Ceratocystis spp. and Botryosphaeriaceae on plantation *Acacia* species in Central Sumatra, Indonesia.

by

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DECLARATION

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

Marthin Tarigan
May 2008

I dedicate this thesis to my wife Maria Peratenta Sembiring,
my daughter Melisa Phebeyola E. Tarigan and
my son Mikha Daniel L. Tarigan

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PREFACE

Demand for forest based products such as fiber, plywood, pulp and paper is increasing internationally and as a result, vast areas of land have been converted to forest plantations. This is especially true in Indonesia where approximately 10 million hectares of commercial plantation forests have been established. Similar to other countries mainly non-native *Pinus* spp., *Eucalyptus* spp. and *Acacia* spp. are planted in Indonesia, with *Acacia mangium* and *A. crassicarpa* covering more than one million hectares.

Plantations are vulnerable to disease and pest attacks due to their homogeneous nature and silvicultural practices that favour attack. Not only are vast areas planted to single species, but trees are also planted at high densities increasing competition and stress. *Acacia* spp. in Indonesia also require pruning, resulting in stress to trees and providing wounds for infection by pathogens. Consequently, a number of disease problems have been reported from plantation grown *Acacia* spp. in Indonesia. Relatively few scientific studies have, however, been undertaken to elucidate the causes of these diseases, or to develop sustainable management plans to reduce the impact and incidence of diseases in *Acacia* plantations in Indonesia. The aim of studies in this dissertation was to address this problem by providing a base of knowledge regarding wilt and canker diseases of *A. mangium* and *A. crassicarpa* in the country.

Chapter 1 of this dissertation provides a review of diseases of *Acacia* spp., including *A. mangium*, *A. crassicarpa*, *A. auriculiformis* and *A. aulacocarpa*, planted in forest plantations in Indonesia. These include leaf diseases, stem diseases and root diseases. The information on the causal agents, disease symptoms, disease status and impact, and where available disease management strategies are discussed. It is shown that little is known regarding plantation diseases in the country, with many diseases being caused by as yet unknown factors. I also provide a brief introduction to the forestry industry in Indonesia, emphasizing the importance of understanding plantation health issues in the country.

Chapter two to four of the dissertation represent studies dealing with the identification of several fungal species isolated from diseased and dying *A. mangium* trees in Indonesia. These fungi were first isolated from trees that had been pruned and were showing signs of wilting and canker in several plantations in Indonesia. To obtain more isolates from *A. mangium*, and to determine if these fungi would also naturally infect *A. crassicarpa*, wounding trials were also conducted. Chapter two describes the isolation and identification of fungal isolates in the *Ceratocystis fimbriata sensu lato* complex using morphology and DNA sequence data. To evaluate the pathogenicity of the isolated fungi, inoculation trials were conducted in the greenhouse and field.

In Chapter 3, *Ceratocystis* spp. in the *C. moniliformis sensu lato* complex are characterized. Three previously undescribed species within *C. moniliformis s.l.*, obtained from wounded *A. mangium*, are characterized and described using morphology and DNA sequence data for the ITS, β-tubulin and Elongation Factor 1-α gene regions. Pathogenicity studies, both in the greenhouse and field, were also conducted to evaluate their virulence to *A. mangium* and *A. crassicarpa.*

The Botryosphaeriaceae appear to be the most common fungi found on plantation grown *Acacia* spp. in Indonesia. Surveys of diseased *A. mangium* in Indonesia resulted in the isolation of three species of Botryosphaeriaceae. In Chapter 4, two previously unknown species are described and the third fungus representing a known species is recognized. Use was made of morphology and comparisons of DNA sequence data for the ITS and Elongation Factor 1-α gene regions to identify the isolates. The ability of these fungi to cause disease on *A. mangium* and *A. crassicarpa* were evaluated in the greenhouse and field.

In commercial *Acacia* plantations, pruning and singling are common tending practices which are regularly applied to 4 - 8 month old trees in order to achieve reduced branching and to promote single stemmed trees. Chapter 5 of this dissertation investigates two pruning methods and two inoculation techniques to understand the impact of different types of wounds on tree infection by two wound-infecting pathogens, *Ceratocystis acaciivora prov. nom.*, described in this dissertation and *Lasiodiplodia theobromae*, on *A. mangium* and *A. crassicarpa* trees. Results of this

chapter provide a practical aspect to minimize the risk of fungal infection to pruning and singling wounds.

Studies presented in this dissertation provide a foundation for future plantation disease management in Indonesia. They identify the cause of previously unknown wilt diseases of *A. mangium*, but also provide practical solutions to reduce the incidence of an important wilt disease. Information in this dissertation should also be valuable to the international forestry, agricultural and scientific communities, providing information on several fungal species, previously unknown to science. It also expands the host and geographic range of an important pathogen in the genus *Ceratocystis*. Considerable research is, however, still required regarding plantation forestry diseases in the country and this study should be seen as strong motivation for continued research in the field of plantation health in Indonesia.

Chapter 1

Diseases of tropical *Acacia* species in forest plantations in Indonesia

1. Introduction

Indonesia is an archipelago country consisting of about 17000 islands that span over 4800 km between the Indomalayan and Australasian realms. The islands lie in the equatorial zone, typically receiving more than 2000 mm of rain per annum and with temperatures ranging between 23°C and 28°C, while the relative humidity ranges between 70 and 90%. The five biggest islands are Sumatra, Java, Kalimantan, Sulawesi and Papua (Fig 1). Indonesia has a high diversity of plants, mammals, birds and reptiles, ranking among the top five countries in the world with respect to its biodiversity (Anonymous 2002, Rhee *et al*. 2004).

Indonesia has approximately 144 million hectares (ha) of forest land (Thompson 1996, Effendy & Hardono 2002) which is located mainly on Sumatra, Kalimantan and Papua islands (Thompson 1996, Anonymous 2002). Indonesian forests rank third in extent, after Brazil and the Democratic Republic of Congo (Anonymous 2002) and has a diversity of forest types. These include dipterocarp and non-dipterocarp forests, monsoon forests, savannah grasslands and mangrove forests (Anonymous 2002).

Plantations of fast-growing non-native tree species expanded rapidly world-wide in the last twenty years. In 2000, the world had about 187 million hectares of plantations with the largest areas in Asia (Carle *et al*. 2002), mostly in China, India, Indonesia, Japan, Thailand and the Islamic Republic of Iran (Carle *et al*. 2002). In terms of the extent, Indonesian plantations rank among the top six countries in the world. The total area of plantations in Indonesia is about 9871000 ha or about 5 % of the world's total plantation area (Carle *et al*. 2002).

In the 1980's the Indonesian government initiated a forest plantation program (Hutan Tanaman Industri – HTI), resulting in large areas of land being converted to plantations of non-native tree species. The total numbers of HTI concessionaires in 2003 numbered 202 units. This included 36 HTI concessions that are managed for the pulp and paper industry, 156 HTI concessions that are managed for the non-pulp and paper industry and 10 mixed plantation forest concessions. The progress of plantation establishment in 2003 was about 2.5 million ha, of which 1.6 million ha were for pulp

and paper HTI and 0.9 millions ha were for non-pulp and paper HTI (Anonymous 2004).

In terms of tree species composition in plantations of the world, *Pinus* spp. and *Eucalyptus* spp. are dominant at about 20% and 10% respectively, with *Acacia* spp. comprising only 4% (Carle *et al*. 2002). In Indonesia, *Acacia* spp. are most commonly planted (Table 1), with *Acacia mangium* Willd. the main tree species used for plantation development (Cossalter & Nair 2000, Nair & Sumardi 2000). This species is native to northern Queensland, the western province of Papua New Guinea and both Papua and Maluku, the eastern islands of Indonesia (Arisman & Hardiyanto 2006).

Acacia mangium was chosen for plantation development because of its rapid growth rate, high pulp yield and its ability to adapt to degraded soils such as ex - grasslands of *Imperata cylindrica* (L.) Beauv. (Arisman & Hardiyanto 2006). As more lowland areas, which have very low pH became available, more *A. crassicarpa* Cunn.: Benth. has been planted. This species has been chosen as it has the ability to adapt to acidic soil and it grows vigorously (Old *et al.* 2000).

Besides *A. mangium* and *A. crassicarpa*, other *Acacia* spp. are also planted to a lesser degree in Asia. These include *A. auriculiformis* A. Cunn: Benth. and *A. aulacocarpa* Cunn.: Benth. Other tree species planted to a lesser extent are *Paraserianthes falcataria* (L.) Nielsen, *Gmelina arborea* Roxb., *Eucalyptus* spp., *Azadirachta excelsa* (Jack) Jacobs, *Hevea braziliensis* (Willd.: Adr. Juss.) Mull. Arg., *Peronema canescens* Jack, *Octomeles sumatrana* Miq., Dipterocarpaceae and *Tectona grandis* L. f. (Table 1) (Cossalter & Nair 2000, Rimbawanto 2002, Lee 2003).

The productivity [mean annual increment (MAI)] of forest plantations, in particular *A. mangium* has increased from 15-20 m^3/ha/year in the late 1980's to 20-25 m^3/ha/year in the mid 1990's (Kaimowitz & Barr 2002). Thus, since a rotation period is 7-8 years, the average volume harvested from a site has increased from 112-150 m^3/ha to 150-190 m^3/ha. This progress has been achieved through tree improvement programmes and improved silvicultural practices. Currently, the productivity of *A. mangium* is being pushed to reach a MAI of 25-30 m^3/ha/year or 190-225 m^3/ha of round wood production (Kaimowitz & Barr 2002) and on the best sites, some forestry

companies can reach a MAI of 40-46 m^3/ha/year (Arisman & Hardiyanto 2006, Golani 2006).

Many factors impact on the productivity and success of forest plantations. Pests and pathogens could be one of the limiting factors, as they could attack at any time during a tree's life cycle. Plantations of single species, with narrow genetic diversity, such as *A. mangium* and *A. crassicarpa*, that are planted over hundreds or thousands of hectares, and in some areas are planted in contiguous stands, are especially at risk when a pest or pathogen to which they are not tolerant, appears (Gales 2002).

Currently, the majority of forest plantations in Indonesia have been developed on the islands of Kalimantan and Sumatra (Figure 1). These areas have more than 2000 mm rainfall annually, with approximately 80% and 28ºC humidity and temperature respectively. The humidity, rainfall and temperature have a major impact on the occurrence of diseases, as many pathogens prefer high humidity and temperatures (Hepting 1963, Coakley *et al*. 1999). These climate conditions are conducive to fungal growth, sporulation, spore germination and infection and influence the rate of spread and response of the host to infection (Hepting 1963, Agrios 1988).

Acacia spp. tend to have multiple stems and branches (Lee & Arentz 1997). Singling and pruning is thus carried out to maintain good tree form and to avoid stem or branch breakage due to wind damage. These practices also lead to avoidance of higher density stands, so that optimum tree growth can be achieved. *Acacia* spp. also produce multiple leaders, especially after severe pest infestations. Corrective pruning of these multiple leaders needs to be taken into account to maintain good tree form. Wounds created from singling and pruning activities enhance the possibility of these trees being infected by pathogens that cause diseases, such as heart rot cause by a complex of *Phellinus noxius* (Corner) G. Cunn. and other unidentified basidiomycetes (Lee & Noraini Sikin 1999).

The ability of *A. mangium* to self prune is low in areas with high rainfall (Lee & Arentz 1997). From a study by Schmitt *et al.* (1995) on the intensity of wound reactions, it was found that *A. mangium* has low intensity wound reactions compared with the reactions of European hardwoods such as *Betula* and *Tilia*. Both these

conditions further increase the risk of pests and diseases on *Acacia* spp. planted in Indonesia.

Besides using degraded areas, such as grasslands dominated by *I. cylindrica*, plantation development in Indonesia also makes use of forest land provided by the Government as concessions, by clear cutting the forests and converting them to plantations. Plantations established in previously mixed hardwood forests tend to have more problems with pests and diseases, such as termites and root rot. This is due to the availability of inoculum and the huge load of debris left in the field as Indonesia applies a "no burning" policy in land preparation (Lee 1999). The source of pest and pathogen populations/inoculum tends to build up and spread faster in plantations with very homogeneous conditions such as plantations that are of the same species and age.

Numerous diseases have been recorded from plantations of tropical *Acacia* spp. (Table 2), some of these from Indonesia. These include foliar diseases, stem cankers, heart and root rot. Currently, the threat of fungal diseases in forest plantations is believed to be higher than that due to insect attacks (Nair 2001). However, both problems are increasing in magnitude and control strategies need to be developed to minimize and, or avoid risk. The aim of this chapter is to provide a review of the diseases of *Acacia* spp. planted in forest plantations in Indonesia. These species include *A. mangium, A. crassicarpa, A. auriculiformis* and *A. aulococarpa*.

2. Diseases of plantation - grown *Acacia* species in Indonesia

Limited studies have been done on the diseases affecting *Acacia* spp. in Indonesia. Some reports of pathogens are based on herbarium specimens, with the result that not much is known regarding the impact of these fungi. Recently, considerable attention has been given to heart and root rot diseases, which are major constraints reducing the quality of timber produced (Barry 2002, Lee 2003, Potter *et al*. 2006). Other diseases on which some reports exist include stem canker diseases, phyllode rust and leaf diseases. The rest of this section summarizes these reports, providing as much information as is available in the literature pertaining to the Indonesian situation.

2.1. Leaf diseases

2.1.1. Phyllode rust

Phyllode rust, caused by *Atelocauda digitata* (G. Wint) Cummins (Basidiomycota: Pucciniales, Pileolariaceae) was first reported from Indonesia in 1980 (Suharti 1980). It has been reported from Java, Madura, Kalimantan and Sumatra (Suharti 1980, Hadi & Nuhamara 1997, Zulfiyah & Gales 1997). Phyllode rust is considered the most serious foliar disease of *Acacia* spp. in Indonesia (Nair & Sumardi 2000).

Phyllode rust is characterized by the occurrence of rust pustules on phyllodes, shoot tips, petioles and fruit. Stool plants or garden hedges, seedlings, young and mature trees are infected by this pathogen (Suharti 1980, Santoso & Suharti 1984, Hadi & Nuhamara 1997, Old *et al*. 2000). The diseased seedlings become chlorotic and stunted. Thousands of seedlings have been destroyed in nurseries because of phyllode rust. Some diseased seedlings could have been dispatched accidentally to the field and allowed the disease to establish under field conditions. In the field, phyllode rust disease continues to develop and causes malformation of the affected parts. Severely infected foliage is shed prematurely. There are no reports on the impact of phyllode rust after out planting, however, phyllode rust in the field seldom causes tree death (Suharti 1980, Old *et al*. 2000).

Phyllode rust affects both seedlings and young trees of *A. auriculiformis* and *A. mangium* (Suharti 1980, Santoso & Suharti 1984, Zulfiyah & Gales 1997, Nair & Sumardi 2000, Old *et al.* 2000). There are no reports of infection on *A. crassicarpa* in Indonesia. Cannon *et al.* (1997) also reported this disease on *A. aulococarpa*, based on herbarium specimens collected from Indonesia.

It has been seen that differences in susceptibility to phyllode rust exist between different *A. mangium* provenances. These variations provide a chance for selecting resistant genotypes to control this disease (Old 1998). Systemic fungicides, such as Baycor and Plantvax respectively, which have been found to be effective to control other rust fungi in the nursery, are likely to also be effective also in controlling phyllode rust (Old *et al*. 2000).

2.1.2. Leaf and shoot blight

Severe foliar and shoot blight caused by *Passalora perplexa* Beilharz, Pascoe, M. J. Wingf. & Crous (Ascomycota: Capnodiales, Mycosphaerellaceae), an anamorph of *Mycosphaerella* and previously known as *Pseudocercospora* sp., was recently reported from *A. crassicarpa* plantations in Indonesia (Beilharz *et al.* 2004). The disease affected phyllodes, petioles and young shoots. Small, elliptical lesions initially develop on phyllodes, becoming larger with time (Fig 2a-b). As the disease develops, the phyllodes of affected trees start curling and malformation of the leaves occurs if the lesions start from the edges of the phyllodes (Beilharz *et al.* 2004). As more shoots become infected, apical growth is affected, resulting in new sprouting, affecting the growth and form of the trees (Gafur *et al.* 2006). The disease can affect young trees severely; however, the impact is less significant on trees older than 12 months (Gafur *et al*. 2006).

To date, *P. perplexa* has been recorded only on *A. crassicarpa*. This is despite the fact that *A. mangium* occurs in the same areas as *A. crassicarpa* (Beilharz *et al.* 2004). In the field, individual *A. crassicarpa* trees show resistance to the disease (Beilharz *et al.* 2004). This has also been shown through selection (Golani 2006). There are no reports of *P. perplexa* on *A. auriculiformis* and *A. aulococarpa*.

Passalora perplexa is a new pathogen of *A. crassicarpa* plantations in Indonesia (Beilharz *et al.* 2004). As *P. perplexa* has been found in plantations and native stands of *A. crassicarpa* in northern Australia, it has been suggested that the fungus may be indigenous to Australia. In this case, it would most likely have been introduced into Indonesia through seed (Old *et al.* 1997).

2.1.3. Foliar spots

A leaf spot disease resembling *Pestalotiopsis* leaf spot (Ascomycota: Xylariales, Amphisphaeriaceae), has been identified from *Acacia* plantations in Kalimantan and Sumatra (Old *et al*. 2000). The occurrence of *Pestalotiopsis* was also recently reported from *A. crassicarpa* seedlings raised in the nursery in Sumatra (Tjahjono 2006). The disease is characterized by scattered dark or reddish brown spots on the phyllodes, forming large necrotic areas (Fig 2d). In other countries, a similar disease is caused by *P. acaciae* (Thumen) Yokoyama & Kaneko and *P. neglecta* (Thum.) Steyaert,

however, the identity of the species in Indonesia is unkown (Old *et al*. 2000). These leaf spot diseases can lead to defoliation when severe infection occurs (Sharma & Florence 1997), but in Indonesia they are not considered important.

2.1.4. Black Mildew

Black mildew, caused by *Meliola* spp. Fr. (Ascomycota: Melioles, Meliolaceae), are common pathogens on *Acacia* spp. in the tropics, including Australia, Malaysia and Indonesia (Old *et al.* 1997, 2000). In Indonesia, *Meliola brisbanensis* Hansford has been reported from *A. aulococarpa* (Cannon *et al*. 1997) and *M. adenanphererae* Cit. & Hansf. on *A. auriculiformis* in Java (Old *et al*. 2000).

The fungi causing black mildew infects leaves, twigs and stems (Old *et al.* 2000). The disease is considered of minor importance on older trees, however, when younger seedlings are affected it can retard the growth (Nair & Sumardi 2000, Old *et al.* 2000). Symptoms are easily recognized as the fungus forms black and radiate colonies on the surfaces of infected tree parts (Fig 2e) (Old *et al*. 2000). The foliage of the lower crown are infected most frequently (Old *et al*. 2000).

2.1.5. Powdery Mildew

Oidium spp. (Ascomycota: Erysiphales, Erysiphaceae) are obligate parasites and the causal agents of powdery mildew disease on *Acacia* spp. and many other hosts (Nair & Sumardi 2000, Old *et al.* 2000). This disease affects mostly the lower crowns of young *Acacia* trees by covering the surfaces of the leaves with powdery white patches of hypae and spores (Fig 2f). Infection leads to yellow blotches on the foliage and often results in defoliation. Trees and foliage growing under shady conditions are more susceptible to the disease (Old *et al.* 2000).

Powdery mildew is considered of minor importance on older trees (Nair & Sumardi 2000), however, special attention needs to be given if powdery mildew occurs on seedlings in the nursery. For example, it has been reported from Thailand that on seedlings, powdery mildew infection can be high, leading to abnormal growth of seedlings, which can lead to thousands of seedlings being destroyed (Tanaka & Chalermpongse 1990). The disease has been reported from *A. auriculiformis* and *A. mangium* in Indonesia (Cannon *et al*. 1997).

2.1.6. Foliar blotch

Foliar blotch caused by *Guignardia* sp. Viala & Ravaz. (Ascomycota: Botryosphaeriales, Botryosphaeriaceae) and its *Phyllosticta* anamorph has been reported on *A. mangium* in South Sumatra (Old *et al.* 1997). This is the first record of the pathogen on *Acacia* spp. in plantations in Indonesia and it was later reported from *A. aulococarpa* herbarium material in Indonesia (Cannon *et al.* 1997). The symptoms are similar to those caused by *Pestalotiopsis neglecta*, but the pycnidia contain spheroidal conidia which are smaller and colourless. Phyllodes can be severely infected, but no major damage occurs to the trees. In terms of disease potential, this disease is considered as moderately important (Cannon *et al.* 1997). *Guignardia* spp. have been reported to cause severe disease on several trees grown in the tropic, however, there is little data available from *Acacia* plantations in Indonesia (Old *et al*. 2000).

2.2. Stem diseases

2.2.1. Pink disease

Erythricium salmonicolor (Berk. & Broome) Burdsall. (Basidiomycota: Polyporales, Phanerochaetaceae), previously known as *Corticium salmonicolor* Berk. & Broome., is a basidiomycete fungus infecting a wide range of hosts in high rainfall areas of the tropics (Lee 1993). The fungus results in a stem and branch canker disease commonly known as Pink disease. This disease is common in Indonesia and has been reported from *A. mangium* plantations in Kalimantan and Sumatra (Hadi & Nuhamara 1997, Zulfiyah & Gales 1997) and from *A. crassicarpa* and *A. aulococarpa* in Kalimantan (Hadi & Nuhamara 1997). *Acacia mangium* was found to be more susceptible to pink disease compared to other *Acacia* spp. such as *A. crassicarpa*, *A. auriculiformis* and *A. aulococarpa* (Hadi & Nuhamara 1997). In *Acacia* plantations, although infected branches often die, trees are seldom killed (Old *et al*. 2000).

Pink disease affects stems and branches and cause branch and stem dieback due to the formation of girdling cankers (Hilton 1958, Florence & Balasundaran 1991). There are four different stages of disease development for pink disease. The cobweb stage is the initial phase of the disease and is characterised by a white felt-like mycelium growing over the stems or branches. The second phase, known as the pustular stage, is

distinguished by isolated masses of pink mycelium on any part of the canker. The third phase, known as the pink or salmon-colored crustose stage, develops as the pathogen invades the stems or branches and is recognised by the occurrence of a tough crust of pink or salmon-coloured mycelium on the surfaces of affected stems and branches. The last phase, known as the necator stage, is distinguished by the development of red pustules on the upper side of the stems or branches (Hilton 1958).

Pink disease is not currently considered a major constraint to plantation forestry in Indonesia. Disease evaluations in the 1990s showed only a two percent disease incidence on *A. mangium* in South Sumatra (Zulfiyah & Gales 1997). Plantations with high density stands had more pink disease problems, as the disease develops more in shady conditions (Zulfiyah & Gales 1997, Hadi & Nuhamara 1997) as a result of *E. salmonicolor* requiring high relative humidity for germination (Seth *et al*. 1978, Schneider-Chrsitians *et al*. 1986). Higher espacement, singling and pruning applications tend to reduce the pink disease problem (Zulfiyah & Gales 1997). However, planting pink disease-resistant varieties is the best way to prevent pink disease occurrence (Florence & Balasundaran 1991, Lee 1993).

2.2.2. Stem canker diseases

Several fungi have been reported to cause stem canker diseases of commercially planted *Acacia* spp. in Indonesia (Fig 3a-d). These include fungi in the Botryosphaeriaceae, especially *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl., and *Phomopsis* spp. (Sacc.) Bubak. (Hadi & Nuhamara 1997). There are no reports available on the impact of stem canker on *Acacia* plantations, however, the incidence of stem canker can be high as it was commonly observed on the main stems of six-year-old *A. auriculiformis* trial plots in Kalimantan (Hadi & Nuhamara 1997).

Phomopsis spp. have been reported as pathogens causing cankers on *Acacia* spp. in many continents (Boa & Lenne 1994, Roux & Wingfield 1997). In Indonesia, the fungus was isolated from cankers on *A. mangium* in Kalimantan, associated with diffuse cankers bearing black stromata (Hadi & Nuhamara 1997). The identity of the species of *Phomopsis* in Indonesia is unkown and no pathogenicity tests with the fungus have been conducted. *Phomopsis* spp. are known to be associated with cankers on *Acacia* spp., however, in countries such as India, *Phomopsis* spp. also cause leaf

spots on *A. auriculiformis, A. aulococarpa* and *A. crassicarpa* (Mohanan & Sharma 1988, Sharma & Florence 1997). Therefore, species of this genus could potentially also causes leaf spot in Indonesian plantations and regular surveys should be conducted to assess this question.

Lasiodiplodia theobromae is a widely distributed pathogen that causes cankers on many tree species in tropical areas (Punithalingam 1979). In *Acacia* plantations in Indonesia, it has been isolated from cankered tissue of *A. mangium*, *A. auriculiformis* and *A. aulococarpa* in West and South Kalimantan. Symptoms of *L. theobromae* infection include gummosis and severe vertical stem cracking, with no obvious stromata of the fungus present. The ages of affected trees vary from two to six-year-old and the cankers are most common on older trees. Cankers are commonly found at the stem base and at the base of forked branches. It was noticed that stems also break on trees with canker symptoms (Hadi & Nuhamara 1997).

Disease surveys for the occurrence of stem canker disease among provenances of *A. mangium*, *A. aulococarpa* and *A. auriculiformis* in West and South Kalimantan indicated that some provenances were very susceptible to stem canker, while others were more tolerant (Hadi & Nuhamara 1997). These differences provide an opportunity for selecting resistant genotypes or provenances to manage these diseases (Hadi & Nuhamara 1997).

It is known that stem cankers are commonly associated with wounds on stems. These wounds could be caused by wind damage, insect damage and silvicultural practices such as singling, pruning and mechanical weeding using machetes (Old *et al*. 2000, Barry *et al*. 2004). Trees subjected to environmental stress, such as infertile soil, extreme climate and improper silvicultural practices are reported to be more susceptible to stem canker diseases than healthy trees (Old *et al.* 2000). This is especially true in the case of stem cankers caused by the Botryosphaeriaceae. Fungi in this group are known to be stress associated pathogens on many tree species, including commercial tree species in the genera *Eucalyptus*, *Pinus* and *Acacia* (Smith *et al*. 1996, Roux & Wingfield 1997, Gezahgne *et al*. 2003, Mohali *et al*. 2007).

The Botryosphaeriaceae are also known to include several species that are endophytic in their hosts, infecting healthy trees and only causing disease under conditions unsuitable for the host tree (Smith *et al*. 1996). In Indonesia, the incidence of stem cankers was higher in *Acacia* trial plots in South Kalimantan compared to trial plots in West Kalimantan and South Sumatra. This could be due to the fact that trial plots in South Kalimantan, besides using Northern Territory provenances that maybe more susceptible to stem canker, were grown on poorer sites which lead to stressed trees (Hadi & Nuhamara 1997, Zulfiyah & Gales 1997).

2.2.3. Heart Rot

Heart rot is considered one of the most common and problematic diseases affecting tropical *Acacia* spp. (Fig 3e-f). Several wood decay fungi have been associated with heart rot of *A. mangium* trees in East Kalimantan and Sumatra. They are *Rigidoporus hypobrunneus* (Petch) Corner, *Phellinus noxius*, *Tinctoporellus epimiltinus* (Berk. & Br.) Ryv. (Lee & Noraini Sikin 1999), *Oudemansiella canarii* (Jung.) Hohn, *Pycnoporus sanguineus* (L.) Murrill. and *Trametes* sp. Fr. (Barry *et al*. 2006, Glen *et al*. 2006).

Rigidiporus hypobrunneus is the most frequently associated with heart rot on *A. mangium* in Kalimantan (Lee & Noraini Sikin 1999). Based on the sporocarps produced, about 43% of the isolates obtained from *A. mangium* trees with heart rot in Kalimantan were identified as *R. hypobrunneus*. This finding was the first report of *R. hypobrunneus* associated with heart rot on *A. mangium* (Lee & Noraini Sikin 1999). Initially the infected wood turns to white rot, bleached cream in colour and hard, but light in weight and later after some time the infected wood becomes very soft in cross section (Lee & Noraini Sikin 1999).

Phellinus noxius is the second most frequently associated fungus with heart rot on *A. mangium* in Kalimantan, Indonesia (Lee & Noraini Sikin 1999). However, *P. noxius* is the most frequently reported pathogen associated with heart rot on *A. mangium* (Lee & Maziah 1993, Hood 2006). The roots and wood of infected trees are pale in colour and recognized by characteristic brown zone lines which demarcate the root into

pockets, known as the honey-comb sheet structure (Lee & Noraini Sikin 1999, Hood 2006).

Tinctoporellus epimiltinus was identified from only one isolate obtained from *A. mangium* trees with heart rot in Kalimantan. The infected wood was spongy and pale in colour. The fungus produces resupinate and rigid fruiting bodies (Lee & Noraini Sikin 1999). At present there is very little information on *T. epimiltinus* caused heart rot disease on *A. mangium*, however, this pathogen has attracted more concern, as it has been reported associated with root rot on *A. mangium* in Malaysia (Old *et al.* 2000).

Oudemansiella canarii, *P. sanguineus* and *Trametes* sp. were all identified from wounding trials on the *A. mangium* trees with heart rot signs in Sumatra (Glen *et al*. 2006). The role of these fungi in heart rot is not clear, however, they are likely to have a role in heart rot as enzyme tests showed the presence of laccase and tyrosinase, indicating white-rot capabilities (Glen *et al*. 2006).

Several factors in Indonesia are conducive to heart rot development. For example, wounds on stems, branches and roots are infection points for heart rot fungi. Activities such as pruning and singling, or slash weeding using machetes commonly result in such wounds in forestry operations in Indonesia (Barry *et al*. 2004). Furthermore, climatic conditions in the country may influence heart rot incidence and spread, as it has been reported that the absence of a dry season reduces the self-pruning ability of *A. mangium* branches, which then create entry points for heart rot fungi (Lee & Arentz 1997). The fact that some *Acacia mangium* provenances produce more branches, and that they have been planted widely, probably also influences heart rot incidence and spread. Tree age is another factor that influences heart rot incidence, as it has been found that less heart rot occurs on 6-year-old compared to 8-year-old trees (Barry *et al.* 2004).

Symptoms on affected trees are not clearly visible unless stems are sectioned to reveal the rotten heart wood of the tree (Zulfiyah & Gales 1997, Old *et al*. 2000, Gales 2002, Barry *et al*. 2004, Rimbawanto 2006). This destructive assessment is very time

consuming and for survey purposes, it is better to observe heart rot symptoms from logs stacked in plantations following harvest (Barry *et al*. 2004)

It is well known that *A. mangium* is prone to develop heart rot, while *A. auriculiformis* and the hybrid of *A. mangium* x *A. auriculiformis* is resistant (Ito & Nanis 1997, Mohammed *et al*. 2006). Different *A. mangium* provenances also have different levels of susceptibility to heart rot. This has been shown in two trials in Sumatra in which the incidence of heart rot on *A. mangium* differed significantly between the provenances (Barry *et al.* 2006). This presents considerable promise for the development of successful breeding programmes to control heart rot disease on *A. mangium* (Ito & Nanis 1997).

Although the incidence of heart rot disease can be high, the impact of the disease is small, especially for plantations grown for pulp and paper. The affected trees typically do not die, however, the rot results in some volume loss (Old *et al*. 2000, Gales 2002).

2.3. Root diseases

Root disease is common on *Acacia* spp. in Indonesian plantations and is caused by a number of basidiomycetous fungi (Old *et al.* 2000, Hood 2006). These include *Rigidoporus microporus* (Sw.) Overeem. [syn. *R. lignosus* (Klotzsch) Imazeki., *Fomes lignosus* (Klotzsch) Bres.], *Junghuhnia vincta* (Berk.) Hood & E.A. Dick. [syn. *Poria vincta* Speg., *Rigidoporus vinctus* (Berk.) Ryvarden.], *Phellinus noxius* and *Ganoderma philippii* (Bres. & Henn. Ex Sacc.) Bres. [syn. *G. pseudoferreum* (Wakef.) Overeem & B.A. Steinm.] (Zulfiyah & Gales 1997, Old *et al*. 2000, Hood 2006, Potter *et al.* 2006). To date no other root rot diseases such as those caused by *Phytophthora* spp. have been recorded in the country.

Rigidoporus microporus is known as a white rot pathogen as it produces white, branching mycelial cords and a white rot (Hood 2006). The disease has been found associated with dying *Acacia* trees in South Sumatra (Zulfiyah & Gales 1997). Infected trees show crown thinning and eventually die, either singly and or in patches with a concentric pattern of spread (Zulfiyah & Gales 1997, Arisman & Hardiyanto 2006). Another white rot disease is caused by *J. vincta*, which produces symptoms similar to those caused by *R. microporus*. The two pathogens can be distinguished

based on their fruiting bodies. *R. microporus* produces a broad fruiting body with an upper surface that is initially orange red to brown and a lower surface that is bright orange to brown, while *J. vincta* produces pinkish or greyish brown fruiting bodies (Hood 2006).

Phellinus noxius causes brown rot of infected trees, covering the roots with a brown mycelium. It also results in a collar-like structure around the bases of the stems. The rotten roots have a pale colour with a typical honey comb sheet of brown zone lines (Hood 2006). *P. noxius* produces a cinnamon brown, broadly attached fruiting body with a blackish crust on the upper surface and a greyish to dark brown colour on the lower surface. *P. noxius* is considered as a less significant root pathogen, but plays an important role in the development of heart rots (Hood 2006).

Root rot caused by *Ganoderma* spp. is the most common form of the disease on *A. mangium* in Indonesia (Lee 2002). *Ganoderma* spp. cause red rot disease on trees, named as such because the roots of infected trees are covered by a reddish brown rhizomorphic "skin", which can be clearly seen when the roots are washed clean (Old *et al*. 2000). Through DNA based molecular methods, *Ganoderma philippii* has been confirmed as the causal agent of root rot disease on *A. mangium* (Glen *et al*. 2006).

Ganoderma root rot has been recorded from *A. auriculiformis*, *A. crassicarpa* and *A. mangium* plantations. Initially the crowns of infected trees turn pale green to yellow, where after the trees wilt completely and eventually tree death follows (Fig 4a) (Old *et al*. 2000). Examination of the root system shows that infected roots are reddish in colour and covered with mycelium (Old *et al*. 2000). In older trees, the fungi produce fruiting bodies around the bases of the stems after the bark and wood starts to decay (Old *et al*. 2000). Fruiting bodies are broadly attached, dark reddish or purplish brown with white margins on the upper surfaces and white or brownish lower surfaces (Figs 4b, c) (Hood 2006).

Infection of *Acacia* trees by *Ganoderma* spp. generally results in tree death (Old *et al*. 2000). It was reported that root rot diseases causes early losses in subsequent plantings. From eight percent of trees in the first rotation to 20 percent in the second rotation can die as a result of root rot (Rimbawanto 2002). This figure clearly

indicates that root rot disease increases over time and as the inoculum builds up. In the second rotation, young trees have been reported to be killed within six months after planting (Old *et al*. 2000). With all these facts, root rot disease caused by *Ganoderma* spp. is considered as a major threat to forest plantations in Indonesia (Potter *et al*. 2006).

It is known that all root rot diseases on *Acacia* spread via root contact between healthy trees and diseased trees (Lee 2000, Old *et al*. 2000). From the infection point, the numbers of dying and dead trees increase, thus expanding the patch size and increasing the source of inoculum. The incidence of root rot is higher in *A. mangium* plantations that are planted in ex-hardwood forest areas when compared to plantations that are planted on ex-grasslands. This is due to a higher inoculum source in ex-hardwood forest areas (Lee 1999).

3. Conclusions

Monocultures of any type, without intensive selection and management programmes, are threatened by the appearance of pest and disease problems. Factors contributing to an increased risk in plantation health problems include off-site planting, uniform unimproved genetic stock and plantation management, such as pruning, that cause wounding. Several factors in Indonesia may impact on pest and disease outbreaks on *Acacia* spp. These trees have been planted and established largely on the Islands of Sumatra and Kalimantan, islands where they did not occur naturally. *Acacia* spp. are thus non-native in the areas where they are grown commercially, adding additional factors that could increase the risk due to diseases.

It is clear from the preceding review that very little information is available regarding the causes of *Acacia* mortality in Indonesia. Over the last few years, heavy mortality has been observed and new diseases have been found in commercial stands of *Acacia* spp. in Sumatra. Few studies have, however, been initiated to identify and study the cause of these diseases or to develop management plans to reduce their impact.

The aim of the thesis that follows this review is to increase the available knowledge regarding fungal pathogens affecting *Acacia* spp., in particular *A. mangium* in Indonesia. During disease surveys conducted by the author, symptoms resembling those caused by Botryospheriaceae and *Ceratocystis* spp. were identified. The

research portions of this thesis focus on these diseases and the identification of their causal agents. Studies were also undertaken to understand the aetiology of the diseases, allowing management plans to be adapted for better disease control.

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Table 1. Tree species and area planted by some Indonesian HTI companies in 2000.

Species	Area (ha)	Percentage
Acacia mangium	443 535	64.2
Other *Acacia* spp.	24 023	3.5
Azadirachta excelsa	18 463	2.7
Dipterocarps	2977	0.4
Eucalyptus spp.	29 821	4.3
Gmelina arborea	47 790	6.7
Hevea braziliensis	8293	1.2
Maesiopsis eminii	282	>0.1
Octomeles sumatrana	4 456	0.7
Paraserianthes falcataria	48401	7.0
Peronema canescens	4963	0.7
Swietenia macrophylla	244	>0.1
Tectona grandis	1966	0.3
Miscellaneous species	55 213	8.0
Total	690 528	100

Source: Cossalter & Nair 2000.

Table 2. Diseases of tropical *Acacia* spp. and their associated fungi.

Disease	Pathogen	Host	Geography	Reference
Foliar diseases				
Phyllode rust	*Atelocauda digitata*	*A. aulacocarpa, A. auriculiformis, A. acrassicarpa, A. mangium*	China, Hawaii, Indonesia, Malaysia, New Zealand, Papua New Guinea,	Suharti 1980, Santosa & Suharti 1984, Old 1998, Old *et al*. 2000
	Cercospora sp. Fresen	*A. auriculiformis, A. mangium.*	Australia, India, Thailand	Pongpanich 1997, Old *et al.* 2000
	Colletotrichum gloeosporioides (Penz.) Sacc.	*A. aulacocarpa, A. auriculiformis, A. crassicarpa A. mangium*	India, Malaysia, Thailand	Maziah 1990, Pongpanich 1997, Sharma & Florence 1997, Old *et al.* 2000
	Cylindrocladium sp. Morgan, *C. quinquespetatum* Boedijn & Reitsma	*A. auriculiformis, A. longifolia* (Andr.) Willd., *A. mangium*	Australia, India, South Africa, Sri Lanka	Bertus 1976, Hagemann & Rose 1988, Abraham *et al*. 1996, Sharma & Florence 1997, Mohanan & Sharma 1988, Old *et al.* 2000
	Exserohilum rostratum (Drechsler) K.J. Leonard & Suggs.	*A. auriculiformis*	India	Mohanan & Sharma 1988
Mildew	*Meliola brisbanensis, M. adenanphererae*	*A. auriculiformis, A. mangium, A. dealbata* Link.	Australia, India, Indonesia, Malaysia	Singh 1980, Old *et al*. 1997, Mohanan & Sharma 1988, Old *et al.* 2000
	Oidium sp.	*A. aulacocarpa, A. auriculiformis, A. crassicarpa A. mangium*	Africa, Australia, China, Hawaii , India, Indonesia, Malaysia, Philippines, Thailand	Maziah 1990, Tanaka & Chalermpongse 1990, Boa & Lenne 1994, Old *et al*. 1997, Old *et al.* 2000
	Passalora perplexa	*A. crassicarpa, A. flavescens* Cunn. Ex Benth	Australia, Indonesia	Cannon *et al*. 1997, Old *et al*. 1997, Beilharz *et al*. 2004

Table 2. (Continued)

	Pestalotiopsis sp., *P. acaciae, P. neglecta*.	*A. aulacocarpa, A. auriculiformis, A. crassicarpa, A. mangium*	Australia, India, Indonesia, Malaysia, Vietnam	Old *et al.*1997, Sharma & Florence 1997, Old *et al.* 2000
	Phaeotrichoconis crotalariae (Salam & Rao) Subram	*A. auriculiformis, A. crassicarpa*	Australia, Florida, India	Mohanan & Sharma 1988, Old *et al*.1996
	Phyllosticta sp., *Guignardia* sp.	*A aulacocarpa, A. mangium*	Australia, Indonesia	Cannon *et al*. 1997, Old *et al*. 1997,
	Phomopsis sp.	*A. aulacocarpa, A. Auriculiformis, A. crassicarpa*	India	Mohanan & Sharma 1988, Sharma & Florence 1997
Heart rot	*Oudemansiella canarii*	*A. mangium*	Indonesia	Glen *et al*. 2006
	Oxyporus latemarginatus (Durieu & Mont.) Donk	*A. mangium*	Malaysia	Lee & Noraini Sikin 1999
	Phellinus noxius	*A. mangium, A. auriculiformis*	India, Indonesia, Malaysia, Thailand	Lee & Maziah 1993, Mehrotra *et al*. 1996, Old *et al.* 2000
	Pycnoporus sanguineus	*A. mangium*	Indonesia	Glen *et al*. 2006
	Rigidiporus hypobrunneus	*A. mangium*	Indonesia, Malaysia	Lee & Noraini Sikin 1999
	Tinctoporellus epimiltinus	*A. mangium*	Indonesia, Malaysia	Lee & Noraini Sikin 1999
	Trametes sp.	*A. mangium*	India, Indonesia	Mehrotra *et al*. 1996, Glen *et al*. 2006

Table 2. (Continued)

Root rot	*Ganoderma philippii*	*A. aulacocarpa, A. auriculiformis, A. crassicarpa, A. mangium*	India, Indonesia, Malaysia, Papua New Guinea	Arentz 1990, Barari 1993, Lee 1997, Old *et al.* 2000, Lee 2002, Glen *et al.* 2006
	Junghuhnia vincta	*A. mangium*	Indonesia	Hood 2006
	Phellinus noxius	*A. aulacocarpa, A. auriculiformis, A. crassicarpa, A. mangium*	Indonesia, Malaysia, Philippine	Khamis 1982, Almonicar 1992, Lee 1997, Old *et al*. 2000, Hood 2006
	Rigidiporus microporus	*A. aulacocarpa, A. auriculiformis, A. crassicarpa, A. mangium*	Indonesia	Lee 1997, Zulfiyah & Gales 1997, Old *et al*. 2000, Hood 2006
Stem cankers/ Pink disease				
	Botryosphaeria sp.	*A. auriculiformis, A. aulococarpa*	Thailand	Pongpanich 1997
	Erythricium salmonicolor	*A. aulacocarpa, A. auriculiformis, A. crassicarpa, A. mangium*	India, Indonesia, Malaysia	Singh 1980, Florence & Balasundaran 1991, Lee 1993, Hadi & Nuhamara 1997, Sharma & Florence 1997, Zulfiyah & Gales 1997
	Lasiodiplodia theobromae	*A. auriculiformis*	Indonesia	Hadi & Nuhamara 1997
	Nattrassia mangiferae (Syd. & P. Syd.) B. Sutton & Dyko	*A. auriculiformis, A. mangium*	India	Sharma & Florence 1997
	Phomopsis sp.	*A. auriculiformis, A. mangium*	Indonesia	Hadi & Nuhamara 1997

Fig 1. Map of Indonesia. Indonesia is an archipelago country in the equatorial zone consisting of about 17000 islands that span over 4800 km between the Indomalayan and Australasian realms. The five biggest Indonesian islands from West to East are Sumatra, Java, Kalimantan, Sulawesi and Papua.
(Source: http://www.smartraveller.gov.au/)

Fig 2. Foliar diseases of tropical *Acacia* spp. and their associated fungi. (a, b). Leaf and shoot blight caused by *Passalora perplexa*, (c). Bacterial leaf streak caused by *Xanthomonas* sp., (d). Leaf spot caused by *Pestlotiopsis* spp., (e). Black mildew caused by *Meliola* spp., (f). Powdery mildew caused by *Oidium* spp.

Fig 3. Stem diseases of tropical *Acacia* spp. and their associated fungi. (a). Shoot die-back caused by *Lasiodiplodia theobromae*, (b). Stem canker caused by *L. theobromae*, (c, d). Branch and stem breakage due to stem canker infection, (e, f). Heart rot infection on 7 year old *A. mangium*.

Fig 4. Root diseases of tropical *Acacia* spp. and their associated fungi. (a). Trees dying in a group, (b, c). Fruiting bodies of *Ganoderma* sp. on stems of *A. mangium*, (d). Stem breakage due to root rot caused by *Ganoderma* sp.

Chapter 2

A new wilt and die-back disease of *Acacia mangium* associated with *Ceratocystis manginecans* and *C. acaciivora prov. nom.* in Indonesia

ABSTRACT

Species of *Ceratocystis* are well-known wound related pathogens of many tree species, including commercially planted *Acacia* spp. Recently, several *Ceratocystis* isolates were collected from wilting *A. mangium* in plantations in Indonesia. The aim of this study was to identify these *Ceratocystis* isolates and to investigate their ability to cause disease on two plantation-grown *Acacia* spp. using greenhouse and field inoculation experiments. For identification, morphological characteristics and comparisons of DNA sequence data for the ITS, β-tubulin and Elongation Factor 1-α gene regions, was used. Two species were identified including *C. manginecans*, a serious pathogen of mango trees in Oman and Pakistan, and a previously undescribed species in the *C. fimbriata sensu lato* complex, described here as *C. acaciivora prov. nom*. This is the first report of *C. manginecans* from a host other than mango. Both *C. manginecans* and *C. acaciivora prov. nom* gave rise to lesions on *A. mangium* and *A. crassicarpa*. However *C. acaciivora prov. nom*. was most pathogenic and the results suggest that this fungus is responsible for the death of trees under natural conditions.

Keywords: *Acacia crassicarpa, Ceratocystis,* forestry, stem canker, wounds.

INTRODUCTION

Species of *Ceratocystis* have been reported to cause root rots, stem rots, vascular stains, cankers and fruit or pod rot on many plants especially trees in tropical parts of the world (Kile 1993). *Ceratocystis fimbriata* Ellis & Halst *sensu lato* (*s.l*), for example causes disease and death of *Eucalyptus* trees in the Republic of Congo and Brazil (Roux *et al*. 2000), *Gmelina arborea* Roxb. and *Colocasia esculenta* (L.) Schott in Brazil (Muchovej *et al*. 1978, Harrington *et al*. 2005) and *Coffea* sp. in Colombia and Venezuela (Pontis 1951). This fungus has also been reported to cause mango decline or 'seca' disease in Brazil (Viegas 1960, Ribeiro 1980, Ploetz 2003) and it is considered one of the most important pathogens of agricultural and tree crops in South America.

In Indonesia, *Ceratocystis* spp. was first noted when *C. fimbriata* (reported as *Rostrella coffeae* Zimm.) was reported in 1900 on *Coffea arabica* L. on the island of Java (Zimmerman 1900). Subsequently, various species of *Ceratocystis* have been reported from other hosts on many Indonesian islands. Examples include *C. fimbriata* [reported as *Sphaeronema fimbriatum* (Ellis & Halst.) Sacc.] from *Hevea brasiliensis* Müll. Arg. in Sumatra, Kalimantan and Java (Wright 1925, Tayler & Stephens 1929, Leefmans 1934), *C. polychroma* M. van Wyk, M.J. Wingf. & E.C.Y. Liew from *Syzygium aromaticum* (L.) Merrill & Perry in Sulawesi (Van Wyk *et al*. 2004) and *C. tribiliformis* M. van Wyk & M.J. Wingf. from *Pinus merkusii* Jungh. & de Vriese in Sumatra (Van Wyk *et al*. 2006a).

As a result of an increased international demand for pulp and paper products, plantation industries are proliferating rapidly in many parts of the world, including Indonesia (Barr 2001, Anonymous 2004). *Acacia mangium* Willd. and *A. crassicarpa* Cunn.: Benth., native to the eastern islands of Indonesia, northern Queensland and the western Province of Papua New Guinea, are two of the most widely planted species in Indonesia (Doran & Skelton 1982, Turnbull 1986, Moran *et al*. 1989). While these trees have displayed excellent growth in the region, they have also been negatively affected by various diseases (Old *et al.* 2000, Potter *et al*. 2006).

Ceratocystis spp. have increasingly been reported as the cause of wilt and canker of plantation-grown *Acacia* spp. in many areas, world-wide. In Brazil, *C. fimbriata s.l.* has been reported to cause canker and wilt of *A. decurrens* Willd. (Ribeiro *et al.* 1988) and in South Africa, *C. albifundus* M.J. Wingf., De Beer & Morris is considered the most important pathogen of plantation-grown *A. mearnsii* De Wild. and *A. decurrens* trees (Morris *et al*. 1993; Wingfield *et al.* 1996; Roux & Wingfield 1997, Roux *et al*. 1999).

During the course of recent disease surveys in *A. mangium* plantations in Indonesia, significant mortality of young trees showing rapid wilt symptoms has been observed. Isolates of *Ceratocystis* species were collected from stained vascular tissue on these trees. The aim of this study was to identify these *Ceratocystis* isolates and to consider their ability to cause disease on two plantation-grown *Acacia* spp. using greenhouse and field inoculation experiments.

MATERIALS AND METHODS

Disease and fungal isolates

Wilt and canker symptoms were commonly observed on young *A. mangium* trees in plantations. The bark and the wood surrounding the cankers was discolored and had a black appearance due to the exudation of gum. The discolored wood typically had a streaked appearance, turning a uniform dark brown to dark blue color with age (Fig 1). Affected trees ranged from 8 to 12 months in age. The disease was observed in several plantation areas, including Teso, Logas and Pelalawan, all in the Riau Province of Sumatra. All diseased trees had been pruned 6 – 12 weeks earlier and lesions appeared to originate from these wounds, suggesting the involvement of a wound colonizing pathogen.

Twenty diseased *A. mangium* trees were selected randomly and sampled in plantations at Teso and Logas. Sections of discolored wood and bark were cut from the leading edges of cankers. These were then wrapped in newspaper to maintain moisture, and taken to the laboratory for examination.

In addition to isolations from naturally infected *A. mangium*, artificially induced wounds were made on the stems of 15 one-year-old *A. mangium* and *A. crassicarpa* at Teso and Pelalawan using a procedure described by Barnes *et al*. (2003). Five weeks after wounding, slices of discolored wood were removed from the wound sites and taken to the laboratory for further study. These were incubated in moist chambers to induce the production of ascomata.

Single spore drops, characteristic of *Ceratocystis* spp., were collected directly from fungal fruiting bodies from the diseased and wound samples onto 2 % (w/v) Malt Extract Agar (MEA) (Biolab, Midrand, South Africa). Where no fruiting structures were visible, a subset of samples was placed in plastic bags containing moistened tissue paper for 4-10 days to induce sporulation. Another subset of samples was baited for *Ceratocystis* spp. using carrot slices (Moller & De Vay 1968). Symptomatic wood pieces were wrapped between carrot slices that had first been immersed for 10 min in a 0.001 g vol^{-1} streptomycin sulfate solution (SIGMA, Steinheim, Germany) and then placed in plastic bags and incubated for 3-5 days, or until fruiting bodies were observed.

Isolates collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates have also been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Delft, Netherlands. Dried specimens were deposited at the National Fungal Herbarium of South Africa (PREM), Pretoria.

DNA extractions

Twelve isolates of the *Ceratocystis* spp. from *A. mangium* were selected randomly and grown on 2 % MEA at 22 ºC for two weeks. The mycelium was scraped off the surface of the agar using sterilized scalpel blades and transferred to 1.5 ml Eppendorf tubes and lyophilized overnight. The lyophilized mycelium was crushed to a fine powder using a glass rod or mechanical grinder (Ball mills machine, Retsch, Haan, Germany). The DNA was extracted using the method described by Van Wyk *et al*. (2006b).

PCR amplification, sequencing and analyses

The Internal Transcribed Spacer regions (ITS), including the 5.8S gene of the ribosomal DNA (rDNA) operon, part of the β-tubulin (βt) and Transcription Elongation factor 1-α (EF1-α) gene regions were amplified using polymerase chain reaction (PCR) and an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). Amplification of the gene regions were made using the primers ITS1 and ITS4 (White *et al.* 1990), βt1a and βt1b (Glass & Donaldson 1995) and EF1-728F and EF1-986R (Carbone & Kohn 1999) respectively. PCR reaction mixtures consisted of 0.5 µL of each primer (10 mM), 2 µL of 10 mM dNTP mixture (2.5 mM of each dNTP), 0.5 µL DNA Fast Start Taq enzyme (Roche Molecular Biochemicals, Almeda California), 2.5 µL of a 10x concentration buffer containing $MgCl_2$ (3.5 mM), 2 µL of DNA template (2-10 ng) and 17 µL sabax water to make up 25 µl total volume reactions. The PCR program, product visualization and DNA purification were carried out following methods described by Van Wyk *et al*. (2006b).

An ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) was used to sequence the purified PCR amplicons in both the forward and reverse directions. Sequencing of each gene region was done using the same primers as those used for the PCR reactions. After cleaning the sequence products, using the same protocol as those used for the PCR reactions, the final sequence products were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems, Foster City, California). Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California) was used to analyze the sequence electropherograms. Using Blast analyses, the sequences obtained were compared with sequences of *Ceratocystis* spp. that are available in GenBank (The National Centre for Biological Information, http://www.ncbi.nlm.nih.gov/) (Table 1). The online version of MAFFT (Katoh *et al*. 2002) version 6 (http://align.bmr.kyushu-u.ac.zp/mafft/online/server/) was used to align the sequences of the Indonesian isolates with published sequences of closely related species identified using Blast and the alignments were then checked manually. A partition homogeneity test was run using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10* to determine whether the data for the three gene regions could be combined into a single data set (Swofford 2002). Data for each data set were analyzed using PAUP version 4.0b10* (Swofford 2002).

Gaps were treated as "fifth base" and trees were obtained via stepwise addition of 1000 replicates with the Mulpar option in effect. Bootstrap confidence intervals, using 1000 replicates, were calculated. Two *Ceratocystis moniliformis* (Hedgc.) C. Moreau isolates were used as the out-group. MrBayes version 3.1.1. (Ronquist & Huelsenbeck 2003) was used to run Bayesian analyses on the combined data set. The Markov Chain Monte Carlo (MCMC) algorithm was used to calculate support of the nodes of the phylogenetic trees based on Bayesian posterior probabilities. The model of nucleotide substitution for each gene region was determined using MrModeltest2 (Nylander 2004), and the models obtained were used in the Bayesian analysis. One million random trees were generated with four chains and sampled every 100th generation following the MCMC procedure. Samples were taken only from trees after convergence and trees outside the point of convergence were discarded.

Culture Characteristics and Morphology

Three isolates from each of the groups identified based on DNA sequence comparisons were grown on 2 % MEA for two weeks at 22 ºC for growth studies. A five mm diameter cork borer was used to cut discs of mycelium from the margins of actively growing cultures. These were placed at the centers of 90 mm diameter Petri dishes containing 2 % MEA. Discs for each isolate were placed at the centre of five plates and incubated at 4 ºC and from 10 ºC to 35 ºC at 5 ºC intervals. Two measurements of colony diameter, at right angles to each other, were taken seven days after incubation and averages for all measurements were computed. The entire experiment was repeated once. Data from both studies were combined and analyzed statistically using analysis of variance (ANOVA).

Two-week-old cultures grown on 2 % MEA were used to describe the morphological characteristics of the isolates. Fruiting structures were mounted in lactic acid (85 %) for observation and measurements. Fifty measurements of all characteristic structures from each representative isolate (extype) and 10 measurements for one additional isolate in each group, were made with a Carl Zeiss microscope and a Zeiss Axiovision camera system (Oberkochen, Germany). The average (mean), standard deviation (stdv), maximum (max) and minimum (min) measurements were calculated and they are presented as follows: (min-) mean minus stdv – mean plus stdv (-max). The color of the cultures was described using Rayner's color charts (Rayner 1970).

Pathogenicity tests

Greenhouse inoculations **–** Pathogenicity tests were carried out on one-year-old *A. mangium* and *A. crassicarpa* seedling plants (~ 15 mm diam.) in a greenhouse. Trees were grown in 20 cm diameter plastic bags containing a mixture of topsoil and compost. The temperature and humidity of the greenhouse was adjusted to ~30 ºC and 65 % respectively for optimum growth of the seedlings. Five isolates (CMW22563, CMW22564, CMW22581, CMW22595, CMW22621) representing Group 1 and four isolates representing Group 2 (CMW21123, CMW21125, CMW21127, CMW21132) identified using DNA sequence data were used for the inoculations. Five seedlings of each *Acacia* sp. was used for each isolate. Wounds were made on the stems of the seedlings using a cork borer (4 mm diam.) and an agar disc taken from an actively growing colony, on 2 % MEA with the mycelium facing downwards, was placed in the wound. These were covered with Parafilm (Pechiney, Menasha, Wisconsin) to reduce contamination and desiccation. Five plants of each tree species were inoculated with sterile MEA plugs to serve as controls.

The Parafilm was removed from the stems of treated plants 10 days after inoculation and the lengths of the lesions produced in the xylem were measured. Pieces of symptomatic stem tissue were taken from the inoculated trees and these were placed into moist chambers as well as baited using carrots as described previously, to induce the production of fruiting structures. Spores from these structures were then transferred to 2 % MEA to verify their identity. Analysis of variance (ANOVA) was calculated for all data obtained using SAS statistical analyses (SAS Version 8.2, 2001).

Field inoculations **–** One-year-old *A. mangium* and *A. crassicarpa* trees growing in plantations in Riau Province of Sumatra, ranging in diameter from 70 mm to 90 mm, were used in field pathogenicity tests. Inoculations were made on the stems, 1.3 m from the ground. Three isolates from each of the two *Ceratocystis* groups, found to be the most pathogenic in the greenhouse tests were used for field inoculations. Twenty trees of each *Acacia* sp. were used for each isolate and an equal number of trees were inoculated as controls. Prior to inoculation, a wound was made on the tree stems using a sterilized cork borer (10 mm diam.) and inoculations were carried out using the same technique as described for the greenhouse trials. Control inoculations were made

using sterile 2 % MEA plugs. After inoculation, the wounds were sealed with masking tape to reduce contamination and desiccation of the inoculum and wounds.

Six weeks after inoculation the tree diameter at the inoculation point and the length of lesions produced on the stems were measured. Pieces of symptomatic tissue from the area associated with the inoculation points for a representative set of trees and isolates were taken and placed in moist chambers to induce sporulation and to confirm that the lesions were associated with the inoculated fungus. The entire field inoculation trial was repeated once. Data obtained were analyzed with analysis of variance (ANOVA) using SAS statistical analyses (SAS Version 8.2, 2001).

RESULTS

Fungal isolates

A total of 77 *Ceratocystis* isolates were obtained from artificially wounded trees and diseased trees. These included 10 isolates from artificially wounded *A. crassicarpa* trees at Teso and 21 isolates from the *A. mangium* trees wounded in Pelalawan. The remaining 46 isolates were from diseased *A. mangium* at Teso and Logas. Within two weeks, they produced mature perithecia containing hat-shaped ascospores and a *Thielaviopsis* anamorph when grown on 2 % MEA.

PCR amplification, sequencing and analyses

For both the ITS and βt gene regions, PCR amplification resulted in fragments of ~500 base pairs (bp) in size, while for the EF1-α gene region, amplification resulted in fragments of ~800 bp in size. Blast searches in GenBank indicated that isolates from Indonesia grouped within the *C. fimbriata s.l.* species complex and were most closely related to *C. manginecans* M. van Wyk, A Al-Adawi, & M.J. Wingf. The partition homogeneity test for the three data sets from ITS, βt and EF1-α resulted in a P value of 0.01, which is an acceptable level (Cunningham 1997, Barker & Lutzoni 2002) to combine the data. The combined data set consisted of a sequence with a total length of 2063 characters, including gaps. The dataset contained 1172 constant characters, 52 parsimony-uninformative characters and 839 parsimony-informative characters. One hundred forty-four most parsimonious trees were obtained of which one (Fig 2) was selected for presentation. This tree had a length of 1630, a consistency index (CI) =

0.7479, a homoplasy index (HI) = 0.2521, a retention index (RI) = 0.7395 and a rescaled consistency index (RC) = 0.6479.

Model test analyses suggested a HKY + G model [Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 2 rates = gamma] for the ITS gene region, GTR + G model [Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 6 rates = gamma] for the βt gene region and for the EF1-α region a HKY + I + G model [Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 2 rates = invgamma]. These models were then used in Bayesian analyses. The first 2000 trees obtained with Bayesian analyses were discarded following the burn-in procedure. Both Bootstrap and Bayesian values were attached to the posterior probability of the branch nodes of the combined dataset (Fig 2).

The isolates from *Acacia* resided in two sub-clades, designated Group 1 and Group 2, (Fig 2) within the larger *C. fimbriata s.l.* species complex and most closely related to *C. manginecans.* The Group 1 (CMW22562, CMW22563, CMW22564, CMW22595, CMW22621) isolates from *Acacia* spp. in Indonesia formed a well resolved and distinct group. They had posterior probability support for the branch nodes of 87 % and 88 % Bootstrap and Bayesian values respectively. Group 2 (CMW21123, CMW21125, CMW21127, CMW21132, CMW22579, CMW22581) had no Bootstrap or Bayesian support and this sub-clade grouped closest to *C. manginecans* with a Bootstrap and Bayesian support of 94 % and 96 % respectively. Based on their low values of support, the Group 2 *Acacia* isolates are considered to represent *C. manginecans.*

C. manginecans and the two clades from Indonesia could be distinguished based on several sequence differences. Indonesian isolates in Group 2 differed from *C. manginecans* in only 3 base points (bp) for the ITS region, none for the βt and 2 bp for the EF1-α, and from those in Group 1 they differed with 9 bp (5 bp single nucleotide and 4 bp a block nucleotide) for the ITS regions, and none for the βt and EF1-α sequences. Group 1 isolates from Indonesia differed from *C. manginecans* in 12 bp, with 5 bp single nucleotide differences and 2 blocks of 3 bp and 4 bp each respectively for the ITS regions, none for the βt and two bp single nucleotides for the EF1-α region.

Culture Characteristics and Morphology

Ceratocystis isolates representing the two sub-clades from *A. mangium* and *A. crassicarpa* in Indonesia, recognized based on DNA sequence comparisons, are similar in culture characteristics and morphology, however, some differences were also found. Group 1 isolates grew slightly faster than those representing Group 2. Isolates of both groups showed similar responses to temperature, all having optimal growth at 25 °C. Group 1 isolates reached a diameter of 34 mm and Group 2 isolates reach 29 mm in seven days. For all isolates growth was limited at 15 °C and no growth occurred at 4 °C, 10 °C and 35 °C (Fig 3). Both of the Indonesian groups and *C. manginecans* have similar responses to temperature, all having optimal growth at 25 °C, however, *C. manginecans* has a lighter culture color. Cultures representing both groups of Indonesian isolates were olive green (23m) and produced both teleomorph and anamorph structures within 1-2 weeks. The cultures placed in sealed plastic boxes produced a weak fruity aroma which disappeared when the boxes had been opened for some time.

Ceratocystis isolates from Indonesia were typical of *Ceratocystis* spp. They had black ascomatal bases which were globose to sub globose in shape. All isolates produced ascomatal necks with divergent ostiolar hyphae at their tips, from which hat shaped ascospores exuded. Group 1 isolates had smaller ascomatal bases and shorter necks than those in Group 2 and *C. manginecans* (Table 2). Group 2 isolates had ascomatal bases and ascomatal necks within the same size ranges when compared to *C. manginecans.*

Pathogenicity tests

Greenhouse inoculations – Ten days after inoculation, all *Ceratocystis* isolates used in the pathogenicity trials produced distinct lesions on *A. crassicarpa* and *A. mangium* seedlings. The lesion lengths ranged from 17 – 345 mm on *A. crassicarpa* and 14 – 151 mm on *A. mangium.* Isolates representing Group 1 produced longer lesions on both *A. crassicarpa* and *A. mangium* compared to those of Group 2 (Fig 4). Two isolates (CMW21123 and CMW21132) representing Group 2 consistently produced only small lesions that were not significantly different to the controls (Fig 4). *Ceratocystis fimbriata s.l.* isolates were consistently re-isolated from the treated plants but they were not retrieved from the controls. Three of the most pathogenic isolates

for Group 1 (CWM22563, CMW22564, CMW22621) and for Group 2 (CMW21125, CMW21127, CMW21132) were selected for field inoculation.

Field inoculations **-** Within six weeks of inoculation, all *Ceratocystis* isolates produced lesions both on *A. mangium* and *A. crassicarpa* trees. The lesion lengths ranged from 100-660 mm on *A. crassicarpa* and 130-680 mm on *A. mangium* in the first trial (Fig 5). In the second trial the lesions ranged from 80-380 mm on *A. crassicarpa* and 70-290 mm on *A. mangium* (Fig 6). Although lesions in the second trial were smaller than those in the first trial, both trials produced similar trends where all isolates in Group 1, except CMW22621, consistently produced longer lesions, both on *A. crassicarpa* and *A. mangium,* than isolates in Group 2 (Figs 5, 6, 7). Isolate CMW21125 (Group 2) consistently produced significantly longer lesions compared to the controls on *A. crassicarpa* and *A. mangium* in both trials. Other isolates (CMW21127 and CMW21132) residing in Group 2 consistently produced lesions that were significantly larger than those of the controls when inoculated into *A. crassicarpa* in both trials, but they produced lesions that were not significantly different to the controls when they were inoculated on *A. mangium* trees. Re-isolation from lesions on inoculated trees consistently yielded *Ceratocystis* isolates that were morphologically similar to the test fungi. No *Ceratocystis* isolates were collected from the control trees.

TAXONOMY

Based on morphology and DNA sequence comparisons, the results show that Group 1 isolates from diseased *A. mangium* in Indonesia represent an undescribed taxon. It is consequently described as follows:

Ceratocystis acaciivora Tarigan, M. Van Wyk & M.J. Wingf. *prov. nom.*

(Fig 8)

Etymology: The name refers to ability of this fungus to infect *Acacia* species.
Stat.conid.: *Thielaviopsis*

Coloniae olivaceae, *mycelium* aerium. *Hyphae* laeves, septis non constrictis. *Bases ascomatum* pallide brunneae, atrobrunneae vel nigrae, globosae vel subglobosae

(105-) 131-175 (-206) µm longae, (107-) 125-167 (-188) µm latae, hyphis ornatae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallentia (301-) 348-448 (-522) µm longa, basi (25-) 33-45 (-53) µm, apice (11-) 13-17 (-20) µm lata, basi discoidea. *Hyphae ostiolares* divergentes hyalinae (30-) 35-49 (-60) µm longae. *Asci* non visi. *Ascosporae* in massis fulvo-flavescentibus mucosis in apicibus collorum ascomatum. Ascosporae lateraliter visae cucullatae hyalinae, non septatae, vaginis inclusae, 5-7 x 3-4 µm cum vagina, 4-6 x 3-4 µm sine illa.

Anamorpha Thielaviopsis: *conidiophorae primariae* in mycelio singulae, hyalinae, basi incrassatae, apicem versus angustatae, (53-) 61-95 (-127) µm longae, basi 4-6 µm, medio (2-) 5-7 (-8) µm, apice 3-6 µm latae. *Conidiophorae secondariae* in mycelio singulae hyalinae (52-) 57-69 (-68) µm longae, basi (3-) 2-4 (-5) µm, apice 4-6 µm latae. *Conidia* biformia: primaria hyalina cylindrica non septata (11-) 14-22 (-29) x 3-5 µm; secondaria hyalina doliiformia non septata (8-) 9-11 (-13) x 4-6 µm. *Chlamydosporae* ovales, parietibus crassis, laeves, brunneae, (10-) 12-14 (-15) x (7-) 8-12 (-14) µm, in agaro inclusae, terminales, singulae vel in catenis.

Typus: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (PREM59884 – holotype, living culture-extype: CMW 22563).

Colonies olive green (23m) in color. *Mycelium* aerial. *Optimal temperature* for growth 25 ºC, growth reduced at 15 ºC, no growth at 35 ºC, 10 ºC and below. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* dark brown to black, globose to sub globose, (105-) 131-175 (-206) µm long, (107-) 125-167 (-188) µm in width, ornamented with hyphae. *Ascomatal necks* dark brown to black becoming lighter toward the apexes, (301-) 348-448 (-522) µm long, (25-) 33-45 (-53) µm wide at the base, (11-) 13-17 (-20) µm wide at the apex. *Ostiolar hyphae* divergent, hyaline, (30-) 35-49 (-60) µm long. *Asci* not observed. *Ascospores* accumulating in buff-yellow (19d) mucilaginous masses at the apices of ascomatal necks. Ascospores cucullate in side view, aseptate, hyaline, invested in sheath, 5-7 x 3-4 µm with sheath, 4-6 x 3-4 µm without sheath.

Thielaviopsis anamorph: *primary conidiophores* occurring singly on mycelium, hyaline, swollen at the bases, tapering toward the apexes, (53-) 61-95 (-127) µm long,

4-6 µm wide at the bases, (2-) 5-7 (-8) µm wide at the middle, 3-6 µm wide at the apices. *Secondary conidiophores* occurring singly on mycelium, hyaline (52-) 57-69 (-68) µm long, (3-) 2-4 (-5) µm wide at the bases, 4-6 µm wide at the apexes. *Conidia* of two types: primary conidia hyaline, aseptate, cylindrical (11-) 14-22 (-29) x 3-5 µm, secondary conidia hyaline, aseptate, barrel-shaped (8-) 9-11 (-13) x 4-6 µm. *Chlamydospores* oval, thick walled, smooth, argus brown (13m), (10-) 12-14 (-15) x (7-) 8-12 (-14) µm, embedded in agar, formed singly or in chains, terminally.

Additional specimen examined: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (culture CMW22564, PREM59885; CMW22621, PREM59886).

DISCUSSION

Two *Ceratocystis* spp. associated with a serious disease of young *A. mangium* trees in the Riau area, Sumatra were characterised. A new *Ceratocystis* sp., described as *C. acaciivora prov. nom.*, as well as isolates indistinguishable from *C. manginecans* were consistently isolated from symptomatic tissue and these fungi were shown to be pathogenic on inoculated trees. They thus appear to be involved in causing the disease although their respective role is not clear. Futhermore, in an associated study (Chapter 4, this thesis), the opportunistic endophytic fungus *Lasiodiplodia theobromae* was isolated from trees showing similar symptoms and it is probable that all three fungi contribute to tree death.

Based on DNA sequence comparisons, the *Ceratocystis* isolates collected from dying *A. mangium* trees were shown to represent two distinct groups. One group of isolates (Group 2) was very closely related to *C. manginecans*, a serious pathogen of mango trees in Oman and Pakistan (Van Wyk *et al*. 2007). The other formed a separate and distinct clade and was described as the new species *C. acaciivora prov. nom*. *Ceratocystis* isolates from *Acacia* also differed based on growth in culture and in the sizes of their ascomatal bases and neck lengths. Isolates residing in Group 2 were morphologically most similar to *C. manginecans*, with ascomatal base and neck lengths similar to those of that species. There were also only minor sequence differences (3 bp different in ITS and 2 bp different in EF1-α) between the isolates from *Acacia* and *C. manginecans* and at least for the present, these are maintained as

representing that species. When compared with *C. manginecans*, *C. acaciivora* isolates differed in the sizes of their ascomatal bases and neck lengths and in sequence data (12 bp for the ITS, 2 bp for the EF1-α region) and these distinct differences justified the establishment of a new species.

Stem wounds such as those associated with pruning appear to be an important component of the disease of *A. mangium* found in this study. Pruning of these trees during the first year of growth to reduce branching and improve stem straightness is a routine procedure (Beadle *et al*. 2007). These wounds are visited by insects such as nitidulid beetles that are known to carry *Ceratocystis* spp. (Appel *et al.* 1990, Hayslett *et al*. 2005) and it is probable that *C. manginecans* and *C. acaciivora* are carried to fresh wounds after pruning. *Acacia mangium* is known to be highly sensitive to wounding and infection by *C. manginecans*, *C. acaciivora* and opportunistic pathogens such as *L. theobromae* appear to lead to rapid tree death. The fact that a similar disease is not seen in *A. crassicarpa* might be explained by the fact that this tree has a greater capacity to sustain wounding than does *A. mangium*. This view is also supported by the fact that *C. manginecans* and *C. acaciivora* were isolated only from artificially induced wounds on the stems of both *A. mangium* and *A. crassicarpa*, and never from naturally diseased *A. crassicarpa* in plantations.

All dying trees from which *C. manginecans* and *C. acaciivora* were isolated were those of *A. mangium* and the disease was not seen on *A. crassicarpa*. Because both species are grown in plantations in close proximity, it was considered interesting to determine their relative susceptibility to infection by *C. manginecans* and *C. acaciivora* in artificial inoculations. These tests showed that both species are equally susceptible to infection. In a recent survey, *C. acaciivora* was also found on dying *A. mangium* in the absence of pruning wounds, but where wood boring beetles had infested stems (Wingfield, unpublished). It is possible that these insects are more closely attracted to *A. mangium* than *A. crassicarpa* and that this difference could account for the absence of disease on the latter species.

The discovery of a fungus that has been identified as *C. manginecans* on *Acacia* trees in Sumatra is interesting. This fungus is known as a serious pathogen of mango in Oman and Pakistan (Van Wyk *et al.* 2007) and this is the first report of the fungus on

a host other than mango. It remains possible that the fungus from *Acacia* Group 2 represents a closely related, but distinct species to C. *manginecans*, although it is not currently possible to resolve this question. Alternatively, it is possible that the mango pathogen was introduced into Indonesia and that it has found a new host on *Acacia*. Mango trees are commonly grown as non-natives for fruit production in Indonesia and the introduction for a pathogen such as *C. manginecans* could easily have occurred with these trees.

The role of C. *manginecans* and *C. acaciivora* as pathogens of young *A. mangium* in plantations requires further study. The fact that this fungus was commonly isolated together with the opportunistic pathogen, *L. theobromae* (Chapter 4) implies that their respective roles are unresolved. *Lasiodiplodia theobromae* is an opportunistic fungus, well known on many trees including *Acacia* spp. in tropical areas of the world (Punithalingam 1976, Old *et al*. 2000). It can easily be isolated from healthy *Acacia* tissue and our impression is that it is a less important pathogen than *C. manginecans* and *C. acaciivora*. The latter fungus also resulted in lesions much longer than those associated with *L. theobromae* in a related study (Chapter 4, this thesis), suggesting that it is the more relevant factor in the death of *A. mangium*.

The disease of *A. mangium* that gave rise to this study is serious and management options to reduce its incidence are required. Clearly, pruning wounds are an important factor in disease development and the negative impact of pruning will require careful consideration. The fact that *C. acaciivora* has also recently been found on dying trees that have not been pruned (Wingfield, unpublished) requires further investigation. Here, the role of wood boring insects as vectors of this pathogen will need to be understood. *Ceratocystis manginecans* and *C. acaciivora* are aggressive pathogens and a deeper understanding of their role in tree death will be important in the future.

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Table 1. *Ceratocystis* isolates considered in phylogenetic analyses in this study.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector
C. acaciivora	CMW22562	EU588655 EU588635 EU588645	*Acacia mangium*	Indonesia	M. Tarigan
C. acaciivora	CMW22563	EU588656 EU588636 EU588646	*A. mangium*	Indonesia	M. Tarigan
C. acaciivora	CMW22564	EU588657 EU588637 EU588647	*A. mangium*	Indonesia	M. Tarigan
C. acaciivora	CMW22595	EU588660 EU588639 EU588649	*A. mangium*	Indonesia	M. Tarigan
C. acaciivora	CMW22621	EU588661 EU588640 EU588650	*A. mangium*	Indonesia	M. Tarigan
C. albifundus	CMW4068	DQ520638 EF070429 EF070400	*A. mearnsii*	RSA	J. Roux
C. albifundus	CMW5329	AF388947 DQ371649 EF070401	*A. mearnsii*	Uganda	J. Roux
C. atrox	CMW19383 CBS120517	EF070414 EF070430 EF070402	*Eucalyptus grandis*	Australia	M.J. Wingfield
C. atrox	CMW19385 CBS120518	EF070415 EF070431 EF070403	*E. grandis*	Australia	M.J. Wingfield
C. cacaofunesta	CMW15051 CBS152.62	DQ520636 EF070427 EF070398	*Theobroma cacao*	Cost Rica	A.J. Hansen
C. cacaofunesta	CMW14809 CBS115169	DQ520637 EF070428 EF070399	*T. cacao*	Ecuador	C. Suarez
C. caryae	CMW14808 CBS115168	EF070423 EF070440 EF070411	*Carya ovata*	U.S.A	J. Johnson

Table 1. (continued)

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector
C. caryae	CMW14793 CBS114716	EF070424 EF070439 EF070412	*C. cordiformis*	U.S.A	J. Johnson
C. fimbriata	CMW15049 CBS141.37	DQ520629 EF070442 EF070394	*Ipomoea batatas*	U.S.A	C.F. Andrus
C. fimbriata	CMW1547	AF264904 EF070443 EF070395	*I. batatas*	Papua New Guinea	E.C. McKenzie
C. fimbriata	CMW8857	AY233868 AY233878 EU241483	*Annana muriata*	Colombia	M.J. Wingfield
C. fimbriata	CMW8856 CBS121793	AY233867 AY233874 EU241484	*Citrus* sp.	Colombia	B. Castro
C. fimbriata	CMW9565	AY233864 AY233870 EU241487	*Citrus* sp.	Colombia	B. Castro
C. fimbriata	CMW9572	AY233863 AY233871 EU241488	*Citrus* sp.	Colombia	M.J. Wingfield
C. fimbriata s.l.	CMW14797 CBS114721	AY953382 EF433307 EF433316	*Mangifera indica*	Brazil	C.J. Baker
C. fimbriata s.l.	CMW15052 CBS600.70	EF433298 EF433306 EF433315	*M. indica*	Brazil	B.M. Figueiredo
C. fimbriatomima	CMW24174 CBS121786	EF190963 EF190951 EF190957	*Eucalyptus* sp.	Venezuela	M.J. Wingfield
C. fimbriatomima	CMW24176 CBS121787	EF190964 EF190952 EF190958	*Eucalyptus* sp.	Venezuela	M.J. Wingfield
C. manginecans	CMW13851	AY953383 EF433308 EF433317	*M. indica*	Oman	M. Deadman

Table 1. (continued)

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector
C. manginecans	CMW13852	AY953384 EF433309 EF433318	*Hypocryphalus mangifera*	Oman	M. Deadman
C. manginecans	CMW22579	EU588658 EU588638 EU588648	*A. mangium*	Indonesia	M. Tarigan
C. manginecans	CMW22581	EU588659 EU604671 EU604670	*A. mangium*	Indonesia	M. Tarigan
C. manginecans	CMW21123	EU588662 EU588641 EU588651	*A. crassicarpa*	Indonesia	M. Tarigan
C. manginecans	CMW21125	EU588663 EU588642 EU588652	*A. crassicarpa*	Indonesia	M. Tarigan
C. manginecans	CMW21127	EU588664 EU588643 EU588653	*A. crassicarpa*	Indonesia	M. Tarigan
C. manginecans	CMW21132	EU588665 EU588644 EU588654	*A. crassicarpa*	Indonesia	M. Tarigan
C. moniliformis	CMW4114	AY528986 AY528997 AY529007	*Schizolobium parahybum*	Ecuador	M.J. Wingfield
C. moniliformis	CMW9990 CBS155.62	NA	*T. cacao*	Costa Rica	A.J. Hansen
C. neglecta	CMW11284	EF127988 NA NA	*Eucalyptus* sp.	Colombia	M.J. Wingfield
C. neglecta	CMW11285	EF127989 NA NA	*Eucalyptus* sp.	Colombia	M.J. Wingfield
C. pirilliformis	CMW6569	AF427105 DQ371652 AY528982	*E. nitens*	Australia	M.J.Wingfield

Table 1. (continued)

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector
C. pirilliformis	CMW6579	AF427105 DQ371653 AY528983	*E. nitens*	Australia	M.J. Wingfield
C. platani	CMW14802 CBS115162	DQ520630 EF070425 EF070396	*Platanus occidentalis*	U.S.A	T.C. Harrington
C. platani	CMW23918	EF426554 EF070426 EF070397	*Platanus sp.*	Greece	M.J. Wingfield
C. polycroma	CMW11424 CBS115778	AY528970 AY528966 AY528978	*Syzygium aromaticum*	Indonesia	M.J. Wingfield
C. polycroma	CMW11436 CBS115777	AY528971 AY528967 AY528979	*S. aromaticum*	Indonesia	M.J. Wingfield
C. populicola	CMW14789 CBS119.78	EF070418 EF070434 EF070406	*Populus* sp.	Poland	J. Gremmen
C. populicola	CMW14819 CBS114725	EF070419 EF070435 EF070407	*Populus* sp.	U.S.A	T. Hinds
C. smalleyi	CMW14800 CBS114724	EF070420 EF070436 EF070408	*Carya cordiformis*	U.S.A	G. Smalley
C. tsitsikammensis	CMW14276	EF408555 EF408569 EF408576	*Rapanea melanophloeos*	South Africa	G.N. Kamgan & J. Roux
C. tsitsikammensis	CMW14278	EF408556 EF408570 EF408577	*R. melanophloeos*	South Africa	G. N. Kamgan & J. Roux
C. variospora	CMW20935 CBS114715	EF070421 EF070437 EF070410	*Quercus alba*	U.S.A	J. Johnson
C. variospora	CMW20936 CBS114714	EF070422 EF070438 EF070410	*Q. robur*	U.S.A	J. Johnson

Table 2. Morphological characteristics of Indonesian *Ceratocystis* isolates collected in this study compared to *C. manginecans* (Van Wyk *et al.* 2007).

	C. acaciivora **CMW22563**	**Group 2** **CMW21132**	***C. manginecans*** **CMW13851**
Cultures			
Color	Olive green (23m)	Olive green (23m)	Grayish olive (21````)
Growth rate	Opt. at 20-30 ºC	Opt. at 25-30 ºC	Opt. at 20-25 ºC
	Growth reduced at 15 ºC, no growth at 4, 10 & 35 ºC	Growth reduced at 15 ºC, no growth at 4, 10 & 35 ºC	No growth at 4, 10 & 35 ºC
Hyphae	Smooth & segmented	Smooth & segmented	Smooth & segmented
Ascomatal Base			
Color	Black	Black	Black
Long	(105-) 131-175 (-206)µm	(126-) 177-239 (-279)µm	(153-) 192-254 (-281)µm
Wide	(107-) 125-167 (-188)µm	(145-) 190-240 (-271)µm	-
Form	Globose to sub globose	Globose to sub globose	Globose
Ascomatal Neck			
Color	Black	Black	Dark brown
Long	(301-) 348-448 (-522)µm	(449-) 544-732 (-854)µm	(514-) 557-635 (-673)µm
Wide (Base)	(25-) 33-45 (-53)µm	(22-) 28-42 (-53)µm	(25-) 32-42 (-48)µm
Wide (Tip)	(11-) 13-17 (-20)µm	(11-) 14-20 (-26)µm	(14-) 16-22 (-26)µm
Ostiolar hyphae			
Shape	Divergent	Divergent	Divergent
Measurements	(30-) 35-49 (-60)µm	(31-) 38-60 (-74)µm	(42-) 45-59 (-69)µm
Asci	Not seen	Not seen	Not seen
Ascospores			
Colour	Hyaline	Hyaline	Hyaline
Shape (Side view)	Hat-shaped	Hat-shaped	Hat-shaped
Measurement with sheath	5-7 µm x 3-4 µm	5-7 µm x 3-4 µm	7-8 µm x 3-4 µm
without sheath	4-6 µm x 3-4 µm	4-6 µm x 3-4 µm	4-5 µm x 3-4 µm

Table 2. (Continued)

	C. acaciivora CMW22563	Group 2 CMW21132	*C. manginecans* CMW13851
Primary Conidiophores			
Length	(53-) 61-95 (-127) µm	(71-) 82-122 (-138) µm	(72-) 81-109 (-144) µm
Width (Base)	4-6 µm	4-6 (-8) µm	5-7 (-9) µm
Width (Middle)	(2-) 5-7 (-8) µm	5-7 (-8) µm	6-8 (-9) µm
Width (Tip)	3-6 µm	3-5 (-7) µm	3-6 µm
Shape	Lageniform	Lageniform	Lageniform
Secondary Conidiophores			
Length	(52-) 57-69 (-68) µm	(41-) 66-104 (-116) µm	(72-) 81-109 (-144) µm
Width (Base)	(3-) 2-4 (-5) µm	(3-) 4-6 (-7) µm	5-7 (-9) µm
Width (Tip)	4-6 µm	(3-) 5-7 µm	3-6 µm
Shape	Tube-like	Tube-like	Tube-like
Primary Conidia			
Shape	Cylindrical	Cylindrical	Cylindrical
Long	(11-) 14-22 (-29) µm	(9-) 12-22 (-33) µm	(15-) 23-29 (-33) µm
Wide	3-5 µm	3-5 µm	3-6 µm
Secondary Conidia			
Shape	Barrel-shaped	Barrel-shaped	Barrel-shaped
Long	(8-) 9-11 (-13) µm	7-11 (-14) µm	(8-) 9-11 (-12) µm
Wide	4-6 µm	(4-) 5-7 (-8) µm	5-7 (-8) µm
Chlamydospores			
Colour	Brown	Brown	Brown
Shape	Globose to sub-globose	Globose to sub-globose	Globose to sub-globose
Long	(10-) 12-14 (-15) µm	(10-) 11-15 (-17) µm	(11-) 12-14 µm
Wide	(7-) 8-12 (-14) µm	(8-) 10-12 (-14) µm	9-11 (-12) µm

Fig 1. Disease symptoms caused by *Ceratocystis acaciivora prov. nom.* and *C. manginecans* on *A. mangium* trees in Indonesia. (a). Stem cankers, wilting and death of trees, (b). Discoloration of bark on main stem of tree, (c). Discolored lesions and gum exudation from lesions, (d). Discolored xylem of infected tree.

Fig 2. A phylogenetic tree based on the combined sequence data of three gene regions; ITS, βt and EF1-α, showing relationships between *C. fimbriata s.l.* used in this study. Isolates in bold were isolated from *A. crassicarpa* and *A. mangium* in Indonesia and sequenced as part of this study. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated at the branches and Bayesian values follow in the brackets. Two isolates of *C. moniliformis* were selected as the out-group.

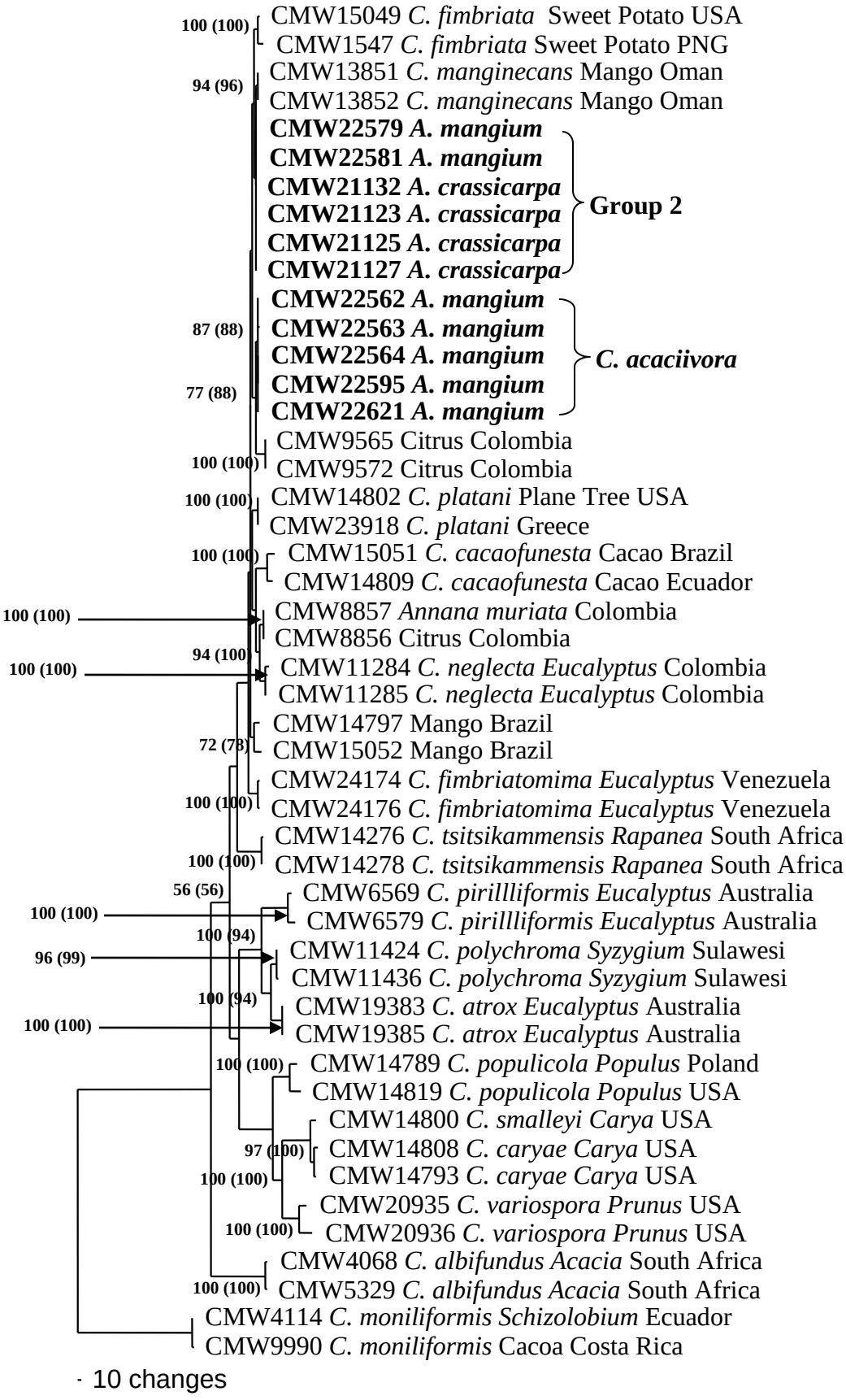
100 (100)
CMW15049 C. fimbriata Sweet Potato USA
CMW1547 C. fimbriata Sweet Potato PNG
94 (96)
CMW13851 C. manginecans Mango Oman
CMW13852 C. manginecans Mango Oman
CMW22579 A. mangium
CMW22581 A. mangium
CMW21132 A. crassicarpa
CMW21123 A. crassicarpa
CMW21125 A. crassicarpa
CMW21127 A. crassicarpa
Group 2
CMW22562 A. mangium
87 (88)
CMW22563 A. mangium
CMW22564 A. mangium
77 (88)
CMW22595 A. mangium
CMW22621 A. mangium
C. acaciivora
CMW9565 Citrus Colombia
100 (100)
CMW9572 Citrus Colombia
100 (100)
CMW14802 C. platani Plane Tree USA
CMW23918 C. platani Greece
100 (100)
CMW15051 C. cacaofunesta Cacao Brazil
CMW14809 C. cacaofunesta Cacao Ecuador
100 (100)
CMW8857 Annana muriata Colombia
CMW8856 Citrus Colombia
94 (100
100 (100)
CMW11284 C. neglecta Eucalyptus Colombia
CMW11285 C. neglecta Eucalyptus Colombia
CMW14797 Mango Brazil
72 (73)
CMW15052 Mango Brazil
CMW24174 C. fimbriatomima Eucalyptus Venezuela
100 (100)
CMW24176 C. fimbriatomima Eucalyptus Venezuela
CMW14276 C. tsitsikammensis Rapanea South Africa
100 (100)
CMW14278 C. tsitsikammensis Rapanea South Africa
56 (56)
CMW6569 C. pirillliformis Eucalyptus Australia
100 (100)
CMW6579 C. pirillliformis Eucalyptus Australia
100 (94)
CMW11424 C. polychroma Syzygium Sulawesi
96 (99)
CMW11436 C. polychroma Syzygium Sulawesi
100 (94)
CMW19383 C. atrox Eucalyptus Australia
100 (100)
CMW19385 C. atrox Eucalyptus Australia
100 (100)
CMW14789 C. populicola Populus Poland
CMW14819 C. populicola Populus USA
CMW14800 C. smalleyi Carya USA
97 (100)
CMW14808 C. caryae Carya USA
100 (100)
CMW14793 C. caryae Carya USA
CMW20935 C. variospora Prunus USA
100 (100)
CMW20936 C. variospora Prunus USA
CMW4068 C. albifundus Acacia South Africa
100 (100)
CMW5329 C. albifundus Acacia South Africa
CMW4114 C. moniliformis Schizolobium Ecuador
CMW9990 C. moniliformis Cacoa Costa Rica
10 changes

Fig 3. Growth of *C. acaciivora prov. nom.* (CMW22563, CMW22564, CMW22621) and *C. manginecans* (CMW21125, CMW21127, CMW21132) on MEA at seven different temperatures (4, 10, 15, 20, 25, 30, 35°C) after 48 hours. Bars on the graphs indicated with the same letter are not statistically significant different (P value = 0.05).

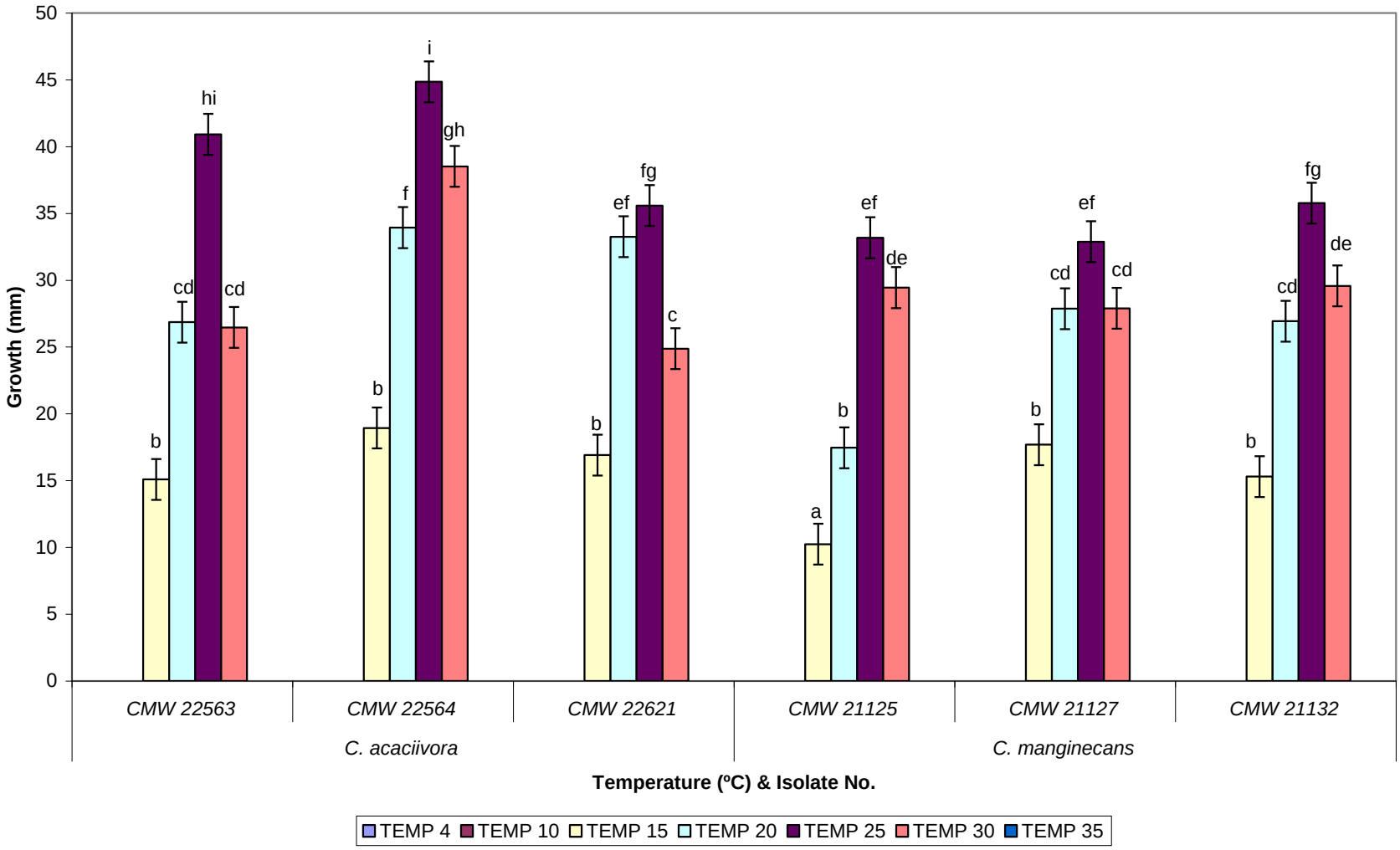
Growth (mm)
50
45
40
35
30
25
20
15
10
5
0
b
cd
hi
cd
b
f
i
gh
b
ef
fg
c
a
b
ef
de
b
cd
ef
cd
b
cd
fg
de
CMW 22563
CMW 22564
CMW 22621
CMW 21125
CMW 21127
CMW 21132
C. acaciivora
C. manginecans
Temperature (ºC) & Isolate No.
TEMP 4
TEMP 10
TEMP 15
TEMP 20
TEMP 25
TEMP 30
TEMP 35

Fig 4. Lesion lengths associated with inoculation of *C. acaciivora prov. nom.* (CMW22563, CMW564, CMW22581, CMW22595, CMW22621) and *C. manginecans* (CMW21123, CMW21125, CMW21127, CMW21132) isolates from *Acacia* spp. in one-year-old *A. mangium* and *A. crassicarpa* seedlings, grown in 20 cm diameter polybags in a greenhouse, 10 days after inoculation. Bars indicated with the same letter are not statistically significant different (P value = 0.05).

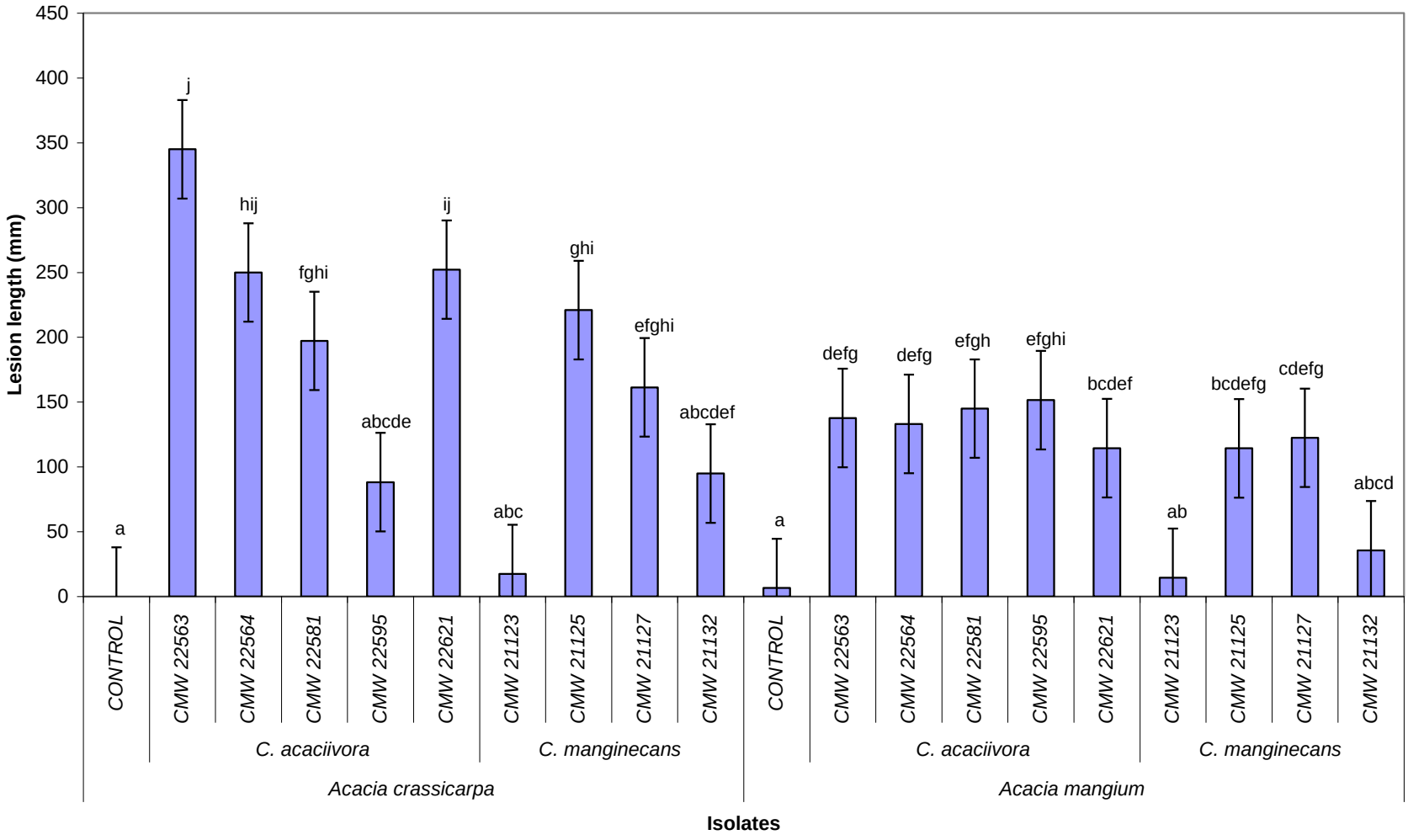
Lesion length (mm)
450
400
350
300
250
200
150
100
50
0
a
j
hij
fghi
abcde
ij
abc
ghi
efghi
abcdef
a
defg
defg
efgh
efghi
bcdef
ab
bcdefg
cdefg
abcd
CONTROL
CMW 22563
CMW 22564
CMW 22581
CMW 22595
CMW 22621
CMW 21123
CMW 21125
CMW 21127
CMW 21132
C. acaciivora
C. manginecans
Acacia crassicarpa
Acacia mangium
Isolates

Fig 5. Lesion lengths associated with the first set of inoculations using *C. acaciivora prov. nom.* (CMW22563, CMW22564, CMW22621) and *C. manginecans* (CMW21125, CMW21127, CMW21132) on one-year-old *A. mangium* and *A. crassicarpa* trees in an Indonesian plantation, six weeks after inoculation. Bars on the graph indicated with the same letter are not significantly different from each other (P value = 0.05).

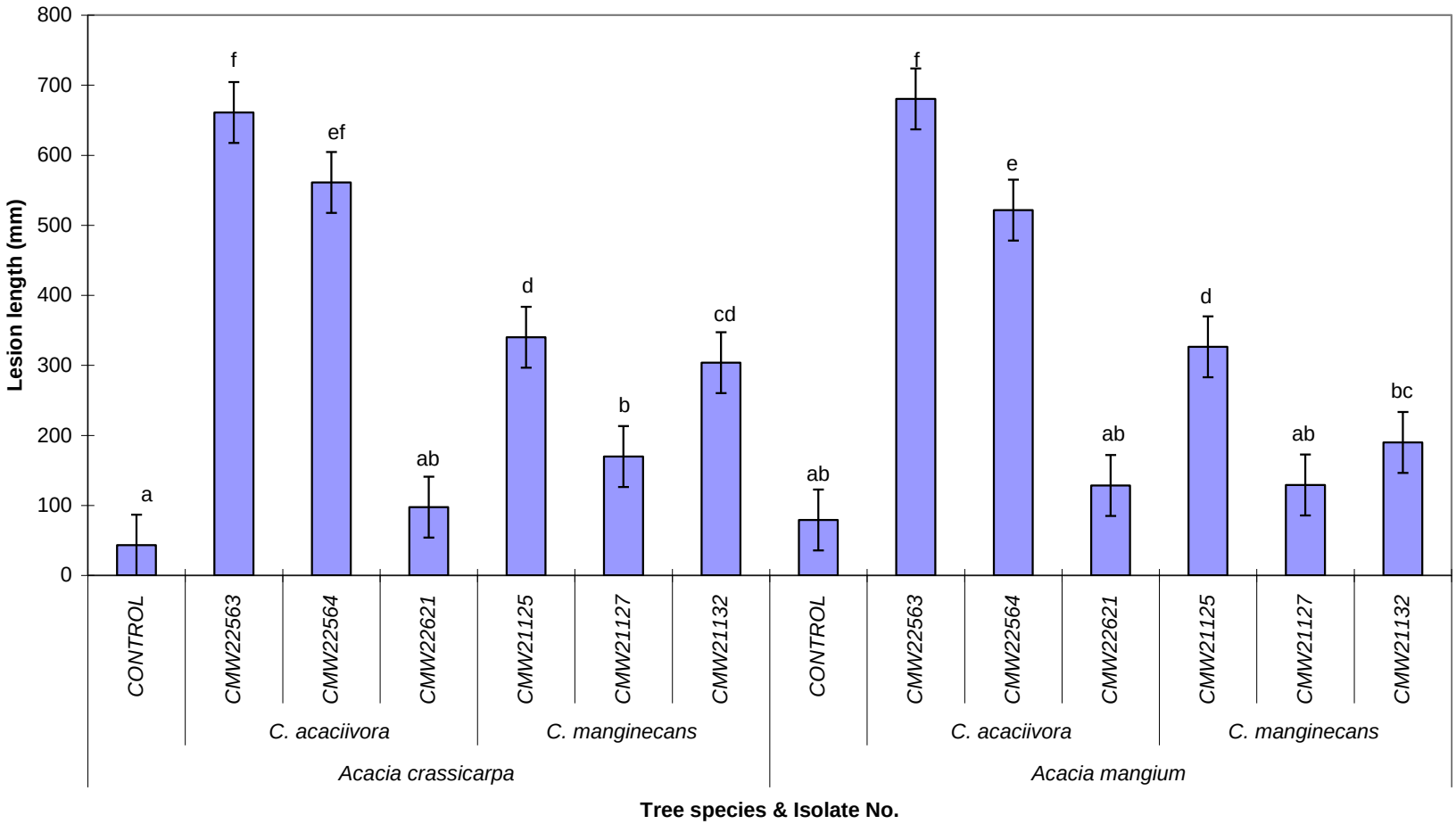
Lesion length (mm)
800
700
600
500
400
300
200
100
0
a
f
ef
ab
d
b
cd
ab
f
e
ab
d
ab
bc
CONTROL
CMW22563
CMW22564
CMW22621
CMW21125
CMW21127
CMW21132
CONTROL
CMW22563
CMW22564
CMW22621
CMW21125
CMW21127
CMW21132
C. acaciivora
C. manginecans
C. acaciivora
C. manginecans
Acacia crassicarpa
Acacia mangium
Tree species & Isolate No.

Fig 6. Lesion lengths associated with the second set of inoculations using *C. acaciivora prov. nom.* (CMW22563, CMW22564, CMW22621) and *C. manginecans* (CMW21125, CMW21127, CMW21132) on one-year-old *A. mangium* and *A. crassicarpa* trees in an Indonesian plantation, six weeks after inoculation. Bars on the graph indicated with the same letter are not significantly different from each other (P value = 0.05).

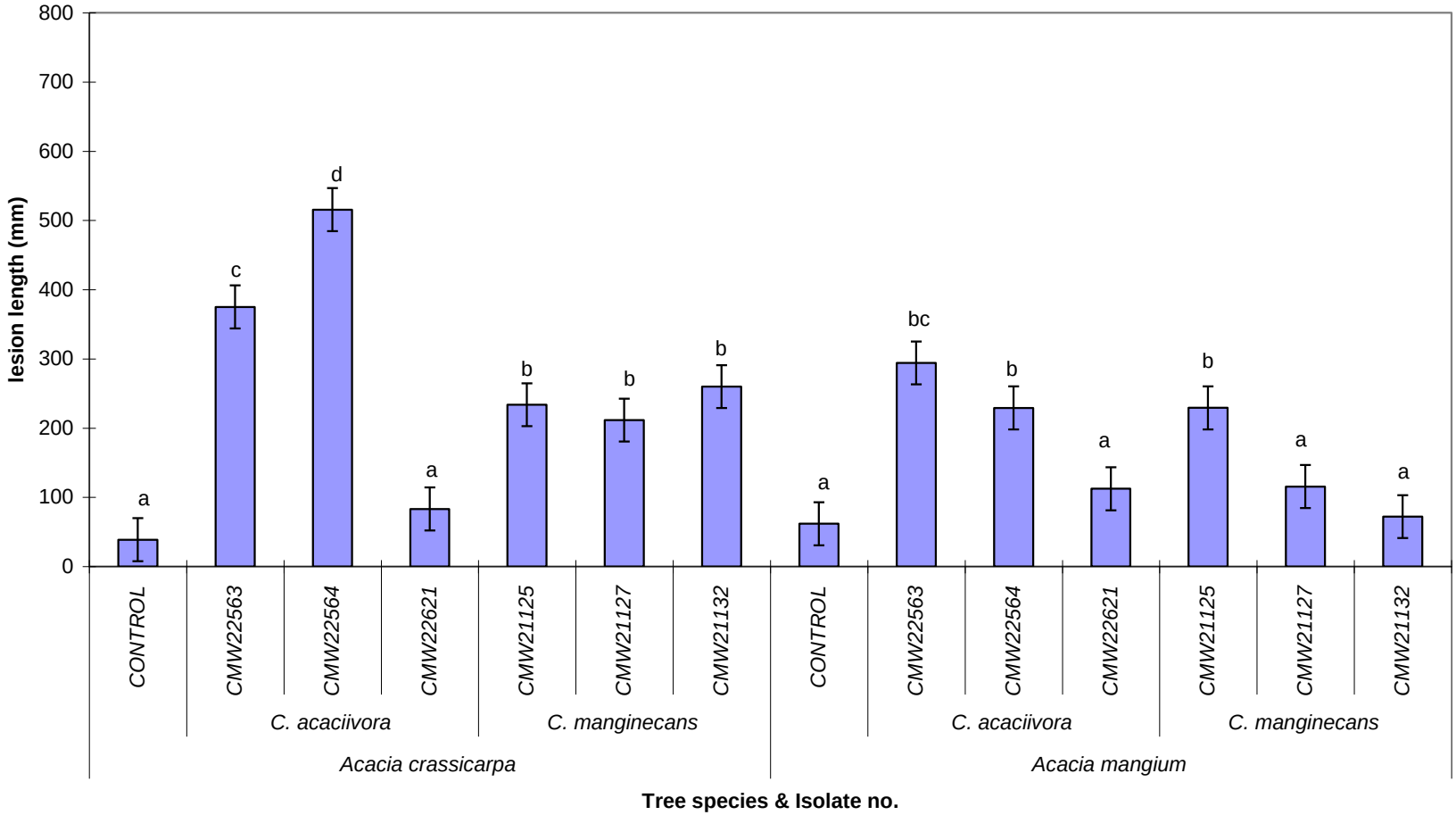
lesion length (mm)
800
700
600
500
400
300
200
100
0
a
c
d
a
b
b
b
a
bc
b
a
b
a
a
CONTROL
CMW22563
CMW22564
CMW22621
CMW21125
CMW21127
CMW21132
CONTROL
CMW22563
CMW22564
CMW22621
CMW21125
CMW21127
CMW21132
C. acaciivora
C. manginecans
C. acaciivora
C. manginecans
Acacia crassicarpa
Acacia mangium
Tree species & Isolate no.

Fig 7. Lesions on the bark and the cambium of one-year-old *A. mangium* trees, six weeks after inoculation with *C. acaciivora prov. nom.* (CMW22563) and *C. manginecans* (CMW21132) in Indonesia. (a). External/bark lesion for control inoculation, (b). Internal/xylem lesion for control inoculation, (c). External/bark lesion caused by *C. acaciivora*, (d). Internal/xylem lesion caused by *C. acaciivora*, (e). External/bark lesion caused by *C. manginecans*, (f). Internal/xylem lesion caused by *C. manginecans.*

Fig 8. Morphological characteristics of *Ceratocystis acaciivora prov. nom.* (CMW22563). (a). Globose ascomata with long neck, (b) Divergent ostiolar hyphae, (c). Hat-shaped ascospore in side view, (d). Chlamydospores, (e). Primary phialides, (f). Secondary phialides, (g). Cylindrical conidia, (h). Barrel-shaped conidia. [Scale bars (a) = 50 µm; (b, e, f) = 10 µm; (c, d, g, h) = 5 µm].

Chapter 3

Three new *Ceratocystis* spp. in the *C. moniliformis* complex from Indonesia and their pathogenicity to *Acacia mangium* and *A. crassicarpa*

ABSTRACT

The genus *Ceratocystis* includes many important tree pathogens and agents of sap stain. These fungi have a global distribution and commonly infect wounds on trees. During a survey of wound-infecting pathogens in the genus *Ceratocystis* on plantation-grown *Acacia mangium* trees in Indonesia, several isolates resembling *C. moniliformis sensu lato* (*s.l.*) were obtained. The aim of this study was to identify these isolates and to determine their virulence to two commercially grown *Acacia* spp. in the country. Use was made of morphology and comparisons of DNA sequence data for the ITS, β-tubulin and Elongation Factor 1-α gene regions to identify the isolates. Three previously undescribed species in the *C. moniliformis s.l.* species complex were identified, described here as *C. inquinans prov. nom*., *C. sumatrana prov. nom*. and *C. microbasis prov. nom*. Pathogenicity trials on *A. mangium* and *A. crassicarpa* in the greenhouse and in the field indicated that all three the species have the potential to cause disease of *A. mangium* and *A. crassicarpa*.

Keywords: Ophiostomatales, tree disease, vascular stain, wounds.

INTRODUCTION

Fungal pathogens represent a serious limiting factor to the success and long-term sustainability of commercial plantations in Kalimantan and Sumatra (Indonesia). These plantations, made up especially of non-native *Acacia mangium* Willd. and *A. crassicarpa* Cunn.: Benth., are vulnerable due to their homogenous nature. The trees have also been separated from their natural enemies and these are likely to appear and proliferate in time (Old *et al.* 2000, Old *et al*. 2003). Likewise, some native pathogens will adapt to infect these trees and the uniform distribution of the trees will enhance their vulnerability, as has already been found in the case of diseases that affect *Eucalyptus* plantations (Old *et al.* 2000, Old *et al*. 2003).

During recent disease surveys of *A. mangium* plantations in Indonesia, significant mortality of young trees were observed. This problem is particularly evident after the trees have been pruned, with infections appearing to initiate at the pruning wounds. Isolations from diseased trees have resulted in the collection of *Ceratocystis manginecans* M. van Wyk, A Al-Adawi, & M.J. Wingf. and *C. acaciivora prov. nom*. that play a role in the death of the trees. This view emerges from the fact that these *Ceratocystis* spp. were isolated from stained wood and stem cankers associated with pruning wounds (Chapter 2).

The genus *Ceratocystis* includes a number of important tree pathogens, including pathogens of Australian *Acacia* spp. For example, *C. albifundus* M.J. Wingf., De Beer & Morris causes wilt and death of *A. mearnsii* De Wild. and *A. decurrens* Willd. trees in Africa (Morris *et al*. 1993, Wingfield *et al.* 1996, Roux & Wingfield 1997, Roux *et al.* 2005). Likewise, *C. fimbriata sensu lato* (*s.l.*) has been reported as the causal agent of canker and wilt of *A. decurrens* in Brazil (Ribeiro *et al.* 1988).

Ceratocystis spp. are known to require wounds for infection (Zalasky 1965, Teviotdale & Harper 1991, Kile 1993). The wounding of trees has thus in recent years been commonly used to bait for *Ceratocystis* spp. (Barnes *et al*. 2003, Roux *et al*. 2005, Kamgan *et al*. 2008). *Acacia mangium* trees grown in plantations in Indonesia are commonly wounded during their cultivation. They tend to have multiple leader stems (Srivastava 1993, Lee & Arentz 1997) and it is standard practice in pulpwood

plantations to prune or single the stems at four to eight months of age (Beadle *et al*. 2007). This practice would allow stems of *A. mangium* to be infected by wound-infecting fungi such as *Ceratocystis* spp.

The genus *Ceratocystis* represents a polyphyletic assemblage of fungi that would most appropriately reside in discrete genera (Wingfield *et al.* 2006). Two important groups of species that are found on hardwood trees are those belonging to the *C. moniliformis* (Hedgc.) C. Moreau species complex and the *C. fimbriata* Ellis & Halst. species complex. Species in the former group are generally considered to be non-pathogenic, whereas those in the latter assemblage include important tree pathogens (Davidson 1935, Bakshi 1951, Hunt 1956, Kile 1993, Wingfield *et al.* 1996). However, these designations have emerged from a very limited number of studies, particularly in the case of the *C. moniliformis* species complex.

The aim of this study was to identify a collection of isolates of *Ceratocystis* belonging to the *C. moniliformis* species complex. These isolates were collected from the stems of *A. mangium* trees that had been artificially wounded. Identifications were based on morphology and comparisons of DNA sequence data. Furthermore, their ability to cause disease on *A. mangium* and *A. crassicarpa* were evaluated using pathogenicity trials in the greenhouse and in the field.

MATERIALS AND METHODS

Fungal isolates

Wounding trials were established on one-year-old *A. mangium* and *A. crassicarpa* trees in the Teso area, Riau Province, following the method described by Barnes *et al.* (2003). Fifteen trees of both species were randomly selected and wounded. Five weeks after establishment, samples of discolored wood were collected from the wounds, wrapped in newspaper to keep them moist, and were taken to the laboratory for examination.

Two methods were used to isolate fungi from the wood and bark material collected. When fungal fruiting bodies, characteristic of *Ceratocystis* spp. were observed, direct isolations were made from single spore drops onto 2 % (w/v) Malt Extract Agar

(MEA, 15 g Agar, 20 g Malt Extract) (Biolab, Midrand, South Africa). When no fruiting bodies were observed, wood tissue was incubated in sealed plastic bags containing moistened tissue paper for 4-10 days, until sporulation was observed, after which single spore masses were lifted from the tips of ascomata. In addition, a carrot baiting technique, described by Moller & DeVay (1968), was used to induce growth and sporulation of *Ceratocystis* spp. Here, symptomatic wood pieces were placed between carrot slices that had first been treated by immersion for 10 min in water containing 0.001 g vol^{-1} streptomycin sulfate (SIGMA, Steinheim, Germany). These carrot baits were then placed in plastic bags and incubated for 3-5 days, or until the development of fruiting bodies on the carrot surfaces.

All isolates collected in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates have been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Delft, Netherlands and dried specimens were deposited at the National Herbarium of South Africa (PREM), Pretoria.

DNA extractions

Nine *Ceratocystis* isolates were selected randomly based on culture morphology and grown on 2 % MEA at 22 ºC for one week. Using sterilized scalpels, the mycelium was scraped from the surface of the agar and transferred into 1.5 ml Eppendorf tubes and lyophilized overnight. The lyophilized fungal mycelium was placed into liquid nitrogen and crushed to a fine powder using a glass rod or mechanical grinder (Ball Mills machine, Retsch, Haan, Germany). The DNA was extracted using the method described by Van Wyk *et al*. (2006a).

PCR amplification, sequencing and analyses

Polymerase chain reaction (PCR) amplification was carried out for three gene regions. These included the Internal Transcribed Spacer regions (ITS), including the 5.8S gene of the ribosomal DNA (rDNA) operon and portions of the β-tubulin (βt) and Transcription Elongation factor 1-α (EF1-α) regions. Each gene region was amplified using the primers ITS1 and ITS4 (White *et al.* 1990), βt1a and βt1b (Glass & Donaldson 1995) and EF1-728F and EF1-986R (Carbone & Kohn 1999) respectively.

The PCR reaction mixtures were 25 µL in total, consisting of 0.5 µL of 10 mM forward and reverse primers, 2 µL of 10 mM dNTP mixture (2.5 mM of each dNTP), 0.5 µL DNA Taq enzyme (Roche Molecular Biochemicals, Almeda California), 2.5 µL of a 10x concentration buffer containing $MgCl_2$ (3.5 mM), 2 µL of DNA (2-10ng) and 17 µL sabax water. The PCR program, electrophoresis conditions and DNA purification procedures were as described by Van Wyk *et al.* (2006a).

Sequencing of purified PCR amplicons was carried out in both directions using the ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit, following the manufacturer's protocols (Applied BioSystems, Foster City, California). Sequencing of each gene region was achieved using the same primers as those used for the PCR reactions. After cleaning the sequence products using the same protocol as that used for the PCR reactions, the final sequence products were run on an ABI PRISM™ 3100 Auto sequencer (Applied BioSystems, Foster City, California). Sequence electropherograms were analyzed using Sequence Navigator version 1.0.1 (Applied BioSystems, Foster City, California).

BLAST analyses were used to compare the sequences obtained with those of *Ceratocystis* spp. that are available in GenBank (The National Centre for Biological Information, http://www.ncbi.nlm.nih.gov/). Thereafter, sequences of known published species were incorporated into data sets for each gene region, together with the isolates from wounds on *A. mangium*, and aligned using the online interface of MAFFT version 6 (http://align.bmr.kyushu-u.ac.zp/mafft/online/server/). Alignments were confirmed manually.

After alignment, sequence data for the Indonesian *Ceratocystis* spp. were analyzed using multiple programs. A partition homogeneity test was run using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10* to determine whether the data from the three gene regions could be combined into a single data set (Swofford 2002). Data for each data set were analyzed using PAUP version 4.0b10* (Swofford 2002). Gaps were treated as "newstate" and trees were obtained via stepwise addition of 1000 replicates. The Mulpar option was in effect. Bootstrap confidence intervals, using 1000 replicates, were calculated. *Ceratocystis virescens* (Davidson) Moreau was used as the out-group taxon. The model of nucleotide substitution for each gene

region was also determined using Mrmodeltest2 (Nylander 2004) and the models obtained were used in Bayesian analysis using MrBayes version 3.1.1. (Ronquist & Huelsenbeck 2003). Bayesian probabilities of Markov Chain Monte Carlo (MCMC) algorithms, combining each model test obtained, were used to calculate support for the phylogenetic trees. Following the MCMC procedure, one million random trees were generated with four chains and sampled every 100th generation. Samples were taken only from trees after convergence and trees outside the point of convergence had been discarded.

Culture Characteristics and Morphology

Two, or where available three, isolates from each of the groups identified using DNA sequence comparisons were grown on 2 % MEA for one week at 22 ºC. These isolates were then used for growth comparisons. A mycelial disc taken from the edge of an actively growing culture was cut using a 5 mm cork borer and placed at the centre of 90 mm Petri dishes containing 2 % MEA. Each isolate was placed on five plates for each treatment and incubated at 4 ºC, 10 ºC, 15 ºC, 20 ºC, 25 ºC, 30 ºC and 35 ºC respectively. Two diameter growth measurements for each colony, at right angles to each other, were taken two days after incubation and averages were computed. The experiment was repeated once and analysis of variance (ANOVA) was calculated for all data obtained using SAS statistical analyses (SAS Version 8.2, 2001).

The morphology of the *Ceratocystis* isolates was described from one-week-old cultures grown on 2 % MEA. The color of cultures was described using Rayner's color charts (Rayner 1970). Fruiting structures were mounted in lactic acid (85 %) for observation and measurement. Fifty measurements of characteristic structures for each isolate representing the main groups and 10 measurements from one additional isolate in each group were made using a Carl Zeiss microscope and a Zeiss Axiovision camera system (Oberkochen, Germany). The average (mean), standard deviation (stdv), maximum (max) and minimum (min) measurements were calculated and are presented as follows: (min-) mean minus stdv – mean plus stdv (-max).

Pathogenicity tests

Greenhouse inoculations - Pathogenicity tests were conducted on the stems of one-year-old *A. mangium* and *A. crassicarpa* seedlings (~ 15 mm diameter size) grown in 20 cm diameter plastic bags containing a mixture of topsoil and compost. The seedlings were placed in a greenhouse where the temperature and humidity was adjusted for optimum growth of the seedlings (30 ºC). One to three isolates representing the various groups of *Ceratocystis* [(Group 1 = CMW21106), (Group 2 = CMW21109, CMW21111, CMW21113), (Group 3 = CMW21115, CMW21117, CMW21118)] identified using DNA sequence data were used for the inoculations. Wounds were made on the stems of the seedlings using a sterilized cork borer and inoculations were carried out by inserting an agar disc taken from the edge of an actively growing colony grown on 2 % MEA into the wound, with the mycelium facing the exposed cambium. Parafilm (Pechiney, Menasha, Wisconsin) was used to seal the wounds after inoculation to avoid contamination and desiccation of the wounds. Five seedlings of each *Acacia* species were inoculated with each of the test fungi and five seedlings were likewise inoculated with sterile MEA plugs to serve as controls.

Ten days after inoculation, the parafilm was removed from the inoculation wounds and the lengths of the lesions in the xylem were measured. A piece of symptomatic stem tissue was taken from the inoculation site for five seedlings representing each test isolate and placed into moist chambers to induce the production of fruiting structures. Spores from these structures were then transferred to 2 % MEA to verify the identity of the inoculated isolate and fulfill the requirements of Koch's postulates. Analysis of variance (ANOVA) was calculated for all data obtained using SAS statistical analyses (SAS Version 8.2, 2001).

Field inoculations - Pathogenicity trials were conducted on ~ one-year-old *A. mangium* and *A. crassicarpa* trees in commercial plantations in Indonesia. Trees ranged from 70 to 90 mm in diameter at breast height. The same isolates as those used in the greenhouse inoculations, were used to inoculate 20 trees of each of the *Acacia* spp. for each *Ceratocystis* isolate. An additional 20 trees of each *Acacia* sp. were inoculated with sterile MEA plugs to serve as controls. For inoculation, a wound was made on the tree stems using a sterilized cork borer (10 mm diameter) and

inoculations were carried out by inserting an agar disc of the same size, taken from the edges of actively growing colonies on 2 % MEA. These were placed into the inoculation point, with the mycelium facing the exposed cambium. The wounds were sealed with wrapping tape after inoculation to avoid contamination and desiccation of the inoculum and wounds.

Six weeks after inoculation, the tree diameter at the inoculation point and the lengths of lesions produced on the stems were measured. Re-isolations were made by taking a piece of symptomatic tissue from the area associated with the inoculation points for five trees of each isolate and placing these in moist chambers to induce sporulation. Spore masses were then taken from the tips of fruiting structures and plated on 2 % MEA to verify the presence of the inoculated fungus. Analysis of variance (ANOVA) was calculated for all data obtained using SAS statistical analyses (SAS Version 8.2, 2001). The entire field experiment was repeated once.

RESULTS

Fungal isolates

Fourteen *Ceratocystis* isolates, resembling *C. moniliformis s.l.* were obtained from three of the fifteen *A. mangium* trees wounded in this study. No isolates were obtained from *A. crassicarpa* trees wounded in this study. Of the fourteen isolates obtained, four were collected from one tree (CMW21105, CMW21106, CMW21107, CMW21108), six isolates (CMW21109, CMW21110, CMW21111, CMW21112, CMW21113, CMW21114) were collected from a second tree and four isolates (CMW21115, CMW21116, CMW21117, CMW21118) were obtained from a third tree. All isolates were obtained using the moist chamber method. No isolates were obtained from carrot baiting. Within two weeks, all isolates produced mature perithecia containing hat-shaped ascospores and a *Thielaviopsis* anamorph. At the time of sample collection, staining of the vascular tissue was observed on the trees in the vicinity of the wounds.

PCR amplification, sequencing and analyses

PCR amplification resulted in fragments of ~500 base pairs (bp) in size for the ITS and βt gene regions, while for the EF1-α gene region, amplification resulted in

fragments of ~300 bp in size. The partition homogeneity test on the three data sets resulted in a P value of 0.02, which is an acceptable level to combine the data (Cunningham 1997, Barker & Lutzoni 2002). Thus, all data sets were combined for further analyses.

The combined data set consisted of 1344 characters including gaps, of which 950 characters were constant, 11 were parsimony-uninformative and 383 parsimony-informative. Three most parsimonious trees were obtained after analyses, one of which was selected for presentation (Fig 1). This tree had a length of 525, a consistency index (CI) = 0.8838, homoplasy index (HI) = 0.1162, retention index (RI) = 0.9250 and rescaled consistency index (RC) = 0.8175.

Model test analysis produced a GTR + G model [Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 6 rates = gamma] for both the ITS and βt data sets, while for EF1-α it produced a HKY + I model [Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 2 rates = propinv]. Burn-in was taken at 2000 trees. Bootstrap values followed by Bayesian values in brackets (Fig 1) were attached to the branch nodes of the combined datasets. Bayesian values provided strong support to the bootstrap values obtained.

Three distinct groups within the *C. moniliformis s.l.* complex were obtained from *A. mangium* in Indonesia. These are designated Group 1 (CMW21106, CMW21107), Group 2 (CMW21109, CMW21111, CMW21113) and Group 3 (CMW21115, CMW21117, CMW21118). All three groups formed well-resolved clades with posterior probability values for the branch nodes of 99 % (99 %), 96 % (99 %) and 82 % (98 %) respectively for group 1, group 2 and group 3 (Fig 1). All of these isolates were phylogenetically most closely related to *C. omanensis* Al-Subhi, M.J. Wingf., M. van Wyk & Deadman. Other clades obtained in the phylogenetic analyses were those for *C. moniliformopsis* Z.Q. Yuan & C. Mohammed [100 % (100 %)], *C. tribiliformis* M. van Wyk & M.J. Wingf. [100 % (98 %)], *C. moniliformis* [100 % (100 %)], *C. bhutanensis* M. van Wyk, M.J. Wingf. & Kirisits [100 % (100 %)] and *C. savannae* Kamgan & Jol. Roux [100 % (100 %)]. These results indicated that the three groups representing *C. moniliformis s.l.* from *A. mangium* in Riau, Sumatra represent distinct and undescribed species.

Culture characteristics and morphology

All three *Ceratocystis* groups emerging from the phylogenetic analyses showed similar growth rates and temperature optima. All isolates showed optimum growth at 30 ºC, with Group 1 isolates reaching an average diameter of 67 mm, Group 2 isolates reaching an average diameter of 56 mm and Group 3 reaching and average diameter of 63 mm in 2 days at 30 ºC. For all isolates, growth was reduced at 35 ºC, 20 ºC and 15 ºC. Very slow growth was observed at 10 ºC and no growth was found at 4 ºC (Fig 2).

Cultures representing Group 1 had hair brown (17````i) aerial mycelium while those in Group 2 and Group 3 had cream buff (19``d) to hair brown (17````i) aerial mycelium. Within 1-2 weeks, both teleomorph and anamorph structures were produced in all cultures. The ascomatal bases were brown, or dark brown to black, in color and globose to sub globose, with ornamentations in the form of conical spines. The ascomatal bases of Group 3 isolates were much smaller and lighter in color than those of Group 1 and Group 2 isolates. All isolates, irrespective of the group to which they belonged had necks with disc-like bases. They also all had divergent ostiolar hyphae at the tips of the ascomatal necks, from which hat shaped ascospores were produced. The ostiolar hyphae of Group 3 isolates were shorter than those of Group 1 and Group 2 isolates. Both primary and secondary phialides were present in the isolates, except for Group 3 which only has primary phialides. Primary phialides produced cylindrical conidia either in chains or singly, while secondary phialides produced barrel shaped conidia, also in chains or singly. None of the isolates representing any of the three groups produced chlamydospores.

Pathogenicity tests

Greenhouse inoculations **-** All isolates inoculated in this study produced lesions on *A. crassicarpa* and *A. mangium* seedlings within ten days of inoculation. The lesion lengths ranged from 21-67 mm on *A. crassicarpa* and 27-66 mm on *A. mangium* (Fig 3). Isolate CMW21106 (Group 1) consistently produced significantly longer lesions when compared to the controls on *A. crassicarpa* and *A. mangium*. Isolates residing in Group 2 and Group 3 also produced longer lesions than the control, however, only one isolate of each group, namely CMW21113 (Group 2) and CMW21115 (Group 3) produced lesions that were significantly different from the controls (P values = 0.05,

R-Square = 0.31; Coeff. Var = 94.1; Root MSE = 35.5). Re-isolations from the lesions consistently yielded *C. moniliformis s.l.* isolates and these were never found associated with the control inoculations.

Field inoculations - All *Ceratocystis* isolates produced lesions on *A. mangium* and *A. crassicarpa* trees within six weeks of inoculation (Fig 4). Lesion lengths ranged from 90-170 mm on *A. crassicarpa* and 160-220 mm on *A. mangium* in the first trial (Fig 5). In the second trial they were between 90-160 mm on *A. crassicarpa* and 140-250 mm on *A. mangium* (Fig 6). Lesion length trends were similar in both trials, with lesions on *A. crassicarpa* trees consistently smaller when compared to those on *A. mangium*. All isolates, other than CMW21113, inoculated on *A. crassicarpa*, resulted in lesions that differed significantly from the controls (P values = 0.05, R-Square = 0.37; Coeff. Var = 42.5; Root MSE = 6.41).

Based on inoculations, *A. mangium* was consistently more susceptible to infection than *A. crassicarpa*. Lesion lengths in the second trial were consistently longer than those in the first trial, with all inoculations other than those associated with CMW21113 significantly longer than those of the controls (P values = 0.05, R-Square = 0.32; Coeff. Var = 51.04; Root MSE = 7.22). Re-isolations from the lesions failed to yield isolates of the inoculated fungi. Trees diameter ranged from 70 – 90 mm and had no impact on the lesion length (P values = 0.05, R-Square = 0.40; Coeff. Var = 14.34; Root MSE = 1.24).

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TAXONOMY

Results of this study have shown that the three *Ceratocystis* spp. belonging to the *C. moniliformis s.l.* species complex isolated from wounded *A. mangium* in Indonesia represent new taxa. They are consequently described as follows:

Ceratocystis inquinans Tarigan, M. van Wyk & M.J. Wingf. *prov. nom*

(Fig 7)

Etymology: The name refers to the ability of this fungus to cause stain. Inquinans means staining.

Stat.conid.: *Thielaviopsis*

Coloniae crinobrunneae, *mycelium* aerium. *Hyphae* laeves, septis non constrictis. *Bases ascomatum* atrobrunneae vel nigrae, globosae vel subglobosae (116-) 149-205 (-236) µm longae, (130-) 161-217 (-270) µm latae, spinis hyphisque ornatae; spinae atrobrunneae vel nigrae (18-) 20-30 (-37) µm longae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallentia (347-) 393-575 (-687) µm longa, basi (66-) 83-119 (-141) µm, apice (14-) 16-20 (-23) µm lata, basi discoidea. *Hyphae ostiolares* divergentes, hyalinae (20-) 24-34 (-38) µm longae. *Asci* non visi. *Ascosporae* in massis fulvo-flavescentibus mucosis in apicibus collorum ascomatum. Ascosporae lateraliter visae cucullatae hyalinae, non septatae, vaginis inclusae, 5-7 x 2-4 µm cum vagina, 4-6 x 2-4 µm sine illa.

Anamorpha Thielaviopsis: *conidiophorae primariae* in mycelio singulae, hyalinae, basi incrassatae, apicem versus angustatae, (18-) 22-32 (-44) µm longae, basi 3-5 µm, medio 2-4 µm, apice 1-3 µm latae. *Conidiophorae secondariae* in mycelio singulae hyalinae (19-) 23-35 (-43) µm longae, basi apiceque (2-) 2-4 (-5) µm latae. *Conidia* biformia: primaria hyalina cylindrica non septata (5-) 6-8 (-11) x (2-) 3-5 (-7) µm; secondaria hyalina doliiformia (4-) 5-7 (-8) x 1-3 µm.

Typus: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (PREM59866 – holotype, living culture: CMW21106).

Colonies hair brown (17````i) in color. *Mycelium* aerial. *Optimal temperature* for growth 25-30 ºC, no growth at 4 ºC, but growth observed at 35 ºC. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* dark brown to black, globose to sub globose, (116-) 149-205 (-236) µm high, (130-) 161-217 (-270) µm wide, ornamented with spines and hyphae, spines dark brown to black, (18-) 20-30 (-37) µm long. *Ascomatal necks* dark brown to black becoming lighter towards the apexes, (347-) 393-575 (-687) µm long, (66-) 83-119 (-141) µm wide at the base, (14-) 16-20 (-23) µm wide at the apex, with a disc-like base. *Ostiolar hyphae* divergent, hyaline, (20-) 24-34 (-38) µm long. *Asci* not observed. *Ascospores* accumulating in buff-yellow (19d) mucilaginous masses at the apexes of the ascomatal necks. Ascospores cucullate in side view, aseptate, hyaline, invested in sheath, 5-7 x 2-4 µm with sheath, 4-6 x 2-4 µm without sheath.

Thielaviopsis anamorph: *primary conidiophores* occurring singly on mycelium, hyaline, swollen at the bases, tapering toward the apexes, (18-) 22-32 (-44) µm long, 3-5 µm wide at the bases, 2-4 µm wide at the middle, 1-3 µm wide at the apices. *Secondary conidiophores* occurring singly on mycelium, hyaline, (19-) 23-35 (-43) µm long, (2-) 2-4 (-5) µm wide at the bases, (2-) 2-4 (-5) µm wide at the apexes. *Conidia* of two types: primary conidia hyaline, aseptate, cylindrical (5-) 6-8 (-11) x (2-) 3-5 (-7) µm, secondary conidia hyaline, aseptate, barrel-shaped (4-) 5-7 (-8) x 1-3 µm. Chlamydospores absent.

Additional specimen examined: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (culture CMW21107, PREM59867).

Ceratocystis sumatrana Tarigan, M. van Wyk & M.J. Wingf. *prov. nom.*

(Fig 8)

Etymology: Sumatrana refers to the Indonesian island Sumatra where this species was discovered.

Stat.conid.: *Thielaviopsis*

Coloniae cremeo-fulvae vel crinobrunneae, *mycelium* aerium. *Hyphae* laeves, septis non constrictis. *Bases ascomatum* atrobrunneae vel nigrae, globosae vel subglobosae (148-) 168-218 (-293) µm longae, (158-) 187-235 (-296) µm latae, spinis hyphisque ornatae; spinae atrobrunneae vel nigrae (5-) 6-12 (-18) µm longae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallentia (323-) 390-574 (-687) µm longa, basi (67-) 81-103 (-128) µm, apice (12-) 15-23 (-32) µm lata, basi discoidea. *Hyphae ostiolares* divergentes hyalinae (21-) 24-32 (-35) µm longae. *Asci* non visi. *Ascosporae* in massis fulvo-flavescentibus mucosis in apicibus collorum ascomatum. Ascosporae lateraliter visae cucullatae hyalinae, non septatae, vaginis inclusae, 5-7 x 3-4 µm cum vagina, 4-6 x 3-4 µm sine illa.

Anamorpha Thielaviopsis: *conidiophorae primariae* in mycelio singulae, hyalinae, basi incrassatae, apicem versus angustatae, (17-) 21-31 (-38) µm longae, basi (2-) 2-4 (-5) µm, medio (2-) 2-4 (-5) µm, apice 1-3 µm latae. *Conidiophorae secondariae* in mycelio singulae hyalinae (23-) 23-37 (-47) µm longae, basi 2-4 µm, apice 3-5 µm

latae. *Conidia* biformia: primaria hyalina cylindrica non septata (5-) 5-7 (-8) x (2-) 2-4 (-5) µm; secondaria hyalina doliiformia non septata (4-) 5-7 (-8) x 1-3 µm.

Typus: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (PREM59870 – holotype, living culture: CMW21113).

Colonies cream-buff (19``d) to hair brown (17````i) in color. *Mycelium* aerial. *Optimal temperature* for growth 25-30 ºC, no growth at 4 ºC, but growth observed at 35 ºC. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* dark brown to black, globose to sub globose, (148-) 168-218 (-293) µm high, µm (158-) 187-235 (-296) µm wide, ornamented with spines and hyphae, spines dark brown to black, (5-) 6-12 (-18) µm long. *Ascomatal necks* dark brown to black and becoming lighter towards the apexes, (323-) 390-574 (-687) µm long, (67-) 81-103 (-128) µm wide at the bases, (12-) 15-23 (-32) µm wide at the apexes, with a disc-like base. *Ostiolar hyphae* divergent, hyaline, (21-) 24-32 (-35) µm long. *Asci* not observed. *Ascospores* accumulating in buff-yellow (19d) mucilaginous masses at the apexes of the ascomatal necks. Ascospores cucullate in side view, aseptate, hyaline, invested in sheath, 5-7 x 3-4 µm with sheath, 4-6 x 3-4 µm without sheath.

Thielaviopsis anamorph: *primary conidiophores* occurring singly on mycelium, hyaline, swollen at the bases, tapering toward the apexes, (17-) 21-31 (-38) µm long, (2-) 2-4 (-5) µm wide at the bases, (2-) 2-4 (-5) µm wide at the middle, 1-3 µm wide at the apexes. *Secondary conidiophores* occurring singly on mycelium, hyaline, (23-) 23-37 (-47) µm long, 2-4 µm wide at the bases, 3-5 µm wide at the apexes. *Conidia* of two types: primary conidia hyaline, aseptate, cylindrical (5-) 5-7 (-8) x (2-) 2-4 (-5) µm, secondary conidia hyaline, aseptate, barrel-shaped (4-) 5-7 (-8) x1-3 µm. Chlamydospores absent.

Additional specimen examined: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (culture CMW21109, PREM59868; CMW21111, PREM59869).

Ceratocystis microbasis Tarigan, M. van Wyk & M.J. Wingf. *prov. nom.*

(Fig 9)

Etymology: The name refers to the small size of the ascomatal bases of this fungus. Microbasis means with a small base.

Stat.conid.: *Thielaviopsis*

Coloniae cremeo-fulvae vel crinobrunneae, *mycelium* aerium. *Hyphae* laeves, septis non constrictis. *Bases ascomatum* pallide brunneae, atrobrunneae vel nigrae, globosae vel subglobosae (65-) 82-122 (-162) µm longae, (82-) 100-146 (-185) µm latae, spinis hyphisque ornatae; spinae atrobrunneae vel nigrae (4-) 6-12 (-19) µm longae. *Colla ascomatum* atrobrunnea vel nigra, apicem versus pallentia (185-) 301-499 (-574) µm longa, basi (41-) 57-81(-95) µm, apice (8-) 10-14 (-16) µm lata, basi discoidea. *Hyphae ostiolares* divergentes hyalinae (9-) 14-22 (-25) µm longae. *Asci* non visi. *Ascosporae* in massis fulvo-flavescentibus mucosis in apicibus collorum ascomatum. Ascosporae lateraliter visae cucullatae hyalinae, non septatae, vaginis inclusae, 5-7 x 2-4 µm cum vagina, 4-6 x 2-4 µm sine illa.

Anamorpha Thielaviopsis: *conidiophorae* in mycelio singulae, hyalinae, basi incrassatae, apicem versus angustatae, (18-) 22-36 (-41) µm longae, basi (2-) 2-4 (-5) µm, medio 2-4 µm, apice 1-2 µm latae. Conidia: hyalina cylindrica non septata (3-)4-6(-11) x 1-3 µm.

Typus: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (PREM59872 – holotype, living culture: CMW21117).

Colonies cream-buff (19``d) to hair brown (17````i) in color. *Mycelium* aerial. *Optimal temperature* for growth 25-30 ºC, no growth at 4 ºC, but growth observed at 35 ºC. *Hyphae* smooth, not constricted at septa. *Ascomatal bases* light brown or dark brown to black, globose to sub globose, (65-) 82-122 (-162) µm high, (82-) 100-146 (-185) µm wide, ornamented with spines and hyphae, spines dark brown to black, (4-) 6-12 (-19) µm long. *Ascomatal necks* dark brown to black and becoming lighter towards the apexes, (185-) 301-499 (-574) µm long, (41-) 57-81 (-95) µm wide at the bases, (8-) 10-14 (-16) µm wide at the apexes, with disc-like bases. *Ostiolar hyphae* divergent, hyaline, (9-) 14-22 (-25) µm long. *Asci* not observed. *Ascospores*

accumulating in buff-yellow (19d) mucilaginous masses at the apices of the ascomatal necks. Ascospores cucullate in side view, aseptate, hyaline, invested in sheath, 5-7 x 2-4 µm with sheath, 4-6 x 2-4 µm without sheath.

Thielaviopsis anamorph: *conidiophores* occurring singly on mycelium, hyaline, swollen at the bases, tapering toward the apexes, (18-) 22-36 (-41) µm long, (2-) 2-4 (-5) µm wide at the bases, 2-4 µm wide at the middle, 1-2 µm wide at the apexes. *Conidia* hyaline, aseptate, cylindrical (3-) 4-6 (-11) x (1-3) µm. Barrel-shaped conidia absent. Chlamydospores absent.

Additional specimen examined: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (culture CMW21115, PREM59871; CMW21118, PREM59873).

DISCUSSION

In this study three previously undescribed *Ceratocystis* spp. belonging to the *C. moniliformis s.l.* species complex were found associated with wounds on *A. mangium* in Indonesia. These three fungi were recognized as undescribed taxa based on DNA sequence comparisions. Their unique natures were also confirmed based on morphology. Furthermore, we were able to show that these fungi are able to infect wounded *A. mangium* and *A. crassicarpa* trees in Indonesia. However, the inability to re-isolate them from inoculated trees and the relatively limited lesions produced in pathogenicity tests suggests that they are not aggressive pathogens.

The new *Ceratocystis* spp. recognized in this study are all morphologically similar to species in the *C. moniliformis* species complex. In this respect, they all have long necks from which hat-shaped ascospores exude. More importantly, they all have globose bases ornamented with spines and their necks attach to the ascomatal bases with a distinct plate-like structure that easily becomes detached from the bases (Davidson 1935). Until recently, all species of *Ceratocystis* with these characteristics were broadly grouped within *C. moniliformis*. However, with the advent of DNA sequence comparisons, *C. moniliformis* has come to be recognized as an aggregate of several cryptic species for which numerous names have been provided (Yuan & Mohammed 2002, Van Wyk *et al*. 2006b).

The three new species from Indonesia are morphologically very similar to each other. They all rapidly produce both teleomorph and anamorph structures in culture, typically within one week. However, the ability to produce these structures diminishes over time. This appears to be a common feature of species in the *C. moniliformis* complex (Van Wyk *et al*. 2004).

The three species described in this study can relatively easily be distinguished from each other. *Ceratocystis inquinans* has hair brown aerial mycelium, which is different to *C. sumatrana* and *C. microbasis*, which both have cream buff to hair brown aerial mycelium. *Ceratocystis inquinans* and *C. sumatrana* have ascomatal bases, ascomatal necks and spines that are dark brown to black, while the ascomata of *C. microbasis* are lighter in color. *Ceratocyctis microbasis* can, furthermore, be distinguished from the other two species by the much smaller size of its teleomorph structures. The anamorph structures of the three new species are practically indistinguishable, however both *C. inquinans* and *C. sumatrana* have secondary conidiophores, while *C. microbasis* has no secondary conidiophores.

Phylogenetically, the species from Indonesia are most closely related to *C. omanensis*, *C. savannae* and *C. bhutanensis*. This is also supported by the similarity of some morphological characteristics of these new species with *C. omanensis*, *C. savannae* and *C. bhutanensis*. However, clear differences exist between these species. *Ceratocystis omanensis* and *C. savannae* have much longer ascomatal necks compared to Indonesian species and *C. bhutanensis* (Van Wyk *et al*. 2004, Al-Subhi *et al*. 2006, Kamgan *et al.* 2008). The Indonesian species produce secondary conidiophores, while *C. savannae* and *C. bhutanensis* have no secondary conidiophores (Van Wyk *et al.* 2004, Kamgan *et al*. 2008). Species from Indonesia have hair brown or cream buff to hair brown colonies, while *C. omanensis* has wood brown colonies, *C. savannae* has smoke grey colonies and *C. bhutanensis* has cream buff to dark olive to black colonies (Van Wyk *et al*. 2004, Al-Subhi *et al*. 2006, Kamgan *et al*. 2008).

The *Ceratocystis* spp. described in this study all have temperature optima at 30 ºC. This is consistent with the area in which they are found where temperatures range between 25 ºC and 30 ºC throughout the year. All isolates also grew well at 35 ºC but

they showed substantially reduced growth at temperatures below 20 ºC and failed to grow at 4 ºC. These growth trends are similar to those reported for *C. omanensis* and *C. savannae* (Al-Subhi *et al*. 2006, Kamgan *et al.* 2008), other species originating from areas with warm climates. In contrast, they are markedly different to *C. bhutanensis* (Van Wyk *et al*. 2004), *C. tribiliformis* (Van Wyk *et al*. 2006b), *C. moniliformis* (Hedgcock 1906) and *C. moniliformopsis* (Yuan & Mohammed 2002) that have lower temperature optima for growth, most of which originate from areas with cool climates.

The three *Ceratocystis* spp. from wounds on *A. mangium* were able to cause relatively short lesions on inoculated trees, but these were significantly different to the controls. However, they appeared to be short-lived in the stems of trees and we were not able to re-isolate them from lesions. This suggests that they are not significant pathogens which is consistent with other members of the *C. moniliformis* species complex (Davidson 1935, Bakshi 1951, Hunt 1956, Al–Subhi *et al*. 2006).

In the field inoculations, lesions produced on *A. mangium* trees were longer than those on *A. crassicarpa*. These results confirmed those of a previous study, which indicated that *A. mangium* is sensitive to wounding (Schmitt *et al.* 1995). Similar results were also obtained in Chapters 2 and 4 of this thesis in which inoculations with other fungi on *A. mangium* produced longer lesions than on *A. crassicarpa*.

This study resulted in the description of three previously unknown *Ceratocystis* spp. from a limited geographic area in Indonesia. This and the fact that three new species were isolated from three trees with one species per tree clearly emphasize the general lack of information on fungal diversity in general, even in relatively well studied environments. Results emerging from this study will be useful to managers of forest plantations as they develop strategies to control pathogens that result in tree death.

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Table 1. Morphological characteristics of Indonesian *Ceratocystis* species collected in this study compared to *C. omanensis* (Al-Subhi *et al.* 2006).

	C. inquinans **CMW 21106**	***C. sumatrana*** **CMW 21113**	***C. microbasis*** **CMW 21117**	***C. omanensis*** **CMW 11048**
Cultures				
Color	hair brown (17````i)	cream-buff (19``d) to hair brown (17````i)	cream-buff (19``d) to hair brown (17````i)	Wood brown (17``)
Growth rate	Opt. at 25-30ºC	Opt. at 25-30ºC	Opt. at 25-30ºC	Opt. at 30-35ºC
	Grows well at 35ºC, no growth at 4ºC	Grows well at 35ºC, no growth at 4ºC	Grows well at 35ºC, No growth at 4ºC	no growth at 4ºC
Hyphae	Smooth	Smooth	Smooth	Smooth
Ascomatal Base				
Color	Dark brown to black	Dark brown to black	Light brown or brown	Dark brown to black
Length	(116-) 149-205 (-236)µm	(148-) 168-218 (-293)µm	(65-) 82-122 (-162)µm	(154-) 206-254 (-279)µm
Width	(130-) 161-217 (-270)µm	(158-) 187-235 (-296)µm	(82-) 100-146 (-185)µm	?
Shape	Globose to sub globose	Globose to sub globose	Globose to sub globose	Globose
Ornamentation	Spines with hyphal hairs	Spines with hyphal hairs	Spines with hyphal hairs	Spines with hyphal hairs
Spines				
Color	Dark brown to black	Dark brown to black	Dark brown to black	Dark brown to black
Length	µm	(5-) 6-12 (-18)µm	(4-) 6-12 (-19)µm	(4-) 9-19 (-26)µm

Table 1. (Continued)

Ascomatal Necks				
Color	Dark brown to black	Dark brown to black	Light brown to brown	Dark brown to black
Disc-form at base	Present	present	present	Present
Length	(347-) 393-575 (-687)µm	(323-) 390-574 (-687)µm	(185-) 301-499 (-574)µm	(385-) 443-819 (-1097)µm
Width (Base)	(66-) 83-119 (-141)µm	(67-) 81-103 (-128)µm	(41-) 57-81 (-95)µm	(30-) 43-57 (-64)µm
Width (Tip)	(14-) 16-20 (-23)µm	(12-) 15-23 (-32)µm	(8-) 10-14 (-16)µm	(14-) 16-22 (-26)µm
Ostiolar hyphae				
Orientation	Divergent	Divergent	Divergent	Divergent
Length	(20-) 24-34 (-38)µm	(21-) 24-32 (-35)µm	(9-) 14-22 (-25)µm	(10-) 18-36 (-50)µm
Asci	Not seen	Not seen	Not seen	Not seen
Ascospores				
Colour	Hyaline	Hyaline	Hyaline	Hyaline
Shape (Side view)	Cucullate	Cucullate	Cucullate	Hat-shaped
Measurements				
with sheath	5-7 x 2-4µm	5-7 x 3-4µm	5-7 x 2-4µm	5-7 x 2-4µm
without sheath	4-6 x 2-4µm	4-6 x 3-4µm	4-6 x 2-4µm	4-6 x 2-4µm
Primary Conidiophores				
Length	(18-) 22-32 (-44)µm	(17-) 21-31 (-38)µm	(18-) 22-36 (-41)µm	(19-) 22-36 (-56)µm
Width (Base)	3-5µm	(2-) 2-4 (-5)µm	(2-) 2-4 (-5)µm	(1-) 2-4 (-5)µm

Table 1. (Continued)

Width (Middle)	2-4µm	(2-) 2-4 (-5)µm	2-4µm	-
Width (Tip)	1-3µm	1-3µm	1-2µm	1-3µm
Shape	Phialides	Phialides	Phialides	Phialides
Secondary Conidiophores				
Length	(19-) 23-35 (-43)µm	(23-) 23-37 (-47)µm	-	-
Width (Base)	(2-) 2-4 (-5)µm	2-4 µm	-	-
Width (Tip)	(2-) 2-4 (-5)µm	3-5µm	-	-
Shape	Phialides	Phialides	-	-
Primary Conidia				
Shape	Cylindrical	Cylindrical	Cylindrical	Cylindrical
Length	(5-) 6-8 (-11) µm	(5-) 5-7 (-8) µm	(3-) 4-6 (-11) µm	6-8 (-9) µm
Width	(2-) 3-5 (-7)µm	(2-) 2-4 (-5)µm	1-3µm	2-3µm
Secondary Conidia				
Shape	Barrel-shaped	Barrel-shaped		Barrel-shaped
Length	(4-) 5-7 (-8) µm	(4-) 5-7 (-8) µm		(5-) 6-8 (-10) µm
Width	1-3µm	1-3µm		3-5µm
Chlamydospores	Absent	Absent	Absent	Absent

Table 2. Isolates considered in the phylogenetic analyses.

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector
C. bhutanensis	CMW8217 CBS114289 PREM57807	AY528957 AY528962 AY528952	*Picea spinulosa*	Bhutan	T. Kirisits & D.B. Chhetri
C. bhutanensis	CMW8242 CBS112907 PREM57809	AY528956 AY528961 AY528951	*P. spinulosa*	Bhutan	T. Kirisits & D.B. Chhetri
Ceratocystis inquinans	CMW21106	EU588587 EU588666 EU588674	*Acacia mangium*	Riau, Indonesia	M. Tarigan
C. inquinans	CMW21107	EU588588 EU588667 EU588675	*A. mangium*	Riau, Indonesia	M. Tarigan
C. microbasis	CMW21115	EU588592 EU588671 EU588679	*A. mangium*	Riau, Indonesia	M. Tarigan
C. microbasis	CMW21117	EU588593 EU588672 EU588680	*A. mangium*	Riau, Indonesia	M. Tarigan
C. microbasis	CMW21118	EU588594 EU588673 EU588681	*A. mangium*	Riau, Indonesia	M. Tarigan
C. moniliformis	CMW4114	AY528997 AY528986 AY529007	*Schizolobium parahybum*	Ecuador	M.J. Wingfield
C. moniliformis	CMW9590 CBS116452	AY431101 AY528985 AY529006	*Eucalyptus grandis*	South Africa	J. Roux
C. moniliformopsis	CMW9986 CBS109441	AY528998 AY528987 AY529008	*E. obliqua*	Australia	Z.Q. Yuan
C. moniliformopsis	CMW10214 CBS115792 ORB33	AY528999 AY528988 AY529009	*E. sieberi*	Australia	M.J. Dudzinski

Table. 2. (Continued)

Species	Isolate no.	GenBank accession no.	Host	Geographical origin	Collector
C. omanensis	CMW11048 CBS115780 PREM57815	DQ074742 DQ074732 DQ074737	*Mangifera indica*	Oman	A.O. Al-Adawi
C. omanensis	CMW11046 CBS118112 PREM57814	DQ074739 DQ074729 DQ074734	*M. indica*	Oman	A.O. Al-Adawi
C. savannae	CMW17300 PREM59423	EF408551 EF408565 EF408572	*Acacia nigrescens*	South Africa	G.N. Kamgan & J. Roux
C. savannae	CMW17298	EF408551 EF408551 EF408551	*Terminalia sericea*	South Africa	G.N. Kamgan & J. Roux
C. sumatrana	CMW21109	EU588589 EU588668 EU588676	*A. mangium*	Riau, Indonesia	M. Tarigan
C. sumatrana	CMW21111	EU588590 EU588669 EU588677	*A. mangium*	Riau, Indonesia	M. Tarigan
C. sumatrana	CMW21113	EU588591 EU588670 EU588678	*A. mangium*	Riau, Indonesia	M. Tarigan
C. tribiliformis	CMW13012 PREM57826	AY529002 AY528992 AY529013	*Pinus merkusii*	Indonesia	M.J. Wingfield
C. tribiliformis	CMW13013 PREM57827 CBS115866	AY529003 AY528993 AY529014	*P. merkusii*	Indonesia	M.J. Wingfield
C.virescens	CMW3276	DQ061281 AY528990 AY529011	*Quercus* sp.	U.S.A	T. Hinds
C. virescens	CMW11164	DQ520639 EF070441 EF070413	*Fagus americanum*	U.S.A	D. Houston

Fig 1. A phylogenetic tree based on the combined sequence data of three gene regions; ITS, βt and EF1-α, showing relationships between *C. moniliformis s.l.* isolates used in this study. Isolates in bold were isolated from *Acacia mangium* in Indonesia and sequenced as part of this study. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above branches and Bayesian values follow in brackets. Two isolates of *C. virescens* were selected as the out-group.

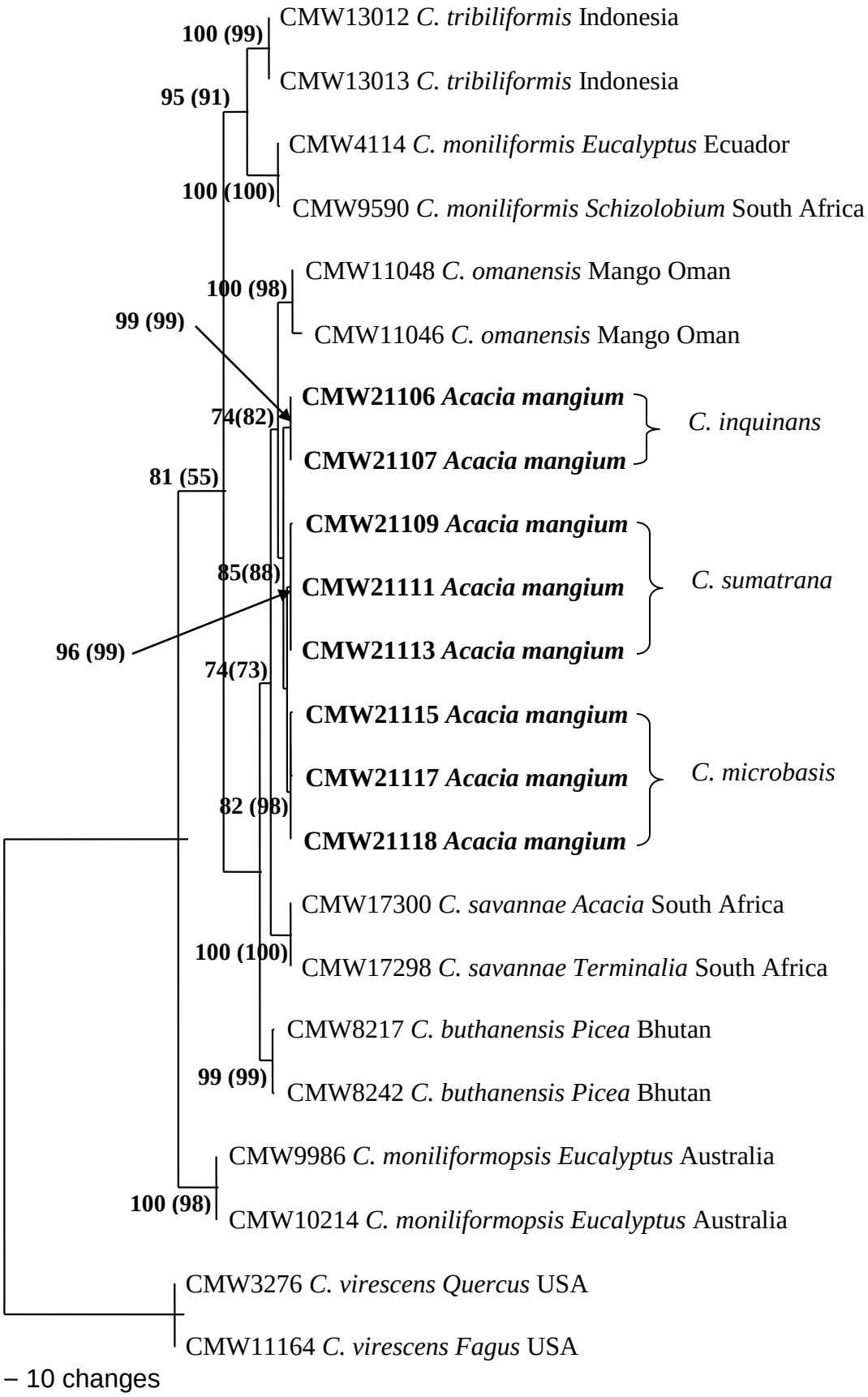
CMW13012 C. tribiliformis Indonesia
100 (99)
CMW13013 C. tribiliformis Indonesia
95 (91)
CMW4114 C. moniliformis Eucalyptus Ecuador
100 (100)
CMW9590 C. moniliformis Schizolobium South Africa
CMW11048 C. omanensis Mango Oman
100 (98)
99 (99)
CMW11046 C. omanensis Mango Oman
CMW21106 Acacia mangium
74(82)
C. inquinans
CMW21107 Acacia mangium
81 (55)
CMW21109 Acacia mangium
85(88)
CMW21111 Acacia mangium
C. sumatrana
96 (99)
CMW21113 Acacia mangium
74(73)
CMW21115 Acacia mangium
CMW21117 Acacia mangium
C. microbasis
82 (98)
CMW21118 Acacia mangium
CMW17300 C. savannae Acacia South Africa
100 (100)
CMW17298 C. savannae Terminalia South Africa
CMW8217 C. buthanensis Picea Bhutan
99 (99)
CMW8242 C. buthanensis Picea Bhutan
CMW9986 C. moniliformopsis Eucalyptus Australia
100 (98)
CMW10214 C. moniliformopsis Eucalyptus Australia
CMW3276 C. virescens Quercus USA
CMW11164 C. virescens Fagus USA
– 10 changes

Fig 2. Growth of *Ceratocystis inquinans* (CMW21106, CMW21107), *C. sumatrana* (CMW21109, CMW21111, CMW21113) and *C. microbasis* (CMW21115, CMW21117, CMW21118) at 7 different temperatures (4, 10, 15, 20, 25, 30, 35°C) after 48 hours incubation. Bars on the graph indicated with the same letter are not significantly different from each other (P value = 0.05).

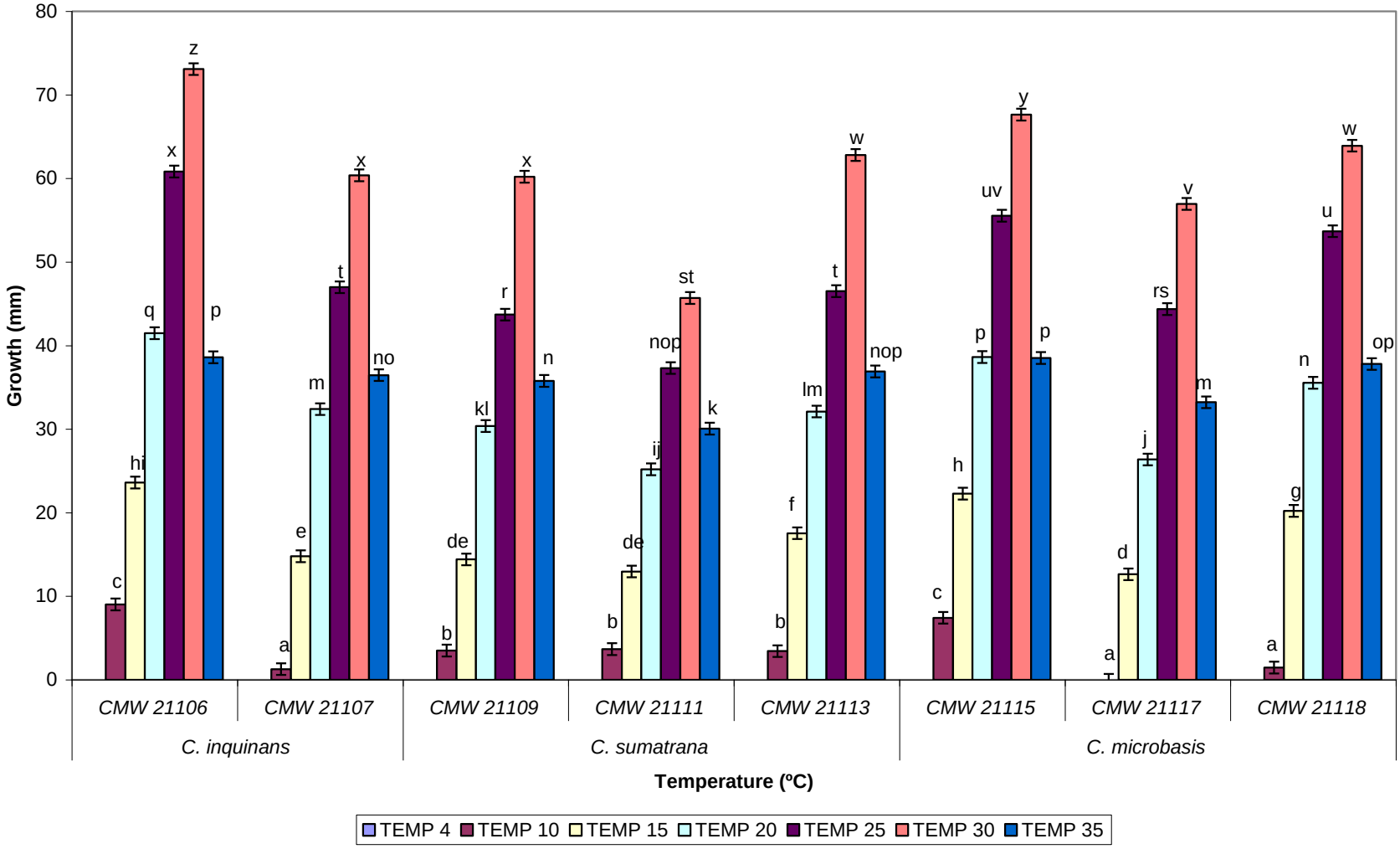
Growth (mm)
0
10
20
30
40
50
60
70
80
c
hi
q
x
z
p
a
e
m
t
x
no
b
de
kl
r
x
n
b
de
ij
nop
st
k
b
f
lm
t
w
nop
c
h
p
uv
y
p
a
d
j
rs
v
m
a
g
n
u
w
op
CMW 21106
CMW 21107
CMW 21109
CMW 21111
CMW 21113
CMW 21115
CMW 21117
CMW 21118
C. inquinans
C. sumatrana
C. microbasis
Temperature (ºC)
TEMP 4
TEMP 10
TEMP 15
TEMP 20
TEMP 25
TEMP 30
TEMP 35

Fig 3. Results of inoculations with *C. inquinans* (CMW21106), *C. sumatrana* (CMW21109, CMW2111, CMW21113) and *C. microbasis* (CMW21115, CMW21117) on one-year-old *A. mangium* and *A. crassicarpa* seedlings grown in 20 cm diameter polybags in the greenhouse, 10 days after inoculation. Bars on the graph indicated with the same letter are not significantly different from each other (P value = 0.05).

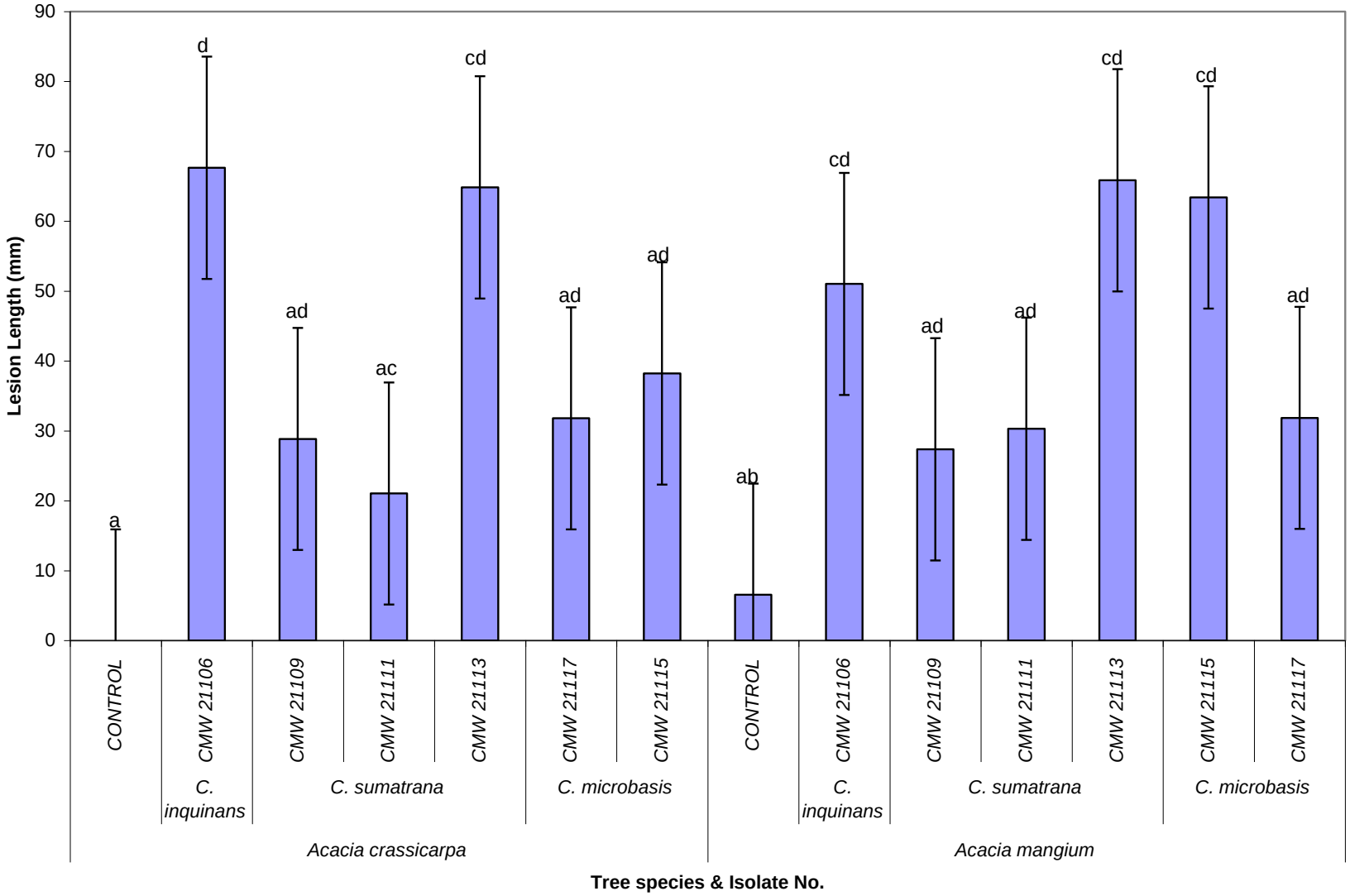
Lesion Length (mm)
90
80
70
60
50
40
30
20
10
0
a
d
ad
ac
cd
ad
ad
ab
cd
ad
ad
cd
cd
ad
CONTROL
CMW 21106
CMW 21109
CMW 21111
CMW 21113
CMW 21117
CMW 21115
CONTROL
CMW 21106
CMW 21109
CMW 21111
CMW 21113
CMW 21115
CMW 21117
C. inquinans
C. sumatrana
C. microbasis
C. inquinans
C. sumatrana
C. microbasis
Acacia crassicarpa
Acacia mangium
Tree species & Isolate No.

Fig 4. Lesions on the bark and the cambium of one-year-old *A. mangium* trees in Indonesia, six weeks after inoculation with *C. inquinans* (CMW21106), *C. sumatrana* (CMW21113) and *C. microbasis* (CMW21117). (a). External/bark lesion for control inoculation, (b). Internal/xylem lesion for control inoculation, (c). External/bark lesion caused by *C. inquinans*, (d). Internal/xylem lesion caused by *C. inquinans*, (e). External/bark lesion caused by *C. sumatrana,* (f). Internal/xylem lesion caused by *C. sumatrana*, (f). External/bark lesion caused by *C. microbasis,* (g). Internal/xylem lesion caused by *C. microbasis.*

Fig 5. Results of a first set of inoculations using *C. inquinans* (CMW21106, CMW21107), *C. sumatrana* (CMW21109, CMW2111, CMW21113) and *C. microbasis* (CMW21115, CMW21117, CMW21118) on one-year-old *A. mangium* and *A. crassicarpa* trees in an Indonesian plantation, six weeks after inoculation. Bars on the graph indicated with the same letter are not significantly different from each other (P value = 0.05).

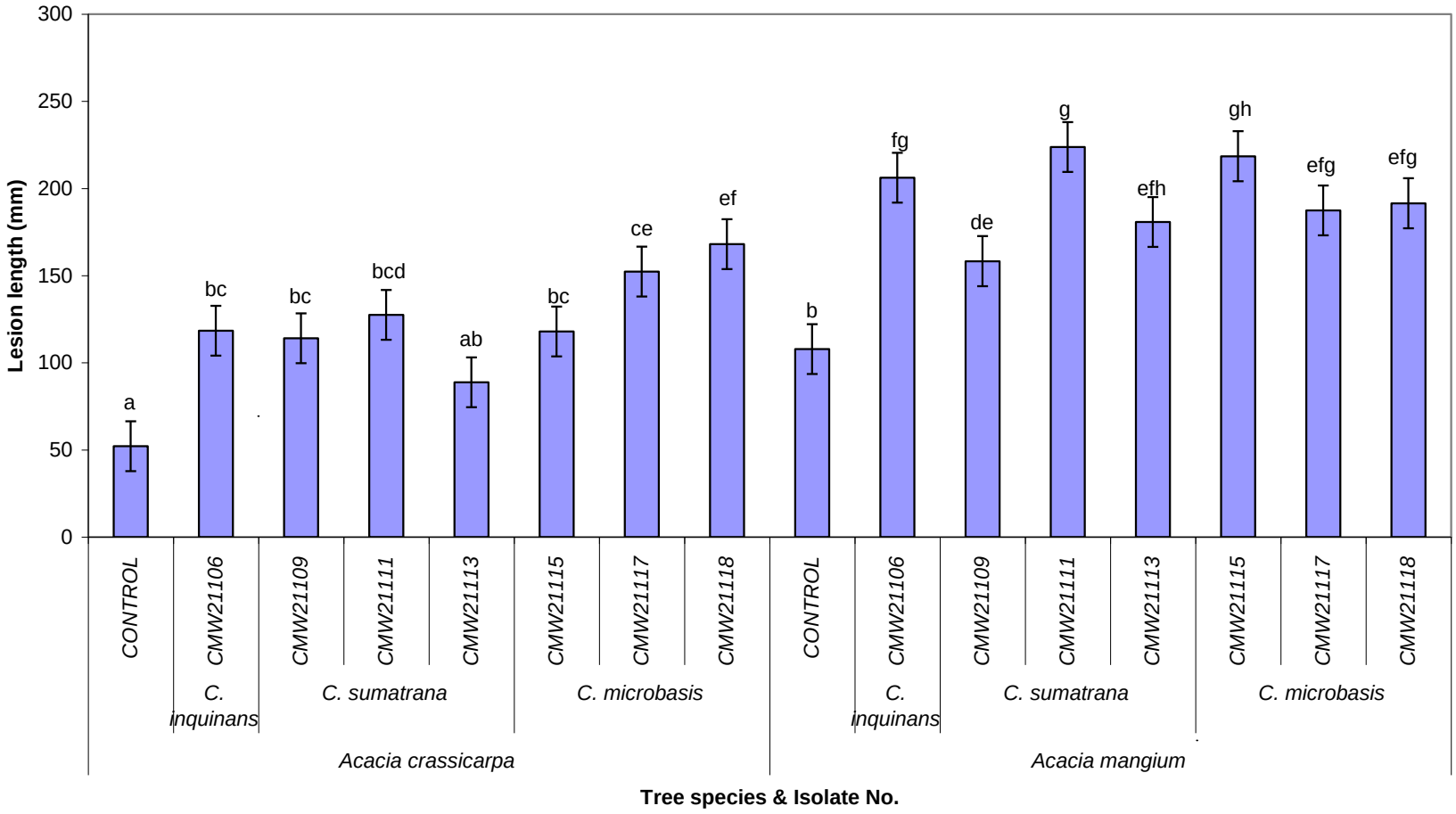
Lesion length (mm)
300
250
200
150
100
50
0
a
bc
bc
bcd
ab
bc
ce
ef
b
fg
de
g
efh
gh
efg
efg
CONTROL
CMW21106
CMW21109
CMW21111
CMW21113
CMW21115
CMW21117
CMW21118
CONTROL
CMW21106
CMW21109
CMW21111
CMW21113
CMW21115
CMW21117
CMW21118
C. inquinans
C. sumatrana
C. microbasis
C. inquinans
C. sumatrana
C. microbasis
Acacia crassicarpa
Acacia mangium
Tree species & Isolate No.

Fig 6. Results of a second set of inoculations using *C. inquinans* (CMW21106, CMW21107), *C. sumatrana* (CMW21109, CMW2111, CMW21113) and *C. microbasis* (CMW21115, CMW21117, CMW21118) on one-year-old *A. mangium* and *A. crassicarpa* trees in an Indonesian plantation, six weeks after inoculation. Bars on the graph indicated with the same letter are not significantly different from each other (P value = 0.05).

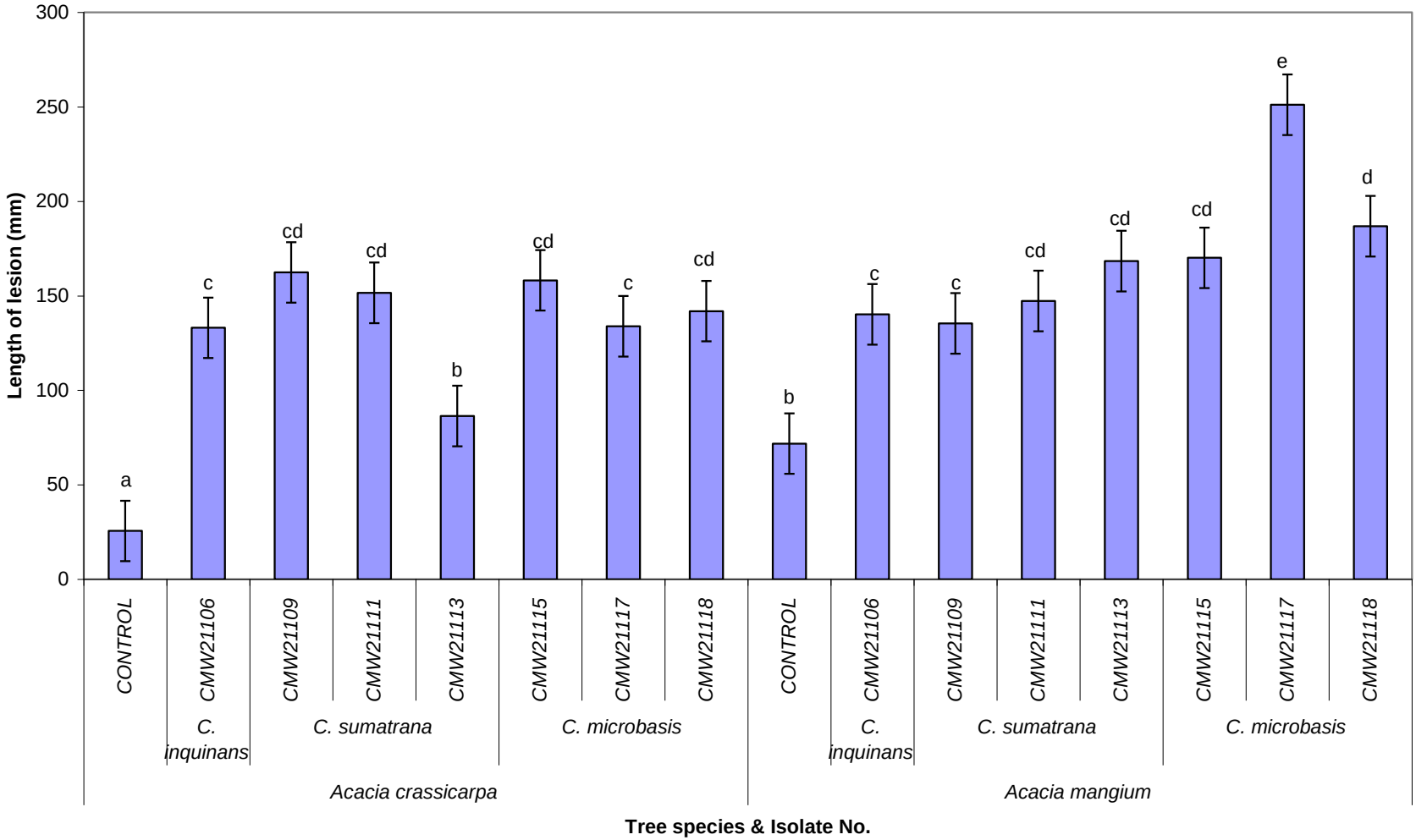
Length of lesion (mm)
300
250
200
150
100
50
0
a
c
cd
cd
b
cd
c
cd
b
c
c
cd
cd
cd
e
d
CONTROL
CMW21106
CMW21109
CMW21111
CMW21113
CMW21115
CMW21117
CMW21118
CONTROL
CMW21106
CMW21109
CMW21111
CMW21113
CMW21115
CMW21117
CMW21118
C. inquinans
C. sumatrana
C. microbasis
C. inquinans
C. sumatrana
C. microbasis
Acacia crassicarpa
Acacia mangium
Tree species & Isolate No.

Fig 7. Morphological characteristics of *Ceratocystis inquinans prov. nom*. (CMW21106). (a). Globose ascomata with long neck, (b). Ascomatal base with conical spines and hyphal ornamentation, (c). Ascomatal neck with disc-shaped attachment at base, (d). Divergent ostiolar hyphae, (e). Hat-shaped ascospores in side view, (f). Primary phialides, (g). Secondary phialides, (h). Barrel-shaped conidia, (i). Cylindrical conidia. [Scale bars (a, c, e) = 50 µm; (d) = 10 µm; (b, f-i) = 5 µm].

Fig 8. Morphological characteristics of *Ceratocystis sumatrana prov. nom.* (CMW21113). (a). Globose ascomata with long neck, (b). Ascomatal base with conical spines and hyphal ornamentation, (c). Ascomatal neck with disc-shaped attachement at base, (d). Divergent ostiolar hyphae, (e). Hat-shaped ascospores in side view, (f). Cylindrical conidia, (g). Barrel-shaped conidia, (h). Primary phialides, (i). Secondary phialides. [Scale bars (a) = 100 µm; (c, d) = 10 µm; (b, e-i) = 5 µm].

Fig 9. Morphological characteristics of *Ceratocystis microbasis prov. nom.* (CMW21117). (a). Globose ascomata with long neck, (b). Ascomatal base with conical spines and hyphal ornamentation, (c). Ascomatal neck with disc-shaped attachment at base, (d). Divergent ostiolar hyphae, (e). Hat-shaped ascospores in side view, (f). Chonidiophore/phialide, (g). Cylindrical conidia. [Scale bars (a) = 100 µm; (c) = 50 µm; (d) = 10 µm; (b, e, f, g) = 5 µm].

Chapter 4

Identification and pathogenicity of Botryosphaeriaceae from diseased *Acacia mangium* in Indonesia

ABSTRACT

Species in the Botryosphaeriaceae are tree pathogens, causing cankers and in some cases death of many tree species globally. A number of species affect trees in commercial plantations of *Acacia*, *Eucalyptus* and *Pinus* spp. During a survey of diseases of *Acacia mangium* trees in plantations in Indonesia, Botryosphaeriaceae were commonly isolated. The aim of this study was to identify these isolates and to consider their pathogenicity to *A. mangium* and *A. crassicarpa* trees. Isolates were identified using morphological characteristics and comparisons of DNA sequence data for the ITS and EF1-α gene regions. Three species were identified including *Lasiodiplodia theobromae* and two previously undescribed species of *Pseudofusicoccum*, described here as *P. sumatranum prov. nom.* and *P. acaciicola prov. nom.* Pathogenicity trials on *A. mangium* and *A. crassicarpa* trees showed that all three species have the potential to cause disease of *A. mangium* and *A. crassicarpa*. However, *L. theobromae* was the most pathogenic and is apparently an important contributing factor to diseases of *Acacia* spp. in Indonesia.

Keywords: Dieback, *Lasiodiplodia*, plantation forestry, *Pseudofusicoccum*, stem canker.

INTRODUCTION

Fungi in the Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) are known to be associated with disease of numerous tree species (Punithalingam 1979, Slippers & Wingfield 2007), including *Acacia* spp. (Hadi & Nuhamara 1997, Pongpanich 1997, Roux & Wingfield 1997, Sharma & Florence 1997, Old *et al.* 2000, Mohali *et al*. 2007) in many parts of the world. Most parts of the trees, including the stems, branches, twigs, fruits and leaves can be infected by these fungi, resulting in stem cankers, shoot blight, fruit rot, die-back and gummosis of the infected hosts (Von Arx 1987, Slippers & Wingfield 2007). The Botryosphaeriaceae are typically known as endophytes that exist in healthy tree tissue and only cause disease under conditions unsuitable for the host tree (Smith *et al*. 1996, Old *et al.* 2000, Slippers & Wingfield 2007). Trees subjected to extreme environmental conditions such as infertile soil, water stress, freezing, wounding or damage caused by insects, other pathogens and improper silvicultural practices such as excessive pruning, are generally affected by diseases caused by the Botryosphaeriaceae (Wene & Schoeneweiss 1980, Pusey 1989, Smith *et al*. 1994, Old *et al*. 2000, Ma *et al*. 2001). For example, *Botryosphaeria dothidea* (Moug.: Fr.) Ces. & De Not. infections on *Eucalyptus* spp. in South Africa are associated with drought, hot wind and frost (Smith *et al*. 1994), while water stress increases the infection of, for example, pistachio trees (Ma *et al*. 2001) by this pathogen.

Acacia mangium Willd., *A. crassicarpa* Cunn.: Benth., *A. auriculiformis* A. Cunn.: Benth. and *A. aulococarpa* Cunn.: Benth. have been established in commercial plantations in Indonesia. These plantations have increased in dramatically magnitude since the 1980's after the Government initiated a forest plantation program known as Hutan Tanaman Industri (HTI) to produce wood that is mainly used for pulp and paper production (Anonymous 2004). *Acacia mangium* and *A. crassicarpa* are the major species planted and they are native to northern Queensland, the western Province of Papua New Guinea, Irian Jaya and Maluku, the eastern islands of Indonesia. As plantation forestry species, *A. mangium* and *A. crassicarpa* form extensive monocultures on the islands of Kalimantan and Sumatra, distant from where they occur naturally. They have thus been separated from their natural enemies.

Emerging pests and diseases, however, present a major threat to their future productivity.

In recent years, a number of diseases caused by fungi have been reported affecting *A. mangium* and *A. crassicarpa* trees in plantations in South East Asia as well as other tropical and sub-tropical regions (Lee 1993, Old *et al*. 1997, Nair & Sumardi 2000, Old *et al*. 2000). Among the causal agents of these diseases, fungi in the Botryosphaeriaceae are commonly associated with cankers and dieback. *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl., has been isolated from cankers on *A. mangium*, *A. auriculiformis* and *A. aulococarpa* trees in Indonesia (Hadi & Nuhamara 1997); *Neofusicoccum mangiferae* (Syd. & P. Syd.) Crous. [reported as *Nattrassia mangiferae* (Syd. & P. Syd.) B. Sutton & Dyko] was associated with *A. mangium* and *A. auriculiformis* in India (Sharma & Florence 1997) and in Thailand an unidentified species of *Botryosphaeria* has been associated with diseased *A. auriculiformis* (Pongpanich 1997).

Species in the Botryosphaeriaceae have previously been separated into two groups, namely those with *Diplodia*-like anamorphs and and those with *Fusicoccum*-like anamorphs (Jacobs & Rehner 1998, Denman *et al*. 2000). However, a recent study by Crous *et al*. (2006), based on the Large Subunit gene sequences revealed 11 clades in the Botryosphaeriaceae. Of these, clade 1 was represented by species of *Diplodia*, *Lasiodiplodia*, *Sphaeropsis* and *Tiarosporella*; clade 2 by *Botryosphaeria dothidea*, *Macrophomopsis* and *Dichomera saubinetii*; clade 3 by *Macrophomina* and *B. mamane*; clade 4 by *Neoscytalidium*; clade 5 by *Dothidotthia* (anamorph *Dothiorella*); clade 6 by *Neofusicoccum* and *Dichomera*; clade 7 by *Pseudofusicoccum*; clade 8 by *B. melanops*, clade 9 by *Saccharata proteae* and clade 10 by *Guignardia* and *Pyllosticta*; while clade 11 included *Camarosporium, P. plevolandica, Byssothecium circinans, Karstenula rhadostoma, Letendraea helminthicola* and *Microdiplodia*.

During disease surveys in *A. mangium* plantations in the Southern part of Sumatra, stem cankers and wilting symptoms typical of those caused by fungi in the Botryosphaeriaceae were observed. Isolates having both *Lasiodiplodia* and *Pseudofusicoccum* conidia were obtained from the disease symptoms. The aims of this study were to identify these Botryosphaeriaceae associated with diseased *A.*

mangium based on morphology and DNA sequence comparisons. Inoculation trials, both in the greenhouse and in the field were carried out to consider the pathogenicity of these fungi on *A. mangium* and *A. crassicarpa*.

MATERIALS AND METHODS

Symptoms and fungal isolates

Fungal isolates were collected from pieces of wood sampled randomly from 40, eight to 12 month-old *A. mangium* trees showing top, branch and stem die-back and cankers (Fig 1) in plantations located in the Teso (LAT 0º2'33"N, LONG 101º24'21 E, 20 trees) and Logas (LAT 0º17'52"S, LONG 101º15'53 E, 20 trees) areas, Riau Province, Indonesia. Disease symptoms observed on the trees included stem cankers, wilting and death of trees. The bark surrounding the cankers was a black colour due to the exudation of gum in response to fungal infection. In the case of severely affected trees this discoloration covered the entire length of the stems. Wood below the cankers was discoloured, often in streaked patterns, but later turning a uniform dark brown to dark blue colour. Diseased trees were scattered throughout the plantations and infection appeared to have commenced shortly after pruning. These trees had been pruned approximately six to eight weeks before sampling and the disease was suspected to be caused by a wound-infecting pathogen.

Small pieces of wood and cambium were cut from the leading edges of lesions associated with the cankers. These were wrapped in newspaper to maintain moisture and transported to the laboratory for further examination. Small pieces (approximately 3 mm^2) of discoloured cambium and wood were plated onto 2 % (w/v) Malt Extract Agar (MEA) (Biolab, Midrand, South Africa) and incubated for 4 - 7 days until fungal growth was observed. Dark, grey-coloured, fluffy mycelium typical of the Botryosphaeriaceae were then transferred to clean MEA plates.

To induce sporulation, isolates from pure cultures were transferred to 2 % Water Agar (WA) (Biolab, Midrand, South Africa) with sterilized pine needles placed on the agar surface (Slippers *et al*. 2005) and incubated for 10 - 14 days under near-UV light or until sporulation was observed on the pine needles. Masses of conidia were collected from pycnidia, transferred to WA and distributed evenly over the surface of the agar

to obtain single spores. These plates were incubated for 4 – 10 hrs until conidia had germinated after which single germinating spores were transferred onto 2 % (w/v) MEA. Some conidia from the pine needles were mounted directly on microscope slides in lactic acid (85 %) in order to study their morphology.

All isolates collected in this study have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates have also been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Delft, Netherlands. Representative specimens were also dried and deposited at the National Fungal Herbarium of South Africa (PREM), Pretoria.

Morphological characteristics

A Carl Zeiss microscope and a Zeiss Axiovision camera (Oberkochen, Germany) was used to examine morphological characteristics of all isolates, which were grouped based on conidial shape, colour and size. Detailed morphological characteristics of isolates representing discrete groups, identified based on DNA sequence comparisons (as described below) were assessed based on measurements of the conidiomata, conidiogenous cells and conidia. All material for microscopic studies was mounted in lactic acid (85 %). Fifty measurements of each characteristic structure were made from each representative isolate selected to serve as a extype specimen for new species and 10 measurements from each isolate selected as a paratype. The average (mean), standard deviation (stdv), maximum (max) and minimum (min) measurements were calculated and measurements in this study are presented as (min-) mean minus stdv – mean plus stdv (-max) for each isolate.

DNA extraction and PCR amplification

Representative isolates for each morphological group were grown on 2 % MEA at 22 ºC for two weeks. Using sterilized scalpels, the mycelium was scraped off the surface of the agar and transferred into 1.5 ml Eppendorf tubes and lyophilized overnight. The lyophilized material was placed in liquid nitrogen and crushed to a fine powder using a glass rod or a mechanical grinder (Ball mills machine, Retsch, Haan, Germany). The DNA was extracted using a modified phenol-chloroform method of Raeder and Broda (1985) as described in Smith *et al*. (2001).

The Internally Transcribed Spacer regions (ITS1, ITS2), including the 5.8S gene, and part of the Transcription Elongation factor 1-α (EF1-α) gene region were amplified using polymerase chain reaction (PCR) on an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). DNA was amplified using the primers ITS1 and ITS4 (White *et al.* 1990) and EF1-728F and EF1-986R (Carbone & Kohn 1999), respectively. PCR reaction mixtures consisted of 0.5 µL of each primer (10 mM), 2 µL of 10 mM dNTP mixture (2.5 mM of each dNTP), 0.5 µL of DNA Taq enzyme (Roche Molecular Biochemicals, Almeda California), 2.5 µL of 10x concentration buffer containing $MgCl_2$ (3.5 mM), 2 µL DNA template (2 -10 ng) and 17 µL Sabax water to make 25 µL total volume reactions. The PCR program was set at 95 ºC for 4 min for DNA denaturation, followed by 10 cycles of denaturation at 94 ºC for 20 s, 55 ºC for 48 s for annealing and 72 ºC for 45 s for elongation. A further 25 cycles at 94 ºC for 20 s, 55 ºC for 40 s with an additional 5 s added after each cycle, followed by 72 ºC for 45 s. The program was completed with a final step of 10 min at 72 ºC. Amplification of the respective gene regions was confirmed by visualizing bands under UV illumination using a 2 % agarose (Roche Diagnostics, Mannheim, Germany) gel stained with ethidium bromide. Amplicons were purified using Sephadex G-50 columns (SIGMA, Steinheim, Germany) following the protocol provided by the manufacturer.

Sequencing and analyses

The ABI PRISM™ Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Foster City, California) was used to sequence the purified PCR amplicons in both the forward and reverse directions. The same primers as those used for the PCR reactions were used for sequencing. The sequenced products were purified using the same protocol as used for the PCR reactions and the final sequence products were run on an ABI PRISM™ 3100 Autosequencer (Applied BioSystems). Sequence Navigator version 1.0.1 (Applied BioSystems) was used to analyse the sequence electropherograms and these were checked for inconsistencies.

The obtained sequences were compared, through Blast analyses, with sequences of Botryosphaeriaceae that were available in GenBank (Table 1). Using the online version of MAFFT (Katoh *et al*. 2002) version 6 (http://align.bmr.kyushu-u.ac.zp/mafft/online/server/), sequences of isolates from *A. mangium* in Indonesia

were aligned with published sequences of closely related species identified using Blast and then checked manually before they were analyzed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10* (Swofford 2002). Gaps were treated as "newstate" and trees were obtained via stepwise addition of 1000 replicates with the Mulpar option in effect. The most parsimonious tree for each data set was obtained through the heuristic search option based on parsimony with tree bisection reconnection. Confidence intervals using 1000 bootstrap replicates were calculated. *Mycosphaerella pini* (A. Funk & A.K. Parker) Arx. was used as the out-group taxon. Both sequence data sets, for the two different gene regions sequenced, were combined after a partition homogeneity test was run and they were then analyzed as described above.

MrBayes version 3.1.1. (Ronquist & Huelsenbeck 2003) was used to run Bayesian analyses on the combined data set and to determine support on the nodes of the phylogenetic trees based on Bayesian posterior probabilities using the Markov Chain Monte Carlo (MCMC) algorithm. The model of nucleotide substitution for each gene region was determined using MrModeltest2 (Nylander 2004) and the models obtained were used in the Bayesian analysis. One million random trees were generated with four chains and sampled every 100^{th} generation following the MCMC procedure. Samples were taken from trees only after convergence and trees outside the point of convergence were discarded.

Culture characteristics

To determine the optimum temperatures for growth, three isolates from each of the groups identified using DNA sequence comparisons (Table 1) were grown on 2 % MEA for two weeks at 22 ºC. A five mm diam sterilized cork borer was used to cut discs of mycelium from the actively growing margins of cultures. These were placed at the centers of 90 mm diam Petri dishes containing 2 % MEA. Discs for each isolate were placed at the centres of five plates each and incubated at 4 ºC and between 10 ºC and 35 ºC at 5 ºC intervals. Two growth measurements of colony diameter, at right angels to each other, were taken two days after incubation and averages for all measurements were computed. The experiment was repeated once. Data from both studies were combined and analyzed using analysis of variance (ANOVA). The color of cultures was described using Rayner's color charts (Rayner 1970).

Pathogenicity tests

Greenhouse inoculations **–** One-year-old *A. mangium* and *A. crassicarpa* seedlings (~15 mm diam) were used in pathogenicity tests. The seedlings were grown in 20 cm diam plastic bags containing a mixture of topsoil and compost and placed in a greenhouse where the temperature and humidity (~30 ºC; 65 %) was adjusted for optimum growth. Five isolates of each Botryosphaeriaceae group, identified using DNA sequence comparisons and morphology were used in the greenhouse pathogenicity test (Table 1). These included the *Lasiodiplodia* group (CMW23003, CMW23018, CMW23026, CMW23031, CMW32073), *Pseudofusicoccum* Group A (CMW23068, CMW23076, CMW23078, CMW23085, CMW23089) and *Pseudofusicoccum* Group B (CMW23072, CMW23075, CMW23079, CMW23081, CMW23084). Each of these isolates was inoculated onto five seedlings of each of *A. mangium* and *A. crassicarpa*. Five additional seedlings for each *Acacia* sp. were inoculated with sterile 2 % MEA plugs to serve as controls.

Bark discs (5 mm diam) were removed from the stems of the seedlings using a cork borer and agar discs of the size taken from an actively growing colony on 2 % MEA were inserted into the wounds, mycelium facing inwards. These were then covered with Parafilm (Pechiney, Menasha, Wisconsin) to reduce desiccation and contamination. Twelve days after inoculation, the Parafilm was removed and the lengths of the lesions on the xylem were measured. A piece of symptomatic wood was taken from the lesions on each tree and placed onto 2 % MEA to verify the presence of the inoculated fungus. Data for lesion lengths were analyzed using SAS statistical analyses (SAS Version 8.2, 2001).

Field inoculations **-** One-year-old *A. mangium* and *A. crassicarpa* trees in a plantation in South Sumatra, Indonesia were inoculated with three isolates from each group of Botryosphaeriaceae found to be the most pathogenic in the greenhouse inoculations. Twenty trees of each *Acacia* sp. were used for each isolate. Prior to inoculation, a wound was made on the tree stems using a sterilized cork borer (10 mm diam) and inoculations were carried out using the same technique as described above for the greenhouse trials. For controls, 20 trees of each *Acacia* sp. was inoculated with sterile 2 % MEA plugs.

Both tree diameter at the point of inoculation and the lengths of lesions were measured six weeks after inoculation. Pieces of symptomatic tissue were taken from the inoculated trees and plated onto 2 % MEA to verify the presence of the inoculated fungi. The entire experiment was repeated once. Analysis of variance (ANOVA) was calculated for all data obtained using SAS statistical analyses (SAS Version 8.2, 2001).

RESULTS

Symptoms and fungal isolates

One hundred and nine Botryosphaeriaceae isolates were obtained from 30 (75 %) of the 40 diseased *A. mangium* trees sampled. Within two weeks of isolation, the isolates had grown on WA amended with pine needles and anamorphic structures formed readily on the needles. Cultures obtained from diseased *A. mangium* trees in Indonesia could be placed in two main groups based on morphology. These were a *Lasiodiplodia* group where isolates were characterized by hyaline conidia which turned brown with age and developed longitudinal striations at maturity, and a *Pseudofusicoccum* group where conidia remained hyaline and aseptate. Of the isolates, 89 (82 %) obtained from 26 trees (65 %) had *Lasiodiplodia* conidia of which 37 isolates originated from the Logas area and 53 isolates from the Teso area. Twenty isolates (18 %), obtained from 10 trees (25 %), had *Pseudofusicoccum* conidia, with 18 isolates originating from the Logas area and only two isolates from the Teso area. Isolates from both conidial groups were found to co-occur on six trees.

The *Pseudofusicoccum* isolates could be further divided into two morphological groups referred to as *Pseudofusicoccum* A and *Pseudofusicoccum* B, based on slight differences in colony colour and conidial sizes (Table 2). Isolates of *Pseudofusicoccum* A had hyaline conidia with an average length of 38 µm and width of 9 µm. The conidia of *Pseudofusicoccum* B were also hyaline but were smaller, on average 36 µm long and 8 µm wide.

Sequencing and analyses

The PCR amplification of the ITS and EF1-α gene regions resulted in fragments of ~500 and ~300 base pairs (bp) in size, respectively. Blast searches in GenBank

indicated that *Lasiodiplodia* isolates were most similar to *L. theobromae*, while those of the *Pseudofusicoccum* group were most similar to *P. stromaticum* (Mohali, Slippers & M.J. Wingf.) Mohali, Slippers & M.J. Wingf.

The partition homogeneity test of the two data sets for the ITS and EF1-α gene regions resulted in a P value of 0.01, which is an acceptable level to combine the data (Cunningham 1997, Barker & Lutzoni 2002) and all datasets were thus combined. The combined data set had a total of 866 characters including gaps, of which 389 were constant characters, 105 were parsimony-uninformative characters and 372 were parsimony-informative characters. Thirty two most parsimonious trees, with similar topologies, were obtained and one of these was selected for presentation (Fig 2). This tree had a length of 1139 steps, a consistency index (CI) = 0.7147, homoplasy index (HI) = 0.2853, retention index (RI) = 0.9478 and rescaled consistency index (RCI) = 0.6774.

A GTR + I + G [Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 6 rates = invgamma] model was produced from model test analysis (MrModeltest2) for the ITS data set and a HKY + G [Prset statefreqpr = dirichlet (1, 1, 1, 1); Lset nst = 2 rates = gamma] model was produced for the EF1-α dataset. Both models were then used in Bayesian analysis. The first 4000 trees were discarded following the burn-in procedure. Both Bootstrap and Bayesian values were attached to the posterior probability of the branch nodes of the combined datasets (Fig 2).

Two distinct clades emerged from the phylogenetic analyses, supporting the morphological observations that two main groups of Botryosphaeriaceae were associated with diseased *A. mangium* in Indonesia. Isolates that resembled *Pseudofusicoccum* spp. grouped most closely, but separate from *P. stromaticum.* Based on both ITS and EF1-α sequences, as well as in the combined tree, these isolates represent two distinct and previously undescribed *Pseudofusicoccum* species (Fig 2). The second group of isolates identified based on morphology were confirmed to represent *L. theobromae* (Fig 2).

Isolates of *Pseudofusicoccum* A (CMW23068, CMW23076, CMW23078, CMW23085, CMW23089) had a bootstrap value of 70 % and had no Bayesian

support. *Pseudofusicoccum* B isolates (CMW23072, CMW23074, CMW23075, CMW23079, CMW23081, CMW23082, CMW23083, CMW23084, CMW23086) had bootstrap values of 99 % and Bayesian values of 100 % (Fig 2). The *L. theobromae* isolates (CMW23003, CMW23008, CMW23018, CMW23026, CMW23031, CMW23073) had bootstrap values of 63 % and Bayesian values of 77 % (Fig 2).

Cultural characteristics

All cultures from *A. mangium* were fast growing, especially those of *L. theobromae*. The optimum temperature range for growth of the *Lasiodiplodia* isolates was 25 - 30 ºC, reaching a diameter of 43 - 64 mm in two days. The growth was reduced at 35 ºC and 20 ºC and very slow growth occurred at 15 ºC, while no growth occurred at 10 ºC or below (Fig 3).

The optimum temperature range for growth of both *Pseudofusicoccum* spp. was 30 - 35 ºC and colonies reached an average diameter of 26 - 39 mm and 34 - 43 mm for *Pseudofusicoccum* A and *Pseudofusicoccum* B, respectively. Growth of these fungi was reduced at 25 ºC and 20 ºC, very slow at 15 ºC and there was no growth at 10 ºC or below (Fig 3).

The color of *Pseudofusicoccum* A colonies was mouse gray (15`````) to olive gray (23`````b) at the surface and dark mouse gray (15`````k) on the reverse side when cultured on 2 % MEA at 25 ºC for 15 days. *Pseudofusicoccum* B isolates were mouse gray (15`````) to dark mouse gray (15`````k) at the surface and dark mouse gray (15`````k) to fuscous black (13````m) as seen from the reverse sides of plates when cultured on 2 % MEA at 25 ºC after 15 days.

Pathogenicity tests

Greenhouse inoculations - All isolates used in the greenhouse pathogenicity tests produced lesions within 12 days after inoculation. All *L. theobromae* isolates (CMW23003, CMW23018, CMW23026, CMW23031, CMW32073) consistently produced longer lesions on both *A. mangium* and *A. crassicarpa* seedlings than the *Pseudofusicoccum* spp., and these were significantly different from the controls (Fig 4). The average lesion length for the *L. theobromae* isolates was 58 mm on *A.*

crassicarpa and 92 mm on *A. mangium*. Other than isolate CMW23018 all cultures of *L. theobromae* produced significantly longer lesions than either of the *Pseudofusicoccum* spp. (P values = 0.05, R-Square = 0.83; Coeff. Var = 41.5; Root MSE = 15.6).

With the exception of CMW23068, isolates of *Pseudofusicoccum* A inoculated onto *A. crassicarpa* produced distinct lesions when compared with the control inoculations (P values = 0.05, R-Square = 0.83; Coeff. Var = 41.5; Root MSE = 15.6). When inoculated onto *A. mangium*, three *Pseudofusicoccum* A isolates also produced significantly longer lesions when compared to the controls and the exceptions were CMW23078 and CMW23085 that were not different to the controls (P values = 0.05, R-Square = 0.83; Coeff. Var = 41.5; Root MSE = 15.6). In contrast, only one isolate (CMW23079) of *Pseudofusicoccum* B inoculated onto *A. crassicarpa* produced a lesion that was significantly different to the controls, and no isolates of *Pseudofusicoccum* B inoculated onto *A. mangium* produced significant lesions when compared to the control inoculations (P values = 0.05, R-Square = 0.83; Coeff. Var = 41.5; Root MSE = 15.6).

Re-isolations from inoculation sites yielded the inoculated fungi while no Botryosphaeriaceae emerged from control inoculations. Based on the results of the greenhouse inoculations, three isolates of *L. theobromae* (CMW23003, CMW23026, CMW23031), *Pseudofusicoccum* A (CMW23068, CMW23078, CMW23089) and *Pseudofusicoccum* B (CMW23072, CMW23079, CMW32084) were selected for field inoculations.

Field inoculations **–** In both pathogenicity tests, all Botryosphaeriaceae isolates produced lesions on *A. mangium* and *A. crassicarpa* trees within six weeks of inoculation (Fig 5). *Lasiodiplodia theobromae* isolates consistently produced lesions on both *A. mangium* and *A. crassicarpa* trees that were longer than those associated with the *Pseudofusicoccum* spp. or controls (P values = 0.05, R-Square = 0.54; Coeff. Var = 36.2; Root MSE = 4.71; Fig 6 and P values = 0.05, R-Square = 0.44; Coeff. Var = 50.6; Root MSE = 6.2; Fig 7 for first and second test respectively). Lesions for *L. theobromae* ranged in size from 17 - 26 cm on *A. mangium* trees and 12 - 16 cm on *A. crassicarpa* trees. While on *Pseudofusicoccum* A the lesions ranged in size from 9 -

16 cm and 6 – 9 cm on *A. mangium* and *A. crassicarpa* trees respectively. Lesions for *Pseudofusicoccum* B ranged in size from 10 – 16 cm and 7 – 13 cm on *A. mangium* and *A. crassicarpa* trees. Most of the *Pseudofusicoccum* A and B isolates inoculated on *A. mangium* were significantly different to the controls, but only a few of these isolates inoculated on *A. crassicarpa* produced lesions significantly different from the controls. Overall, lesions on *A. mangium* trees were longer than those on *A. crassicarpa* trees for all the fungi tested (P value = 0.05). Fungi used in inoculations were re-isolated from lesions. Occasionally isolates with dark mycelium, identified as *L. theobromae* based on ITS sequences, emerged from the control inoculations.

TAXONOMY

The *Pseudofusicoccum* isolates from diseased *A. mangium* in Riau Province, Indonesia are distinct from other described species of Botryosphaeriaceae examined in this study. The results show that these isolates represent two previously undescribed *Pseudofusicoccum* spp. The following descriptions are provided for these new species:

Pseudofusicoccum sumatranum Tarigan, Slippers & M.J. Wingf. *prov. nom.*

(Fig 8)

Etymology: Sumatranum refers to the Indonesian island of Sumatra where this species was first discovered.

Coloniae cultae laxae, supra murinae vel olivaceo-griseae, infra atromurinae post 15 d. in MEA ad 25° C. *Conidiomata* mediocriter 388 µm diametro, solitaria in foliis pinorum subimmersa, papillata, hyphis tecta, parietibus pycnidiorum e textura angulare atro-brunnea factis, ad regionem conidiogenam gracilescenti hyalinescentique. *Cellulae conidiogenae* hyalinae, laeves, cylindricae (10–) 11–17 (–18) x 3–5 µm. *Conidia* hyalina strato mucoso tenue tecta, parietibus tenuis, non septata, laevia, contento tenue granulare, ellipsoidea, recta vel subirregularia, apice basique rotundata, (30–) 33–43 (–47) x (7–) 8-10 (-11) µm.

Teleomorpha non visa.

Typus: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (PREM59875 – holotype, living culture: CMW23076).

Cultures fluffy, mouse gray (15`````) to olive gray (23`````b) (surface) and dark mouse gray (15`````k) (reverse) after 15 d on MEA at 25 ºC. Colonies reaching 14 - 23 mm diam on MEA after 2 d in the light at 25 ºC. Cardinal temperatures for growth; min. 15 ºC, optimum range 30 - 35 ºC. *Conidiomata* average 388 µm diam, solitary, semi immersed on pine needles, papillate, covered with hyphae (Fig 8), pycnidial walls consisting of a dark brown *textura angularis*, becoming thinner and hyaline towards the conidiogenous region. *Conidiogenous cells* hyaline, smooth, cylindrical, (9–) 11–15 (–16) x (2-) 3–5 µm (average of 50 conidiogenous cells 13 x 4 µm). *Conidia* hyaline, covered in thin mucus layer, thin walled, aseptate, smooth with fine granular content, ellipsoid, straight to slightly irregular, both apex and base rounded (Fig 7), (30–) 33–43 (–47) x (7–) 8-10 (-11) µm (average of 50 conidia 38.0 x 9.0 µm).

Teleomorph: Not observed.

Additional specimens examined: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (paratype PREM59874, living culture CMW23068; paratype PREM59876, living culture CMW23078; paratype PREM59877, living culture CMW23085; paratype PREM59878, living culture CMW23089).

Pseudofusicoccum acaciicola Tarigan, Slippers & M.J. Wingf. *prov. nom.*

(Fig 9)

Etymology: Acaciicola reffers to the genus name of the tree from which this fungus was first discovered. Acaciicola means dweller on *Acacia.*

Coloniae cultae laxae, supra murinae vel atromurinae, infra atromurinae vel fusconigrae post 15 d. in MEA ad 25° C. *Conidiomata* mediocriter 266 µm diametro, solitaria in foliis pinorum subimmersa, papillata, hyphis tecta, parietibus pycnidiorum e textura angulare atro-brunnea factis, ad regionem conidiogenam gracilescenti hyalinescentique. *Cellulae conidiogenae* hyalinae, laeves, cylindricae (10–) 11–15 (–17) x (1.5–) 2–3 µm. *Conidia* hyalina strato mucoso tenue tecta, parietibus tenuis, non

septata, laevia, contento tenue granulare, ellipsoidea, recta vel subirregularia, apice basique rotundata, (29–) 33–39 (–47) x (-6) 7-9 (10-) µm.

Teleomorpha non visa.
Typus: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (PREM59879 – holotype, living culture: CMW 23072).

Cultures fluffy, mouse gray (15`````) to dark mouse gray (15`````k) (surface) and dark mouse gray (15`````k) to fuscous black (13````m) (reverse) after 15 d on MEA at 25 ºC. Colonies reaching 18-26 mm diam on MEA after 2 d in the light at 25 ºC. Cardinal temperatures for growth; min. 15 ºC, optimum 30-35 ºC. *Conidiomata* 266 µm diam, solitary, semi immersed on pine needles, papillate, covered with hyphae, pycnidial walls consisting of a dark brown *textura angularis*, becoming thinner and hyaline towards the conidiogenous region (Fig 9). *Conidiogenous cells* hyaline, smooth, cylindrical (Fig 9), (6–) 8–12 (–14) x 2–4 µm (average of 50 conidiogenous cells 10 x 3 µm). *Conidia* hyaline, covered in thin mucus layer, thin walled, aseptate, smooth with fine granular content, ellipsoid, straight to slightly irregular, apex and base both rounded (Fig 9), (29–) 33–39 (–47) x (-6) 7-9 (10-) µm (average of 50 conidia 36 x 8 µm).

Teleomorph: Not observed.

Additional specimens examined: Indonesia: Sumatra, isolated from *Acacia mangium*, 2005, M. Tarigan (paratype PREM59880, living culture CMW23075; paratype PREM59881, living culture CMW23079; paratype PREM59882, living culture CMW23081; paratype PREM59883, living culture CMW23084).

DISCUSSION

Three species of the Botryosphaeriaceae were collected from diseased *A. mangium* plantations in this study. These were identified as *L. theobromae* and two newly described species, *Pseudofusicoccum sumatranum* and *P. acaciicola* (referred to *Pseudofusicoccum* A and B). Both *Pseudofusicoccum* spp. were most closely related to *P. stromaticum. Lasiodiplodia theobromae* and the *Pseudofusicoccum* spp.

occasionally occurred simultaneously on trees confirming that co-infection of different Botryosphaeriaceae is possible on trees (Shear *et al*. 1925, Brown & Britton 1986, Pavlic *et al*. 2007).

Lasiodiplodia theobromae was the most commonly isolated fungus associated with the die-back disease of young *A. mangium* trees. This is not surprising as the fungus is well-known as a pathogen of woody plants in the tropics (Punithalingam 1979). *Lasiodiplodia theobromae* is well-known on *Acacia* spp. in Indonesia and it has been reported from *A. mangium*, *A. auriculiformis* and *A. aulococarpa* trees in West and South Kalimantan (Hadi & Nuhamara 1997). Results of the present study, including pathogenicity tests in the greenhouse and field, strongly suggest that *L. theobromae* contributes to die-back and death of young *A. mangium* trees in Riau, Central Sumatra.

Lasiodiplodia theobromae was first described in 1892 by Patouillard (Patouillard & de Lagerheim 1892) and since then it has been reported on many woody crops in the tropics and sub-tropics (Punithalingam 1979). More recently, it has been shown that there are numerous species morphologically similar to *L. theobromae* (Pavlic *et al*. 2004, Burgess *et al*. 2006) that could easily be confused with it. For this reason, it was necessary to confirm the identity of isolates from *A. mangium* based on DNA sequence comparisons. Isolates from Indonesia collected in this study, however, grouped with other *L. theobromae* isolates with relatively low bootstrap and posterior probability values. This, together with previous reports (Alves *et al.* 2007, Alves *et al.* 2008), suggest that *L. theobromae* represents a species complex. This question deserves further consideration, but fell outside the scope of the present study.

Discovery of two previously undescribed *Pseudofusicoccum* spp. on *A. mangium* is consistent with the recent recognition of many new species in the Botryosphaeriaceae in studies using DNA sequence comparisons. In the past, species of *Pseudofusicoccum* have often been attributed to morphologically similar species such as *Botryosphaeria dothidea* and *Neofusicoccum ribis* (Slippers, Crous & M. J. Wingf.) Crous, Slippers & A. J. L. Phillips (Mohali *et al*. 2006, Crous *et al*. 2006). Analyses of DNA sequence data, in conjunction with morphological characteristics have, however, led to the recognition of several cryptic taxa within the

Botryosphaeriaceae (Denman *et al.* 1999, Zhou & Stanosz 2001, Denman *et al.* 2003, De Wet *et al*. 2003, Slippers *et al*. 2004). The two *Pseudofusicoccum* spp. described in the current study could easily have been overlooked if it were not for the faceted approach to the identification of Botryosphaeriaceae used in this study. The fact that these two species were obtained in much lower numbers than *L. theobromae* could also have easily lead to them being overlooked. Great care should thus be taken in similar studies to ensure a true indication of the species diversity and interaction on trees.

Pseudofusicoccum sumatranum and *P. acaciicola* are closely related to each other and to the recently described *P. stromaticum*. The conidia of these three species are covered by thin mucus layers, which is a distinguishing character of this genus (Crous *et al.* 2006). *Pseudofusicoccum sumatranum* and *P. acaciicola* from Indonesia, however, have much larger conidia than those of *P. stromaticum*. They can also be distinguished from *P. stromaticum* based on the fact that their cultures are dark grey in colour compared to the greenish olivaceous cultures of *P. stromaticum* (Mohali *et al*. 2006). *Pseudofusicoccum sumatranum* and *P. acaciicola* have papillate, solitary and semi immersed conidiomata on pine needles, while *P. stromaticum* has superficial, multilocular and eustromatic conidiomata (Mohali *et al*. 2006). *Pseudofusicoccum sumatranum* and *P. acaciicola* can also be distinguished from each other based on both DNA sequence data and differences in morphology. In this regard *P. sumatranum* has larger conidia than those of *P. acaciicola*. Furthermore, colonies of *P. sumatranum* are lighter in colour than those of *P. acaciicola*.

In pathogenicity trials *P. sumatranum and P. acaciicola* produced lesions on both *A. mangium* and *A. crassicarpa*. However, they were much smaller than those of *L. theobromae*, and not all isolates produced lesions statistically different to those of the controls. Their low levels of pathogenicity, and slower growth in culture as compared to *L. theobromae*, are consistent with the fact that they were only occasionally isolated from cankered and dying *A. mangium* trees. The low level of pathogenicity of *Pseudofusicoccum* spp. in this study is similar to the results of pathogenicity trials of *P. stromaticum* by Mohali (2006), suggesting that species of *Pseudofusicoccum* are weak pathogens.

The occasional re-isolation of *L. theobromae* from tissue associated with control inoculations was not surprising. It is well known that the Botryosphaeriaceae live as endophytes in healthy trees and they cause disease when trees are predisposed to their development (Smith *et al.* 1996, Pavlic *et al*. 2004, Slippers & Wingfield 2007). Wounds made in the inoculation tests would have produced the stress necessary for the development of *L. theobromae* propagules existing as endophytes. Despite their obvious background presence, they did not confound the overall outcome of the inoculation tests, which clearly established the pathogenicity of *L. theobromae* on both *A. mangium* and *A. crassicarpa*. The presence of inoculated species could be confirmed through re-isolation to clarify results.

This study emerged from the appearance of a serious canker and die-back disease of *A. mangium* in Riau, Central Sumatra, plantations. Although this disease has not been observed on *A. crassicarpa*, trees of this species were inoculated because it is widely planted in the area and there was concern that it might emerge as susceptible to the disease in the future. However, inoculations showed clearly that *A. mangium* is substantially more susceptible to *L. theobromae* and the two new *Pseudofusicoccum* spp. than is *A. crassicarpa*. The higher level of susceptibility of *A. mangium* could be related to the fact that this tree responds poorly to wounding (Schmitt *et al.* 1995). Disease avoidance may thus be achieved by planting *A. crassicarpa* in areas where *A. mangium* are most commonly affected. Disease tolerance may also be found in provenances or clones of *A. mangium* consistent with the findings of Hadi & Nuhamara (1997), who showed that *A. mangium* provenances differ in their susceptibility to stem cankers. Trials to select canker-tolerant *A. mangium* planting stock should thus be considered, using the techniques and species identified in this study.

Lasiodiplodia theobromae appears to be a common and important pathogen of *A. mangium* in Riau, Central Sumatra. In this study, infections were mainly associated with trees after they had been pruned. The Botryosphaeriaceae are known to be wound and stress-related pathogens (Smith *et al*. 1994, Ma *et al.* 2001, Van Niekerk *et al*. 2006), and pruning clearly increases the risk of *Acacia* plantations to infection. While pruning to reduce branches and to gain single stems is a basic practice in *Acacia* forestry, every effort should be made to refine pruning in order to minimize

stem damage and stress. This, together with the planting of trees having lower levels of susceptibility to *L. theobromae* should provide a reasonable solution to what is currently a serious disease problem.

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Table 1. Isolates considered in the phylogenetic analyses.

Identity	Culture no.	Other no.	Host	Location	Collector	GenBank Accession no. ITS	GenBank Accession no. EF-1α
Botryosphaeria dothidea	CMW9075	ICMP8019	*Populus sp.*	New Zealand	G.J. Samuels	AY236950	AY236899
B. dothidea	CMW8000	CBS115476	*Prunus sp.*	Switzerland	B. Slippers	AY236949	AY236898
Diplodia mutila	CMW7060	CBS431.82	*Fraxinus excelsior*	Netherlands	H.A. van der Aa	AY236955	AY236904
D. seriata	CMW7774		*Ribes sp.*	New York, USA	B. Slippers/G. Hundler	AY236953	AY236902
D. seriata	CMW8230	920729	*Picea glauca*	Canada	J. Reid	AY972104	DQ280418
Lasiodiplodia crassispora		WAC12533	*Santalum album*	Kununurra, Australia	T.I. Burgess/B. Dell	DQ103552	DQ103559
L. crassispora		WAC12534	*S. album*	Kununurra, Australia	T.I. Burgess/B. Dell	DQ103550	DQ103557
L. crassispora		WAC12535	*S. album*	Kununurra, Australia	T.I. Burgess/B. Dell	DQ103551	DQ103558
L. gonubiensis	CMW14077	CBS115812	*Syzygium cordatum*	Eastern Cape, S. Africa	D. Pavlic	AY639595	DQ103566
L. gonubiensis	CMW14078	CBS116355	*S. cordatum*	Eastern Cape, S. Africa	D. Pavlic	AY639594	DQ103567
L. rubropurpurea		WAC12537	*E. grandis*	Tully, Queensland	T.I. Burgess/G. Pegg	DQ103554	DQ103572
L. rubropurpurea		WAC12538	*E. grandis*	Tully, Queensland	T.I. Burgess/G. Pegg	DQ103555	DQ103573
L. rubropurpurea		WAC12539	*E. grandis*	Tully, Queensland	T.I. Burgess/G. Pegg	DQ103556	DQ103574
L. theobromae	CMW9074		*Pinus sp.*	Mexico	T.I. Burgess	DQ103533	DQ103565
L. theobromae	CMW18420	BOT979	*Casuarina cunninghamii*	Uganda	J. Roux	DQ103534	DQ103564
L. theobromae	CMW18421	BOT1279	*E. urophylla*	Mexico	M.J. Wingfield	DQ103542	DQ103560
L. theobromae	CMW23003		*Acacia mangium*	Riau, Indonesia	M. Tarigan	EU588629	EU588609
L. theobromae	CMW23008		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588630	EU588610
L. theobromae	CMW23018		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588633	EU588613
L. theobromae	CMW23026		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588634	EU588614
L. theobromae	CMW23031		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588631	EU588611
L. theobromae	CMW23073		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588632	EU588612
L. theobromae	CMW18422	BOT1479	*P. patula*	Mpumalanga, S. Africa	W. de Beer	DQ103544	DQ103562
L. venezuelensis		WAC13513	*A. mangium*	Acarigua, Venezuela	S. Mohali	DQ103549	DQ103570
L. venezuelensis		WAC12540	*A. mangium*	Acarigua, Venezuela	S. Mohali	DQ103547	DQ103568
L. venezuelensis		WAC12541	*A. mangium*	Acarigua, Venezuela	S. Mohali	DQ103548	DQ103569
Mycosphaerella pini	CMW15077		*P. ponderosa*	Idaho, USA	L.M. Carris	AY808299	AY808264
Neofusicoccum andinum	CMW13446	CBS117452	*Eucalyptus sp.*	Merida, Venezuela	S. Mohali	DQ306263	DQ306264
N. andinum	CMW13455	CBS117453	*Eucalyptus sp.*	Merida, Venezuela	S. Mohali	AY693976	AY693977
N. australe	CMW6837		*Acacia sp.*	Batemans Bay, NSW, Australia	M.J. Wingfield	AY339262	AY339270

Table 1. (Continued)

Identity	Culture no.	Other no.	Host	Location	Collector	GenBank Accession no. ITS	GenBank Accession no. EF-1α
N. australe	CMW9073		*Acacia sp.*	Melbourne, Victoria, Australia	J. Roux/ D. Guest	AY339261	AY339269
N. eucalyptorum	CMW11705		*E. nitens*	S. Africa	B. Slippers	AY339256	AY339264
N. eucalyptorum	CMW10125	CBS115791	*E. grandis*	Mpumalanga, S. Africa	H. Smith	AF283686	AY236891
N. eucalypticola	CMW6539	CBS115679	*E. grandis*	Orbost, Victoria, Australia	M.J. Wingfield	AY615141	AY615133
N. eucalypticola	CMW6543	CBS115770	*Eucalyptus sp.*	Orbost, Victoria, Australia	M.J. Wingfield	AY615140	AY615132
N. luteum	CMW10309	CAP002	*Vitis vinifera*	Portugal	A.J.L. Philips	AY339258	AY339266
N. luteum	CMW10310	CAP037	*V. vinifera*	Portugal	A.J.L. Philips	AY339259	AY339267
N. mangiferae	CMW7024	BRIP24101	*Mangifera indica*	Australia	G.I. Johnson	AY615185	DQ093221
N. mangiferae	CMW7797	BRIP24083	*M. indica*	Australia	G.I. Johnson	AY615186	DQ093220
N. parvum	CMW6235		*Tibouchina lepidota*	Melbourne, Victoria, Australia	M.J. Wingfield	AY615136	AY615128
N. parvum	CMW9081	ICMP8003	*P. nigra*	New Zealand	G.J. Samuels	AY236943	AY236888
N. ribis	CMW7054	CBS121.26	*Ribes sp.*	New York, USA	N.E. Stevens	AF241177	AY236879
N. ribis	CMW7772	CBS115475	*Ribes sp.*	New York, USA	B. Slippers/ G. Hundler	AY236935	AY236877
Pseudofusicoccum acaciicola	CMW23072		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588615	EU588595
P. acaciicola	CMW23074		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588620	EU588600
P. acaciicola	CMW23075		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588619	EU588599
P. acaciicola	CMW23079		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588617	EU588597
P. acaciicola	CMW23081		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588618	EU588598
P. acaciicola	CMW23082		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588622	EU588602
P. acaciicola	CMW23083		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588621	EU588601
P. acaciicola	CMW23084		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588616	EU588596
P. acaciicola	CMW23086		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588623	EU588603
P. stromaticum	CMW13434		*E.urophylla*	Acarigua, Venezuela	S. Mohali	AY693974	AY693975
P. stromaticum	CMW13435		*E.urophylla*	Acarigua, Venezuela	S. Mohali	DQ436935	DQ436936
P. sumatranum	CMW23068		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588626	EU588606
P. sumatranum	CMW23076		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588624	EU588604
P. sumatranum	CMW23078		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588627	EU588607
P. sumatranum	CMW23085		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588628	EU588608
P. sumatranum	CMW23089		*A. mangium*	Riau, Indonesia	M. Tarigan	EU588625	EU588605

Table 2. Morphological characteristics of two new *Pseudofusicoccum sumatranum* and *P. acaciicola* from *A. mangium* in Indonesia compared to the closely related species *P. stromaticum* (Mohali *et al*. 2006).

	P. sumatranum **CMW23076**	***P. acaciicola*** **CMW23072**	***P. stromaticum*** **CMW13366**
Cultures			
Color (surface)	Mouse gray (15`````) to olive gray (23`````b)	Mouse gray (15`````) to dark mouse gray (15`````k)	Greenish olivaceous (23```b)
Color (reverse)	Dark mouse gray (15`````k)	Dark mouse gray (15`````k) to fuscous black (13````m)	Olivaceous (21``k)
Growth rate	Opt. at 30-35ºC Little or no growth at 10ºC 14-23mm diam on MEA after 2 days in the light at 25ºC	Opt. at 30-35ºC Little or no growth at 10ºC 18-26mm diam on MEA after 2 days in the light at 25ºC	Opt. at 30-35ºC Little or no growth at 10ºC 70-75mm diam on MEA after 4 days in the dark at 25ºC
Hyphae	Fluffy	Fluffy	Fluffy
Conidia			
Color	Hyaline	Hyaline	Hyaline
Length	(30-) 33-43 (-47) µm	(29-) 33-39 (-47) µm	(19-) 20-23 (-24) µm
Width	(7-) 8-10 (-11) µm	(6-) 7-9 (-10) µm	(4-) 5-6 µm
Averages	38 x 9 µm	36 x 8 µm	21.5 x 5.5 µm
Conidiomata			
Size	388 µm diam, soliter	266 µm diam, soliter	Large, multilocular
Shape	semi immerse, papillate	semi immerse, papillate	Superficial, eustromatic
Conidiogenous cells			
Color	Hyaline, smooth	Hyaline, smooth	Hyaline, smooth
Shape	Cylindrical	Cylindrical	Cylindrical
Length	(9-) 11-15 (-16) µm	(6-) 8-12 (-14) µm	(10-) 11-15 (-17) µm
Width	(2-) 3-5 µm	2-4 µm	2-3 µm
Averages	13 x 4 µm	10 x 3 µm	13 x 2.5 µm

Fig 1. Disease symptoms of the Botryosphaeriaceae on *A. mangium* trees in Indonesia.. (a). Stem cankers, wilting and death of trees, (b). Tip die-back, (c). Discoloration of bark on main stem of tree and gum exudation from lesions, (d). Discolored xylem of infected tree.

Fig 2. A phylogenetic tree based on the combined sequence data of two gene regions, ITS and EF1-α, showing relationships between Botryosphaeriaceae used in this study. Isolates in bold were from *Acacia mangium* in Indonesia and sequenced as part of this study. The phylogram was obtained using the heuristic search option based on parsimony. Bootstrap values are indicated above branches and Bayesian values follow in the brackets. *Mycosphaerella pini* was selected as the out-group.

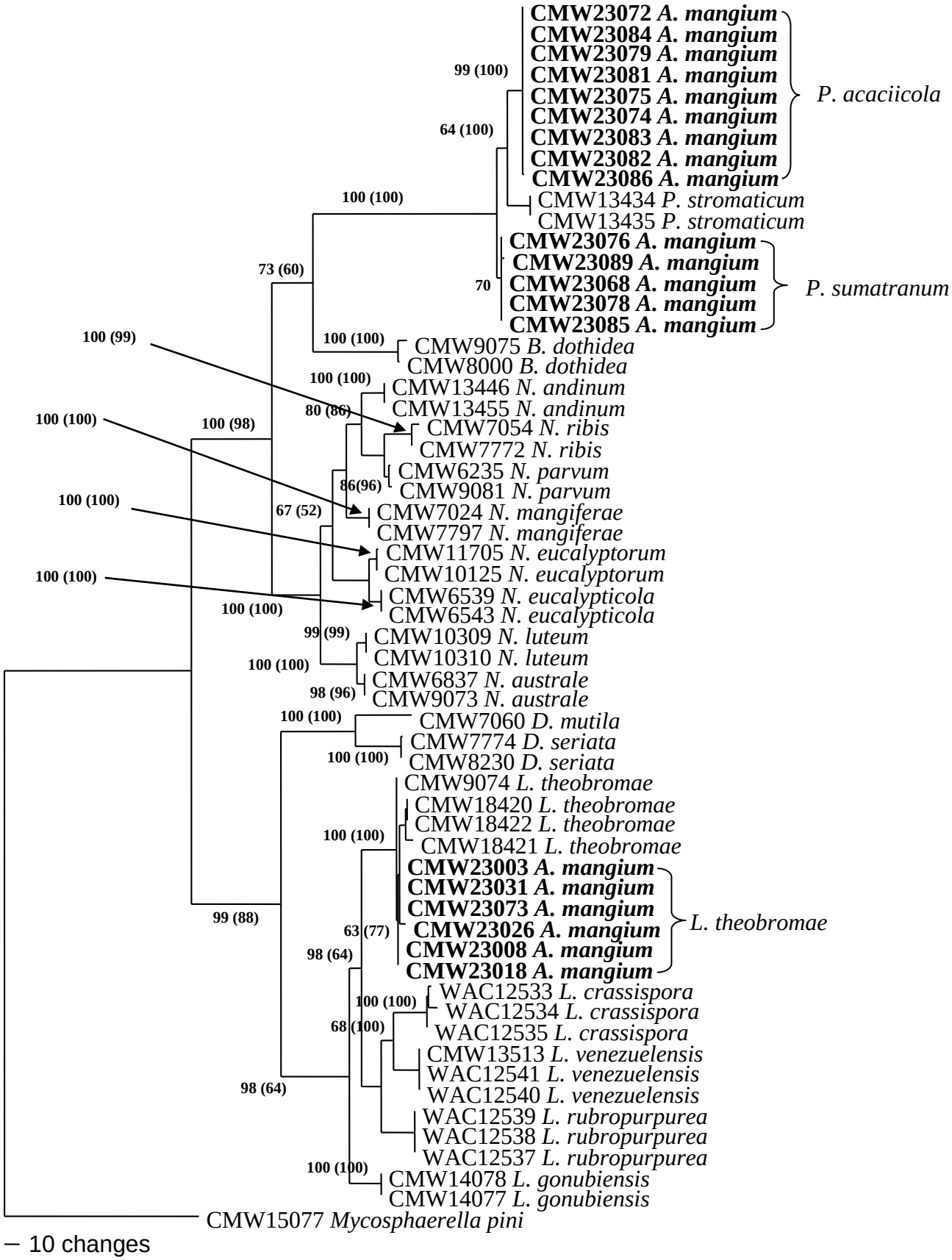
CMW23072 A. mangium
CMW23084 A. mangium
CMW23079 A. mangium
CMW23081 A. mangium
CMW23075 A. mangium
CMW23074 A. mangium
CMW23083 A. mangium
CMW23082 A. mangium
CMW23086 A. mangium
P. acaciicola
CMW13434 P. stromaticum
CMW13435 P. stromaticum
CMW23076 A. mangium
CMW23089 A. mangium
CMW23068 A. mangium
CMW23078 A. mangium
CMW23085 A. mangium
P. sumatranum
CMW9075 B. dothidea
CMW8000 B. dothidea
CMW13446 N. andinum
CMW13455 N. andinum
CMW7054 N. ribis
CMW7772 N. ribis
CMW6235 N. parvum
CMW9081 N. parvum
CMW7024 N. mangiferae
CMW7797 N. mangiferae
CMW11705 N. eucalyptorum
CMW10125 N. eucalyptorum
CMW6539 N. eucalypticola
CMW6543 N. eucalypticola
CMW10309 N. luteum
CMW10310 N. luteum
CMW6837 N. australe
CMW9073 N. australe
CMW7060 D. mutila
CMW7774 D. seriata
CMW8230 D. seriata
CMW9074 L. theobromae
CMW18420 L. theobromae
CMW18422 L. theobromae
CMW18421 L. theobromae
CMW23003 A. mangium
CMW23031 A. mangium
CMW23073 A. mangium
CMW23026 A. mangium
CMW23008 A. mangium
CMW23018 A. mangium
L. theobromae
WAC12533 L. crassispora
WAC12534 L. crassispora
WAC12535 L. crassispora
CMW13513 L. venezuelensis
WAC12541 L. venezuelensis
WAC12540 L. venezuelensis
WAC12539 L. rubropurpurea
WAC12538 L. rubropurpurea
WAC12537 L. rubropurpurea
CMW14078 L. gonubiensis
CMW14077 L. gonubiensis
CMW15077 Mycosphaerella pini
99 (100)
64 (100)
100 (100)
73 (60)
70
100 (99)
100 (100)
100 (100)
80 (86)
100 (98)
100 (100)
86(96)
67 (52)
100 (100)
100 (100)
100 (100)
99 (99)
100 (100)
98 (96)
100 (100)
100 (100)
99 (88)
100 (100)
63 (77)
98 (64)
100 (100)
68 (100)
98 (64)
100 (100)
10 changes

Fig 3. Growth of three species in the Botryosphaeriaceae, *L. theobromae* (CMW23003, CMW23026, CMW23031), *Pseudofusicoccum sumatranum* (CMW23068, CMW23076, CMW23085) and *P. acaciicola* (CMW23072, CMW23079, CMW23081) at 7 different temperatures (0, 10, 15, 20, 25, 30, 35 ºC) after 48 hours incubation. No growth observed at 10 ºC and below. Bars on the graph indicated with the same letter are not significantly different from each other (P value = 0.05).

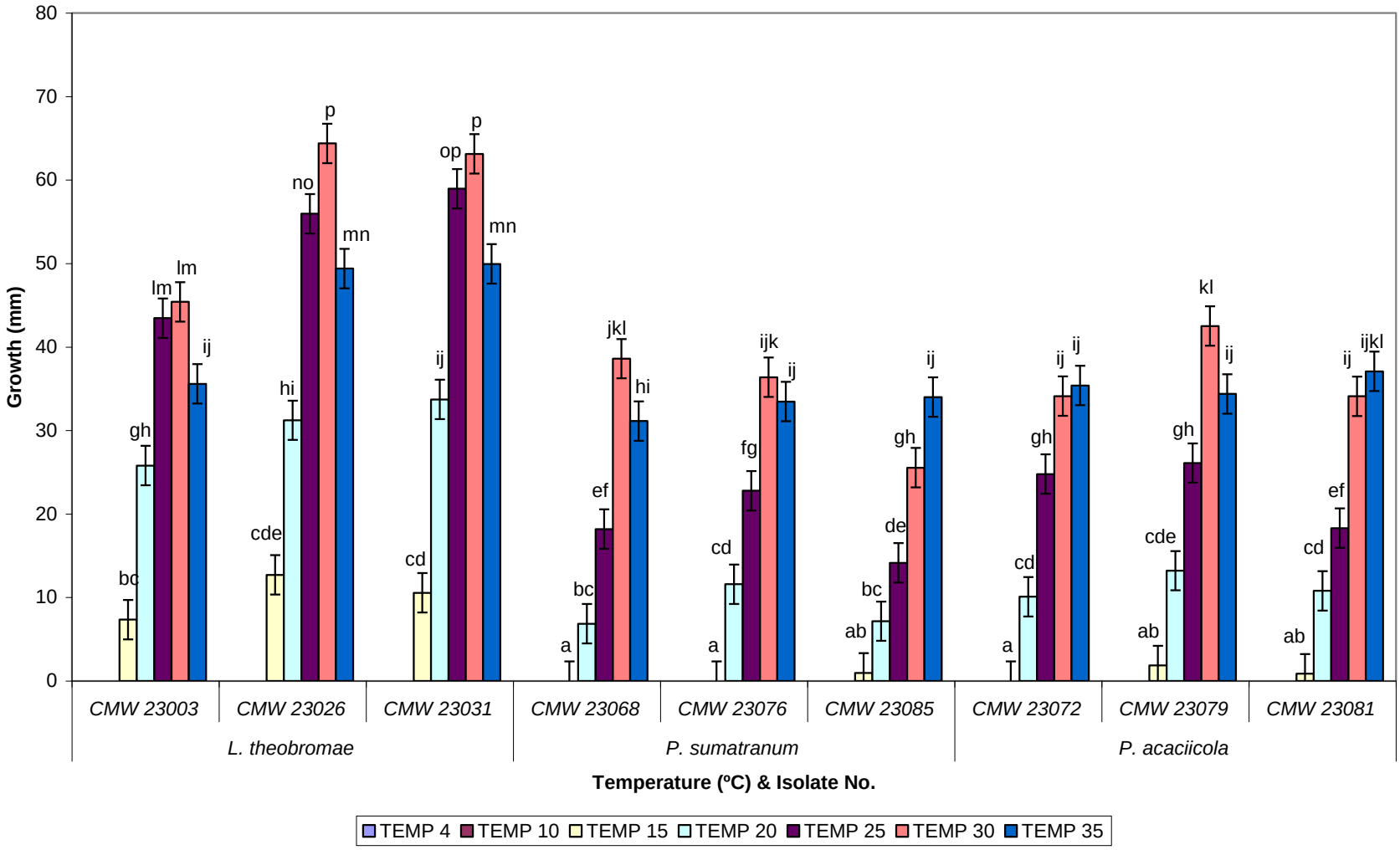
Growth (mm)
0
10
20
30
40
50
60
70
80
bc
gh
lm
lm
ij
cde
hi
no
p
mn
cd
ij
op
p
mn
a
bc
ef
jkl
hi
a
cd
fg
ijk
ij
ab
bc
de
gh
ij
a
cd
gh
ij
ij
ab
cde
gh
kl
ij
ab
cd
ef
ij
ijkl
CMW 23003
CMW 23026
CMW 23031
CMW 23068
CMW 23076
CMW 23085
CMW 23072
CMW 23079
CMW 23081
L. theobromae
P. sumatranum
P. acaciicola
Temperature (ºC) & Isolate No.
TEMP 4
TEMP 10
TEMP 15
TEMP 20
TEMP 25
TEMP 30
TEMP 35

Fig 4. Results of pathogenicity tests with *L. theobromae* (CMW23003, CMW23018, CMW23026, CMW23031, CMW32073), *Pseudofusicoccum sumatranum* (CMW23068, CMW23076, CMW23078, CMW23085, CMW23089) and *P. acaciicola* (CMW23072, CMW23075, CMW23079, CMW23081, CMW32084) on one-year-old *A. mangium* and *A. crassicarpa* seedlings grown in 20 cm diam polybags in a greenhouse, 12 days after inoculation. Bars indicated with the same letter are not statistically significantly different from each other (P value = 0.05).

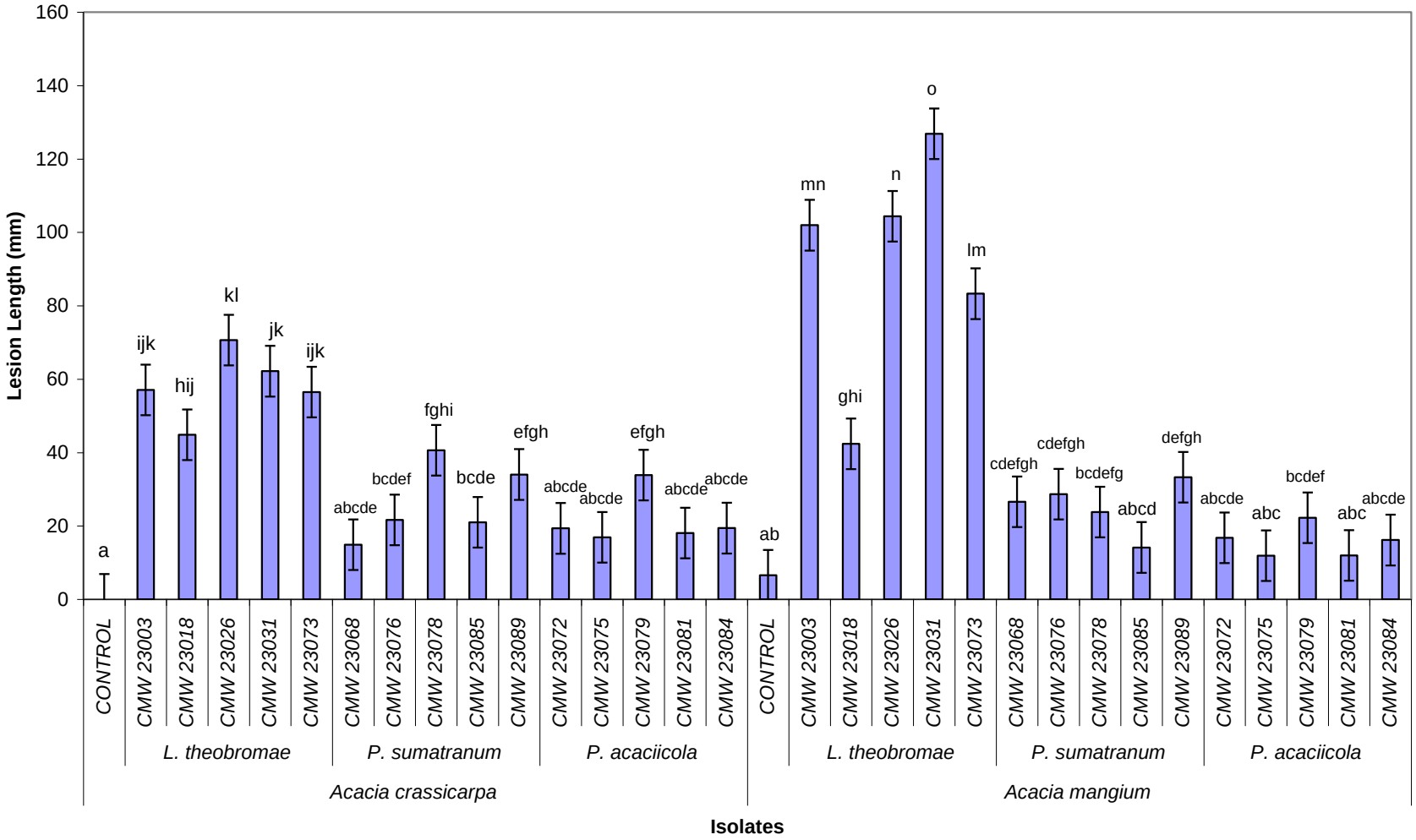
Lesion Length (mm)
160
140
120
100
80
60
40
20
0
a
ijk
hij
kl
jk
ijk
abcde
bcdef
fghi
bcde
efgh
abcde
abcde
efgh
abcde
abcde
ab
mn
ghi
n
o
lm
cdefgh
cdefgh
bcdefg
abcd
defgh
abcde
abc
bcdef
abc
abcde
CONTROL
CMW 23003
CMW 23018
CMW 23026
CMW 23031
CMW 23073
CMW 23068
CMW 23076
CMW 23078
CMW 23085
CMW 23089
CMW 23072
CMW 23075
CMW 23079
CMW 23081
CMW 23084
CONTROL
CMW 23003
CMW 23018
CMW 23026
CMW 23031
CMW 23073
CMW 23068
CMW 23076
CMW 23078
CMW 23085
CMW 23089
CMW 23072
CMW 23075
CMW 23079
CMW 23081
CMW 23084
L. theobromae
P. sumatranum
P. acaciicola
L. theobromae
P. sumatranum
P. acaciicola
Acacia crassicarpa
Acacia mangium
Isolates

Fig 5. Lesions on the bark and the cambium of one-year-old *A. mangium*, six weeks after inoculation with Botryosphaeriaceae. (a). External/bark lesion for control inoculation, (b). Internal/xylem lesion for control inoculation, (c). External/bark lesion caused by *L. theobromae*, (d). Internal/xylem lesion caused by *L. theobromae*, (e). External/bark lesion caused by *P. sumatranum*, (f). Internal/xylem lesion caused by *P. sumatranum*, (g). External/bark lesion caused by *P. acaciicola*, (h). Internal/xylem lesion caused by *P. acaciicola*.

Fig 6. Results of a first set of inoculations using *L. theobromae* (CMW23003, CMW23026, CMW23031), *Pseudofusicoccum sumatranum* (CMW23068, CMW23078, CMW23089) and *P. acaciicola* (CMW23072, CMW23079, CMW32084) on 1-year-old *A. mangium* and *A. crassicarpa* trees in Indonesian plantations, six weeks after inoculation. Bars indicated with the same letter are not statistically significantly different to each other (P value = 0.05).

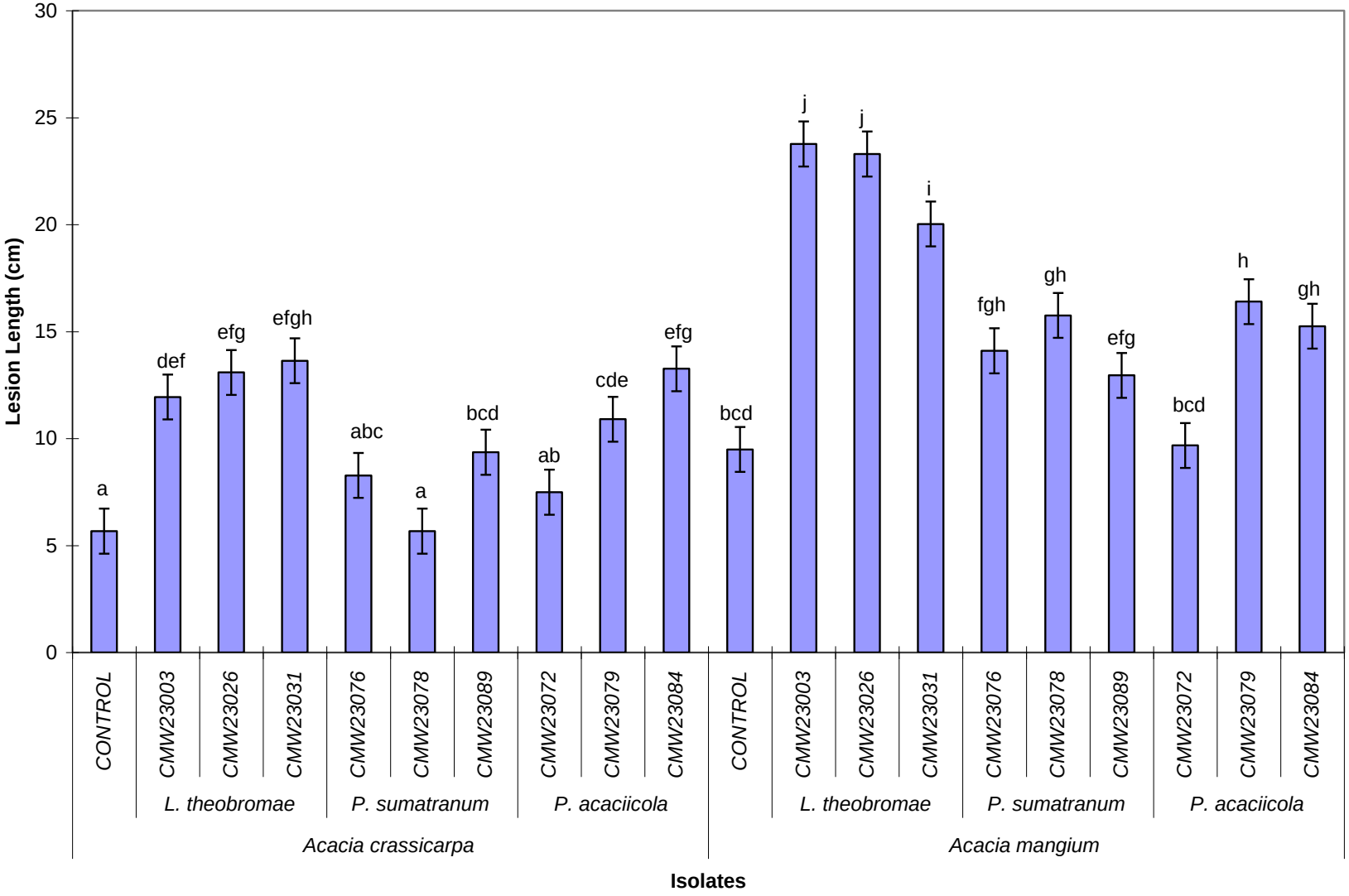
Lesion Length (cm)
30
25
20
15
10
5
0
a
def
efg
efgh
abc
a
bcd
ab
cde
efg
bcd
j
j
i
fgh
gh
efg
bcd
h
gh
CONTROL
CMW23003
CMW23026
CMW23031
CMW23076
CMW23078
CMW23089
CMW23072
CMW23079
CMW23084
CONTROL
CMW23003
CMW23026
CMW23031
CMW23076
CMW23078
CMW23089
CMW23072
CMW23079
CMW23084
L. theobromae
P. sumatranum
P. acaciicola
L. theobromae
P. sumatranum
P. acaciicola
Acacia crassicarpa
Acacia mangium
Isolates

Fig 7. Results of a second set of inoculations using *L. theobromae* (CMW23003, CMW23026, CMW23031), *Pseudofusicoccum sumatranum* (CMW23068, CMW23078, CMW23089) and *P. acaciicola* (CMW23072, CMW23079, CMW32084) on 1-year-old *A. mangium* and *A. crassicarpa* trees in Indonesian plantations, six weeks after inoculation. Bars indicated with the same letter are not statistically significantly different to each other (P value = 0.05).

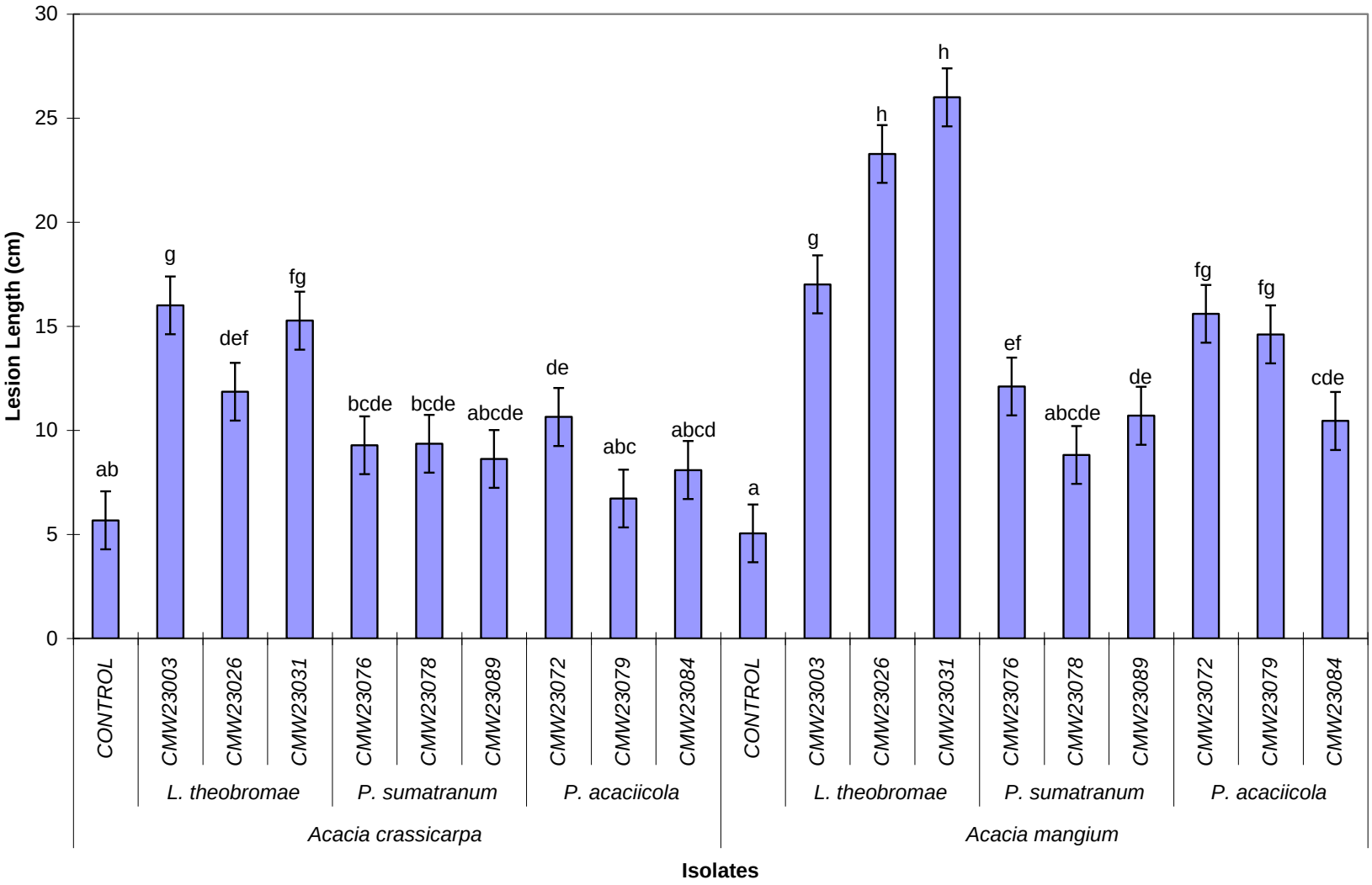
Lesion Length (cm)
30
25
20
15
10
5
0
ab
g
def
fg
bcde
bcde
abcde
de
abc
abcd
a
g
h
h
ef
abcde
de
fg
fg
cde
CONTROL
CMW23003
CMW23026
CMW23031
CMW23076
CMW23078
CMW23089
CMW23072
CMW23079
CMW23084
CONTROL
CMW23003
CMW23026
CMW23031
CMW23076
CMW23078
CMW23089
CMW23072
CMW23079
CMW23084
L. theobromae
P. sumatranum
P. acaciicola
L. theobromae
P. sumatranum
P. acaciicola
Acacia crassicarpa
Acacia mangium
Isolates

Fig 8. Morphological characteristics of *Pseudofusicoccum sumatranum prov. nom*. (CMW23076). (a). Conidiomata on pine needle, (b). Conidiogenous cell and conidia; (c). Conidia with thin walls. [Scale bars (a) = 500 µm; (b, c) = 10 µm].

Fig 9. Morphological characteristics of *Pseudofusicoccum acaciicola prov. nom.* (CMW23072). (a). Conidiomata on pine needle, (b). Conidiogenous cell, (c). Conidia with thin walls. [Scale bar (a) = 500 µm; (b, c) = 10 µm].

Chapter 5

Influence of pruning quality on infection of *Acacia mangium* and *A. crassicarpa* by *Ceratocystis acaciivora prov. nom.* and *Lasiodiplodia theobromae*

ABSTRACT

Pruning and singling are common tending practices in commercial *Acacia* plantations because these trees tend to have multiple stems. The wounds resulting from pruning are susceptible to infection by pathogens. *Ceratocystis acaciivora prov. nom.* and *Lasiodiplodia theobromae* have recently been shown to be important pathogens of *A. mangium* in Indonesia, where they are commonly associated with wounds on trees. The aim of this study was to determine the impact of different wound types on infection of *A. mangium* and *A. crassicarpa* by these two pathogens. Isolates of *C. acaciivora prov. nom.* and *L. theobromae*, found to be the most pathogenic in a prior study, were used to inoculate pruning wounds. Results showed that pruning conducted in a manner to reduce stem damage, resulted in lower levels of fungal infection. Where pruning resulted in tearing of the bark, there were greater levels of infection and disease occurred even without artificial inoculation. Inoculation of pruning wounds on *A. mangium* and *A. crassicarpa* showed that both fungi have the potential to cause disease. However, *C. acaciivora prov. nom.* was most pathogenic. Results of this study showed conclusively that careful pruning will result in lower levels of disease in young *A. mangium* and *A. crassicarpa* plantations in Indonesia.

Keywords: Botryosphaeriaceae, *Ceratocystis*, disease management, silviculture, wound-infecting pathogens**.**

INTRODUCTION

Plantations of *Acacia mangium* Willd. and *A. crassicarpa* Cunn.: Benth. have expanded rapidly in Indonesia since the 1980's, specifically to provide raw material for Indonesian pulp and paper industries (Barr 2001, Anonymous 2004). These *Acacia* spp., however, tend to have poor stem form, with multiple stems and branches (Srivastava 1993, Lee & Arentz 1997). Singling and pruning is thus carried out to improve tree form and to increase tree strength reducing stem or branch breakage, particularly after strong winds (Beadle *et al.* 2007). Furthermore, the capacity of *A. mangium* trees to self prune is low in areas with high rainfall (Lee & Arentz 1997) and pruning is necessary especially in Indonesia. These practices also reduce the density of stands so that optimum tree growth can be achieved (Neilsen & Gerrand 1999).

Wounds resulting from singling and pruning activities provide infection sites for pathogens (Gales 2002, Lee 2002). For example, heart rot caused by a complex of *Phellinus noxius* (Corner) G. Cunn. and other unidentified basidiomycetes are common on *Acacia* spp. in Malaysia and Indonesia (Lee & Noraini Sikin 1999). A recent study to determine the cause of death of young *A. mangium* trees in Indonesian plantations (Chapters 2 and 4) has shown an association between pruning wounds and disease caused by *Ceratocystis acaciivora prom. nom.* and *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl.

In order to develop management guidelines for pruning *Acacia* spp. in Indonesia, a study was undertaken to consider the effect of the quality of pruning wounds on disease development. For this purpose, pathogenic isolates of *C. acaciivora prov. nom.* (Chapter 2) and *L. theobromae* (Chapter 4) were used in inoculations of pruning wounds of different quality on *A. crassicarpa* and *A. mangium* in Indonesia.

MATERIALS AND METHODS

One-year-old *A. mangium* and *A. crassicarpa* trees in Riau province, Indonesia were used in pruning wound inoculations. Trees ranged in diameter from 70 mm to 90 mm at approximately 1.3 m above the ground level.

Isolates of *C. acaciivora prov. nom.* (CMW22563) and *L. theobromae* (CMW23003), identified as highly pathogenic in previous studies (Chapters 2 and 4) were selected for the inoculation experiments. Isolates were grown on 2 % (w/v) Malt Extract Agar (MEA) (Biolab, Midrand, South Africa) for two weeks prior to inoculation.

Two pruning methods were used. In one case, branches were pruned above the branch collar taking care not to tear the bark (Fig 1a). In the alternative pruning technique, branches were pruned on the branch collar and the bark was torn to create a flap (Fig 1b). Pruning wounds were made using a handsaw, similar to that used in routine pruning activities.

Pruning wounds were inoculated either by spreading inoculum over the wound surface or alternatively by spraying the inoculum onto the wounds. Where inoculum was spread over the wounds, 10 mm diameter agar plugs [2 % MEA (Malt Extract Agar, Biolab, Midrand, South Africa)], taken from the edges of actively growing fungal colonies, were spread over the surface of freshly made wounds. Alternatively, 1 ml of a fungal suspension ($>10^{-4}$ spores per ml of sterile water) was sprayed onto the surface of freshly made wounds. For the controls, pruning wounds were sprayed with sterile water or inoculated with a sterile plug of 2 % MEA.

Twenty trees of *A. crassicarpa* and *A. mangium* were used in each of the four treatments. In all, 240 trees of each species were used. These included twenty trees of each species for each of the two wounding techniques, and twenty trees for each species inoculated by either spreading or spraying the inoculum. Twenty control trees were included for each treatment, giving a total of 80 control trees for the entire experiment. After five weeks, the lengths of the lesions in the cambium associated with the pruning sites were measured. Data obtained were analyzed with analysis of variance (ANOVA) using SAS statistical analyses (SAS Version 8.2, 2001).

After measuring lesions, isolations were made from tissue associated with the pruning wounds. For *C. acaciivora prov. nom.,* pieces of symptomatic tissue from the areas associated with the inoculation points were collected and placed in moist chambers to induce sporulation. Spore masses were taken from the tips of fruiting structures and plated onto 2 % MEA to verify the presence of the inoculated fungus. For the *L.*

theobromae and control treatments, pieces of tissue were taken from the pruning sites and these were plated onto 2 % MEA. The identity of the inoculated fungi was also confirmed by selecting representative isolates and subjecting them to DNA sequence comparisons based on the Internal Transcribed Spacer region (ITS1, ITS4) including the 5.8S rRNA operon as previously described (Chapter 2).

RESULTS

Inoculation of *A. mangium* and *A. crassicarpa* trees with *C. acaciivora prov. nom.* and *L. theobromae* resulted in significant lesions after five weeks. In general, the *C. acaciivora prov. nom.* isolate produced significantly larger lesions than those associated with *L. theobromae*, except on *A. crassicarpa* where inoculum was spread onto the wounds. The *L. theobromae* isolate did not produce lesions significantly different to those of the controls, except on *A. crassicarpa* where the inoculum was spread across the wounds and where the rough pruning was applied. In general, the lesions on *A. mangium* trees were longer than those on the *A. crassicarpa* trees (Fig 2).

Both the careful pruning and the rough pruning methods produced lesions on *A. mangium* and *A. crassicarpa*. However, all treatments using the rough pruning method, including the control, produced much larger lesions than those associated with careful pruning (Figs 2, 3). The mycelium spread and spray application techniques both gave rise to large lesions on *A. mangium* and *A. crassicarpa* where rough pruning was applied. No, or only small lesions developed on *A. mangium* and *A. crassicarpa* where careful pruning was applied (Figs 2, 3).

Where careful pruning was used, the average lesion lengths associated with *C. acaciivora prov. nom.* inoculation was 81 mm and 69 mm on *A. crassicarpa* and 118 mm and 160 mm on *A. mangium* for the spray and spread techniques respectively (Fig 2). These were much smaller than those where rough pruning was applied where lesion lengths produced an average of 406 mm and 315 mm on *A. crassicarpa* and 450 mm and 343 mm on *A. mangium* for the spray and spread techniques, respectively (Fig 2). *Lasiodiplodia theobromae* inoculation gave rise to lesions with an average length of 16 mm and 16.3 mm on *A. crassicarpa* and 11 mm and 17 mm on *A. mangium* for the spray and spread techniques, where careful pruning was used. In contrast, where rough pruning

was applied, lesions lengths averaged 240 mm and 372 mm on *A. crassicarpa* and 256 mm and 185 mm on *A. mangium* for the spray and spread techniques respectively (P values = 0.05; R-Square = 0.697; Coeff. Var. = 56.67; Root MSE = 9.74).

Re-isolation from lesions on trees inoculated with *C. acaciivora prov. nom.* consistently yielded the inoculated fungus. This fungus was never isolated from wounds on the control trees. Where trees were inoculated with *L. theobromae*, it was also re-isolated consistently, however, it was also isolated from some of the wounds on the control trees.

DISCUSSION

Results of this study have shown clearly that the quality of pruning has a significant effect on the infection of *A. mangium* and *A. crassicarpa* by two fungal pathogens that are associated with stem disease development after pruning. These results have practical implications for the management of *A. mangium* and *A. crassicarpa* diseases in plantations in Indonesia where pruning is routinely used to improve stem form and growth. Furthermore, this study clearly supports the results of previous investigations where it has been shown that *C. acaciivora* and *L. theobromae* are important pathogens that kill *A. mangium* after pruning in Indonesia.

Pathogenic isolates of *C. acaciivora* and *L. theobromae*, collected from diseased *A. mangium* in previous studies (Chapters 2, 4), were used to inoculate pruning wounds on both *A. mangium* and *A. crassicarpa*. Both fungi produced significant lesions within five weeks of being applied to wounds created by rough pruning. These results, as well as those obtained in Chapter 2 and 4 of this dissertation confirm that *C. acaciivora* and *L. theobromae* are important pathogens of *A. mangium*, causing disease and death of trees after infection via pruning wounds. We have also shown that after wounding, *A. crassicarpa* can be equally vulnerable to infection and disease, even though damage equivalent to that on *A. mangium* has not been observed in plantations.

Lesion lengths after inoculation on *A. crassicarpa* were smaller than those on *A. mangium*. These results confirm that *A. mangium* trees respond poorly to wounding, as previously described (Schmitt *et al.* 1995). Results of this study also confirmed those of previous inoculation experiments on *A. mangium* and *A. crassicarpa* (Chapters 2, 3, 4)

where lesions on *A. crassicarpa* were smaller than those on *A. mangium*. The results, furthermore, correlate well with field observations, where serious canker and die-back is more commonly found on *A. mangium* than on *A. crassicarpa* trees.

Wound type was shown to play an important role in lesion development on both *A. mangium* and *A. crassicarpa*. Results clearly showed that even in the absence of inoculation, significantly longer lesions develop on pruning wounds that have broken the branch collar and exposed larger areas of wood than on carefully pruned trees. This is most likely due to the fact that roughly pruned wounds allow more opportunities for opportunistic pathogens to infect and develop. Poorly pruned trees also have bark flaps under which fungi can develop and they are most likely more heavily stressed than undamaged trees.

Lasiodiplodia theobromae is a well known opportunist and latent pathogen (Burgess *et al*. 2006, Punithalingam 1976). The fungus can easily be isolated from the bark of healthy *A. mangium* and *A. crassicarpa* (Wingfield, unpublished) and it has the ability to cause disease on stressed tissues such as those that are found on roughly pruned trees. The fact that *L. theobromae* was isolated from pruning wounds on control trees supports this view. As confirmed in Chapter 4 of this thesis, *L. theobromae* appears to be a common pathogen in Riau, and is known to be a wound and stress-related pathogen (Smith *et al*. 1994, Van Niekerk *et al*. 2006). It commonly exists as an endophyte in healthy trees (Smith *et al.* 1996, Slippers & Wingfield 2007). Isolation of *L. theobromae* from the controls during re-isolation was thus not un-expected.

No differences in lesion development were observed between the two different inoculation methods used in this study. Spreading mycelium over the wound surfaces or application of inoculum by spraying both gave rise to substantial lesions. However, application of the inoculum by spreading it over the wounds was preferable as it was easier to quantify the amount of inoculum being applied. This was particularly true in the case of *L. theobromae* that does not readily produce spores in culture.

Results of this study have shown clearly that careful pruning can reduce the incidence of stem disease in *Acacia* plantations in Indonesia. This, together with sound selection and breeding strategies, will ensure the success of future plantings. Poor silvicultural

practices, such as excessive pruning and rough pruning should be actively avoided. Late pruning also results in large branches that are difficult to prune without significant damage to the stems and this practice should be avoided.

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Fig 1. Two pruning methods used in this study. (a) Careful pruning where the branches were pruned above the branch collar without tearing the bark, (b) Rough pruning where the branch collar was cut and the bark was torn.

Fig 2. Lesion lengths on *Acacia mangium* and *A. crassicarpa* pruning wounds after inoculation with pathogenic isolates of *Ceratocystis acaciivora* (CMW22563) and *Lasiodiplodia theobromae* (CMW23003), five weeks after inoculation. Bars on the graph bearing the same letter are not significantly different from each other (P value = 0.05).

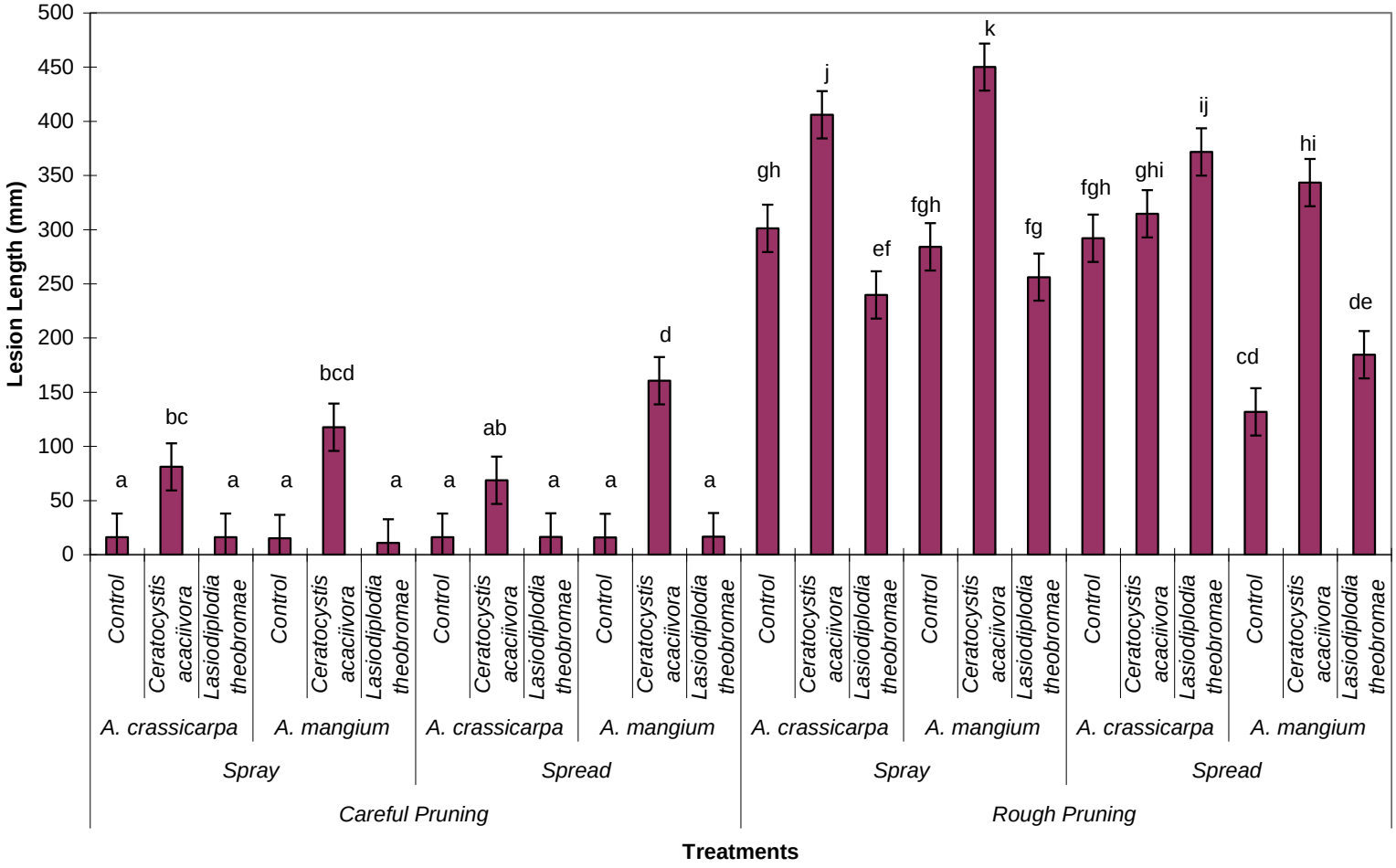
Lesion Length (mm)
500
450
400
350
300
250
200
150
100
50
0
a
bc
a
a
bcd
a
a
ab
a
a
d
a
gh
j
ef
fgh
k
fg
fgh
ghi
ij
cd
hi
de
Control
Ceratocystis acacivora
Lasiodiplodia theobromae
A. crassicarpa
A. mangium
Spray
Spread
Careful Pruning
Rough Pruning
Treatments

Fig 3. Lesion development associated with pruning wounds on *A mangium* and *A. crassicarpa* after inoculation with *C. acaciivora prov. nom.* (CMW22563) and *L. theobromae* (CMW23003), five weeks after inoculation, (a). Control inoculation after careful pruning, (b). Control wound after rough pruning, (c). Lesion caused by. *L. theobromae* after careful pruning, (d). Lesion caused by *L. theobromae* after rough pruning, (e). Lesions caused by *C. acaciivora prov. nom.* after careful pruning, (f). Lesion caused by *C. acaciivora prov. nom.* after rough pruning.

SUMMARY

The use of *Acacia* spp. in plantation forestry increased dramatically in Indonesia since the 1980's after the Indonesian Government initiated a plantation development program known as Hutan Tanaman Industri (HTI). The aim of this programme is to supply raw material to especially the pulp and paper industries. Currently, more than one million hectares of *Acacia* plantations have been established, mainly on the islands of Kalimantan and Sumatra. These *Acacia* plantations, with *A. mangium* and *A. crassicarpa* as the main species, display robust growth with mean annual increments (MAI) of 25-30 m^3/ha/year and 40-46 m^3/ha/year on the average and the best site respectively. However, diseases limit the success of forestry plantations in Indonesia.

During disease surveys in *Acacia* plantations in Indonesia, symptoms resembling those caused by Botryosphaeriaceae and *Ceratocystis* spp. were identified and representative samples were collected from wounded and diseased *A. mangium* trees. Studies in this dissertation described the causal agents of the disease and provided some practical solutions to reduce the incidence of the disease. Seven fungal species were discovered, five of them are new to science, one representing a first report for *Acacia* spp. and one other species is confirmed as the most common fungal species in *Acacia* plantations in Indonesia.

The first chapter of this dissertation provided a background to the forestry industry in Indonesia and details were given on the diseases affecting plantation *Acacia* spp. in Indonesia. The review clearly shows that diseases such as stem diseases and root diseases can have a severe impact resulting in retarded growth of trees, reduced timber quality, and in severe cases they result in tree death. The most common disease reported from *Acacia* plantations prior to this dissertation was heart rot and root rot.

The second chapter outlines the discovery of *Ceratocystis acaciivora prov. nom.*, a new species, and *C. manginecans*, a recently described species that causes a serious disease of mango trees in Oman and Pakistan, associated with disease of *A. mangium* in Indonesia. Identification of these fungi was achieved using morphological characteristics and comparison of DNA sequence data for the ITS, β-tubulin and

Elongation Factor 1-α gene regions. From pathogenicity trials on *A. mangium* and *A. crassicarpa*, both in the greenhouse and the field, it was clear that these fungi are pathogens.

In chapter 3 of the dissertation, three previously undescribed *Ceratocystis* species in the *C. monililiformis sensu lato* complex are identified. These have been given the names *C. inquinans prov. nom., C. sumatrana prov. nom.* and *C. microbasis prov. nom.* and were obtained from wounded *A. mangium* trees. The virulence of these three new species was tested on *A. mangium* and *A. crassicarpa* in the greenhouse and in the field. The results indicate that all three species are mild pathogens but they have the potential to cause disease on *A. mangium* and *A. crassicarpa*.

The fourth chapter of this dissertation reports on the occurrence of three species in the Botryosphaeriaceae on plantation-grown *Acacia* spp. in Indonesia. Two of the species are shown to be new to science and were provided with the names *Pseudofusicoccum sumatranum prov. nom.* and *P. acaciicola prov. nom.* The third species was identified as the well-known tree pathogen, *Lasiodiplodia theobromae*. Pathogenicity results indicated that all three species have the potential to cause disease of *A. mangium* and *A. crassicarpa*. However, *L. theobromae* was the most pathogenic and was the most common species isolated.

In the fifth chapter, the impact of different wound and inoculum types on infection by *C. acaciivora prov. nom.* and *L. theobromae*, the two most pathogenic wound-infecting pathogens on *A. mangium* and *A. crassicarpa*, is evaluated. Clear differences were found between careful and rough pruning techniques. The results indicated that improved management practices will minimize disease risk in plantations.