficult to isolate, have not been undertaken. However, our preliminary sequencing data show that the fungus from *Eucalyptus* in the Republic of Congo is most similar to *C. fimbriata* isolates from South America and *C. fimbriata* from *A. mearnsii* in South Africa. These data might imply that the fungus originated in South America where *C. fimbriata* is a well-known pathogen of a wide range of crops. Further phylogenetic and biogeographic studies are planned to consider this question more completely.

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Table 1: List of *Ceratocystis* isolates used in DNA sequence comparisons.

SPECIES	ISOLATE NUMBERS[a]	ORIGIN	GENEBANK ASSENCION NUMBER/ ISOLATE NUMBER
Ceratocystis adiposa	CMW1622	Japan	AF043606
C. albofundus	CMW2475	South Africa	F043605
"	PREM51639	"	
C. coerulescens	C666	Norway	U756618
C. eucalypti	C639	Australia	U75627
C. fagacearum	CMW2651	USA	AFO43598
C. fimbriata	**CMW4769**	**Republic of Congo**	
"	**CMW4783**	"	
"	**CMW4101**	**South Africa**	
"	**CMW4900**	**Brazil**	
"	**CMW4901**	"	
"	CMW2220	Europe	AFO43604
"	PREM51830	"	
"	C854	USA	AFO07749
C. paradoxa	CMW1546	New Zealand	AFO43607
C. virescens	CMW0460	USA	U75625

[a] CMW numbers represent cultures maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

PREM - Official designation of the National Collection of fungi, Pretoria, South Africa.

C - Culture collection of T.C. Harrington, Department of Plant Pathology, Iowa State University.

Table 2: Lesions produced on *E. grandis* X *E. camaldulensis* clones in glasshouse inoculation trials.

ISOLATE	LESION LENGTH (mm)[a]
CMW 4786	50.7
CMW 4769	47.15
CMW 4781	40.15
Control	9.0

[a] Each value represents an average of 20 measurements for the 3 isolates tested and 10 measurements for the controls.

Figures 1-4: Symptoms caused by *Ceratocystis fimbriata* on *Eucalyptus* clones.

Fig. 1: Streaking of the xylem of a ~ 2 year-old *E. urophylla* X *E. pellita* (UP) clone.

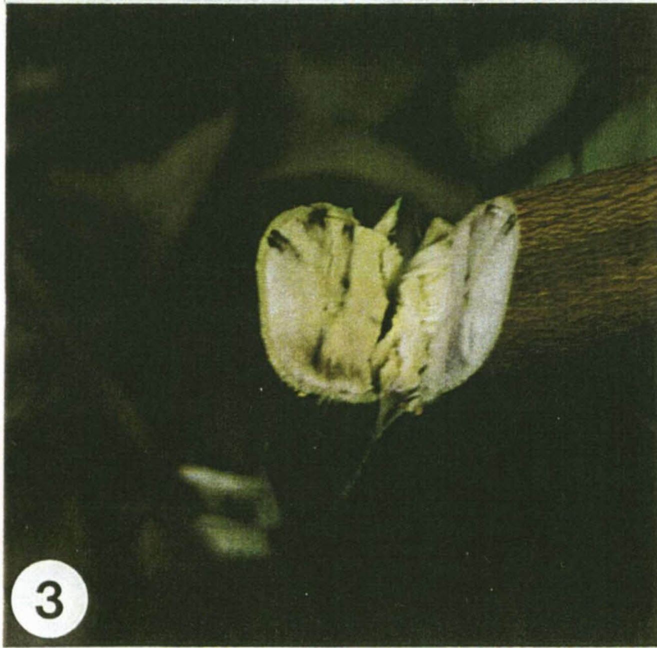
Fig. 2: Die-back of ~ 6-month-old coppice stems of a UP clone.

Fig. 3: Discolouration of the xylem of UP coppice stem after infection with *C. fimbriata*.

Fig. 4: Lesion produced on *E. grandis* X *E. camaldulensis* clone during greenhouse inoculation trials with *C. fimbriata*.

1

2


3

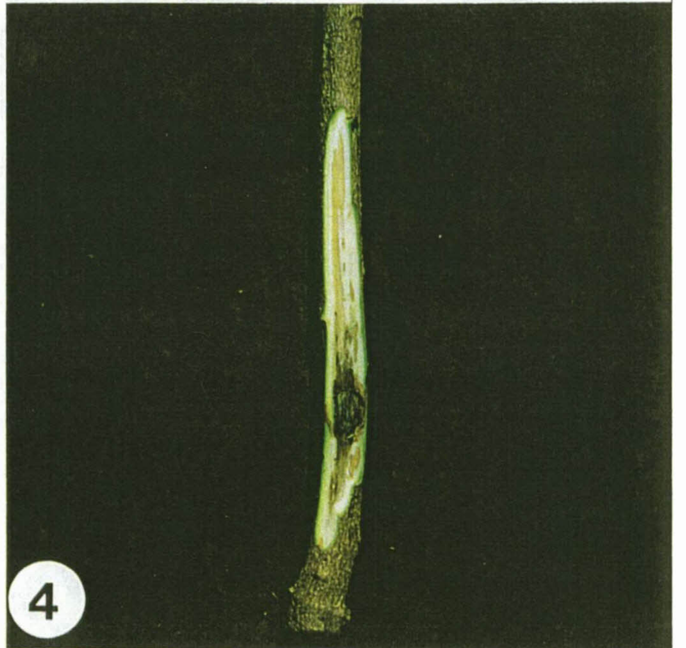

4

Figure 5: Aligned nucleotide sequences for the ITS region of the rRNA operon for different species of *Ceratocystis*. N indicates unknown basis, a dash (-) indicates a gap in the sequence alignment and (.) indicates bases identical to the corresponding base in the sequence of *C. albofundus* isolate number ALBOFUNDUS2475.

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                          10         20         30         40         50         60         70

ALBOFUNDUS2475       GCTGCCTTG- --GTGGGTG- TCT-G-TAGT GGTGTTAA-C CTCTTTTTAT ----AAGGGG GCAGCCC-AC
ALBOFUNDUS51639      .........- --.......- .N.-.-.... ........-. .......... ----...... .......-..
ACACIA4101           C.ATGTG..A AC..ACCCTA ...T.-.... .AGA.G..TG ..G.-..GG TGGT-....- -----..TT.
IPOMOEA854           -.ATGTG..A AC..ACC-TA ...T.-.... .AGA.G..TG ..G.-..GG TGGT-....- -----..TT.
PLANE2220            C.ATGTG..A AC..ACC-TA ...T.-.... .AGA.G..TG ..G.-..GG TGGT-....- -----..TT.
PLANE51830           ---T---..A ---.------ ...T.-.... .AGA.G..TG G.G..G.-GG TGGT-..-T. .-----..TT.
CONGO4783            C.ATGTG..A AC..AC--TA ...-.-.... .AGA.G..T- -.G--...GG TGGT-...-- -----..TT.
CONGO4769            C.ATGTG..A -C..-CC-TA ...-.-.... -AGA.G..TG ..G.-..GG TGGT-....- -----..TT.
BRAZIL4900           C.ATGTG..A AC..ACCCTA ...T.-.... .AGA.G..TG ..G.-..GG TGGT-....- -----..TT.
BRAZIL4901           ---TGTG..A AC..ACCCTA ...T.-.... .AGA.G..TG ..G.-..GG TGGT-....- -----..TT.
VIRESCENS0460        C.ATATG..A ACA.ACC-TA .-.------- -------.-G ..GC-...GG C-----..-- ------.TTG
EUCALYPTI639         C.ATATG..A ACA.ACC-T- ..-------- -------.-G ..GC-...GG C-----..-- ------.TTG
COERULESCENS666      C.ATATG..A ACA.ACC--T ..-------- -------.-G ..GC-...GG C-----..-- ------.TTG
PARADOXA1546         C.ATTTG..A ACT.ACC--T ...-.----- ---------G ..GC-...GG C-----..T- -----..TTG
ADIPOSA1622          C.ATTTG..A ACA.ACC-TA ...T--.-- ---------- ..GC-...GG C-GT--..T- ------.TT.
FAGACEARUM2651       C.ATTTG..A ACA.ACC-.A .-.TTT.TT. CTCTAAT.-- ..GC-...GG C------..A ------.TT.
PETRIELLA            C.CTTTG..A ACC.TACC-A --.T.T..-- --.....---G .CTCGGC-GG -GGTT.---- --C--..CCA
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                          80         90        100        110        120        130        140

ALBOFUNDUS2475     TACCGC-TAG -CCACC---- ----AGCAGC ATACA--AG- TCTTTTACCA CTAT---AAA -C-CTTCTGT
ALBOFUNDUS51639    ......-... -.....---- ---....... ......--.- .......... ....---... -.-.......
ACACIA4101         .-GAAGAG.. GG....G--C TGCC.....- -.--TT..-  ....-CG... ..G.---... -.T...TAT.
IPOMOEA854         .-GAAGGG.. GG....G--C TGCC.....- -.--TT..-  ...--C.... ....---... -.T...T.--
PLANE2220          .-GAAGGG-- --....G--C TGCC.....- -.---T..-  ...--CG... ..G.---... -.T...----
PLANE51830         .-GAAG---- --....G--C TGCC.....- -.---T..-  ...--CG... ..G.---... -.T...----
CONGO4783          .-GAAGAG.. G-....G--C TGCC.....- -.---T..-  ...--CG... ..G.AAA... A.T...TAT.
CONGO4769          .-GAAGAG.. GG....G--C TGCC.....- -.---T..-  ...--CG... ..G.AAA... A.T...TAT.
BRAZIL4900         .-GAAGAG.. GG....G--C TGCC.....- -.--TT..-  ....-CG... ..G.---... -.T...T-T.
BRAZIL4901         .-GAAGAG.. GG....G--C TGCC.....- -.--TT..G  ....CGG... ..G.---... -.T...T-T.
VIRESCENS0460      GTAACA---- --..AGTC-- TGCCG.T..- -.-------- --...----- --------.. A.T...T.T.
EUCALYPTI639       GTAACA---- --..AGTC-T TGCCG.T..- -.-------- --...----. .--------.. -.T...T.T-
COERULESCENS666    GTAAAA---- --..AGTC-- TGCCG.T... --.TTT-.-- ---------- --------.. A.T...T---
PARADOXA1546       G------G.T T--------- TGCCG.T..- ---------- ---------.. .-.AAC-... -.T...T---
ADIPOSA1622        GG-----G.T TG-------- TGCCG.T..- -.-------- --...----. .--------.. -.T...T.--
FAGACEARUM2651     .TT-CTTC.. GGG.TGTTTC TGCC..T..- -..TTT-.-- ---------.. ----------. A.T...T.T-
PETRIELLA          A.--..T.CT C..-G.CGG- ---C....-. -.-------- ---------- --------.. ---....-.
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                                150        160        170        180        190        200        210
ALBOFUNDUS2475      --AT-ATT-T TTTAAAA--T TTTT-AAAA- --ATTGCTGA GTGGCAT--A A-CTATAAAA A-AAGTTAAA
ALBOFUNDUS51639     --.-....-. .......--. ....-....- --........ .......--. .-........ .-........
ACACIA4101          --..T...T. -C..--GA-. ...------- -C........ .......--. .-........ .-........
IPOMOEA854          --..T...T. -C..--GA-. ...------- -C........ .......--. .-........ .-........
PLANE2220           --..-...T. -CC.--GA-. ....T----- -C........ .......--. .-........ --........
PLANE51830          --..-...T. .CC.--GA-. ....T----- -C........ .......--. .-........ --........
CONGO4783           --.C----T. .C..--GA-. ...------- -C........ .......--. .-........ --........
CONGO4769           --.C----T. .C..--GA-. ...------- -C........ .......--. .-........ .-........
BRAZIL4900          --..T...T. -C..--GA-. ...------- -C........ .......--. .-........ .-........
BRAZIL4901          --..T...T. -C..--GA-. ...------- -C........ .......--. .-........ .-........
VIRESCENS0460       TT---...-- -C....GAA. .----.---T TC........ .......T-. .-.-.-..-- -T........
EUCALYPTI639        ------..T- -C..G.GAA. .----.---T TC........ .......T-. .-.-.-..-- -T........
COERULESCENS666     --.-.-..T- -C..G.GAA. .----.---T TC........ .......T-. .-.-.-..-- -T........
PARADOXA1546        --.-.-..T- -C..G.GAA. .----.---T TC........ .......T-. .-.--....- -T........
ADIPOSA1622         --.--...T- -C..G.GAA. .----.---T TC........ ..T....TT. .-.---...- -T.-......
FAGACEARUM2651      --.--...T- -C..G.GAA. .----.---T TC........ ..T....TT. .-.-.-...- -T.-......
PETRIELLA           --.--...T. A.AGCG--GA .----.T.-- -C-..-.... A.ACA..--. --.---.... -C..A.A...
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                      220        230        240        250        260        270        280

ALBOFUNDUS2475    ACTTTCAACA ACGGATCTCT TGGCTCTAGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA
ALBOFUNDUS51639   .......... .......... .......... .......... .......... .......... ..........
ACACIA4101        .......... .......... .......... .......... .......... .......... ..........
IPOMOEA854        .......... .......... .......... .......... .......... .......... ..........
PLANE2220         .......... .......... .......... .......... .......... .......... ..........
PLANE51830        .......... .......... .......... .N........ .......... .......... ..........
CONGO4783         .......... .......... .......... .......... .......... .......... ..........
CONGO4769         .......... .......... .......... .......... .......... .......... ..........
BRAZIL4900        .......... .......... .......... .......... .......... .......... ..........
BRAZIL4901        .......... .......... .......... .......... .......... .......... ..........
VIRESCENS0460     .......... .......... .......... .......... .......... .........C ..........
EUCALYPTI639      .......... .......... .......... .......... .......... .........C ..........
COERULESCENS666   .......... .......... .......... .......... .......... .........C ..........
PARADOXA1546      .......... .......... .......... .......... .......... .........C ..........
ADIPOSA1622       .......... .......... .......... .......... .......... .......... ..........
FAGACEARUM2651    .......... .......... .......... .......... .......... .......... ..........
PETRIELLA         .......... .......... ...T...G.. .......... .......... .......... ..........
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                               290        300        310        320        330        340        350
ALBOFUNDUS2475      TTGCAGAATT CAGTGAATCA TCGAATCTTT GAACGCACAT TGCCCCTGG- TAGTATTCTG CCAGGCATGC
ALBOFUNDUS51639     .......... ........N. ......N... .......... ..NN.....- .......... ..........
ACACIA4101          .......... .......... .......... .......... ...G.....G C......... ..........
IPOMOEA854          .......... .......... .......... .......... ...G.....- C......... ..........
PLANE2220           .......... .......... .......... .......... .........- .......... ..........
PLANE51830          .......... .......... ...NN...N. .......... ...G.....- C......... ..........
CONGO4783           .......... .......... .......... .......... ...G.....- C......... ..........
CONGO4769           .......... .......... .......... .......... ...G.....- C......... ..........
BRAZIL4900          .......... .......... .......... .......... ...G.....- C......... ..........
BRAZIL4901          .......... .......... .......... .......... ...G.....- C......... ..........
VIRESCENS0460       .......... .......... .......... .......... ...G.....- C......... ..........
EUCALYPTI639        .......... .......... .......... .......... ...G.....- C......... ..........
COERULESCENS666     .......... .......... .......... .......... ...G.....- C......... ..........
PARADOXA1546        .......... .......... .......... .......... ...G.....- C......... .....T....
ADIPOSA1622         .......... .......... .......... .......... ...G.....- C......... ..........
FAGACEARUM2651      .......... .......... .......... .......... ...G...A.- C......... .T........
PETRIELLA           .......... .......... .......... .......... ...G..C..- C....A.... ..G.......
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                              360        370        380        390        400        410        420

ALBOFUNDUS2475      CTGTCCGA-G CGTCATTTCA CCACTCAA-G ACTT-GCTTT --AGTT-TTG GT-GTT-GGA GGTCCTGTTC
ALBOFUNDUS51639     ....N...-. .......... ........-. ....-..... --....-... ..-...-... ..........
ACACIA4101          ........-. .......... ........-. ...---.... T--...C... .GC...G... ..........
IPOMOEA854          ........-. .......... ........-. .-..--A..C T---..C... .-C...-... ..........
PLANE2220           ........-. .......... ........G. ...CC--... ---...C... .-C...-... ..........
PLANE51830          ........-. .......... ........G. ...CC--... ---...C... .-C...-... ..........
CONGO4783           ........A. .......... ........-. .-..--A..C T---..C... .-C...-... ..........
CONGO4769           ........-. .......... ........-. .-..--A..C T---..C... .-C...-... ..........
BRAZIL4900          ........-. .......... ........-. ...---.... T--...C... .-C...-... ..........
BRAZIL4901          ........-. .......... ........-. ...---.... T--...C... .-C...-... A.........
VIRESCENS0460       ........-. .......... ........-. -..CC....- ---.G.G..- -------... ..A......-
EUCALYPTI639        ........-. .......... ........-. -..CC....- ---.G.G..- -------... ..A.-.CG-.
COERULESCENS666     ........-. .......... ........-. -..CT....- ---.G.G..- -------... ..A..-----
PARADOXA1546        ........-. .......... ........-. -..CT....- ---.GCG..- -------... ..A..-----
ADIPOSA1622         ........-. .......... ........-. -..CT....- ---.G.G..- -------... ..A..-----
FAGACEARUM2651      ........-. .......... ........-. -.C.T....- ---.G.G..- -------... ..A..-----
PETRIELLA           ........-. .......... A.C...G.-. C..AA.-... T---..AAA- --C-.--.A. ..AT.G..GT
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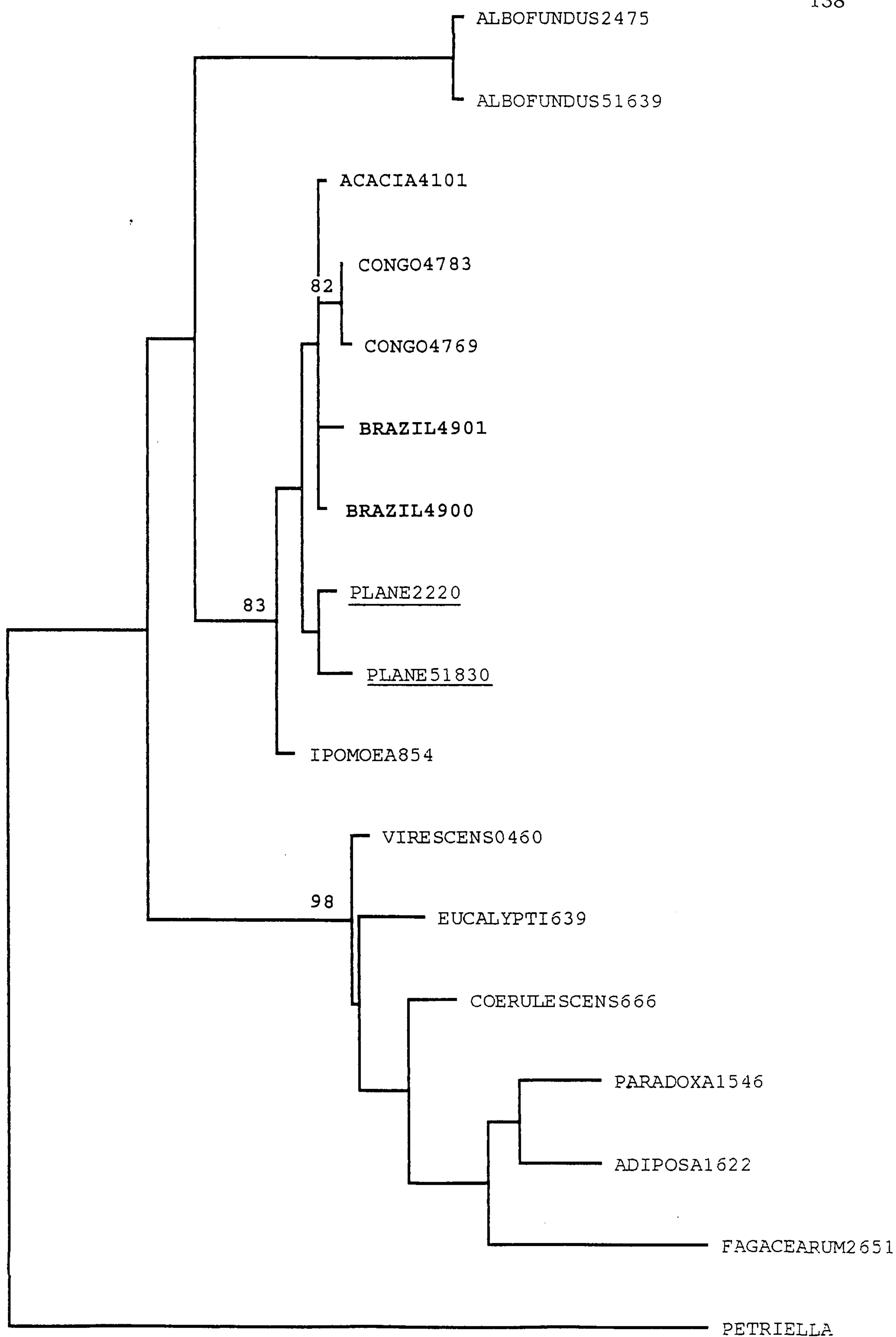
                   430        440        450        460        470        480        490
ALBOFUNDUS2475     -TT----ACC CTTC-----T GAA-CAGGCC -GCCGAAATG CATCGGCTGT TATTTTTACT TGCCAACTCC
ALBOFUNDUS51639    -..----... ...------C ...-...... -......... .......... .......... ..........
ACACIA4101         -.------.. .--.-----. ...-...... -......... T......... ...----... ..........
IPOMOEA854         -.------.. .--.-----. ...-...... -......... .......... ...----... ..........
PLANE2220          -.------.. .--.-----. ...-...... -......... T......... ...----... ..........
PLANE51830         -.------.. .--.-----. ...-...... -......... T......... ...----... ..........
CONGO4783          -.------.. .--.-----. ...-...-.. -......... T......... ...----... ..........
CONGO4769          -.------.. .--.-----. ...-...... -......... T......... ...----... ..........
BRAZIL4900         -.------.. .--.-----. ...-...... -......... T........G ...----... ..........
BRAZIL4901         -..-----.. .--------. ...-...... -......... T......... ...----... ..........
VIRESCENS0460      G..TTC---- ---------- -..-...... -A........ .......... ...----... ...-.G..T.
EUCALYPTI639       A.-------- ...-A----. ..TG.G.... G-........ .......... ...----... ...-.G..T.
COERULESCENS666    -----CGCAT ...------- ----GC..G. CA........ .......... ...----.T. ...-.G..T.
PARADOXA1546       -----CGC-- G..-TGC--- ------..G. C......... A......... ..----.... ...-.G..T.
ADIPOSA1622        -----CGC-- ----TG---- TC.AGC..G. C......... .......... AG----..T. ...-.G..T.
FAGACEARUM2651     -----C-CA- ...GT-CA-- -C.AGC-.G. CA........ .......... -.G---..T. ...-.G..T.
PETRIELLA          T-----G--- G--G--G-CG CT.-...CGG TT.T----.C GG-A.CAGC. GTAGG--C.C ..-A..TA.-
```

```
                    500        510        520        530        540        550        560
ALBOFUNDUS2475      CC-TGTGTAG TACAAGATTT T-TTAAATTT TTACGCTTT- GGAGTGCTTG TGTAACAT-G CCGT--TAAA
ALBOFUNDUS51639     ..-....... .......... .-........ .........- .......... ........-. ....--....
ACACIA4101          ..-....... ..T..A.-.. .C.--..... .-..A....- .A...T.... .......C-. :..C--....
IPOMOEA854          ..-....... ..T..A.-.. .C.--..... ....A....- .A...T.... .......C-. ...C--....
PLANE2220           ..-....... ..T..A.-.. .C.--..... ....A....- .A...T.... .......C-. ...C--....
PLANE51830          ..-....... ..T..A.-.. .C.--..... ....A....- .A...T.... .......C-. G..C--....
CONGO4783           .--....... ..T..A---. .C.---.... .-..A...-- .A...----- ---------- ----------
CONGO4769           ..-....... ..T..A--.. .C.--..... --..A...-- .A...-.... ...T...C-. ...---...-
BRAZIL4900          ..-....... ..T..A.-.. .C.--..... ....A....- .A...T.... .......C-. ...C--....
BRAZIL4901          ..-....... ..T..A.-.. .C.--..... ....A....- .A...T.... .......C-. ...C--....
VIRESCENS0460       ..-....... --T..T.-- -C.---.-- ....A....- .A.ACT.... .ACT....-. ...---....
EUCALYPTI639        ..-....... --T..T.... ------.-- .........- .A.ACT.... .ACT....-. ...---....
COERULESCENS666     ..-....... --T..T.... ------.--. .........- .A.ACT...A .AC.....-. ...---.T..
PARADOXA1546        ..-..C.... --T.---.. .------.-G .........- .A.ACT.... .ACT....-. ...---.T..
ADIPOSA1622         ..-..C.... --T..A.C.. .G.------G .........- .A.CCT.... .AC.....-. ...---....
FAGACEARUM2651      ..-..C.... ..-..AC... .G.------G ........-C .A.ACT.... .ACG....T. ...---....
PETRIELLA           --------.. .GGCG.-.CC CGCCGC---- -GG..----- ------...- --CT-----. .-..AG....
```

Figure 6: Phylogram generated using the Heuristic search option, with no branch swopping, in PAUP. Bootstrap confidence intervals are indicated at the branches of the tree. Isolates sequenced in this study appear in bold type.


ALBOFUNDUS2475
ALBOFUNDUS51639
ACACIA4101
CONGO4783
82
CONGO4769
BRAZIL4901
BRAZIL4900
PLANE2220
83
PLANE51830
IPOMOEA854
VIRESCENS0460
98
EUCALYPTI639
COERULESCENS666
PARADOXA1546
ADIPOSA1622
FAGACEARUM2651
PETRIELLA

CHAPTER 5

MOLECULAR COMPARISON OF A SEIRIDIUM SPECIES FROM ACACIA MEARNSII WITH THE CYPRESS CANKER PATHOGENS

5

MOLECULAR COMPARISON OF A *SEIRIDIUM* SPECIES FROM *ACACIA MEARNSII* WITH THE CYPRESS CANKER PATHOGENS

ABSTRACT

During a disease survey of *Acacia mearnsii* (black wattle) in South Africa, isolates of an unknown *Seiridium* sp. were collected from stem cankers. This species is morphologically indistinguishable from *Seiridium* spp. responsible for Cypress (*Cupressus* spp.) canker. The latter disease occurs in South Africa and many other parts of the world. The aim of this study was to compare isolates of the *Seiridium* sp. from *A. mearnsii* with those of *S. cardinale*, *S. cupressi* and *S. unicorne* associated with cypress canker. The ribosomal RNA operon was used for the molecular comparisons. Representative isolates from *Cupressus* spp. and *A. mearnsii* were also inoculated into *Cupressus lusitanica* and *A. mearnsii* to determine their relative pathogenicity to these hosts. Results obtained from this study suggest that the *Seiridium* sp. from *A. mearnsii* is very closely related to those causing cypress canker. Data also provide further support for the view that only one *Seiridium* sp., or closely related species that has only recently speciated, is associated with cypress canker.

INTRODUCTION

The genus *Seiridium* Nees ex Fr. is characterized by the formation of six celled, appendaged conidia in acervuli (Sutton, 1980). The genus is best known for the species causing cypress canker of Cupressaceae in many parts of the world (Swart, 1973; Graniti, 1986; Wingfield & du Toit, 1986). It has been suggested that cypress canker originated in the United States, where the host is native (Swart, 1973), but the disease has been reported from Africa, Australia, New Zealand, Japan and Europe (Rudd Jones, 1953; Nattrass, Booth & Sutton, 1963; Swart, 1973; Raddi & Panconessi, 1981; Boesewinkel, 1983; Tabata, 1991; Tisserat, 1991). Hosts of these pathogens include species of *Cupressus*, *Chamaecyparis*, *Juniperus* and *Thuja* (Swart, 1973), as well as species of *Rhus*, *Vitis*, *Malus*, *Taxodium* and many more (Boesewinkel, 1983; Graniti, 1986; Tisserat, 1991).

The taxonomy of *Seiridium unicorne* (Cke & Ell.) Sutton, *S. cardinale* (Wagener) Sutton & Gibson and *S. cupressi* (Guba) Boesewinkel has been the subject of considerable debate, for many years. The number of species causing cypress canker, and the correct name to use for this species, or species, have been the subject of many debates (Guba, 1961; Swart, 1973; Boesewinkel, 1983; Graniti, 1986).

The first record of cypress canker reports the causal agent as *Pestalozzia unicornis* Cke & Ell. from diseased *Chamaecyparis thyoides* (L.) in North America in 1878 (Boesewinkel, 1983). Later, two other fungi, from Africa and New Zealand, were, however, also described as the cause of cypress canker, using the names of *Monochaetia unicornis* (Cke & Ell.) and *Coryneam cardinale* (Swart, 1973; Graniti, 1986). The species name "*cupressi*" first appeared in connection with cypress canker in 1961 (Guba, 1961).

The main criteria by which the three *Seiridium* spp. are separated include the orientation of the appendages and differences in the development of the appendages. Boesewinkel (1983) reported that the appendages of *S. unicorne* are formed at right angles to the

median septum, either endogenously or exogenously. Appendages of *S. cupressi* follow the curve of the conidia and may be formed either endogenously or exogenously. *Seiridium cardinale* lacks appendages, or when they are present, they are much shorter than those of the other two species. Chou (1989), confirmed previous reports (Swart, 1973), that the appendages in all species are formed only endogenously. Considerable differences in cultural appearance and geographic distribution were also noted between the three species (Boesewinkel, 1983). There have, however, been many arguments for the existence of only two species (Sutton, 1980; Chou, 1989), and also the view that only one morphologically variable species is associated with cypress canker (Swart, 1973; Viljoen, Wingfield & Wingfield, 1993; Roux, 1996).

During disease surveys of *Acacia mearnsii* de Wild in South Africa, a species of *Seiridium* was regularly isolated from stem cankers and wood of diseased trees (Roux & Wingfield, 1997). The aim of this study was to compare isolates of the *Seiridium* sp. from *A. mearnsii* with cypress isolates, based on sequence data of the rRNA operon and morphological comparisons. The pathogenicity of isolates from *A. mearnsii* and *Cupressus* spp. on *A. mearnsii* and *Cupressus lusitanica* was also compared.

MATERIALS AND METHODS

Isolates

Isolates used in these studies originated from diseased *A. mearnsii* trees, as well as from cankers on *Cupressus* spp. from New Zealand, Greece, Italy, Portugal and South Africa (Table 1). These isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Morphological comparisons

Isolates from *A. mearnsii* were grown on Potato Dextrose Agar (PDA) (39 g Difco PDA in 1000 ml distilled H_2O) at 25°C and the morphology was examined using a Zeiss Axioskop light microscope. Measurements (fifty of each characteristic structure) were made on mature conidia with 4 coloured median cells and intact apical and basal appendages only. These measurements were compared with characteristics of *Seiridium* spp. associated with cypress canker (Table 2) (Sutton, 1980; Boesewinkel, 1983; Graniti, 1986).

Growth rates were determined on PDA at 10, 15, 20, 25 and 30°C respectively. The PDA plates were inoculated with 4 mm discs removed from the margins of actively growing colonies and placed, face down, at the center of the plates. Colony diameters were measured after 10 days. A total of 5 plates were incubated for each isolate at each temperature. Two diameter measurements were obtained from each colony, perpendicular to each other. A total of 10 measurements were thus taken at each temperature for every isolate studied, and the mean growth computed.

DNA isolation and amplification

Cultures used for DNA sequence comparisons included 6 isolates from cypress canker and 6 isolates from *A. mearnsii* (Table 1). Isolates were grown in liquid culture of 20 ml 2% MEA (Biolab Malt Extract Agar) in 50 ml Erlenmeyer flasks on shaking incubators at 25°C for 10 days. The mycelium was collected, by removing the MEA, and then lyophilized. DNA was isolated using the technique of Raeder & Broda (1985) with some amendments. Small pieces of lyophilized mycelium were transferred to sterile 1.5 ml Eppendorf tubes and 100 µl of Extraction buffer (200 mM Tris-HCL, pH 8.5; 250 mM NaCl; 25 mM EDTA; 0. 5% SDS) added. The tubes were emersed in liquid nitrogen and the mycelium ground into a fine powder with a mini pestle. Another 400 µl of extraction buffer was then added and the mixture was incubated in a water bath at 65°C for 5 min.

The aqueous phase was collected after centrifugation and phenol/chloroform extractions were performed until the interface was completely clean. The aqueous phase was further extracted with chloroform and the DNA precipitated with sodium acetate (3 M) and 0.45 vol. isopropanol at -20°C. The pellets were rinsed with 70% ethanol, dried and the DNA resuspended in 100 μl water.

The internal transcribed spacer regions of the 5.8S gene of the ribosomal RNA operon (rRNA) were amplified using the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988). Primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') were used for the amplification reactions (White *et al.* 1990). PCR reactions were carried out on a Omnigene Temperature Cycler (Hybaid, Middlesex, UK). An initial denaturation step was performed at 96°C for 5 min, after which the temperature was lowered to 90 °C until the *Taq* polymerase (Boehringer Mannheim, South Africa) was added. Primer annealing was done at 55°C for 30 sec, chain elongation at 72°C for 1 min and denaturation at 92°C for 1 min. These steps were repeated 35 times. Final chain elongation took place at 72 °C for 5 min, followed by 2 min at 37°C. All PCR products were visualized under UV light on a 1% agarose gel stained with ethidium bromide. The amplified DNA fragments were purified using the Magic PCR Preps Purification System (Promega Corporation, Madison, USA).

DNA sequencing

Purified PCR products were sequenced in both directions using an ABI PRISM™ 377 Autosequencer (Perkin-Elmer). Sequence reactions were carried out with an ABI PRISM™ Dye Terminator Cycle Sequencing Kit with Amplitaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, U.K.). The sequences obtained were aligned manually by the insertion of gaps.

The DNA sequence data were analyzed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1985) and DNA BOOTSTRAP analysis (bootstrap confidence

intervals on DNA parsimony) (Felsenstein, 1988). The heuristic search option of PAUP, with no branch swopping, was used to find the most parsimonious tree. DNA sequence data for *Pestalotiopsis maculans* (Corda) Nag Raj (CBS 322.76) was used as outgroup, since the genus *Pestalotiopsis* belongs to the same sub-order as *Seiridium* (Blastostromatineae), with pigmented conidia produced in acervuli (Sutton, 1980). Sequence data were also analyzed using Unweighted Pair-Group Mean Arithmetic Analysis (UPGMA) to confirm PAUP results (PHYLLIP Version 3.572C) (Felsenstein, 1993). Distances between isolates were calculated using DNADIST and UPGMA was used to analyze the distance matrix and generate a dendogram. Data obtained in this study was also compared with data from a previous study in which *Seiridium* species from *Curpressaseae* were considered (Viljoen *et al.*, 1993). This was done to compare the *A. mearnsii* isolates with more isolates from *Cupressus* hosts.

Pathogenicity trials

To compare the pathogenicity of *Seiridium* isolates from *Cupressus* and *A. mearnsii*, *C. lusitanica* and *A. mearnsii* trees were inoculated with 3 test isolates. Isolates CMW4152 and CMW4149 from *A. mearnsii* and CMW4723 from *C. lusitanica* were used in the pathogenicity trials.

During January 1998, twenty 18-month-old *A. mearnsii* trees were inoculated with each isolate. The trial was established on the Bloemendal Experimental Farm, Pietermaritzburg, South Africa (29° 32. 93S; 30° 27. 33E). Isolates were grown on 2% potato dextrose agar (PDA) (Difco) at 25°C for 2 weeks. Mycelial plugs, 9 mm in diameter, were removed from each culture and inoculated into the trees by removing a piece of bark of equal size, so that the fungus came into contact with the cambium. Twenty trees were inoculated with sterile agar to serve as controls. Each wound was sealed with masking tape to prevent desiccation of the inoculum and wound. Lesion lengths were measured after 6 weeks. The trial was repeated during February 1998.

Side branches, approximately 2 to 3 cm in diameter, of 10 *C. lusitanica* trees were inoculated with the same isolates during January 1998. The same inoculation procedure as was used for inoculating the *A. mearnsii* trees was used. For each isolate, 2 side branches were inoculated per tree. Control inoculations with sterile agar were included on 1 branch of each tree. Lesion lengths were measured after 8 weeks, by removing the outer layer of bark and measuring the size of lesions in the cambium. Data obtained from the pathogenicity trials were statistically analyzed for differences between isolates using Tukey's Studentized range ($P=0.05$).

RESULTS

Morphological comparisons

Isolates of the *Seiridium* sp. from *A. mearnsii* have conidia with appendages most commonly arranged at an angle following the curve of the conidium, or arranged perpendicular to the median septum (Fig. 1). Conidial sizes of the *A. mearnsii* isolates ranged from 23 - 40 µm in length and 4 - 10 µm in width. This makes them similar in size, and indistinguishable from *S. unicorne* isolates associated with Cypress canker. Considerable variation was, however, found in the appendage morphology of the isolates examined, with conidia from the same acervuli having appendages oriented perpendicular to the median septum, or following the curve of the conidium (Fig. 2). Within these acervuli, conidia with very short appendages (± 1-2 µm) were also found.

In growth studies, most isolates from *A. mearnsii* and from *Cupressus* spp. had growth optima at 25°C. The exceptions were CMW3904 and CMW2092 at 15°C and CMW4251 at 20°C. *Acacia* isolates grew poorly, or did not grow, at 30°C, while all the *Cupressus* isolates grew at this temperature (Table 3).

DNA Sequence analysis

DNA amplification resulted in a single DNA fragment. After alignment and insertion of gaps, 402 base pairs were used in further analysis (Fig. 4). The Heuristic search option was used in PAUP to produce a single tree. Values for the consistency index (CI), homoplasy index (HI) and retention index (RI) were 0.834, 0.61 and 0.412 respectively (Fig. 5). No differences were found between the three authenticated *Seiridium* spp. or between the *A. mearnsii* isolates and those from *Cupressus* spp. (Fig. 5). With the exception of isolates CMW4151 and JP1758, the *A. mearnsii* isolates formed a sub-clade on their own. Isolate JP1758, from *A. mearnsii*, grouped with isolate CMW2109 from *Cupressus*.

The bootstrap analysis showed that the *Seiridium* isolates group together with a bootstrap value of 100%. Comparison of the sequences obtained from this study, with published sequences of Viljoen *et al.* (1993), confirmed that the *A. mearnsii* isolates group with the isolates of *Seiridium* spp. from *Cupressus*. The *A. mearnsii* isolates grouped in the same clade as *S. cardinale* from Italy and *S. cupressi* from Greece.

When data were analyzed using UPGMA, isolates clustered in two major clades (Fig. 6). There were no obvious differences between isolates from *A. mearnsii* and *Cupressus*. *Seiridium cardinale* from Italy (CMW1644) formed a clade together with isolate CMW4150 from *A. mearnsii*. All other isolates formed one larger and well resolved clade.

Pathogenicity trials

Lesions were produced on both *A. mearnsii* and *C. lusitanica*, by both the *Acacia* and *Cupressus* isolates (Table 4, Fig. 3). On *A. mearnsii*, lesions produced by the two *Acacia* isolates differed significantly from those of the *Cupressus* isolate in the January 1998 inoculations (*P=0.05*) (Table 4). Isolates CMW4149 and CMW4152, from *A. mearnsii*,

produced larger lesions than isolate CMW4723 from *Cupressus*. In the February 1998 inoculations, lesions produced by CMW4149 from *A. mearnsii,* again differed significantly from that of CMW4723 from *C. lusitanica*. There were, however, no significant difference between isolates CMW4723 and CMW4152. All lesions associated with *Seiridium* isolates differed significantly from those of control inoculations. Lesions produced by all isolates tested, were also much larger on *C. lusitanica* than on *A. mearnsii*.

DISCUSSION

In this study, evidence is provided to support the view that the *Seiridium* sp. from *A. mearnsii* is the same as the species, or species complex, causing cypress canker. Results also support the view that only one species of *Seiridium*, with morphological variation, causes cypress canker (Swart, 1973; Viljoen *et al.*, 1993). This study has further shown that these *Seiridium* spp. all have the ability to cause lesions on both *A. mearnsii* and *C. lusitanica,* when inoculated into these trees.

Seiridium unicorne is reported to have a wide host range beyond the *Cupressaceae*, including *Rhus, Tamarix, Pyrus, Malus* and others (Guba, 1961; Boesewinkel, 1983; Graniti, 1986). It is, therefore, not surprising that this species was isolated from *A. mearnsii*. In contrast, *S. cardinale,* has only been found in association with Cypress canker (Boesewinkel, 1983; Graniti, 1986). This, together with the morphological differences, has been used to support the view that these fungi represent distinct species. The findings of Viljoen *et al.* (1993), that *S. cardinale, S. unicorne* and *S. cupressi,* probably represent morphological variants of a single species, were, therefore, somewhat inconsistent with published morphological, cultural and geographical data. In this study, we have shown that based on sequence data, it is not possible to distinguish isolates of the *Seiridium* sp. from *A. mearnsii*, which in its most common morphological manifestation, is identical to *S. unicorne*. The wide host range that has previously been reported for *S.*

unicorne also supports our view that the fungus is the same as the one that we commonly isolate from *A. mearnsii*.

S. cardinale is known to occur only on *Cupressus* (Boesewinkel, 1983; Graniti, 1986) and its spores are generally, but not always, free of appendages (Sutton & Gibson, 1972; Boesewinkel, 1983). This suggests that the fungus is different to other *Seiridium* spp. on *Cupressus*. The fact that our sequence data suggest that it is the same as *S. unicorne* and the *Seiridium* sp. from *A. mearnsii*, is enigmatic.

The use of appendage orientation as a taxonomic character appears to be unreliable. It has been reported by Viljoen *et al.* (1993) and Roux (1996) that conidia with differing appendage orientation could be found within the same acervuli. Similar findings have been made for the genus *Seimatosporium* Cda., where conidia without appendages are often found amongst those with appendages (Swart, 1973). This may explain the presence of non-appendaged conidia in *S. cardinale,* and supports the view of Swart (1973) that *S. cardinale*, *S. cupressi* and *S. unicorne* represent a single, morphologically variable species.

From pathogenicity tests, it was shown that the *Seiridium* spp. considered in this study are capable of causing lesions of similar magnitude on *A. mearnsii* and on *Cupressus lusitanica*. These data are reasonably unequivocal for inoculations on *A. mearnsii*, where sufficient trees were available to allow a reasonable number of replication and repetition of the trial. The smaller number of inoculations on *Cupressus* arose due to the fact that these trees are valuable ornamentals in South Africa and not commonly available for inoculations. Results should be viewed with a knowledge of this constraint. However, the fact that both the *Acacia* and *Cupressus* isolates of *Seiridium* produced lesions on both *A. mearnsii* and *C. lusitanica* supports molecular and morphological data, suggesting that the *Seiridium* from *A. mearnsii* belongs to the same, or a very similar taxon as the fungus causing cypress canker. The fact that the *Acacia* isolates produced larger lesions on *A. mearnsii* than did the *Cupressus* isolate, suggests that adaptation to their new host has occurred. It is possible that these isolates might have spread to *A. mearnsii* from

Cupressus or visa versa, which would account for the fact that lesions were also formed on *C. lusitanica*.

Molecular techniques, if used correctly, can be valuable in clarifying difficulties in taxonomy (Crawfordt *et al.*, 1996; Wingfield *et al.*, 1996). Direct sequencing of target areas of DNA, using PCR have proven especially useful. This technique has a high level of resolution and allows the sequencing of both strands of DNA (Bruns, White & Taylor, 1991). The ribosomal RNA operon is an extremely useful source of genetic data for taxonomic comparisons (Blanz & Unseld, 1986; Kurtzman, 1992). The internally transcribed spacer region (ITS) of the rRNA operon is variable and can be used at low taxonomic levels such as to distinguish different species of fungi (Chambers, Dutta & Crouch, 1986; Bruns *et al.*, 1991; Hibbet, 1992; Mitchell, Roberts & Moss, 1995; Wingfield *et al.*, 1996; Witthuhn *et al.*, 1998).

The fact that sequence data fails to separate species of *Seiridium* that have minor, yet obvious, morphological differences, or different hosts, might suggest that these fungi are very similar, but that they have speciated recently. This would be similar to the situation with *Ceratocystis laricicola* Redfurn & Minter and *C. polonica* (Siemaszko) Moreau, which are morphologically identical fungi that occur on different hosts and have different insect vectors (Harrington *et al.*, 1996). They cannot be separated based on analysis of ITS sequence data (Witthuhn *et al.*, 1998), but differ at one isozyme locus (Harrington *et al.*, 1996). Comparison of *Seiridium* spp. considered in this study, using a range of techniques, including sequences of other parts of the genome, should provide even more insight into the evolution of this group of pathogens.

For the present, all evidence suggests that the fungus from *A. mearnsii* and those causing canker of *Cupressus* spp. are the same. These results reiterate the conclusions of Viljoen *et al.* (1993), that *S. cardinale*, *S. cupressi* and *S. unicorne* represent one phylogenetic entity. We, therefore, suggest that the *Seiridium* sp. from *A. mearnsii* be known as *S. cardinale*, since this species name was first used for the cypress canker causing fungus in

the genus *Seiridium*. It is clear that morphological characteristics used for differentiating species in *Seiridium* deserve re-evaluation. If these species have recently undergone speciation, the degree of morphological variation, if any, that is sufficient to define taxa must be determined.

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Table 1. Fungal isolates used in the comparison of the *Seiridium* from *A. mearnsii* with *Seiridium* spp. associated with cypress canker.

SPECIES	CULTURE NUMBER[a]	HOST	ORIGIN
Seiridium unicorne	CMW2109	*C. horizontalis*	South Africa
"	CMW1648	"	Portugal
"	CMW1502	*C. glabra*	South Africa
"	CMW806	*C. lusitanica*	"
"	CMW692	"	New Zealand
S. cardinale	CMW1644	"	Italy
"	CMW2092	*C. horizontalis*	South Africa
"	CMW2133	*Cupressus* sp.	Chile
"	CMW690	*Cupressus* sp.	South Africa
Lepteutypa cupressi	CMW1646		Greece
Seiridium sp.	CMW4148	*Acacia mearnsii*	South Africa
"	CMW4149	"	"
"	CMW4150	"	"
"	CMW4151	"	"
"	CMW4152	"	"
"	CMW4153	"	"
"	CMW4154	"	"
"	CMW4155	"	"
"	CMW4157	"	"
"	CMW4159	"	"
"	JP1758	"	"
"	CMW3904	"	"
"	CMW4723	*Cupressus lusitanica*	"
Pestalotiopsis maculans	CBS322.76	*Camelia* sp.	France

[a] Isolates are maintained in the culture collection of the Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Table 2: Comparison of important morphological characteristics of *Seiridium cardinale*, *S. unicorne*, *S. cupressi* and a *Seiridium* sp. from *A. mearnsii*.

FUNGUS	MORPHOLOGICAL CHARACTERISTICS[a]					SOURCE OF DATA
	CONIDIUM LENGTH	CONIDIUM WIDTH	MEDIAN CELLS	APICAL APPENDAGE	BASAL APPENDAGE	
S. cardinale	21 - 30	8 - 9	-[b]	1	(1)	Sutton, 1980
S. unicorne	24 - 28	8 - 9	18 - 22	11 - 19	8 - 13	Sutton, 1980
S. unicorne	(22-)25 - 27(31)	7 - 10	17 - 23	5 - 12	5 - 12	Boesewinkel, 1983
S. cardinale	(21-)24 - 30(-31)	8 - 10	-[b]	-[b]	-[b]	Boesewinkel, 1983
S. cupressi	(25-)27 - 32(-37)	7 - 10	19 - 27	-[b]	-[b]	Boesewinkel, 1983
S. cardinale	21 - 30	8 - 9	17 - 21	-[b]	-[b]	Sutton & Gibson, 1972
Seiridium sp. CMW4150	26 - 34 (30)	6 - 7 (6.2)	17 - 24 (21)	4 - 9 (7)	4 - 10 (5)	This study
Seiridium sp. CMW3904	23 - 30 (27)	6 - 9 (8)	16 - 22 (20)	3 - 12 (8)	3 - 11 (7)	"
Seiridium sp. CMW4155	24 - 38 (29)	6 - 7 (7)	17 - 24 (20)	4 - 12 (7)	3 - 12 (6)	"
Seiridium sp. CMW3913	24 - 35 (31)	6 - 7 (6)	17 - 26 (23)	4 - 10 (8)	4 - 9 (7)	"
Seiridium sp. CMW4159	24 - 31 (27)	4 - 9 (7)	17 - 23 (20)	4 - 13 (8)	3 - 9 (5)	"
Seiridium sp. JP 1758	29 - 40 (33)	7 - 10 (8)	20 - 28 (24)	4 - 10 (8)	3 - 8 (6)	"

[a] All measurements are in µm

[b] Values not published

Table 3. Growth of *S. cardinale* (CMW609, CMW2092), and *S. unicorne* (CMW806, CMW1502, CMW2133) isolates from *Cupressus* hosts, compared with those of *Seiridium* isolates from *A. mearnsii.*

ISOLATES[a]	TRIAL 1 [b,c]				TRIAL 2 [b,c]			
	15°C	20°C	25°C	30°C	15°C	20°C	25°C	30°C
CMW4150	**25.97 bc**	**29.39 bc**	**27.00 de**	**12.51 d**	**21.41 bc**	**28.00 bc**	**31.33 c**	**0 d**
CMW3904	**23.72 c**	**23.39 c**	**21.79 f**	**7.11 f**	**22.70 b**	**22.21 c**	**20.01 e**	**0 d**
CMW4155	**8.93 d**	**11.41 d**	**14.84 g**	**7.00 f**	**0 f**	**12.73 d**	**20.70 de**	**0 d**
CMW4151	**26.38 ab**	**31.22 ab**	**32.81 c**	**0 g**	**23.81 ab**	**30.63 ab**	**34.38 bc**	**0 d**
CMW4157	**13.52 d**	**13.45 d**	**13.88 g**	**0 g**	**8.25 e**	**12.20 d**	**16.96 f**	**0 d**
CMW4152	**22.40 c**	**23.52 c**	**20.95 f**	**7.72 f**	**18.87 c**	**23.60 c**	**20.32 de**	**0 d**
JP1758	**15.59 c**	**24.24 c**	**29.02 d**	**0 g**	**19.91 c**	**29.10 ab**	**31.44 c**	**0 d**
CMW4153	**26.40 ab**	**32.49 ab**	**25.32 e**	**10.32 e**	**24.72 ab**	**28.61 ab**	**32.36 c**	**0 d**
CMW4154	**26.51 abc**	**29.83 abc**	**27.38 de**	**0 g**	**25.82 a**	**31.12 ab**	**36.73 b**	**0 d**
CMW690	26.89 **a**	33.41 **a**	40.30 **a**	28.52 **b**	25.91 **a**	32.97 **a**	52.86 **a**	10.65 **c**
CMW806	16.54 **ab**	30.07 **ab**	36.28 **b**	36.89 **a**	11.89 **d**	28.21 **b**	31.15 **c**	29.35 **a**
CMW1502	10.76 **d**	11.39 **d**	12.21 **g**	10.90 **e**	6.96 **e**	10.51 **d**	10.93 **g**	14.15 **b**
CMW2092	27.36 **bc**	26.43 **bc**	23.44 **ef**	18.67 **c**	26.09 **a**	24.53 **bc**	22.81 **d**	9.66 **c**
CMW2133	9.32 **d**	13.01 **d**	13.85 **g**	13.37 **d**	0 **f**	12.83 **d**	13.51 **g**	9.49 **c**

[a] Growth was measured after incubating cultures for 10 days in the dark. Each bar represents the mean of 20 measurements. Isolates from *A. mearnsii* are in bold.

[b] Each value represents an average of 10 measurements.

[c] Each value with a different letter differs significantly from the others for that specific temperature range.

Table 4: Lesion lengths associated with inoculations using *Seiridium* isolates from *A. mearnsii* and *Cupressus* spp.

ISOLATE	HOST	LESION LENGTH		
		C. lusitanica [a, b]	***A. mearnsii*** [b, c] **January 1998**	***A. mearnsii*** [b, d] **February 1998**
CMW4723	*C. lusitanica*	33.1a	14.1c	18.95b
CMW4152	*A. mearnsii*	31.2a	19.75b	17.4b
CMW4149	*A. mearnsii*	31.2a	22.2a	22.55a
CONTROL		17.0b	10.0d	10c

[a] Each value is an average of 17 measurements. CV= 26.58%.

[b] Values followed by different letters differ significantly at $P=0.05$.

[c] Each value is an average of 20 measurements. CV= 16.5%.

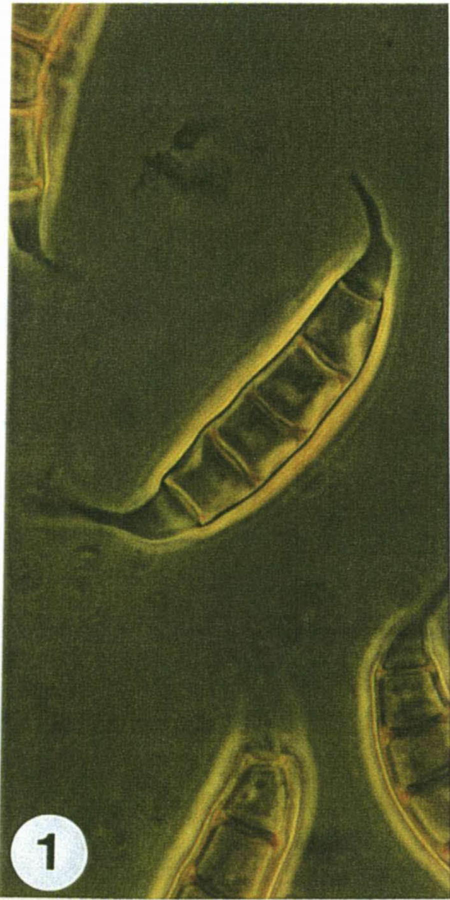
[d] Each value is an average of 20 measurements. CV=17.2%.

Figures 1, 2: Conidial morphology of *Seiridium* isolates from *A. mearnsii*.

Fig. 1: Conidium with appendages at a right angle to the median septum.

Fig. 2: Conidium with appendages following the curve of the conidium.

Figure 3: Lesions produced on *C. lusitanica* after inoculation with *Seiridium* spp. from *C. lusitanica* (A) and *A. mearnsii* (B). The third branch represents the control inoculation.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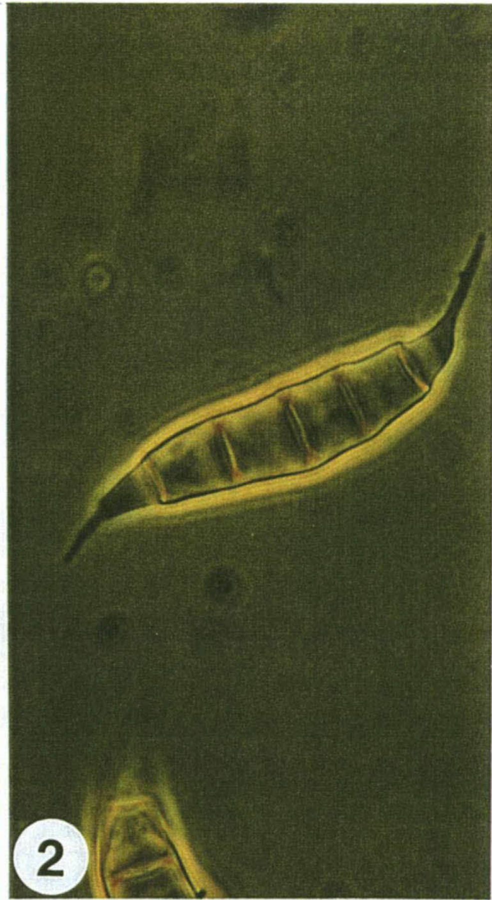
2

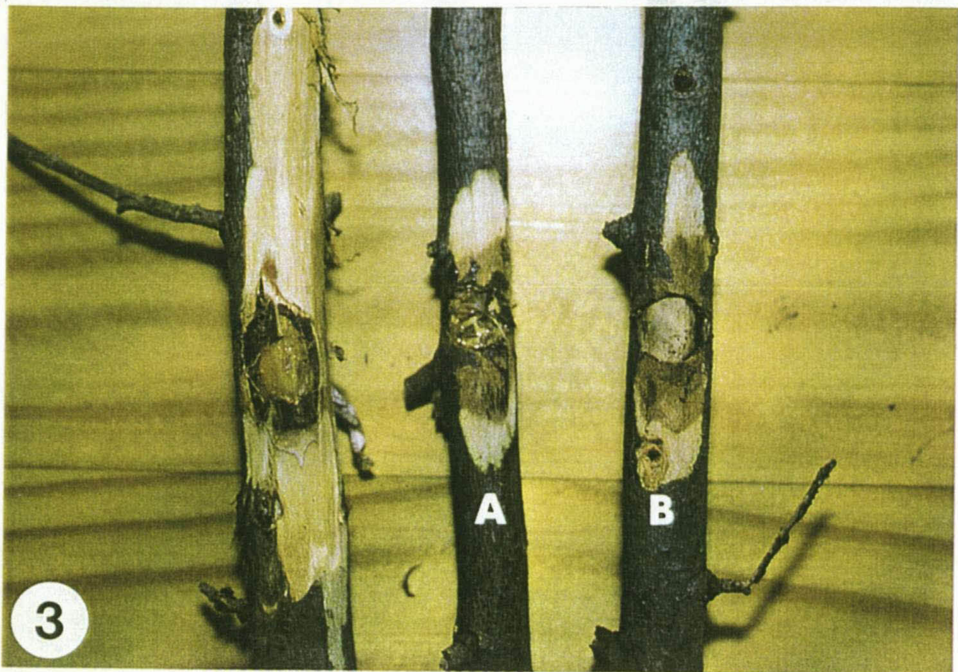
A
B
3

Figure 4: Alignment of 402 bases of the ITS regions of the ribosomal RNA operon for 6 *Seiridium* isolates associated with cypress canker and six isolates from *A. mearnsii*. N indicates an unknown base and gaps (-) indicate spaces necessary for the alignment of the sequence. Dots indicate bases identical to the corresponding base in the *P. maculans* isolate.

```
                                10         20         30         40         50         60         70         80

A.MEARNSII(4150)        ---------- ------AAAA -GCTACCCTG TACCT-ACCT GG-AAACAGC CTACCTGGAA GCNATCCGGG CTGGCCTACC
A.MEARNSII(4149)        TTTGTTGCCT CG-GC-.G.. A......... .....-.... ..-....... ........-. ..G....... ..........
A.MEARNSII(4151)        TTTGTTGCCT CG-GC-.G.G A..C....-. .....-..T. ..-....... .......... -.G...T... ..........
A.MEARNSII(4152)        -TTG---CCT CG-GC-.G.. -......... .....-.... ..-....... .......... ..G....... ..........
A.MEARNSII(4148)        TTTGTTGCCT CG-GC-.GG. -......... .....-.... ..-..-.... ........-. ..G....... ..........
A.MEARNSII(1758)        TTTGTTGCCT CG-GC-.GG. -......... .....-.... ..-....... ........G. ..G.....A. ..........
SEIRIDIUM(4723)         ---------- ----C-.G.. -......-.. .....-.... ..-..-.... ........-. ..G..T.... .....T....
S.UNICORNE-LINCOLN      TTTGTTGCCT CGAGC-.G.. -....A.... .....-.... ..-..-.... ........-. ..G....... ..........
S.CARDINALE-ITALY       TTTGTTGCCT CG-GC-.G.. --......-. .T..-..... ..-..-.... ........-. ..G......- ..........
L.CUPRESSI-GREECE       TTTGTTGCCT CGN-CG.G.. --......-. .T..-..... ..-..-.... ........-. ..G......- ..........
S.UNICORNE-PORTUGAL     TTTGTTGCCT C-AGC-.G.. -......-.. .....-.... ..-....... ........-. ..G....... ..........
S.UNICORNE-SA           TTTGTTGCCT CG-GC-.G.. -......-.. .....-.... ..-..-.... ........-. ..G....... ..........
P.MACULANS              ATTGTTGCCT CG-GC-.... A-......G. .....T.... T.G....G.. .....CT.T. ..G--..--- -----T....

                                90        100        110        120        130        140        150        160

A.MEARNSII(4150)        -TGGAAACGG TCTGG-TGGT CGA----CTG CCGGTGGACC ATTCAACTCT TGTTATTTTA TTGTAATCTG AGCGTCTTAT
A.MEARNSII(4149)        -......... .....-.... ...----... .......... .......... .......... .......... ..........
A.MEARNSII(4151)        -......... .....-.... ...----... .......... .......... .......A.. .......... ..........
A.MEARNSII(4152)        -.....-... .....-.... ...----... .......... .......... .......... .......... ..........
A.MEARNSII(4148)        -......... .....-.... ...----... .......... .......... .......... .......... ..........
A.MEARNSII(1758)        -......... ...C.G.... ...A---... .......... .......... .......... .......... ..........
SEIRIDIUM(4723)         -.....-... .....-.... ...----... .......... .......... .......... .......... ..........
S.UNICORNE-LINCOLN      -.....-... .....-.... ...----... .......... .......... .......... .......... ..........
S.CARDINALE-ITALY       -.....-... .....-.... ...----... .......... .......... .......... .......... ..........
L.CUPRESSI-GREECE       -......... .....-.... ...----... .......... .......... .......... .......... ..........
S.UNICORNE-PORTUGAL     -.....-... .....-.... ...----... .......... .......... .......... .......... ..........
S.UNICORNE-SA           -.....-... .....-.... ...----... .......... .......... .......... .......... ..........
P.MACULANS              C.....-... CT.ACCCT.. --.ACGG... .........T .CCA...... .......... ....T..... ..........
```

```
                              170        180        190        200        210        220        230        240

A.MEARNSII(4150)     TTTAATAAGT CAAAACTTTC AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAAAA-CGC AGCGAAATGC GATAAGTAAT
A.MEARNSII(4149)     .......... .......... .......... .......... .......... ...G..-... .......... ..........
A.MEARNSII(4151)     .......... .......... .......... .......... .......... ......-... .......... ..........
A.MEARNSII(4152)     .......... .......... .......... .......... .......... ...G..-... .......... ..........
A.MEARNSII(4148)     .......... .......... .......... .......... .......... ...G..-... .......... ..........
A.MEARNSII(1758)     .......... .......... .......... .......... ....N..... ...G..-... .......... ..........
SEIRIDIUM(4723)      .......... .......... .......... .......... .......... ...G..-... .......... ..........
S.UNICORNE-LINCOLN   .......... .......... .......... .......... .......... ...G..-... .......... ..........
S.CARDINALE-ITALY    .......... .......... .......... .......... .......... ......-... .......... ..........
L.CUPRESSI-GREECE    .......... .......... .......... .......... .......... ...G..-... .......... ..........
S.UNICORNE-PORTUGAL  .......... .......... .......... .......... .......... ......A... .......... ..........
S.UNICORNE-SA        .......... .......... .......... .......... .......... ...G..-... .......... ..........
P.MACULANS           .......... .......... .......... .......... .......... -.....A... .......... ..........

                              250        260        270        280        290        300        310        320

A.MEARNSII(4150)     GTGAATTGCA GAATTCAGTG AATCATCGAA TCTTTGAACG CACATTGCGC CCATTAGTAT TCTAGTGGGC ATGCCTGTTC
A.MEARNSII(4149)     .......... .......... .......... .......... .......... .......... .......... ..........
A.MEARNSII(4151)     .......... .......... .......... .......... .......... .......... .......... ..........
A.MEARNSII(4152)     .......... A......... .......... .......... .......... .......... .......... ..........
A.MEARNSII(4148)     .......... A......... .......... .......... .......... .......... .......... ..........
A.MEARNSII(1758)     ..T....... .......... .......... .......... .......... .......... .......... ..........
SEIRIDIUM(4723)      .......... .......... .......... .......... .......... .......... .......... ..........
S.UNICORNE-LINCOLN   .......... .......... .......... .......... .......... .......... .......... ..........
S.CARDINALE-ITALY    .......... .......... .......... .......... .......... .......... .......... ..........
L.CUPRESSI-GREECE    .......... .......... .......... .......... .......... .......... .......... ..........
S.UNICORNE-PORTUGAL  .......... .......... .......... .......... .......... .......... .......... ..........
S.UNICORNE-SA        ..T....... .......... .......... .......... .......... .......... .......... ..........
P.MACULANS           .......... A......... .......... .......... .......... .......... .......... ..........
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```
                                330        340        350        360        370        380        390        400

A.MEARNSII(4150)      GAGCGTCATT TCAACCCTTA AGCCTAGCTT AGTATTGGGA ATCTACTGTA TTG--TAG-- ----TTCCTC AAATCCAACG
A.MEARNSII(4149)      .......... .......... .......... .......... .......... ...--...-- ----...... ..........
A.MEARNSII(4151)      .......... .......... .......... .......... G......... C..--...-- ----...... ..........
A.MEARNSII(4152)      .......... .......... .......... .......... .......... ...--...-- ----...... ..........
A.MEARNSII(4148)      ..A....... .......... .......... .......... -......... ...--...-- ----...... ..........
A.MEARNSII(1758)      .......... .......... .......... .......... G......... C..--...-- ----...... N.........
SEIRIDIUM(4723)       .......... .......... .......... .......... GC........ ...--...-- ----...... ..........
S.UNICORNE-LINCOLN    .......... .......... .......... .......... G......... ...--...-- ----...... ..........
S.CARDINALE-ITALY     ..A....... .......... .......... .......... G......... ...G-...-- ----...... ..........
L.CUPRESSI-GREECE     ..N....... .......... .......... .......... G......... ...--...-- ----...... ..........
S.UNICORNE-PORTUGAL   .......... .......... .......... .......... G......... ...T-...-- ----...... ..........
S.UNICORNE-SA         ..A....... .......... ......-... .N........ N......... ...TN----- ----...... C.........
P.MACULANS            .......... .......... .......... ...G...... GC......CT ..TGC...CT GTAGC....G ....A.....


A.MEARNSII(4150)      GC
A.MEARNSII(4149)      ..
A.MEARNSII(4151)      ..
A.MEARNSII(4152)      ..
A.MEARNSII(4148)      ..
A.MEARNSII(1758)      ..
SEIRIDIUM(4723)       ..
S.UNICORNE-LINCOLN    ..
S.CARDINALE-ITALY     ..
L.CUPRESSI-GREECE     .G
S.UNICORNE-PORTUGAL   ..
S.UNICORNE-SA         ..
P.MACULANS            ..
```

Figure 5: Phylogram produced using the Heuristic search option of PAUP. Midpoint rooting was used. Bootstrap values are expressed as % confidence intervals.

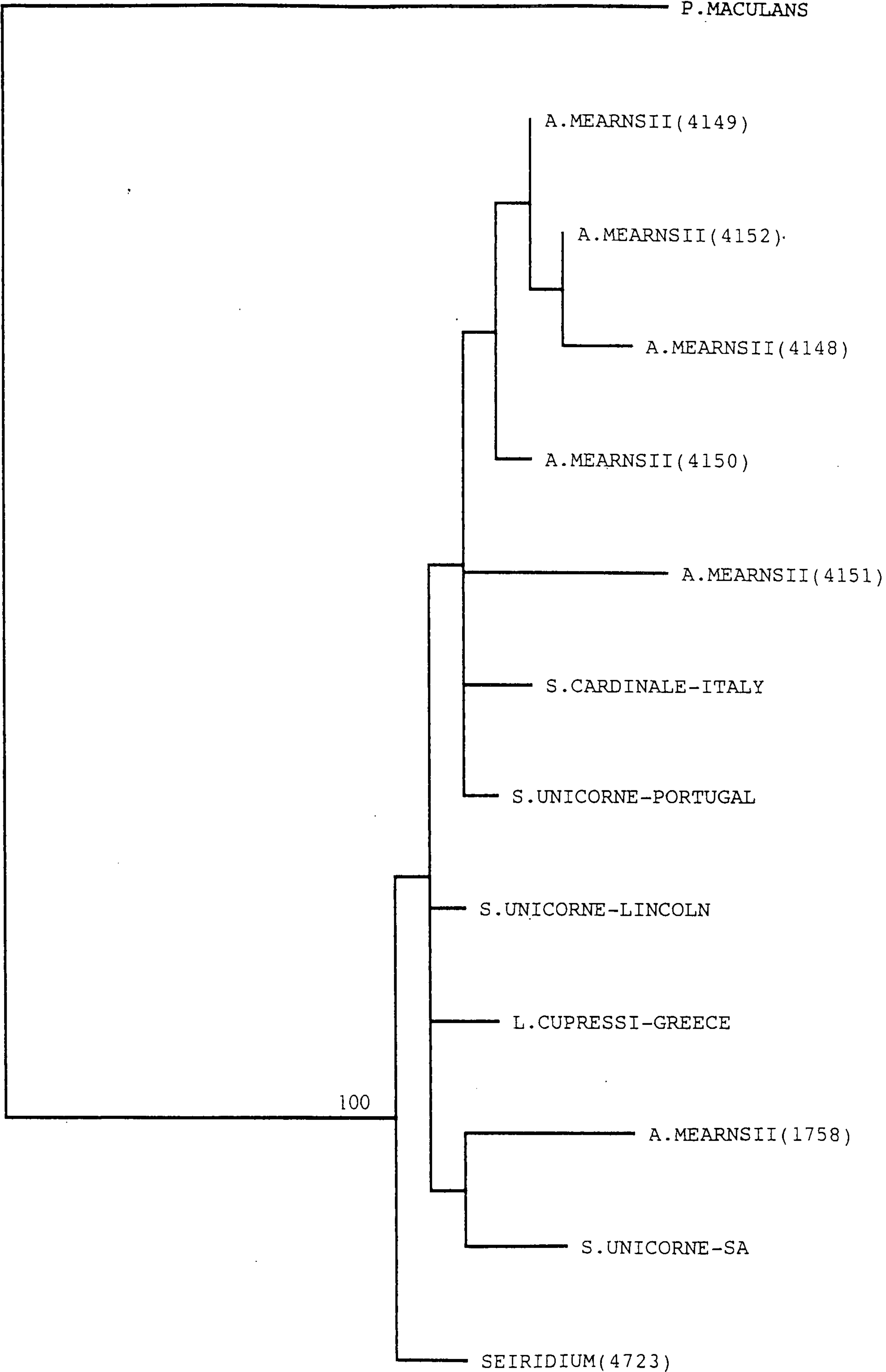
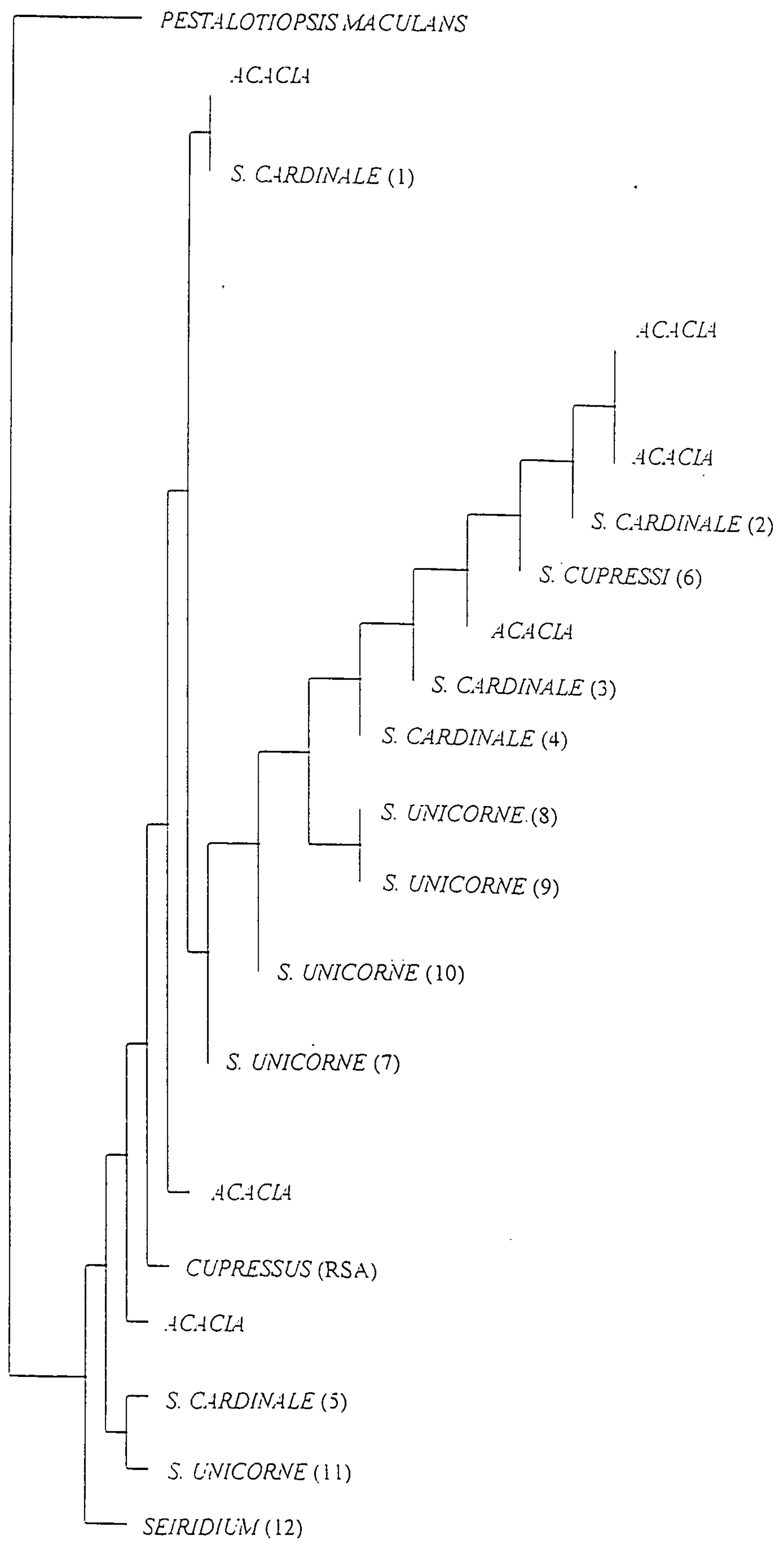

P.MACULANS
A.MEARNSII(4149)
A.MEARNSII(4152)
A.MEARNSII(4148)
A.MEARNSII(4150)
A.MEARNSII(4151)
S.CARDINALE-ITALY
S.UNICORNE-PORTUGAL
S.UNICORNE-LINCOLN
L.CUPRESSI-GREECE
100
A.MEARNSII(1758)
S.UNICORNE-SA
SEIRIDIUM(4723)

Figure 6: Dendogram generated from UPGMA analysis of the ITS region of the ribosomal RNA operon. *Seiridium cardinale* (1) – Italy; *S. cardinale* (2) – New Zealand; *S. cardinale* (3, 4, 5, 9) - South Africa; *S. cupressi* (6) – Greece; *S. unicorne* (7) – Portugal; *S. unicorne (8)* – New Zealand; *S. unicorne* (9) - South Africa; *S. unicorne* (10) – Lesotho; *S. unicorne* (11) – South Africa; *Seiridium* sp. (12) – South Africa, *Cupressus* (RSA) - *C. lusitanica*, South Africa. Three digit numbers refers to CMW numbers (Table 1).


PESTALOTIOPSIS MACULANS
ACACIA
S. CARDINALE (1)
ACACIA
ACACIA
S. CARDINALE (2)
S. CUPRESSI (6)
ACACIA
S. CARDINALE (3)
S. CARDINALE (4)
S. UNICORNE (8)
S. UNICORNE (9)
S. UNICORNE (10)
S. UNICORNE (7)
ACACIA
CUPRESSUS (RSA)
ACACIA
S. CARDINALE (5)
S. UNICORNE (11)
SEIRIDIUM (12)

CHAPTER 6

ENDOPHYTIC FUNGI ASSOCIATED WITH ACACIA MEARNSII IN SOUTH AFRICA

6

ENDOPHYTIC FUNGI ASSOCIATED WITH *ACACIA MEARNSII* IN SOUTH AFRICA

ABSTRACT

Many pathogens of plants can exist as latent or endophytic infections within their host, without any external symptom or sign of disease. Under certain conditions, such as drought or mechanical damage, these organisms are, however, activated, spreading through the entire host, leading to disease and death. To determine which fungi can occur as endophytes of the economically important *Acacia mearnsii* in South Africa, a study was undertaken to identify pathogens with the ability to live as symptomless endophytes in this tree species. Isolations were made from the leaves and stems of healthy trees in plantations in the KwaZulu-Natal Province. Both summer and winter collections were made. The plant material was surface sterilized and isolations made on agar amended with streptomycin. Thirty genera of fungi were isolated. The most abundantly isolated endophytes were species of *Fusarium, Nigrospora, Nodulisporium, Pestalotiopsis* and *Xylaria*. A number of potential pathogens of *A. mearnsii* were also isolated. These included *Botryosphaeria dothidea, Cylindrocladium candelabrum, Diplodia* spp., *Fusarium graminearum* and *Lasiodiplodia theobromae*. No obvious differences in endophytic assemblages were found between winter and summer sampling. Further studies are planned to determine the relative importance of these fungi in the health of *A. mearnsii* trees.

INTRODUCTION

Interest in fungal endophytes of vascular plants has increased considerably in recent years and fungal endophytes have been found in all plants that have been investigated. Varying opinions exist as to what the correct definition of an endophyte is (Wenström, 1994). These could be all organisms capable of colonizing internal plant tissues (Wilsòn, 1995), but it was also suggested that they should include only non-pathogenic organisms, thus excluding latent pathogens (Petrini, 1991). As knowledge of endophytes has increased, the definition has changed to include latent pathogens that can live in their host for some time without causing disease (Petrini, 1991). The Dictionary of The Fungi (1995) defines endophytes as "fungi or bacteria that form symptomless infections, for part of, or all their life cycle, within the healthy leaves and stems of plants" (Hawksworth *et al.*, 1995). More recently, endophytes have been described as being either parasitic or symbiotic and contained entirely within the plant (Sinclair & Cerkauskas, 1996). For the purpose of this paper we have considered endophytes as any organism capable of infecting its host and surviving within it, without causing any outward signs or symptoms of disease or infection. This thus includes pathogenic fungi and those that may never lead to any negative effect to their host.

A number of different effects, both positive and negative, on the host plant have been attributed to endophytic organisms. Endophytes have been reported to protect their hosts against natural enemies such as herbivores and pathogenic microbes (Carroll 1988; Latch, 1993). Vertically transmitted grass endophytes, such as *Neothyphodium* spp. (formally *Acremonium* spp.), for example, are important to the fitness of their hosts, providing them with increased insect resistance, drought tolerance and resistance to herbivory (Siegel, Latch & Johnson, 1985; Petrini *et al.*, 1992; Zhi-Qiang *et al.*, 1993; Bacon & Hill, 1996). Animals grazing on tall fescue (*Festuca arundinacea* Schreb.) and perennial rye grass (*Lolium perenne* L.) often show symptoms of toxicoses and "staggers", caused by the fungal endophytes within these grasses (Siegel *et al.*, 1985).

Endophytes have been reported to provide genetic, physiological and biochemical advantages (Zhi-Qiang *et al.*, 1993). This is achieved by inducing biochemical changes in response to infection by the endophytes and may lead to enhanced resistance to insects and pathogenic fungi, longevity and increased photosynthetic capacities of infected plant cells (White 1988; Sinclair & Cerkauskas, 1996). Endophyte infection may thus lead to changes in plant physiology, morphology and phenology (Siegel *et al.*, 1987; Bacon & Hill, 1996; Wilson, 1999).

Many endophytes are latent pathogens and a number of serious tree and plant diseases are caused by them (Kulik, 1984; Sinclair, 1991; Johnson *et al.*, 1992; Smith, Wingfield & Petrini, 1996a; Stone & White, 1997). The period of latency depends on a wide variety of factors, including environmental conditions such as drought and cold, the genetic constitution of the host plant and the virulence of the endophyte (Cerkauskas & Sinclair, 1980; Petrini, 1991; Sinclair, 1991; Sieber & Dorworth, 1994; Agrios, 1997). Several forest pathogens in South Africa are capable of infecting their hosts without causing immediate symptoms of disease. *Botryosphaeria dothidea* (Moug.) Ces. & De Not. is a well known pathogen of many woody hosts (Ramos *et al.*, 1991; Johnson *et al.*, 1992) and causes severe cankers and die-back of *Eucalyptus* spp. (Smith, Kemp & Wingfield, 1994). It is also one of the most economically important pathogens of *Eucalyptus* spp. in South Africa (Wingfield & Kemp, 1993; Smith *et al.*, 1994). Recent studies on Botryosphaeria canker in South African eucalypt plantations have shown that the causal agent, *B. dothidea*, occurs as a symptomless endophyte in *Eucalyptus grandis* Hill ex Maid., *E. camuldulensis* Dehnh., *E. smithii* R.T. Bak. and *E. nitens* (Deane et Maid.) Maid. (Smith, Wingfield & Petrini, 1996a ; Smith *et al.*, 1996b). Similarly, *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & B. Sutton, which is a serious pathogen of *Pinus* spp., has recently been shown to exist as a symptomless endophyte in *Pinus patula* Schl. et Cham. and *P. radiata* D. Don. in South Africa (Smith *et al.*, 1996b).

In a disease survey of *Acacia mearnsii* de Wild (black wattle) in South Africa, isolates of *B. dothidea* and an unidentified *Sphaeropsis* sp. were obtained from discoloured wood on

dying trees (Roux, Wingfield & Morris, 1997; Roux & Wingfield, 1997). These isolates have subsequently been found to be pathogenic to both *A. mearnsii* and an *E. grandis* clone (Roux & Wingfield, 1997; Roux *et al.*, 1997; Roux, unpublished). These pathogens also belong to genera of fungi that are well known endophytes. For this reason it was of interest to gain a more complete view of the endophytic fungi that occur on *A. mearnsii* in South Africa. The aim of this study was, therefore, to conduct isolations from symptomless *A. mearnsii* trees in order to gain information on the occurrence of possible pathogenic fungi in these trees. We were particularly interested in the possible endophytic occurrence of species of *Botryosphaeria* and *Sphaeropsis*. This study will, however, also provide the first list of endophytic fungi of *A. mearnsii* and will thus contribute towards the mycological data available on this tree.

MATERIALS & METHODS

Collection sites

Endophytes were isolated from 38 *A. mearnsii* trees grown in commercial plantations on the Bloemendal Experimental Farm (29° 32. 93S; 30° 27. 33E) in the Pietermaritzburg area of the KwaZulu-Natal Province. All trees were grown from commercially produced seed and ranged between 2 and 3 years of age. Trees were planted with a 1.5 m spacing and were approximately 4 to 6 meters tall. The older branches from the lower part of the stems were collected, to increase the possibility that endophytic infections would have taken place. Branches were between a half and one centimeter in diameter and were dark green in colour. A total of 90 branches were sampled at 4 different collection times, i.e. February 1996 (3 trees, 5 branches/tree), July 1996 (20 trees, 2 branches/tree), August 1996 (10 trees, 2 branches/tree) and January 1998 (5 trees, 3 branches/tree). Two collections were made during winter (July and August) and 2 during summer (January and February). All branches collected were free of visible disease symptoms and were collected from asymptomatic trees. The sampled branches were sealed in plastic bags and

refrigerated until isolations could be undertaken. Isolations were conducted within 48 hours of sampling.

Isolation methods

Branch and rachi samples (Fig. 1) were split lengthwise into 2 sections, using sterile scissors and sterilized by immersion in 96% ethanol for 1 min, undiluted household bleach (3.5 hyperchlorite) for 5 min, 96% ethanol for 1 min and then rinsed thoroughly in sterile distilled water. The material was then transferred to malt extract agar (MEA) (2 g/L Biolab malt extract, 15 g/L Biolab Agar), amended with 0.1 % streptomycin sulfate (Sigma) to suppress bacterial growth. Petri dishes were incubated at 25°C and isolates were transferred to fresh plates, until the original Petri dishes were completely overgrown, making recognition of individual isolates impossible. Rapidly growing fungi could be transferred from the original plates within 48 hours. Many of the isolates obtained did not sporulate on MEA and were subsequently transferred to water agar (2% Biolab agar) to which sterile pieces of *A. mearnsii* wood had been added. This technique generally stimulated the production of fungal fruiting structures.

For the isolation of endophytes from the rachi, 5 rachi were selected per branch and 4 segments (1.5 cm long) were collected per rachis. This was done by removing the pinnules from the rachis and then splitting them length wise. The epidermis was left intact, because it is extremely thin and impossible to remove without damaging the entire structure. Care was taken to ensure that each segment had three wounded sides; one at each end of the rachis, and the length wise wound created by the splitting of the rachis. This was to provide many wounds to allow endophytes to emerge.

Branch samples were treated in a similar fashion to the rachi. Ten segments (1.5 cm long) were collected per branch (5 branches split length wise). For the branches the bark was, however, removed using a scalpel, creating wounds on all sides of the branch. Five segments were incubated per Petri dish. In a pilot trial, isolations were also attempted

from pinnules, but less than 1% of the structures sampled yielded any fungi. It was, therefore, decided not to sample pinnules for endophytes.

The overall rate of infection was determined for all isolates using the technique described by Carroll & Carroll (1978). This value represents the percentage of infection by each fungal taxon as a percentage of the total number of isolates obtained.

RESULTS

Two hundred and three isolates were obtained from branches and 608 from rachi. Of these, 30 fungal taxa were identified. A total of 438 isolates did not sporulate and were not identified (Table 1).

Nigrospora oryzae (Berk. & Br.) Petch. was the most abundantly isolated fungus (9.9%). The majority of fungi isolated were black/gray in appearance. Many of these isolates did, however, not sporulate and were grouped together as "black isolates" (6.2%). Those black cultures that could be induced to sporulate were either *B. dothidea*, *N. oryzae* or *Diplodia* spp. The non-sporulating black isolates were divided into those that have a "flat" mycelial appearance and those that had a "fluffy" mycelium. Other commonly isolated taxa were species of *Fusarium* (5.4%) (*F. graminearum* Schwabe. and unknown *Fusarium* spp.), *Nodulisporium* sp. (4.4%) and *Xylaria* spp. (3.8%).

A number of the light coloured isolates that were collected, sporulated, but could not be identified. These isolates are grouped as "unidentified". They included many isolates producing small, hyaline ameroconidia in stromata or pycnidia. Some of the light coloured isolates did not sporulate and were grouped together with those designated as "unidentified". In total, these two groups accounted for 50.6% of the isolates collected.

There were no distinct differences in the frequency of occurrence of endophytes collected during the summer and the winter (Table 2). The majority of endophytic fungi, such as

Cylindrocladium candelabrum Viégas and species of *Fusarium*, *Pestalotiopsis* and *Xylaria* were isolated during both the summer and winter months. A number of the less frequently isolated taxa were, however, found in only one of the isolation periods. For example, *B. dothidea* isolates were detected only in the summer, while *Diplodia* sp. A and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. were isolated only during the winter.

The endophytes isolated in this study showed no apparent preference for the tissue types examined. The most abundant isolates could be found in both the xylem and rachi, while some of the rare isolates, such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (rachi), *L. theobromae* (rachi) and *B. dothidea* (side branches), were found only in one tissue type.

DISCUSSION

This survey of *A. mearnsii* endophytes has provided a valuable insight into the role of healthy plant tissue as a source for disease causing fungi. Knowledge of the endophytic fungal flora of *A. mearnsii,* will provide valuable information regarding the development and occurrence of diseases of these trees. This is especially important in understanding disease development associated with latent pathogens that become problematic during unfavourable environmental conditions. This is particularly applicable to *A. mearnsii* in South Africa, that is often planted on poor sites with shallow, rocky soil. Areas in which these trees are planted in South Africa, also often suffer from severe cold stress and regularly experience periods of drought. These conditions predispose the trees to infection by opportunistic pathogens which also appear to be amongst the common endophytes.

Most of the fungal taxa isolated from *A. mearnsii* in this study are known to be endophytic on other plant species. As has been shown in other studies of endophytes (Carroll & Carroll, 1978; Petrini, 1996), a few dominant taxa were found, with the majority of the taxa having a low level of occurrence. *Nigrospora oryzae*, the most frequently isolated

taxon in this study, is a common endophyte of bracken (*Pteridium aquilinum* (L.) Kuhn) (Petrini & Fisher, 1993) *Eucalyptus* spp., *Musa* spp. and many plant other species (Petrini & Fisher, 1993; Smith *et al.*, 1996a; Brown, Hyde & Guest, 1998).

Two different *Fusarium* spp. were isolated in the study and these included *F. graminearum,* which is a pathogen of maize and wheat (Marasas *et al.*, 1988). *Fusarium* spp. are common endophytes of many plant species. Endophytic *Fusarium* spp. include *Fusarium avenaceum* (Fr.) Sacc occurring on wheat and bracken (Petrini & Fisher, 1993; Crous, Petrini & Marais, 1995), *F. oxysporum* Schlecht. Emend. Snyd. & Hans. and *F. solani* (Mart.) Appel & Wollenw. Emend. Snyd. & Hans. on soybeans (Carroll, 1990) and *F. moniliforme* Sheldon on *Eucalyptus nitens* and corn (Foley, 1962; Carroll, 1990; Leslie *et al.*, 1990; Fisher, Petrini & Sutton, 1993). All of these *Fusarium* spp. are capable of causing disease under unfavourable environmental conditions.

A number of the other endophytic taxa collected in this study are known pathogens of trees and could play a role in disease development. These include *L. theobromae* (syn.: *Botryodiplodia theobromae* Pat., *Diplodia natalensis* Pole Evans.), the cause of stem canker and death of *Eucalyptus* spp. (Sharma, Mohanan & Florence, 1984), blue-stain and die-back of *Pinus* spp. (Cilliers, Swart & Wingfield, 1993), root and collar rot of *Acacia* spp. (Lenné, 1992; Lee, 1993), as well as other diseases of trees and agronomic crops (Punithalingam, 1980). The fungus is a weak pathogen associated with unfavourable environmental conditions such as heat and lack of moisture (Sharma *et al.*, 1984; Mullen *et al.*, 1991). Its role in disease of *A. mearnsii* has yet to be determined.

Species of *Cylindrocladium* are well-known pathogens of forest plantation trees, causing losses to *Acacia, Eucalyptus* and *Pinus* plants (Gibson, 1975; Ahmad, 1987; Crous, Phillips & Wingfield, 1991). This genus has a worldwide distribution and leads to root rot, damping off and stem and leaf lesions of infected seedlings in nurseries (Gibson, 1975; Lee, 1993; Crous *et al.*, 1991). It has also been reported as the cause of tree mortality in recently established plantations of *A. mearnsii* and *Eucalyptus* spp. (Crous *et al.*, 1991).

Cylindrocladium spp. have also regularly been isolated from diseased *A. mearnsii* seedlings in South Africa and its isolation as a endophyte of this tree is of interest and concern.

Seven isolates of *Glomerella cingulata* (Stonem.) Spaulding & v. Schrenk were isolated, mostly from rachi. This fungus has been described as a serious pathogen of *Acacia* seedlings under moist conditions (Gibson, 1975; Ahmad, 1987; Lenne, 1993; Lee, 1993). It affects a number of different species of *Acacia*, causing serious leaf drop and in many cases girdling branches and stems of young seedlings. It has not previously been reported from *A. mearnsii*, but *G. acaciae* (K. Ito & Shibukawa) K. Ito has been reported as the cause of anthracnose in Japan (Hodges, 1964). The role of *G. cingulata* in disease of *A. mearnsii* in South Africa deserves investigation.

Two species of *Diplodia* were isolated as endophytes of *A. mearnsii* in this study. *Diplodia* sp. A has a dark colony colour, while *Diplodia* sp. B has a white to cream colony colour with a distinctly fruity aroma. *Diplodia* sp. A resembles *D. pinea* (Desm.) Kickx, Petrak & Sydow f. sp. *cupressi* Solel, Madar, Kimchi & Golan (Roux & Wingfield, 1997), a stress related pathogen of *Cupressus sempervirens* L. in Israel and South Africa (Solel *et al.*, 1987; Linde, Kemp & Wingfield, 1998). *Diplodia* sp. B could not be identified to the species level using morphological characteristics and the identity as well as importance of both species needs further assessment.

The small number of isolates of *B. dothidea* obtained in this study might not accurately reflect its level of occurrence as an endophyte in *A. mearnsii*. The large number of non-sporulating black isolates most likely include many more isolates of this fungus, than those that could be identified with certainty. This can only be verified once isolates can be induced to sporulate, or through the application of molecular techniques. The same may be true for the frequency of species of *Sphaeropsis*, *Diplodia* and *L. theobromae,* which may all be represented by some of the dark, non-sporulating isolates. *Nigrospora* isolates

sporulated readily suggesting that none, or very few, of the remaining black-coloured isolates represent this fungus.

Xylariaceous fungi including *Xylaria* and *Nodulisporium* spp. are common endophytes of many plant species (Petrini & Petrini, 1985; Redlin & Carris, 1996) and were regularly isolated from *A. mearnsii* in this study. These fungi have been reported as pathogens on trees and other plants (Chapela, Petrini & Bielser, 1993). Their significance as endophytes and possible contribution to disease of *A. mearnsii* is uncertain. The identification of *Xylaria* spp. is notoriously difficult because the teleomorph states are rare in culture, with the anamorph states being the most frequently isolated (Petrini & Petrini, 1985; Rodriques, 1996). In this study it was possible only to identify these species to the genus level and further studies are required to identify them further. Such studies should also provide clues to their relative importance.

Many endophytes show specificity to the plant tissues that they inhabit (Fisher *et al.*, 1993). This is often correlated with the age of the tissue (Petrini *et al.*, 1992). In other studies, fungi such as *Verticicladium trifidum* Preuss, *Rhinocladiella atrovirens* Nannf. and others thus showed a distinct preference for specific host tissues, with *R. atrovirens* being isolated only from the xylem, while *V. trifidum* was isolated mostly from surface tissues (Petrini & Fisher, 1988). No obvious tissue specificity could be found for any of the taxa in this study. Only some of the less frequently isolated taxa appeared to be restricted to specific tissues, but because of their low frequencies of isolation, this observation is probably not relevant.

Using different modes of tissue preparation can affect the genera of fungi isolated from plant material (Petrini *et al.*, 1992). Xylariaceous fungi, in particular, may be under-represented in this study, since pretreatment of the tissue by drying can increase its occurrence (Petrini *et al.*, 1992). Numbers provided in this study for species of *Botryosphaeria*, *Sphaeropsis*, *Xylaria* and other fungi may thus be an under-representation of their true abundance as endophytes of *A. mearnsii*. Petrini *et al.* (1992)

determined that sampling between 30-40 individuals of a given tree/plant species may yield up to 80% of the total endophyte taxa present. This is, however, dependent on the site and also on the host examined (Petrini *et al.*, 1992). This study has thus fulfilled the requirement regarding the number of host plants to be sampled. The data represented here should give a reasonable approximation of the fungal endophytes of *A. mearnsii* in a South African plantation, although we recognise that this value is dependent on the time of year, the location and the size of the sampling unit.

This study provides a valuable preliminary list of endophytic fungi of *A. mearnsii*. We were especially interested in the possibility that species of *Botryosphaeria* and *Sphaeropsis* might occur as latent pathogens in these trees. A number of fungi with known pathogenic abilities, especially when associated with environmental stress, such as drought and frost damage, are recorded. The possible role of these fungi in diseases of *A. mearnsii* must still be determined. With the exception of *Diplodia* sp. A, isolates of *B. dothidea*, *Fusarium*, *Seiridium* and *Sphaeropsis*, produced lesions when inoculated into the stems of trees during field pathogenicity trials on 18-month-old trees (Roux & Wingfield, 1997). Further investigations regarding the pathogenicity of *B. dothidea* are needed and results of pathogenicity trials with *F. graminearum* on *A. mearnsii* are reported elsewhere in this thesis.

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Table 1: Fungal taxa, and their frequency, isolated from healthy *Acacia mearnsii* tissue.

Fungal taxa	Number of isolates		Rate of Infection
	Side branch	Rachi	(%) [a]
Alternaria sp.	2	1	0.37
Botryosphaeria dothidea	3	-	0.37
Chaetomium sp.	-	3	0.37
Cladosporium sp.	-	1	0.12
Colletotrichum gloeosporioides	-	1	0.12
Curvularia sp.	-	1	0.12
Cylindrocladium candelabrum	2	15	2.10
Cytospora sp.	-	1	0.12
Diplodia sp. A	3	1	0.49
Diplodia sp. B	6	1	0.86
Drechslera sp.	1		0.12
Epicoccum sp.	5	9	1.73
Fusarium graminearum	4	7	1.36
Fusarium spp.	15	18	4.07
Gelasinospora cerealis Dowding	-	5	0.62
Glomerella cingulata	1	6	0.86
Lasiodiplodia theobromae	-	2	0.25
Micropshaeropsis sp.	1	-	0.12
Nigrospora oryzae	22	58	9.86
Nodulisporium sp.	10	26	4.44
Periconia sp.		11	1.36
Pestalotiopsis sp. (3)	13	22	4.32
Pestalotiopsis sp. (2)	-	1	0.12
Phomopsis archerii	1	15	1.97

Fungal taxa	**Number of isolates**		**Rate of Infection (%)** [a]
	Side branch	**Rachi**	
Pithomyces chartarum (Berk. & Curt.) M. B. Ellis	-	1	0.12
Seiridium cardinale (Wagener) Sutton & Gibson	1	1	0.25
Sordaria sp.	-	4	0.49
Sporormiella sp.	6	12	2.22
Virgaliella sp.	-	1	0.12
Xylaria sp.	6	25	3.82
Black isolates - fluffy	8	23	3.82
Black isolates - flat	2	17	2.34
Unidentified	91	319	50.55
Total	**203**	**608**	**100**

[a] Rate of infection calculated as described by Carroll & Carroll (1978).

Table 2: Distribution of endophytic isolates obtained from *A. mearnsii* in winter and summer isolations

Fungal taxa	**Number of isolates**			
	Winter		**Summer**	
	Stem	**Rachi**	**Stem**	**Rachi**
Alternaria sp.	1	-	1	1
Botryosphaeria dothidea	-	-	3	-
Chaetomium sp.	-	-	-	3
Cladosporium sp.	-	-	-	1
Colletotrichum gloeosporioides	-	1	-	-
Curvularia sp.	-	1	-	-
Cylindrocladium candelabrum	1	8	1	7
Cytospora sp.	-	1	-	-
Drechslera sp.	-	-	1	1
Diplodia sp. A	3	1	-	-
Diplodia sp. B	6	-	-	1
Epicoccum sp.	4	6	1	3
Fusarium graminearum	2	2	2	5
Fusarium spp.	12	6	3	12
Gelasinospora cerealis	-	3	-	2
Glomerella cingulata	1	4	-	2
Lasiodiplodia theobromae	-	2	-	-
Micropshaeropsis sp.	1	-	-	-
Nigrospora oryzae	17	30	5	28
Nodulisporium sp.	10	20	-	6
Periconia sp.	-	-	-	11
Pestalotiopsis sp. (3)	8	6	5	16
Pestalotiopsis sp. (2)	-	-	-	1

Fungal taxa	Number of isolates			
	Winter		Summer	
	Stem	Rachi	Stem	Rachi
Phomopsis archerii	1	8	-	7
Pithomyces chartarum	-	-	-	1
Seiridium cardinale	1	-	-	1
Sordaria sp.	-	4	-	-
Sporormiella sp.	4	3	2	9
Virgaliella sp.	-	1	-	-
Xylaria sp.	6	15	-	10
Black isolates - fluffy	5	23	3	-
Black isolates - flat	-	4	2	13
Unidentified	84	261	7	58
Total	**167**	**410**	**36**	**198**

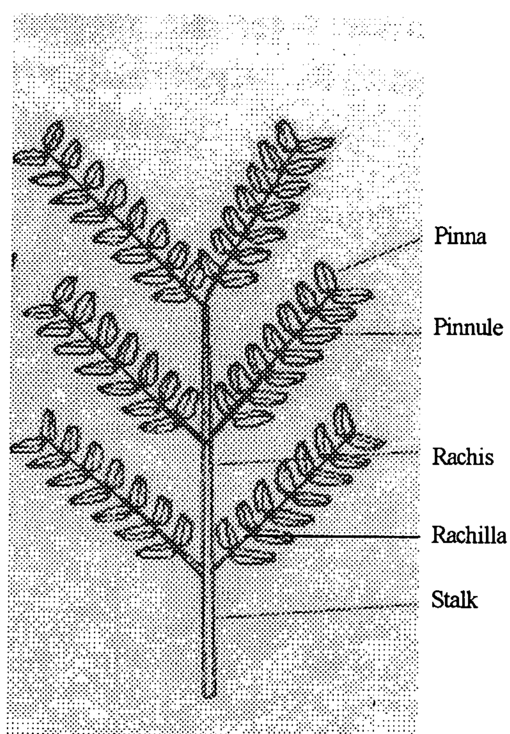

Figure 1: Diagram of a bipinnate leaf of *Acacia mearnsii*

Diagram taken from Carruthers *et al.* (1997)

CHAPTER 7

FIRST REPORT OF FUSARIUM GRAMINEARUM FROM ACACIA MEARNSII DE WILD.

7

FUSARIUM GRAMINEARUM, A PATHOGEN OF THE PLANTATION TREE *ACACIA MEARNSII*

ABSTRACT

During a survey of diseases of *Acacia mearnsii* in South Africa, isolates of an unknown and non-sporulating red fungus were collected. Symptoms associated with this fungus included branch die-back and stem cankers. The fungus was also isolated as an endophyte from healthy plant tissue during a survey of endophytes of *A. mearnsii*. The unidentified fungus was identified using DNA sequence analysis and its relative pathogenicity to *A. mearnsii* was determined in pathogenicity trials. Pathogenicity tests were conducted by inoculating 18-month-old *A. mearnsii* trees in a plantation. None of the isolates of this pathogen produced spores, making its identification, based on morphology, impossible. Sequencing of the beta tubulin gene and analysis of the data led us to identify the fungus as *Fusarium graminearum*. All the isolates tested produced significant lesions on *A. mearnsii*. *Fusarium graminearum* is a well-known pathogen of maize and wheat, with a world-wide distribution. Its occurrence on *A. mearnsii* is thus intriguing, and as far as we are aware, this is the first report of the fungus associated with a disease of a woody host.

INTRODUCTION

South Africa has nearly 1.5 million hectares of plantations of exotic forest trees, of which *Acacia mearnsii* plantations encompass about 7%. (Anonymous, 1998). Despite the relatively small proportion of land planted to *A. mearnsii*, the industry is one of the most popular amongst private farmers, that constitute about 75% of the industry (Anonymous, 1994). Both the wood and the bark of *A. mearnsii* are harvested. The wood is used for the production of paper and pulp and the tannins are extracted from the bark to be used in the manufacture of adhesives and for leather tanning (Sherry, 1971; Anonymous, 1994; Anonymous 1997).

Interest in the diseases affecting *A. mearnsii* gained prominence in 1988 with the outbreak of a new wilt disease, known as Ceratocystis wilt (Morris, Wingfield & de Beer, 1993). Subsequently, intensive disease surveys have been conducted in an effort to identify all pathogens infecting these trees (Roux & Wingfield, 1997). During these surveys, an unidentified and non-sporulating fungus, with a red mycelium in culture, was isolated from stem cankers (Roux & Wingfield, 1997). This fungus was also isolated from apparently healthy plant material in a study of the endophytes occurring in non-symptomatic *A. mearnsii* tissue (Chapter 6). Based on superficial morphological and cultural characteristics, this fungus was tentatively identified as a species of *Fusarium*.

Fusarium spp. are well-known pathogens of a wide range of plants including vegetables, agricultural crops and forestry trees (Boyer, 1961; Nelson, Toussoun & Cook, 1981; Lamprecht, Knox-Davies & Marasas, 1984; Kumar & Nath, 1988; Marasas *et al.*, 1988; Solel, Runion & Bruck, 1988). Diseases caused by *Fusarium* spp. are associated not only with crop losses, but also with the mycotoxins produced by some species (Versonder & Hesseltine, 1981; Desjardins & Hohn, 1997). Disease symptoms include damping-off of young plants (Lamprecht *et al.*, 1984; Huang & Kuhlman, 1990; Lenné, 1992; Viljoen, Wingfield & Crous, 1992), root disease (Cook, 1968; Lamprecht *et al.*, 1984; Lenné, 1992; Viljoen, Wingfield & Marasas, 1994), stem cankers (Hepting & Roth, 1946;

Boyer, 1961; Lenné, 1992) and wilting and top death (Hepting & Roth, 1946; Kumar & Nath, 1988). The best known *Fusarium* sp., pathogenic to mature plantation trees is *F. circinatum* Nirenberg et O'Donnell (Syn.: *F. subglutinans* f. sp. *pini* (Wollenw. & Reinking) Nelson, Toussoun & Marasas), that causes the serious disease known as pitch canker (Hepting & Roth, 1946; Dwinell, Kuhlman & Blakeslee, 1981; Correll *et al.*, 1991; Nirenberg & O'Donnell, 1998). This fungus is also a serious pathogen in forestry nurseries in South Africa (Viljoen *et al.*, 1994).

A number of *Fusarium* spp. have been reported from diseased *A. mearnsii* in South Africa and elsewhere (Stephens & Goldschmidt, 1938; Zeijlemaker, 1968; Bakshi, 1976; Lee, 1993). Most of these reports are from nurseries where species such as *F. oxysporum* Schlecht and *F. solani* (Mart.) Saac. cause damping-off of young seedlings (Bakshi, 1976; Lenné, 1992; Lee, 1993). *Fusarium* spp. have also been reported from other commercially grown plantation *Acacia* spp. In Malaysia, an unidentified *Fusarium* sp. is reported to be associated with leaf spot and lesions of *A. mangium* (Lee, 1993). In South Africa, an unknown species was isolated from a serious wilt disease in the 1930's (Stephens and Goldschmidt, 1938). *Fusarium oxysporum* has also been isolated regularly from trees suffering from a disease known as "black butt" (Zeijlemaker, 1968).

Fusarium spp. can be secondary, opportunistic pathogens of *A. mearnsii*. There are many records of *Fusarium* spp. playing active roles in disease associated with other pathogens (Schilling, Möller & Geiger, 1996) and as secondary, opportunistic agents of disease (Skelly & Wood, 1966). In a recent survey of diseased *A. mearnsii*, additional isolates of the unidentified red, non-sporulating *Fusarium* sp. that had been found in previous surveys, were collected. The objective of this study was to confirm the pathogenicity of this fungus to *A. mearnsii* and then to determine its identity based on DNA sequence data.

MATERIALS & METHODS

Symptoms and Isolations

Cankers from which the non-sporulating red coloured fungus were isolated occurred on side branches (Fig. 1a) and on the main stems of trees. Isolations were made from the leading edges of lesions onto 2% malt extract agar (MEA) (20 g/L Biolab Malt Extract, 15 g/L Biolab Agar). Symptoms from which isolations were made also included basal cankers, associated with black butt disease (Zeijlemaker, 1971), blister and mottle lesions associated with Ceratocystis wilt (Morris *et al.*, 1993) and mechanical wounds on stems and branches. A representative set of isolates that were used in further tests, as well as isolates of other fungi used for comparative purposes (Table 1) are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Pathogenicity tests

Inoculation experiments on *A. mearnsii* were conducted at the Bloemendal Field Experiment Station [South African Wattle Growers Union (SAWGU) and the Institute for Commercial Forestry Research (ICFR)] (29° 32. 93S, 30° 27. 33E). During January of 1998, 4 strains (CMW4375; CMW4490, CMW4492, CMW4493) of the unidentified fungus from *A. mearnsii* were each inoculated into twenty 18-month-old *A. mearnsii* trees. Twenty trees were also included as controls. The entire experiment was repeated in February of the same year.

All cultures were grown on 3.9% potato dextrose agar (PDA) (Merck Potato Dextrose Agar). Actively growing isolates were inoculated by removing a 9 mm diameter bark plug from the trees, by using a cork borer. Mycelial plugs, of equal dimension, were placed into each wound with the mycelium facing inwards. For the controls, trees were inoculated with sterile agar plugs to simulate the same process that was used for the fungal

inoculations. All inoculation wounds were covered with masking tape to prevent desiccation of the wounds and the inoculum. Lesions were measured after 6 weeks and statistical differences in lesion length were determined using Tukey's studentised range test (*P=0.05*).

Pathogen identification

Morphological characteristics

None of the isolates, associated with disease symptoms could be induced to sporulate. This is despite the fact that the cultures were subjected to different light regimes and grown on a wide range of different media. They were incubated under UV light, continuous darkness, continuous light and alternating day/night cycles, as well as at different temperatures on different growth media. Growth media tested included MEA, PDA and water agar to which sterilised *A. mearnsii* twigs had been added. Since we believed that this fungus most closely resembled a *Fusarium* sp., it was also transferred to carnation leaf agar (CLA) (Nelson, Toussoun & Marasas, 1983) in a further attempt to induce sporulation.

ß-tubulin sequencing

Sequencing of the ß-tubulin gene has proven most effective in distinguishing between species of *Fusarium* (O'Donnell & Cigelnik, 1997; O'Donnell, Cigelnik & Nirenberg, 1998). Three isolates of the fungus from *A. mearnsii* had also been tentatively identified as *F. graminearum* by Dr. K. O'Donnell (National Center for Agricultural Utilisation Research, Peoria, Illinois) based on ß-tubulin sequence data. This was an unusual diagnosis and we, therefore, undertook a study to confirm these results.

Three isolates from *A. mearnsii* were selected and their ß-tubulin genes sequenced and compared to 3 isolates of *F. graminearum* from wheat in South Africa and 2 isolates of *F.*

crookwellense Burgess, Nelson & Toussoun (Table 1). *Fusarium crookwellense* was selected as outgroup for sequencing since it has been shown to be a closely related species, and is morphologically difficult to distinguish from *F. graminearum* (Burgess, Nelson & Toussoun, 1982; Sydenham *et al.*, 1991).

DNA Isolation

For the isolation of DNA, isolates were grown on 2% MEA plates until these were covered with mycelium. Because these isolates form a thick mat of aerial mycelium, mycelium was scraped directly from the surface of the agar in Petri dishes. Care was taken not to include agar when collecting the mycelium. Mycelium was transferred to sterile 1.5 mL Eppendorf tubes and 200 µl of CTAB added. The tubes were immersed in liquid nitrogen and the mycelium ground until fine. An additional 800 µl of CTAB was added for each sample and the samples were incubated in a warm water bath at 60°C for five min. Samples were centrifuged at 12 000 g for 30 min and the supernatant transferred to sterile Eppendorf tubes. Equal volumes of phenol and chloroform (500 µl each) was added and mixed with the samples. Samples were centrifuged at 12 000 g for 5 min and the supernatant transferred to sterile Eppendorf tubes. The phenol/chloroform washes were repeated until the interphase was clean.

DNA was precipitated overnight at -20°C by the addition of 50 µl sodium acetate (NaAc) (3M) and 300 µl isopropanol. Samples were centrifuged at 13 000 g for 30 min to collect the DNA. DNA was then washed with 70% ice cold ethanol and centrifuged at 10000 g for 10 min. Pellets were dried in a SpeedVac (Savant SC 100) and the DNA resuspended in 100 µl sterile SABAX water and stored at -20°C.

ß-tubulin amplification

Primers T1 (5'-AACATGCGTGAGATTGTAAGT-3') and T22 (5'-TCTGGATGTTGTTGGGAATCC-3') were used to amplify the ß-tubulin gene

(O'Donnell & Cigelnik, 1997). Reactions were run on a Touchdown Thermocycler (Hybaid) for 40 cycles at: 94°C for 30 sec, 50°C for 30 sec and 72°C for 90 sec. PCR reactions contained 0.2 mM DNTP's, 0.3 ng/µl primer, 1 mM $MgCl_2$, 10X Buffer with $MgCl_2$ and PCR polymerase (Expand™, Boehringer Mannheim, South Africa). The PCR products were stained with ethidium bromide and visualised under UV light on 1% agarose gels.

Sequence analysis

All PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Germany). Sequence reactions were carried out with an ABI PRISM™ Dye Terminator Cycle Sequencing Kit with Amplitaq® DNA polymerase, FS (Perkin-Elmer, Warrington, UK). An ABI PRISM™377 DNA autosequencer (Perkin-Elmer) was used for the sequencing. Primers T1 and T22 were used for sequencing. The obtained sequences were manually aligned by the insertion of gaps. Phylogenetic relationships among isolates were determined using PAUP (Phylogenetic Analysis Using Parsimony; Swofford, 1985) and bootstrap analysis (Bootstrap confidence intervals on DNA parsimony) (Felsenstein, 1988).

RESULTS

Pathogenicity tests

Lesions were produced by all the isolates tested in this study (Table 2). The largest lesions for the January inoculations were an average of 38.1 cm in length (CMW4492) and for the February inoculations 37.8 cm (CMW4490 and CMW4375). Isolate CMW4493 produced the smallest lesions in both experiments (21.7 cm and 28.8 cm). Lesions were characterised by black discolouration of the outer bark surface and the formation of sunken cankers, spreading from the point of inoculation. Extensive death of the xylem, spreading from the inoculation point was also observed (Fig. 1b). No lesions were

produced in the control inoculations and all the inoculation wounds were rapidly covered by callus tissue.

Pathogen identification

None of the isolates of the unidentified pathogen could be induced to sporulate in culture, despite the various techniques used. Sequencing of the ß-tubulin gene of the unidentified *A. mearnsii* pathogen and various isolates of *F. graminearum* and *F. crookwellense* made it possible to analyse a total of 583 base pairs for the T1 primer and 526 bp's for the T22 primer. Sequences were manually aligned by the insertion of gaps (Fig. 2, 4). The heuristic search option, with no branch swopping, produced a single tree for each primer (Fig. 3, 5). Values for the Consistency Index (CI), Retention Index (RI) and Homoplasy Index (HI) for T1 was 0.818, 0.818 and 0.182 respectively and for T22 it was 0.880, 0.824 and 0.120. Bootstrap analysis using the Heuristic option with no branch swopping grouped the fungus from *A. mearnsii* with *Fusarium graminearum* Schwabe from wheat with a confidence interval of 95% for the T1 primer (Fig. 3) and 87% for the T2 primer (Fig. 5). The two isolates of *F. crookwellense* formed a clade separate from the *F. graminearum* isolates. Based on these data and the superficial morphological similarity of isolates to those of *F. graminearum*, we feel confident that the *A. mearnsii* pathogen represents this species.

DISCUSSION

In this study we have clearly shown that the unknown, non-sporulating, fungus associated with die-back and canker symptoms of *A. mearnsii* (Roux & Wingfield, 1997) is *F. graminearum*. This is an intriguing discovery as the fungus has, to the best of our knowledge, never previously been associated with a tree disease. In contrast, *F. graminearum* is a well-known pathogen of maize and wheat in may parts of the world, including South Africa (Cook, 1968; Marasas *et al.*, 1987; Blaney & Dodman, 1988; Marasas *et al.*, 1988a; Marasas *et al.*, 1988; Ouellet & Seifert, 1993).

The role of *F. graminearum* as a pathogen of *A. mearnsii* is not clear. The fungus is generally isolated from canker and die-back symptoms attributed to other primary pathogens of *A. mearnsii*, such as *Ceratocystis albofundus* Wingfield, De Beer & Morris. It has been isolated from the major *A. mearnsii* growing areas in the KwaZulu-Natal Midlands, as well as from the South Eastern Mpumalanga Province. It thus has a wide distribution in the commercial growing areas, where it was isolated from collar rots and stem cankers.

Fusarium graminearum was also isolated from asymptomatic tissues of *A. mearnsii* (Chapter 6) and is thus believed to be an endophyte of this tree. Nevertheless, pathogenicity tests conducted in this study have provided unequivocal evidence to show that *F. graminearum* can cause well defined lesions on trees inoculated with isolates of this fungus from *A. mearnsii*. In a previous study by Roux & Wingfield (1997), inoculation with a single isolate of *F. graminearum* produced only small lesions in artificial inoculation trials. The same isolate was used in this study, as well as three additional isolates. Results showed that all isolates are capable of causing lesions on *A. mearnsii*. All isolates produced considerably larger lesions than produced in the earlier study by Roux & Wingfield (1997). This could possibly be explained by differences in environmental conditions between the earlier inoculations and the 1998 inoculations, as well as by genetic differences in the trees inoculated.

We believe that *F. graminearum* is probably a latent pathogen of *A. mearnsii* that contributes to disease development after the onset of stress. This will not be unique for this genus, since many other *Fusarium* species have been reported as endophytes, capable of causing disease under conducive conditions (Carroll, 1990). It is also common for *Fusarium* diseases in forest nurseries to be more severe under periods of environmental stress (Tint, 1945; Bloomberg, 1981).

Fusarium graminearum (teleomorph: *Gibberella zea* (Schw.) Petch) is an economically important plant pathogen with a world wide distribution (Nelson *et al.*, 1981; Blaney & Dodman, 1988; Marasas *et al.*, 1988; Ouellet & Seifert, 1993). It is a common inhabitant of soil (Marasas *et al.*, 1988) and a recognised pathogen of maize (Marasas *et al.*, 1987) and wheat (Marasas *et al.*, 1988a) in South Africa. It has also been reported from diseased *Medicago* spp. and from *Panicum coloratum* L. in this country (Lamprecht *et al.*, 1984, Marasas *et al.* 1987). On wheat *F. graminearum* causes crown rot and head scab and on maize it causes cob and stalk rot (Blaney & Dodman, 1988). *Fusarium graminearum* is well-known for its ability to produce mycotoxins on infested grain, causing disease of cattle (Hart, Braselton & Stebbins, 1982; Blaney & Dodman, 1988; Desjardins & Hohn, 1997). These mycotoxins are also thought to play an important role in disease development on plants (Desjardins & Hohn, 1997).

Fusarium graminearum exists in two populations, known as group I and group II (Francis & Burgess, 1977; Nelson *et al.*, 1981). Group I isolates produce no perithecia on their host and occur mainly on wheat, causing diseases of the crowns of plants (Francis & Burgess, 1977; Cook, 1981). Group II isolates readily produce perithecia on their hosts and occur mainly on maize, causing disease of the aerial parts of plants (Francis & Burgess, 1977; Cook, 1981). Francis and Burgess (1977) also reported differences in the cultural characteristics of the two groups. From these descriptions and the fact that they do not sporulate in culture, the *A. mearnsii* isolates most closely resemble Group I isolates with the abundant mustard yellow, dense floccose aerial mycelium which rapidly fills Petri dishes.

Group I isolates of *F. graminearum* have been described as being mainly soil borne pathogens and they, therefore, do not sporulate abundantly (Francis & Burgess, 1977). This would be consistent with our observation that isolates from *A. mearnsii* have failed to sporulate in culture. Sequence data in this study confirm a close relationship with wheat isolates of *F. graminearum*. It will now be of interest to also consider isolates from maize and this question will be addressed as soon as relevant isolates become available.

In this study we have been able to highlight one of the many advantages of being able to utilise DNA sequencing techniques in fungal identification. Despite considerable effort, the fungus associated with disease symptoms considered in this study could not be induced to sporulate. The cultures, however, superficially resembled a species of *Fusarium*. In the absence of sequence data we would not have been able to confirm the identification of what is an intriguing new pathogen of *A. mearnsii*.

This report of *F. graminearum* from diseased *A. mearnsii* is enigmatic and, as far as we can tell, represents the first report of this fungus as a pathogen of a woody host. The importance of *F. graminearum* as a pathogen of *A. mearnsii* needs further evaluation. Indications are, however, that *F. graminearum* plays an important role in disease development on *A. mearnsii* in South Africa and we have found that it has a wide distribution on this plant in the country.

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Table 1: List of *Fusarium* species used in DNA sequencing and pathogenicity studies.

ISOLATE NUMBER	ORIGIN	HOST
Fusarium graminearum		
CMW4375	Piet Retief, South Africa	*Acacia mearnsii*
CMW4490	Dalton, South Africa	"
CMW4492	"	"
CMW4493	"	"
F. graminearum		
MRC4517	Bethlehem, South Africa	Wheat, crown rot
MRC4977	Caledon, South Africa	"
MRC5049	George, South Africa	Wheat, head blight
F. crookwellense		
MRC2878	Michigan, USA	Soil
MRC3926	Bethlehem, South Africa	Wheat

[a]CMW numbers are housed in the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

MRC numbers are housed in the culture collection of the Medical Research Council, Tygerberg, Cape Town, South Africa.

Table 2: Lesions produced by *Fusarium graminearum* isolates on *Acacia mearnsii*.

ISOLATE NUMBER	LESION LENGTH (mm)	
	January[a]	**February**[a]
CMW4492	38.1a	34.5b
CMW4375	35.4a	37.8b
CMW4490	30.8ab	37.8b
CMW4493	21.7c	28.8bc
Control	10d	10d

[a] Each value represents an average of 20 measurements.

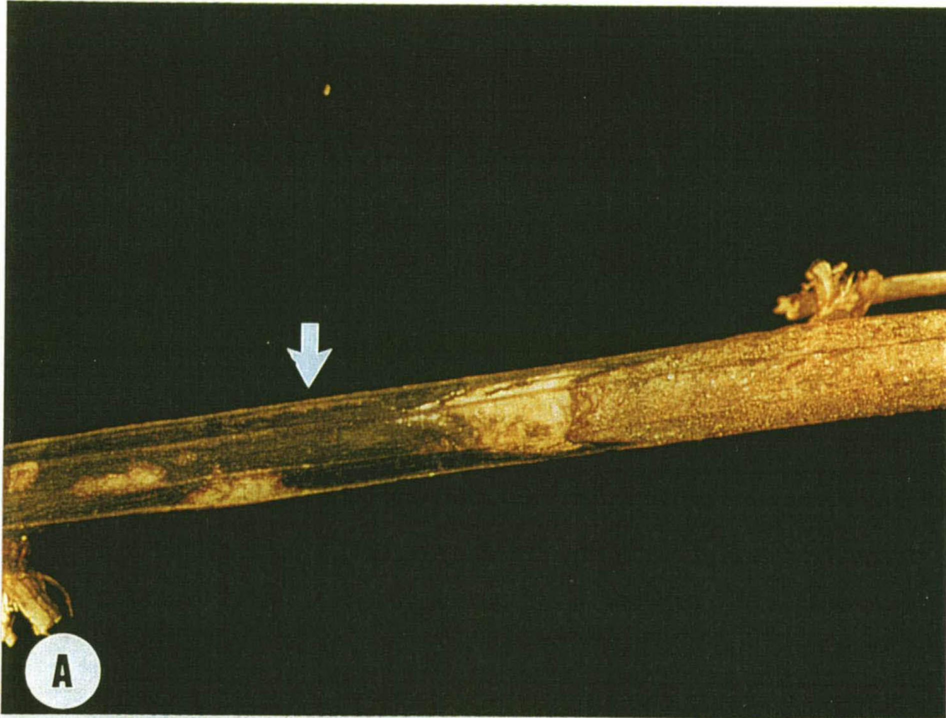
CV= 29.52% (January)

CV= 25.93%(February)

[a] Values followed by different letters differ significantly at $P=0.05$

Figure 1: Lesions associated with *Fusarium graminearum* on *Acacia mearnsii*.

Fig. 1a: Lesion on a side branch from which *F. graminearum* was isolated.
Fig. 1b: Lesion produced on a stem after inoculation.


A

B

Figure 2: Aligned nucleotide sequences for isolates of *Fusarium graminearum* and *F. crookwellense* useing the T1 primer. Homologous base pairs are indicated by a dot (.), gaps by dashes (-) and missing data by N.

```
                          10         20         30         40         50         60         70

CROOKWELLENSE   ---------A CTGACGCTCT GTCACTCAAC CAAACTGACT TTTTCTTTCT T----AGGTC CACGTCCAGG
CROOKWELLENSE   -------TG. ........T. .......... .......... .......... .----..... ..........
ACACIA          ---------- .......... .........T .......... .......... .----..... ..........
ACACIA          -------T-. .......... ........TT .......... .......... .----..... ..........
ACACIA          -ACTC-CTG. ........T. .........T .......... .......... .----..... ..........
GRAMINEARUM     ---------- .......C.. .........T .......... ....-..... .CCCC..... ..........
GRAMINEARUM     -------TG. .......CT. .........T .......... ....-..... .CCCC..... ..........
GRAMINEARUM     AACTCTCTG. ........T. .......... .......... .......... -C---..... ..........

                          80         90        100        110        120        130        140

CROOKWELLENSE   TCGGCCAATG TGTAAGGGCG AACCCACCAC CAAAAAAAAA CTTTGAGGGA CATATGTTGA GTTGATGAAT
CROOKWELLENSE   .......... .......... .......... .......... .......... .......... ..........
ACACIA          .......... .......... .......... ........-- .......... .......... ..........
ACACIA          .......... .......... .......... ........-- .......... .......... ..........
ACACIA          .......... .......... .......... ........-- .......... .......... ..........
GRAMINEARUM     .......... .......... .......... ........-- .......... .......... ..........
GRAMINEARUM     .......... .......... .......... ........-- .......... .......... ..........
GRAMINEARUM     .......... .......... .......... .........C ...-...... .......... ..........

                         150        160        170        180        190        200        210

CROOKWELLENSE   CTTTGTAGGG CAACCAAGTC GGTTCCAGCT TCTGGTGAGT CTTTTTTTAT AAGCTTTAAG CTCAATTGAA
CROOKWELLENSE   .......... .......... .......... .......... .......... .......... ..........
ACACIA          .......... .......... .......... ......A... .......... .......... ..........
ACACIA          .......... .......... .......... ......A... .......... .......... ..........
ACACIA          .......... .......... .......... ......A... .......... .......... ..........
GRAMINEARUM     .......... .......... .......... ......A... ...C...... .......G.. ..........
GRAMINEARUM     .......... .......... .......... ......A... ...C...... .......G.. ..........
GRAMINEARUM     .......... .......... .......... ......A... .......... .......... ..........
```

```
                     220        230        240        250        260        270        280

CROOKWELLENSE   TCCTTGGAAC CTAGATCTAA CCGTATTTCC AGGTCCACCG TCTCCAAGGA GCACGGCATT GATGGCAGCG
CROOKWELLENSE   .......... .......... .......... .......... .......... .......... ..........
ACACIA          ......A... .......... ........T. .......... .......... ......T... ..........
ACACIA          ......A... .......... ........T. .......... .......... ......T... ..........
ACACIA          ......A... .......... ........T. .......... .......... ......T... ..........
GRAMINEARUM     .......... .......... .......... .......... .......... .......... ..........
GRAMINEARUM     .......... .......... .......... .......... .......... .......... ..........
GRAMINEARUM     ......A... .......... .......... .......... .......... ......T... ..........

                     290        300        310        320        330        340        350

CROOKWELLENSE   GCGCGTGAGT CAACAACGTC ACCGACTTTA TGCCTCACAC TTTTGTCTAA CCTGAGCATT AGATACCACG
CROOKWELLENSE   .......... .......... .......... .......... .......... .......... ..........
ACACIA          .......... .......... .......... .......... .......... .....A...C ..........
ACACIA          .......... .......... .......... .......... .......... .....A...C ..........
ACACIA          .......... .......... .......... .......... .......... .....A...C ..........
GRAMINEARUM     .......... .......... .......... .......... .......... .....A...C ..........
GRAMINEARUM     .......... .......... .......... .......... .......... .....A...C ..........
GRAMINEARUM     .......... .......... .......... .......... .......... .....A...C ..........

                     360        370        380        390        400        410        420

CROOKWELLENSE   GAACTTCAGA CCAGCAAGCG TGAGCGCATC AA-CGTCTAC TTTG-TGAGG TGGAGTACCA ATTGCA-T-T
CROOKWELLENSE   .......... .......... .......... ..-....... ....C.T... .......... ......-.-.
ACACIA          .......... .......... .......... ..A....... ....-..... ..-....... ......G.A.
ACACIA          .......... .......... .......... ..A....... ....N..... .......... ......G.A.
ACACIA          .......... ......-... .......... ..-....... ....-..... ..-....... ......G.A.
GRAMINEARUM     .......... ......-... .......... ..-....... ....C..... ..C-...... ......G.-.
GRAMINEARUM     .......... .......... .......... ..-....... ....-....- ..C-...... ......G.-.
GRAMINEARUM     .......... ......-... .......... ..-....... ....-..... ..-....... ....G-----
```

```
                        430        440        450        460        470        480        490

CROOKWELLENSE     GCA------- ------ATTG CAAATGCAAA TT-CA----- A-CGTGATTC CTAACTTTCT CAGGGCGGCA
CROOKWELLENSE     ...------- ------.... .......... ..-.------ .A.......- .......... ..........
ACACIA            ---------- ------.... ...T...... ..-.------ .-........ .......... ..........
ACACIA            ---------- ------.... ...T...... ..-.------ .A........ .......... ..........
ACACIA            ---------- ------.... ...T...... ..-.------ .A........ -......... ..........
GRAMINEARUM       ...GTCGCAG TCGCAT.... ..-T.....- ..G..TATTC .A........ -......... .........-.
GRAMINEARUM       ...GTCGCAG TCGCAT.... ..-T.....- ..G..TATTC .A........ -......... ....-.....
GRAMINEARUM       -..GT----- ----AT.... ...T...... ..-.------ -A........ -......... ....-...-.

                        500        510        520        530        540        550        560

CROOKWELLENSE     CACA------ ---------- ---------- ---------- ---------- ---------- ----------
CROOKWELLENSE     ....A----- ---------- ---------- ---------- ---------- ---------- ----------
ACACIA            ....--GTAC GTCCCCGTGC TGT-CTGGTC GATCTTT-GA AGTCGGCCCA -GATGCCATC CGCGCCGGCC
ACACIA            ....A----- ---------- ---------- ---------- ---------- ---------- ----------
ACACIA            ....-GGTAC GTCCCCGTGC TGT-CTGGTC GATCTTT-GA -GTCGCC--A -GATGCCATC -GCGC-----
GRAMINEARUM       ....ANNNAC NTCCCCGTGC TGT-CTGGTC GATCTT--GA -GTCGGCC-A -GATGC-ATC -GCGCCGGCC
GRAMINEARUM       ....--GTAC GTCCCCGTGC TGTTCTGGTC GATCTTT-GA AGTCGGCC-A -GATGCCATC -GCGC-GGC-
GRAMINEARUM       ....--GNAC GTCCCCGTGC TGT-CTGGTC GAT-TTT-GA -GTCGCC--A -GATGCCATC -GCGCCGGC-

                        570        580

CROOKWELLENSE     ---------- ---------- ---
CROOKWELLENSE     ---------- ---------- ---
ACACIA            TTAGG----- ---------- ---
ACACIA            ---------- ---------- ---
ACACIA            ---------- ---------- ---
GRAMINEARUM       TTAGGCAAGC TCTTCGCCGA CAC
GRAMINEARUM       TTAGGCA-GC T--TCGCCGA CAC
GRAMINEARUM       TTN-GC---- ---------- ---
```

Figure 3: Phylogenetic tree generated for the T1 primer using the Heuristic search option of PAUP. Bootstrap values (1000 replicates) were computed with the branch and bound option.

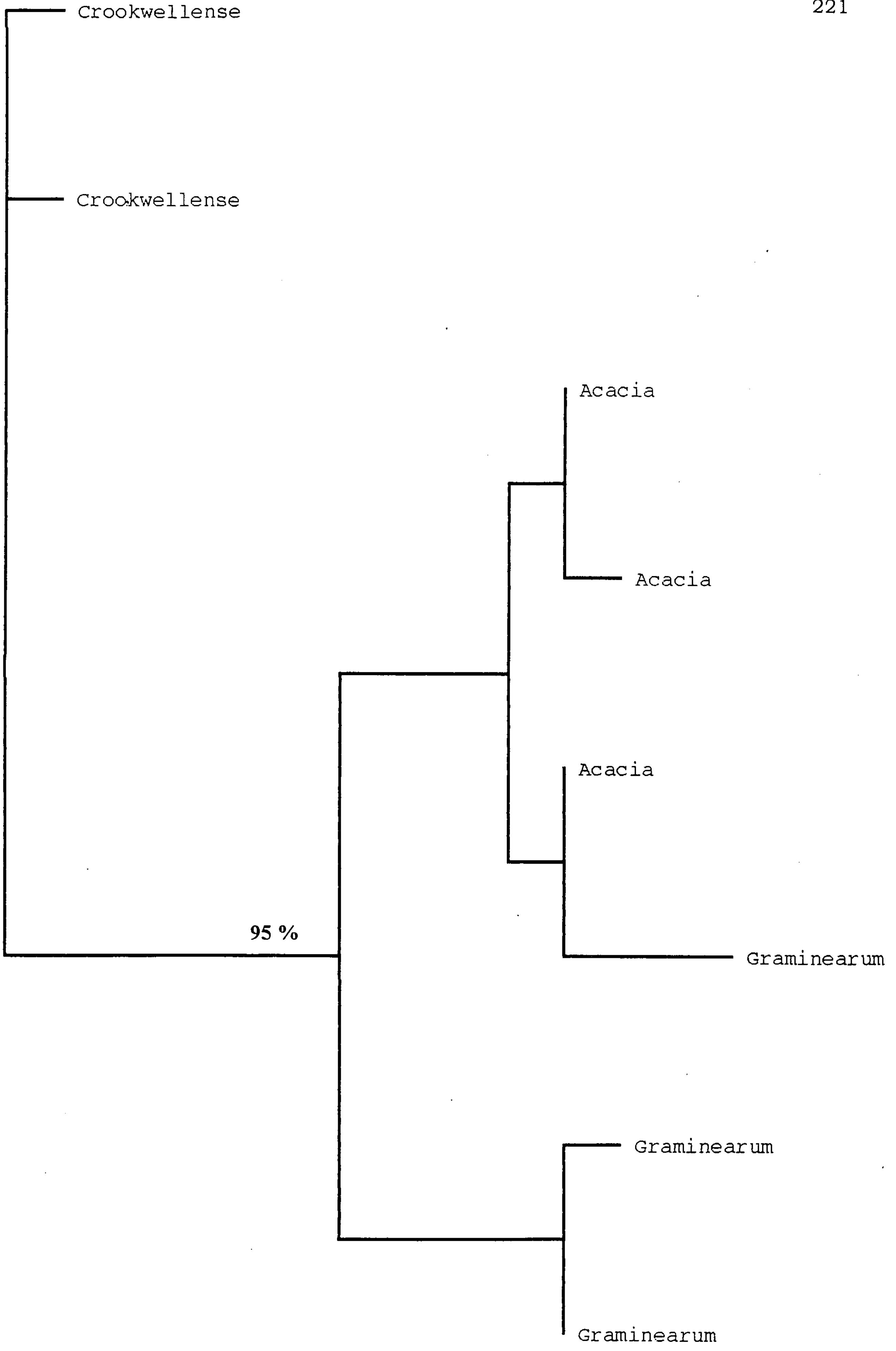

Crookwellense
Crookwellense
Acacia
Acacia
Acacia
95 %
Graminearum
Graminearum
Graminearum

Figure 4: Aligned nucleotide sequences for isolates of *Fusarium graminearum* and *F. crookwellense* useing the T22 primer. Homologous base pairs are indicated by a dot (.), gaps by dashes (-) and missing data by N.

```
                    10         20         30         40         50         60         70

CROOKWELLENSE   ---------- ---------- ---------- --TGTATCGA TAACGAGG-C TCTGTACGAT A-TCTACGAG
CROOKWELLENSE   ---------- ---------- ---------T TC........ ........-. .......... .-........
ACACIA          ---------- ---------- ---------- ---------- ---------- ---------- ----------
ACACIA          ---------- ---------- -GACGAGACT TC........ ........G. .......... .A........
ACACIA          ---------- ---------- -GACGAGACT TC........ ........-. .......... .-........
GRAMINEARUM     ---------- ---------- -----AGACT TC........ ..CGAG..-. .......... .-.-......
GRAMINEARUM     TGA-CAGCTG TCGAGA--TN TNAC-AGACT TC........ ........-. .......... .-........
GRAMINEARUM     ---------- ---------- ---------- --........ ..-.....-. .........N .-.-......

                    80         90        100        110        120        130        140

CROOKWELLENSE   AGACCCTTCA A-GATCGCCG ATCCTT-CGT ACGCC-GATC TCAACTACCC TGATTTCCAC CGGTCATGGC
CROOKWELLENSE   .......... .-........ ......T... .....C.... .......... .......... ..........
ACACIA          -......... .-........ ......-... .....C.... .......-.. .....-.... ..........
ACACIA          .AGA..C... .-........ ......-... .....C.... .........- .......... .-........
ACACIA          .-GA..C... .-........ ......-... ...NN-.... .........- .......... .-........
GRAMINEARUM     .-GA..C... --........ ......-... .....C.... .........- .......... .-........
GRAMINEARUM     .-GA..-... .-........ ......-... .....-.... ........-- .......... .-........
GRAMINEARUM     .-GA..-... --........ ......-... .....C.... .........- .......... .-........

                   150        160        170        180        190        200        210

CROOKWELLENSE   TGGTGTGACG ACCATGTTTC CGATTCCCCG GACAGCTCAA CTCGGATCTG CGAAAGCTCG CTGTTAACAT
CROOKWELLENSE   .......... .......... .......... .......... .......... .......... ..........
ACACIA          C......... .......... .......... .......... ..G....... .......... ..........
ACACIA          C......... ..-....... .......... .......... ..N....... .......... ..........
ACACIA          C......... ..-....... .......... .......... ..N....... .......... ..........
GRAMINEARUM     .......... ..-....... .......... .......... .......... .......... ..........
GRAMINEARUM     .......... ..-....... .......... .......... .......... .......... ..........
GRAMINEARUM     C......... ..A....... .......... .......... .......... .......... ..........
```

```
                    220        230        240        250        260        270        280

CROOKWELLENSE   GATTCCGTTC CCCCGACTTC ACTT-CTTCA TGGTCGGATT TGCCCCTCTG ACTGGTCGCA ACATGAAGAC
CROOKWELLENSE   .......... .......... ....-..... .......... .......... .......... ..........
ACACIA          .......... .......... ....T..... .......... .......... .......... ..........
ACACIA          .......... .......... ....-..... .......... .......... .......... ..........
ACACIA          .......... .......... ....-..... .......... .......... .......... ..........
GRAMINEARUM     .......... .......... ....-..... .......... .......... .......... ..........
GRAMINEARUM     .......... .......... ....-..... .......... .......... .......... ..........
GRAMINEARUM     .......... .......... ....-..... .......... .......... .......... ..........

                    290        300        310        320        330        340        350

CROOKWELLENSE   CTTCCAGCAC GTTACCGTCC CCGGCCTTGC TCAGCAGATT TTCGACAACA AGAACATCAT GGCCGCTGCC
CROOKWELLENSE   .......... .......... .......... .......... .......... .......... ..........
ACACIA          .......... .......... .......... .......... ..T....... .......... ..........
ACACIA          .......... .......... .......... .......... ..T....... .......... ..........
ACACIA          .......... .......... .......... .......... ..T....... .......... ..........
GRAMINEARUM     .......... .......... ....T..... .......... .......... .......... ..........
GRAMINEARUM     .......... .......... ....T..... .......... .......... .......... ..........
GRAMINEARUM     T......... .......... .......... .......... .......... .......... ..........

                    360        370        380        390        400        410        420

CROOKWELLENSE   GATTTCCGCA ACGGACGATA CCTCGCTTGT TCCGCTATCT TGTAAGTTTT GAAACCGACC AAATTTCAAA
CROOKWELLENSE   .......... .......... .......... .......... .......... .......... ..........
ACACIA          .......... .......... .......... .....C.... .......... .....T.... ..........
ACACIA          .......... .......... .......... .....C.... .......... .....T.... ..........
ACACIA          .......... .......... .......... .....C.... .......... .....T.... ..........
GRAMINEARUM     .......... .......... .......... .....C.... .......... .....T.... ..........
GRAMINEARUM     .......... .......... .......... .....C.... .......... .....T.... ..........
GRAMINEARUM     .......... .......... .......... .....C.... .......... .....T.... ..........
```

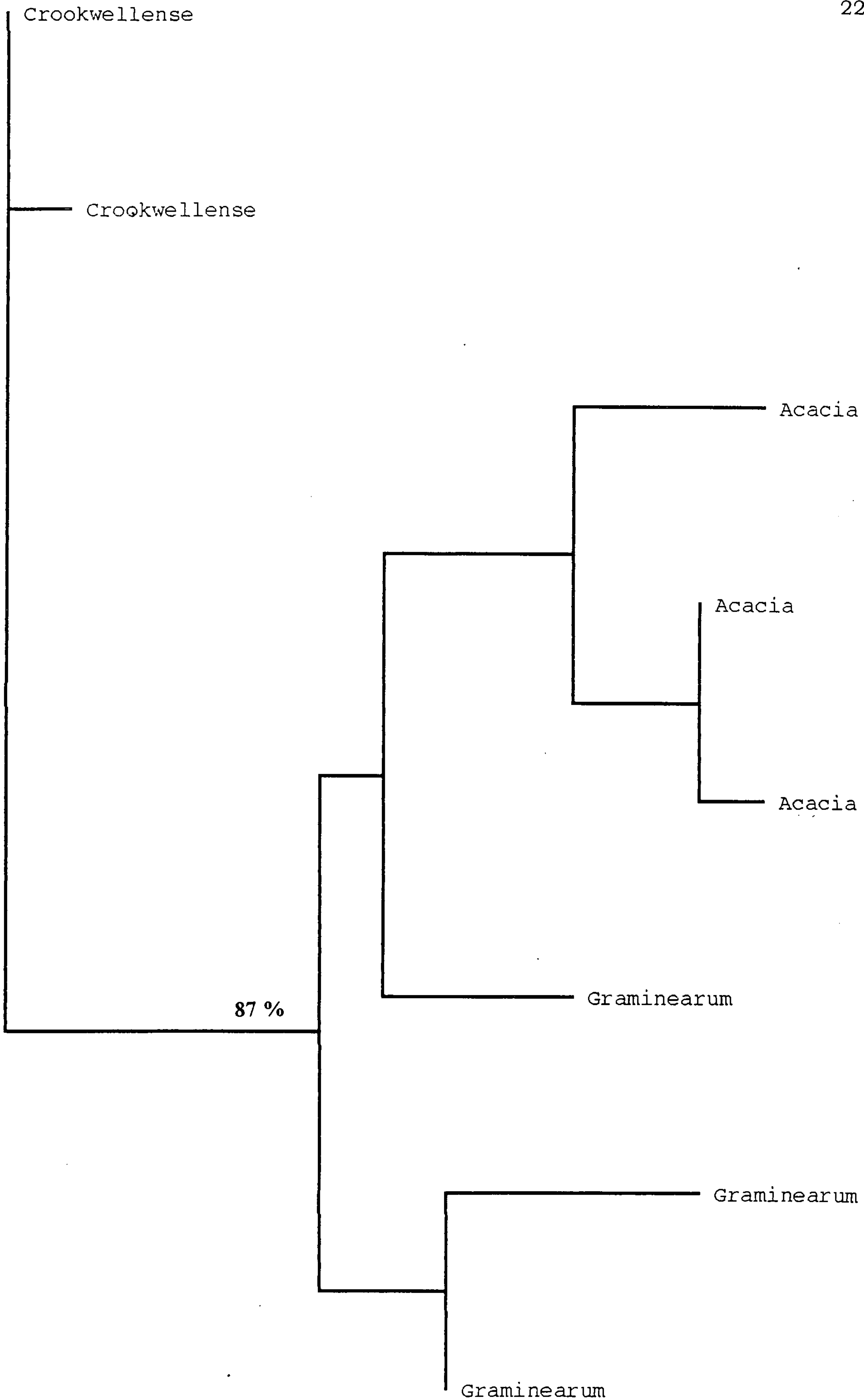
```
                     430        440        450        460        470        480        490

CROOKWELLENSE    AACACCAAAA CTAATGAAAT TCCCAGCCGC GGACGTCTCT CAACAAAGGA GATCGAGGAC CAGATGCTCA
CROOKWELLENSE    .......... .......... .......... .......... .......... .......... ..........
ACACIA           .......... .......... .........T .......... .......... .......... ..........
ACACIA           .......... .......... .........T .......... .......... .......... ..........
ACACIA           .......... .......... .........T .......... .......... .......... ..........
GRAMINEARUM      .......... .......... .......... .......... .......... .......... ..........
GRAMINEARUM      .......... .......... .......... .......... .......... .......... ..........
GRAMINEARUM      .....A.... .......... .......... .......... .......... .......... ..........

                     500        510        520

CROOKWELLENSE    AGGTTCAGAC CAAGAACTCC GA-------- ------
CROOKWELLENSE    .......... .......... ..GTACTTCG CGACTA
ACACIA           .......... .......... ..GTACTTNG CGACTA
ACACIA           .......... .......... ..GTACTTGT CGACTA
ACACIA           .......... .......... ..GTACATG- CGACTA
GRAMINEARUM      .......... .......... ..GTAC-TTG CGACTA
GRAMINEARUM      .......... .......... ..GTACTNTG CGACTA
GRAMINEARUM      .......... .......... ..GTACNTGN CGACTA
```

Figure 5: Phylogenetic tree generated for the T22 primer using the Heuristic search option of PAUP. Bootstrap values (1000 replicates) were computed with the branch and bound option.


Crookwellense
Crookwellense
Acacia
Acacia
Acacia
Graminearum
87 %
Graminearum
Graminearum

SUMMARY

The *Acacia mearnsii* industry is a relatively small, though very profitable industry in South Africa. Wood derived from *A. mearnsii* is currently in greater demand than that of either pine or eucalyptus in South Africa. Despite the importance of this industry, very little attention has been given to the genetic improvement, disease tolerance or general improvement of *A. mearnsii* as a forestry species. The result has been that, during the last few decades, pathogens have become adapted to, and spread through plantations of this tree. Although relatively little research has been conducted on the impact of pathogens on *A. mearnsii*, this situation has changed during the past nine years, and particularly since the identification of Ceratocystis wilt.

The planting of exotics has many advantages over native plants. In South Africa, exotic forestry species, such as *Eucalyptus* spp., *Pinus* spp. and *A. mearnsii* were introduced to halt the uncontrolled logging of native forests. These native forests were logged mainly for furniture and building material, but also for fuel wood, resulting in the near complete destruction of South Africa's native forests. The introduced exotics prevented the further destruction of these forests and soon became a large industry. This was particularly due to the fact that it was found that they also had a superior growth rate when compared to native species. This accelerated growth rate brought rapid results from breeding trials and, thus, a relatively rapid improvement of the material planted. Because they had been separated from their natural enemies, these trees were also initially disease free.

The *A. mearnsii* industry has, and will continue, to face many problems and challenges from pests and diseases. After the initial phase in which the tree was removed from the pathogens affecting it in it's native range, it faced attacks by native South African pests and diseases. These can spread from native *Acacia* species, or from any other native plants in the same, or even different families. Exotic, monoculture industries are also constantly under threat from the introduction of pathogens from other countries, including the country of origin. This can be done by the introduction of new germ plasm or on any

other plant species or plant material brought into a country. Because *A. mearnsii* is now planted as a monoculture, in contrast to it's native situation, diseases and pests can potentially be much more severe and will spread more rapidly and widely throughout even aged and genetically uniform stands.

Propagation of *A. mearnsii* has, recently, advanced considerably and this is concurrent with increased demand for this wood on world markets. Lessons learned from eucalypt and pine forestry need, however, to be heeded to save unnecessary losses and time. With the advent of vegetative propagation of *A. mearnsii* in South Africa, it is important to include disease screening trials at the early stages of the development of clones. In order to do this, a knowledge of all possible pathogens of *A. mearnsii* is needed. This includes pathogens known in South Africa and those that occur beyond the borders of the country. It is also necessary to have a detailed knowledge of the biology and population structure of these pathogens in order to gain an impression of the possible success of control measures.

This thesis is a compilation of work conducted on some of the known pathogens of *A. mearnsii* in South Africa. It also includes a large component dealing with the identification and clarification of previously unknown pathogens of *A. mearnsii*. It, therefore, does not focus only on diseases of *A. mearnsii*, but includes a chapter on a disease of *Eucalyptus*. The causal agent of this disease has, however, also recently been found on *A. mearnsii* in South Africa and this chapter aims at elucidating the possible origin of the isolates from South Africa. It also illustrates the potential threat of this pathogen to the *A. mearnsii* industry.

South Africa is a semi-arid country that regularly suffers from severe drought. Forestry activities in the country are also mainly restricted to areas with poorer soil and where agriculture cannot be pursued on a profitable basis. Factors such as drought, hail, frost and sub-optimal soil conditions can all contribute to increased stress on trees. Under these conditions, many fungi can act as opportunistic pathogens, causing large scale losses.

They often live as endophytes within their hosts, not causing any negative affect until the onset of stress. At this stage, they spread throughout trees, preventing them from recovering from the stress condition and leading to cankers and tree death. Careful management, particularly site/species matching, is required to minimise losses caused by these pathogens.

This thesis provides a basis for future research on the development of management strategies to control diseases of *A. mearnsii* in South Africa. Information, however, also provide valuable knowledge for forestry industries outside South Africa by highlighting the threat of exotic pathogens and the importance of strict quarantine measures to prevent the spread of pathogens. This is true for the movement of not only *A. mearnsii* material, but as was seen here, the movement of any forestry products, since many pathogens have a wide host range. Although the thesis is comprised of a series of individual entities, these all provide information regarding the hygiene of *A. mearnsii* plantations. This thesis thus aims at identifying future focus points for intensive research, while at the same time focusing on those pathogens that have been known to the South African industry for a longer period of time.

Chapter one provides a review of the available literature on diseases affecting not only *A. mearnsii*, but also other *Acacia* spp. important to the forestry industry, world wide. It also highlights some of the uses of these species in the countries where they are planted. The multi-purpose use of *Acacia* spp. is an important aspect emerging from this review. In many countries, *Acacia* spp. are not only planted as forestry species but are also used for soil reclamation, nitrogen fixation and fodder. The main focus of the chapter, however, is on the *A. mearnsii* industry in South Africa, with a brief discussion on all the diseases currently known to occur in the country. It is concluded that much research is still needed to reduce the impact of these diseases and to ensure that the Industry functions optimally.

Ceratocystis albofundus must be considered as one of the most important pathogens of *Acacia* spp., world-wide. Currently this pathogen occurs only in South Africa, but if it is to spread to other countries, large scale losses will be incurred. It may also affect, not only *A. mearnsii*, but most likely many other plant species. Breeding programmes for *A. mearnsii* in South Africa focus strongly on this pathogen. In Chapter two, the population diversity of *C. albofundus* was investigated and compared with data for other *Ceratocystis* spp., using nuclear and mitochondrial DNA fingerprinting. It was found that the *C. albofundus* population has a greater genetic diversity than any of the species with which it was compared. This will thus mean that intensive breeding programmes will be necessary to ensure durability of disease tolerance. It also supports previous hypotheses that *C. albofundus* is native to South Africa and may be a temperate species, not found in tropical areas where its close relative, *C. fimbriata*, commonly occurs.

The first unequivocal report of *C. fimbriata* and *Ch. elegans* from *A. mearnsii* is presented in Chapter three. Both these fungi were isolated from dying trees with typical symptoms of Ceratocystis wilt caused by *C. albofundus*. Both were shown to be capable of causing disease to seedlings under green house conditions. It was, however, found that *C. albofundus* is more virulent than either *Ch. elegans* or *C. fimbriata*. Both isolates were identified using molecular and morphological approaches. Unfortunately only one isolate of each exists and surveys to obtain additional samples continue to be a priority.

The first report of a wilt disease of *Eucalyptus*, caused by *Ceratocystis fimbriata* in the Republic of the Congo in West Africa is recorded in Chapter four. This is not only the first report of *C. fimbriata* as a pathogen of *Eucalyptus* in Africa but is also one of the few unequivocal reports of this fungus from the continent. Pathogenicity of *C. fimbriata* on *Eucalyptus* spp. was confirmed in glass house tests. In this Chapter, *C. fimbriata* and *C. albofundus* from *A. mearnsii*, and *C. fimbriata* from *Eucalyptus* in Brazil were also compared to the *C. fimbriata* from the Congo. Comparison of the ITS region of the rRNA operon showed that isolates from all three areas grouped together in a clade of *C. fimbriata*, separate from European isolates. Sequence data showed that *C. fimbriata* from

A. mearnsii in South Africa is nearly identical to the fungi from *Eucalyptus* in Brazil and Congo, suggesting that they may have a common origin. These findings stress the importance of sound quarantine measures to prevent the introduction of potentially devastating pathogens to South Africa. It is not yet known why *C. fimbriata* has not caused more diseases on *A. mearnsii* or *Eucalyptus* spp. in the country, but the situation will need to be monitored closely.

Apart from *C. albofundus*, there are many other fungi that cause disease of *A. mearnsii* in South Africa. Chapter five reports on a species of *Seiridium* that was isolated from stem cankers on *A. mearnsii*. Morphological and molecular comparisons, as well as pathogenicity studies have shown that the species from *A. mearnsii* is similar to those species responsible for Cypress canker in many parts of the world. It also confirms previous reports that the taxonomy of the three *Seiridium* spp. causing cypress canker needs re-evaluation, since molecular data support the view that the three species, represent a single taxon. Pathogenicity trials on mature *Cuppressus lusitanica* and on *A. mearnsii* trees showed that both the cypress and *A. mearnsii* isolates are capable of causing lesions on both hosts.

Many of the fungi isolated from diseased *A. mearnsii* during the current and previous studies of diseases resulted in the isolation of fungi, commonly found as latent pathogens on other forest trees. Chapter six encompassed a survey of the endophytic fungi of *A. mearnsii*, with the specific aim of identifying possible pathogens. Thirty different fungal taxa were found as endophytes of the xylem and rachi. These included *F. graminearum* and *Botryosphaeria dothidea*, which are known pathogens. During periods of environmental stress, these fungi can apparently cause disease. This is especially true because *A. mearnsii* is often planted on marginal sites in South Africa.

Chapter seven represents the first report of *Fusarium graminearum* from *A. mearnsii* and presents evidence for the fungus being involved in disease of *A. mearnsii*. This pathogen was first isolated during 1994-95 disease surveys, but was not identified due to the fact

that cultures on artificial media did not sporulate. In the current study, additional isolates were obtained from stem cankers and die-back symptoms and the fungus was identified based on ß-tubulin gene sequences. Field inoculations using *F. graminearum* showed extensive lesion formation in the xylem. Previously, this *Fusarium* sp. was known only as a pathogen of maize and wheat in various parts of the world. Results of this study are, therefore, enigmatic and intriguing.

OPSOMMING

Die *Acacia mearnsii* industrie is 'n klein, dog uiters winsgewende industrie in Suid-Afrika. Hout vanaf hierdie boom is tans in meer aanvraag as enige van die ander bosbouspesies in Suid-Afrika. Min aandag is egter geskenk aan die genetiese verbetering, ontwikkeling van siekte weerstandbiedende spesies/klone en die algemene verbetering van hierdie boom as 'n bosbouspesie. Dit het tot die gevolg gehad dat siektes die kans gekry het om aan te pas, te versprei en ernstige probleme te veroorsaak op *A. mearnsii*. Hierdie situasie het egter gedurende die afgelope paar jaar verander, veral na die identifikasie van Ceratocystis wilt aan die einde van die 1980's.

Die verbouing van uitheemse boomspesies, in teenstelling met inheemse spesies, het baie voordele. In Suid-Afrika is uitheemse bosbouspesies aanvanklik geplant om die totale vernietiging van die land se inheemse woude te voorkom. Hierdie woude is teen 'n ontstellende tempo afgekap om aan boumateriaal, meubel- en vuurmaakhout te voorsien. Uitheemse bosbouspesies het nie net vinniger groeitempos as die inheemse spesies nie, maar het aanvanklik min siektes gehad. Na die aanvanklike fase waartydens die uitheemse spesies van hul natuurlike vyande verwyder was, het Suid-Afrikaanse peste en siektes by hierdie bome begin aanpas. Dit het ook vir die *A. mearnsii* boom gegeld. Patogene kan van 'n verskeidenheid ander gashere versprei na *A. mearnsii*. Dit sluit uitheemse organismes wat vanaf die boom se land van oorsprong na Suid-Afrika versprei, asook organismes vanaf ander lande af, in. Omdat *A. mearnsii* nou as mono-kultuur aangeplant word, in teenstelling met sy land van oorsprong, kan peste en patogene teen 'n baie vinniger tempo versprei onder die geneties uniforme plantasies in Suid-Afrika.

Die produksie van *A. mearnsii* het aansienlik verbeter, net soos die aanvraag van hierdie hout verhoog het op die internasionale markte. Met die ontwikkeling van vegetatiewe voortplanting van *A. mearnsii* d.m.v steggies, is dit egter van uiterste belang om in 'n vroeë fase van die proses alle nageslag teen siektes te toets. Hiervoor is 'n kennis van al die moontlike patogene van *A. mearnsii* van belang. Dit sluit beide die patogene in Suid-

Afrika, sowel as patogene in ander lande in. Hierdie kennis moet ook insluit inligting oor die biologie en populasie diversiteit van die patogene. Hierdie inligting sal 'n aanduiding gee van die moontlike sukses van beheermaatreëls.

Hierdie tesis is 'n samestelling van werk wat gedoen is op sommige van die bekende patogene van *A. mearnsii*. Dit sluit ook'n groot gedeelte in wat handel oor die identifikasie van voorheen onbekende patogene van hierdie boom. Dit sluit 'n hoofstuk in oor 'n nuwe siekte van *Eucalyptus* spp., aangesien die patogeen ook op *A. mearnsii* gevind is tydens hierdie studie. Laasgenoemde hoostuk ondersoek die moontlike konneksie tussen die oorsprong van die isolate op *A. mearnsii* i.v.m die van *Eucalyptus* spp.

Suid-Afrika is 'n land wat gereeld deur ernstige droogtes geteister word. Die meeste bosboupraktyke is egter beperk tot areas van swakker grondkwaliteit en ongereelde reënval. Buiten droogte stres, dra hael, ryp en die swak grond by tot verhoogte streskondisies vir bosbou. Onder hierdie toestande kan opportunistiese patogene grootskaalse verliese tot gevolg hê. Hierdie organismes leef dikwels as endofiete in hul gashere, waar hul aanvanklik geen siektes veroorsaak nie. Sodra die boom egter onder stres verkeer, versprei hulle in die boom en verhoed hulle die boom om te herstel van die strestoestande. Hulle lei ook dikwels tot die uiteindelike dood van die boom. Goeie bestuurspraktyke, veral die korrelasie an area met spesie, word benodig om verliese deur hierdie patogene te verminder.

Hierdie tesis lê die grondslag vir verdere navorsing oor die verbetering en bestuur van strategieë teen siektes van *A. mearnsii*. Die potensiële bedreiging deur uitheemse patogene en die noodsaaklikheid van streng kwarentyn work ook uitgelig. Al bestaan die tesis uit 'n reeks individuele hoofstukke, handel almal oor die patogene van *A. mearnsii* en hul impak op hierdie boom. Hierdie tesis lig fokuspunte uit vir verdere navorsing en verskaf ook meer inligting oor die patogene van *A. mearnsii* wat reeds aan die wetenskap bekend is.

Hoofstuk een verskaf 'n literatuuroorsig van die siektes van *A. mearnsii* sowel as van ander *Acacia* spp. wat belangrik is vir die bosboubedryf wêreldwyd. Hierdie hoofstuk lig ook sommige van die ander gebruike van hierdie bome, in die lande waar hulle verbou word, uit. Dit is duidelik dat die multi-gebruikspotensiaal van hierdie genus 'n belangrike aspek vorm van sy gebruik as bosbouspesie. In die meeste lande word *Acacias* byvoorbeeld ook geplant om te help met grondherwinning, stikstofvaslegging en as veevoer. Hoofstuk een se hoof fokuspunt is egter die *A. mearnsii* industrie in Suid-Afrika en die siektes van hierdie boom in die land word bespreek. Dit is duidelik dat, om te verseker dat die industrie maksimaal funksioneer, baie navorsing nog benodig word aangaande die siektes van hierdie spesie.

Ceratocystis albofundus kan as een van die ernstigste siektes van *Acacia* spp. in die wêreld beskou word. Telingsprogramme met *A. mearnsii* in Suid-Afrika fokus daarom op hierdie ernstige patogeen. In Hoofstuk twee het ons die populasiediversiteit van hierdie patogeen in Suid-Afrika ondersoek en die data vergelyk met die van ander *Ceratocystis* spp. Dit is gedoen deur gebruik te maak van nukluêre en mitochondriale DNA profiele. Daar is gevind dat *C. albofundus* 'n hoër populasiediversiteit as enige van die ander drie *Ceratocystis* spp. het. Dit beteken dat intensiewe telingsprogramme benodig sal word om die duursaamheid van siekteweerstand te verseker. Resultate in hierdie hoofstuk ondersteun ook vorige hipotesisse dat *C. albofundus* inheems aan Suid-Afrika is. Dit is heel moontlik dat *C. albofundus* 'n spesie vanaf 'n gematigde klimaat is, wat in teenstelling met die nabyverwante *C. fimbriata*, nie in tropiese areas gevind word nie.

In Hoofstuk drie verskyn die eerste onteenseglike aanmelding van *C. fimbriata* vanaf *A. mearnsii*. Hierdie hoofstuk verteenwoordig ook die eerste aanmelding van *Chalara elegans* vanaf *A. mearnsii*. Beide hierdie fungi is identifiseer deur gebruik te maak van morfologiese en molekulêre tegnieke. Ongelukkig kon slegs een isolaat van elk verkry word en verdere opnames om meer isolate te kry is 'n prioriteit vir toekomstige navorsing. Beide spesies is in staat om siekte van jong saailinge in die glashuis te versaak.

Ceratocystis albofundus was egter die virulentste van die spesies wat getoets is vir patogenisiteit.

Hoofstuk vier verteenwoordig die eerste aanmelding van *C. fimbriata* as die oorsaak van 'n ernstige verwelksiekte van *Eucalyptus* in Afrika. Die patogenisiteit van *C. fimbriata* op *Eucalyptus* is in die glashuis bevestig. In hierdie hoofstuk is *C. fimbriata* vanaf *A. mearnsii* en *Eucalyptus* in Brazil, asook *C. albofundus*, met Congo isolate vergelyk. Vergelyking van die ITS gebied van die rRNA operon van isolate vanaf die Congo, Brazil en Suid Afrika toon dat isolate vanaf al drie areas saam groepeer as *C. fimbriata*, maar apart van Europese *C. fimbriata* isolate. Dit is ook gevind dat *C. fimbriata* vanaf *A. mearnsii* in Suid-Afrika feitlik identiese DNA profiele het as *C. fimbriata* isolate vanaf *Eucalyptus* in Brazil en Wes-Afrika. Dit is dus heel waarskynlik dat hierdie isolate 'n gemeenskaplike oorsprong mag deel.

Buiten vir *C. albofundus*, is daar verskeie ander fungi wat ook siektes van *A. mearnsii* veroorsaak in Suid-Afrika. Hoofstuk vyf handel oor 'n *Seiridium* sp. wat vanaf stam kankers geisoleer is. Morfologiese en molekulêre, asook patogenisiteits toetse toon dat die spesie vanaf *A. mearnsii* soortgelyk is aan die *Seiridium* spp. wat verantwoordelik is vir sipreskanker in verskeie lande van die wêreld. Resultate verkry in hierdie studie bevestig vorige verslae dat die taksonomie van die drie spesies verantwoordelik vir sipreskanker herevaluering benodig. Molekulêre data toon dat die drie sipreskanker spesies in werklikheid een spesie verteenwoordig. Inokulasies van sytakke van volwasse *Cupressus lusitanica* en van *A. mearnsii* toon dat beide die sipres en *Acacia* isolate patogenies is op hul onderskeie gashere.

Baie van die fungi wat gedurende siekteopnames geisoleer is, is bekend as latente patogene van ander bosbou spesies. In Hoofstuk ses is 'n opname van die endofitiese fungi van *A. mearnsii* gedoen, met die spesifieke doel om potensiële patogene te identifiseer. Dertig verskillende taxa is as endofiete geisoleer vanuit die xileem en ragisse van gesonde bome. Hierdie taxa het onder andere *F. graminearum* en *Botryosphaeria*

dothidea ingesluit. Die rol van hierdie fungi gedurende tydperke van ongunstige omgewingstoestande is van uiterste belang omdat *A. mearnsii* dikwels op marginale gebiede geplant word en dus onder gereelde stres verkeer. Hierdie stres kan tot gevolg hê dat latente patogene aktief raak en siekte en dood van die bome veroorsaak.

Fusarium graminearum is 'n bekende en ernstige patogeen van mielies en koring, maar in Hoofstuk sewe word dit vir die eerste keer as patogeen van *A. mearnsii* aangemeld. Dit is vir die eerste keer gedurende 1994-95 ġeisoleer tydens siekteopnames, maar is nie geidentifiseer nie aangesien die isolate vanaf *A. mearnsii* nie in kultuur sporuleer nie. In die heidige studie is meer isolate vanaf stamkankers verkry en identifiseer deur vergelyking van die ß-tubulien geen. Veldinokulasies met *F. graminearum* het ekstensiewe kankerformasie in die xileem tot gevolg gehad.

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