Mycosphaerella Leaf Disease on Eucalypts in Western Australia – the diversity and impact

By

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Declaration

The work described in this thesis was undertaken while I was an enrolled student for the degree of Doctor of Philosophy at Murdoch University, Western Australia. I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a decree at any tertiary education institution. To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged.

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Abstract

Eucalyptus plantation forestry in Western Australia (WA) is a relatively young industry and by the end of 2008, the total plantation estate (softwood and hardwood) was over 950 000 ha. The predominant plantation species is *Eucalyptus globulus*, native to south-eastern Australia. In Western Australia (WA), the most serious foliar disease of eucalypt plantations is Mycosphaerella Leaf Disease (MLD). However, little systematic sampling for MLD has been carried out in WA to determine its impact on plantations, yields, species involved or whether they are introduced or not. The overall aim of this thesis was to investigate MLD in south-western Australia with a particular focus on the species diversity, taxonomy and the impact on early growth on *E. globulus.*

The increase in the number of *Mycosphaerella* and *Teratosphaeria* species associated with Mycosphaerella leaf disease (MLD) in *E. globulus* plantations in WA in the past decade has raised concern about the possible movement of pathogens between the native forests and plantations and vice versa. A survey of necrotic leaf spots collected from plantation and endemic eucalypts from WA and Queensland was conducted. Overall, ten new *Eucalyptus* host records for *Mycosphaerella*/ *Teratosphaeria* species were isolated from WA and five from Queensland. Significantly, *M. nubilosa* was isolated from *E. grandis* x *resinifera* and *E. urophylla* x *globulus* in WA. This is the first time *M. nubilosa* has been isolated from *Eucalyptus* hosts within the series Resinifera (see Chapter 2).

An assessment of the number of fungi that may be contributing to MLD in *E. globulus* plantations in WA was undertaken (Chapter 3) and the changes in the number of species and their incidence since the first surveys were conducted. Four new records of *Mycosphaerella* were identified in this study; *M. ellipsoidea*, *P. fori*, *M. suttoniae* and *M. tasmaniensis*. *Mycosphaerella ellipsoidea* and *P. fori* are first records for Australia, and *M. suttoniae* and *M. tasmaniensis* are first records for WA. The current work shows an increase in the number of *Mycosphaerella* species associated with plantation eucalypts in WA and Australia. With the exception of *M. cryptica*, none of these species were known in WA prior to the commencement of large-scale *E. globulus* plantations, and with *M. cryptica* as the exception, none have a known impact on the major native eucalypts in the region.

The ITS region of the type material of *T. parva*, *M. grandis* and *M. gregaria* using culture and herbarium specimens was sequenced and compared to existing sequences from GenBank (Chapter 4). This was the first study to examine and sequence the type material of *M. grandis*, *T. parva* and *M. gregaria*. As the sequences of the ITS region of *M. grandis* and *T. parva* were identical it was concluded that *M. grandis* be reduced to synonymy with *T. parva*. *Mycosphaerella aurantia*, *M. buckinghamiae* and *M. africana* also match the type sequence of *M. gregaria*. Therefore, these should all be synonymised to *M. gregaria*. Also, this study was the first to describe ITS sequence variation within the same *Mycosphaerella* isolate.

The aim of Chapter 5 was to identify the infection pathway at the leaf surface using scanning electron microscopy and to determine the pathogenicity of *M. marksii* on *E. globulus*. The use of glycerol as a surfactant and its effect on ascospore viability was also assessed. However, this study was unable to confirm pathogenicity of *M. marksii* on *E. globulus* seedlings under laboratory conditions. However, *M. marksii* ascospores were able to germinate and enter *E. globulus* stoma 3–6 days after initial infection.

Species-specific primers were successfully designed and tested for three *Mycosphaerella* species that occur on *E. globulus* in WA (Chapter 6). Meteorological conditions appeared to determine the defoliation of juvenile foliage and not MLD as levels of MLD remained relatively low throughout the trial period. The MLD levels increased throughout spring as warm wet conditions favoured the development of disease especially on the flush of new juvenile foliage. Also, new foliage emerged after late summer rainfall. As disease pressure mounted, the trees responded through defoliation. As temperatures increased and the juvenile foliage aged, there is likely to have been an increase in the defoliation of leaves. Therefore, by mid-summer defoliation levels reached a similar level to disease and insect damage. Following leaf defoliation and the emergence of new juvenile and adult leaves, the relative amount of disease on the trees decreased. This is because most of the disease was present on the older juvenile foliage which was shed. Field observations can be a reliable indication of disease progression. Although field observations at a branch level over exaggerated levels of MLD when there was a higher level of foliage, there was still a similar trend in the amount of disease when

compared to the ASSESS program. Some experience in disease monitoring would indicate a more accurate assessment of MLD. It is interesting to note that the assessors tended to overestimate disease when MLD was at a higher level, and this also included the author.

Infection studies of *Uwebraunia dekkeri* were conducted to confirm how this species enters *E. globulus* leaves and to determine its pathogenicity (Chapter 7). This study demonstrated that conidia of *U. dekkeri* could infect *E. globulus* leaves and that it is not a hyperparasite of *M. cryptica* or *M. nubilosa*. Conidiogenesis was both percurrent and sympodial and the phenomenon of anastomosis was observed for the first time on the leaf surface.

The impact that MLD has on the wood volume has previously not been investigated in WA (Chapter 8). Through the application of pesticides and fungicides in the early stages of establishment at two plantations near Albany, tree volumes were significantly increased. However, the increase in wood volume would be offset by the pesticide and application costs. This study demonstrated that monitoring for pests and disease would be more effective than spraying of chemical treatments for the first three years. The regular use of chemical treatments is expensive to maintain and is proving to be environmentally unacceptable by some communities. This study also showed that spraying for low levels of MLD had little effect on disease incidence and/ or volume increase in *E. globulus* plantations in WA. The most important factors for a healthy plantation appear to be site selection, preparation and tree genetics.

This study was the first to investigate the impact of MLD on the growth of *Eucalyptus globulus* plantations in WA. As part of this study, the biology, taxonomy and pathogenicity of the main species present in WA were investigated. The key findings were: **i**) the number, abundance and distribution of *Mycosphaerella*/ *Teratosphaeria* species in WA is not static and plantations should be continually monitored for the presence of new potentially threatening species; **ii**) spraying for MLD, although effective in reducing the prevalence and impact on growth, was not economically viable; and **iii**) intragenomic variation of the ribosomal genome may explain sequence variation observed in single spore isolates of *Mycosphaerella*/ *Teratosphaeria* and this has taxonomic implications. Further work would identify the impact the new records are having on the plantation estate and also if these species have the potential to spread into the neighbouring endemic forests. This study has provided a broader understanding of MLD in WA and the development of tools that could be used for further study.

Publications arising from the current thesis

Peer reviewed journals

Jackson SL, Bayliss K (2011) Spore traps need improvement to fulfil plant biosecurity requirements. *Plant Pathology* **60**, 801–811.

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Maxwell A, Dell B, Hardy GEStJ and **Jackson SL** (2003) Is *Mycosphaerella* a threat to the *Eucalyptus* estate in Western Australia? A bio-geographical perspective. Poster presented by G. Hardy at ICPP Christchurch, 2003.

List of Abbreviations

ANOVA	Analysis of variance
AQIS	Australian Quarantine and Inspection Service
BLAST	Basic Local Alignment Search Tool
CDI	Crown damage index
DBHOB	Diameter at breast height over bark
DIW	Deionised water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetra acetic acid
EF-1α	Translation elongation factor 1-alpha
FSC	Forest Stewardship Council
hr	Hour
IPMG	Integrated Pest Management Group
ISO	International Organisation for Standardisation
ITC	Integrated Tree Cropping Ltd
ITS	Internal transcribed spacer
LDI	Leaf-chew damage index
LSU	Large subunit
MANOVA	Multivariate analysis of variance
MDI	Mycosphaerella damage index
MEA	Malt extract agar
min	Minute

MLD	Mycosphaerella leaf disease
MURU	Murdoch University Culture Collection
PCR	Polymerase chain reaction
NCBI	National Centre for Biotechnology Information
rDNA	Ribosomal DNA
RH	Relative humidity
SE	Standard error of the mean
SEM	Scanning electron microscopy
sp., spp. (plural)	Species
TAE buffer	Tris base, acetic acid and ethylenediaminetetra acetic acid
UV	Ultraviolet
w/v	Weight per volume
WA	Western Australia
YFEL	Youngest fully expanded leaves

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

In Australia, the hardwood plantation industry has been through a period of rapid expansion. In Western Australia (WA) at the end of 2008, the total plantation estate (softwood and hardwood) was over 950 000 ha (Gavran and Parsons 2009) and continues to grow. One of the greatest concerns regarding the sustainability of the plantation industry in Australia comes from the presence of biotic diseases caused by fungi and other agents (Park *et al.* 2000). Another important biological threat comes from insects, as some have the capacity to completely defoliate plantations, provide entry points for disease, as well as spread pathogens between trees and plantations. Fungal pathogens causing disease in plantations usually express visible symptoms such as necrotic leaf spots, and stem or trunk cankers (Crous 1998; Keane *et al*. 2000). In WA the most serious fungal disease of plantation eucalypts is Mycosphaerella Leaf Disease (MLD) (Maxwell *et al.* 2003). In Queensland, the threat comes from anamorphs linked to *Mycosphaerella* and *Teratosphaeria*, as well as canker and shoot blight pathogens (Andjic *et al.* 2007; Pegg *et al.* 2008). Elsewhere in Australia, MLD is a cause for concern (Maxwell *et al.* 2003; Carnegie 2007b). It is caused by various *Mycosphaerella* and *Teratosphaeria* species and/or several anamorphs. At present little information exists on the biology of these pathogens, their interaction with host species, or the effect of the environment, including the potential risk to endemic eucalypt species. This literature review introduces the eucalypt plantation sector in Australia, and then examines the key threats to their continued production before defining the aims and objectives of the thesis.

1.2 Overview of the *Eucalyptus* plantation industry—an Australian perspective

As the reliance on wood products continues to increase, the pressure on native forests around the world needs to be alleviated by a shift in focus to plantation species. Currently, over twelve countries plant eucalypts on a major scale, with Australia only a recent contributor. This is possibly due to Australia's low population size, where the demand for wood products has been met by native forests (Turnbull 2000). In 2005, plantation forestry made up less than 5% (3.8%) of the world's forest or 140 million ha; however, between the years 2000–2005 the area of plantations increased by 2.8 million ha annually (FAO 2005). Of the 4 billion ha of forests around the world, 33% is used for wood production, fibre and non-wood products (FAO 2005).

In Australia, at the end of 2008 there were 1.97 million ha of plantations, compared to 1.74 million in 2005, with 39% of the products exported to Japan (Parsons *et al.* 2006; ABARES 2009). Hardwood plantations made up 48% of total plantations in 2008, up from 15% in 1994 (Gavran and Parsons 2009). Of the hardwood plantations, *Eucalyptus globulus* made up 62% of the estate, followed by *E. nitens* (19%) (ABARES 2009). In WA, 59 845 ha were planted in 2000 compared to 5403 ha in 2003. This fall has been attributed to the Australian government changing taxation laws, which were subsequently reviewed and changed in 2004. Consequently, 2004 saw a slight increase in the area of trees planted, 7829 ha, the majority (65%) through managed investment schemes (National Forestry Inventory

2005). In 2008 there was more than 300 000 ha of hardwood plantations in Western Australia (Gavran and Parsons 2009).

During 2006, there was an estimated 120 000 people employed in timber production and forest product industries, which accounted for less than 1% of Australia's total employment (ForestWorks 2006). This figure should no doubt increase as plantations mature and harvesting operations and replanting regimes progress.

As a change in values towards the environment is adopted by countries such as Japan, plantation companies in Australia now face the task of attaining certification of exported wood products. The Forest Stewardship Council (FSC) is a non-government organisation that promotes responsible practices of the world's forests. FSC accreditation assures the buyer that the wood product has been managed sustainably and the company has conformed to tight guidelines regarding management of natural ecosystems, chemical control and social impacts (Turnbull 1999). In Australia, a national standard was implemented in 2002 as the Australian Forestry Standard (AFS). The AFS has nine criteria and forty requirements that must be addressed to obtain certification (The Australian Forestry Standard 2007).

1.2.1 Eucalyptus globulus in Western Australia

In WA, *E. globulus* is the preferred plantation species due to its rapid growth rate (15-20 m^3 ha^{-1} yr^{-1}), its ability to coppice up to three times, the short rotation period (average 10 years), its pulping qualities and the compatibility of environmental conditions (Eldridge *et al*. 1994; Morgan 1994). Numerous provenances have been

established in WA from both Victoria and Tasmania. Grown as an exotic, it was thought to be less susceptible to pests and pathogens; however, many Western Australian eucalypt species belong to the subgenus *Symphyomyrtus*. Hence, there is the possibility that plantations, particularly those grown near native stands of eucalypts, could be at risk of disease (Morgan 1994) and *vice versa*.

1.3 Commercial plantation eucalypts

1.3.1 Classification of eucalypts

The classification of species within the eucalypt group has undergone many changes since they were first described over two hundred years ago. Recent studies by Hill and Johnson (1995), Ladiges *et al.* (1995) and Brooker (2000), provide two alternative classification systems for eucalypts. Brooker (2000) believes that *Corymbia* and *Angophora* belong as subgenera under the genus *Eucalyptus*. In contrast, Hill and Johnson (1995) and Ladiges *et al.* (1995) place *Corymbia* and *Angophora* as two separate genera. The classification system of Hill and Johnson (1995) and Ladiges *et al*. (1995) is used in this thesis.

Within the genus *Eucalyptus* there are three major lineages; *Eudesmia*, *Monocalyptus* and *Symphyomyrtus*. *Symphyomyrtus* is the largest subgenus with over three hundred species and is subdivided into six major groups summarised in Potts and Pederick (2000). The most popular plantation species come from the *Maidenaria*, *Exsertaria* and *Transversaria* (Eldridge *et al*. 1994). In south-western Australia, the predominant tree species in native forests are *E. marginata* (jarrah), *E.*

diversicolor (karri), *E. gomphocephala* (tuart) and *Corymbia calophylla* (syn. *E. calophylla*) (marri) (Table 1.1). *Eucalyptus diversicolor* is within the section *Transversaria*, along with the major plantation species: *E. grandis, E. saligna, E. botryoides, E. robusta, E. resinifera, E. pellita* and *E. urophylla*. *Eucalyptus marginata* belongs to the *Monocalyptus* subgenus, of which there are few examples of plantation eucalypts, however, it does include important native forest timber species (Potts and Pederick 2000).

1.3.2 Where are eucalypts grown in the world?

Planted forests make up 7% of the total world forests, increasing by 5 million ha per year from 2005–2010 (FAO 2010). In 2005 there was an estimated 18 million ha of eucalypt plantations in 90 countries (FAO 2005). Four species and their hybrids from the subgenus *Symphyomyrtus*, namely *E. grandis*, *E. urophylla, E. camaldulensis* and *E. globulus*, account for about 80% of the eucalypt plantations worldwide and are grown as exotic species in tropical, sub-tropical and temperate climatic zones (Rockwood *et al*. 2008). The product is mainly used for timber, pulp and fibre production, however, they are also used for domestic uses such as poles, furniture and fuel, particularly in the least modernised countries (Turnbull 1999; FAO 2005).

Table 1.1 Classification of the major *Eucalyptus* species used for plantation forestry in Australia, including important endemic timber species to Western Australia (*) (Pryor and Johnson 1971).

Subgenus	Section	Series	Subseries	Species
Symphyomyrtus	*Transversaria*	*Diversicolores*		*E. diversicolor**
		Salignae	*Salinosae*	*E. grandis*
				E. saligna
				E. botryoides
			Robustosae	*E. robusta*
		Resiniferae		*E. resinifera*
				E. pellita
				E. urophylla
	Aenigmataria	*Corynocalyces*		*E. cladocalyx*
		Reduncae	*Wandoonosae*	*E. wandoo**
		Gomphocephalae		*E. gomphocephala**
	Exsertaria	*Exsertae*	*Tereticornosae*	*E. tereticornis*
			Camaldulensosae	*E. camaldulensis*
				E. rudis
	Maidenaria	*Ovatae*	*Ovatosae*	*E. ovata*
		Globulares	*Bridgesianosae*	*E. dunnii*
			Globulosae	*E. maidenii*
				E. globulus
			Nitentosae	*E. nitens*
		Viminales	*Viminalosae*	*E. viminalis*
Monocalyptus	*Renantheria*	*Obliquae*	*Obliquosae*	*E. obliqua*
		Pauciflorae	*Delegatensosae*	*E. delegatensis*
	Jarraria	*Jacksonianae*		*E. jacksonii**
		Marginatae		*E. marginata**

1.3.3 Hybrids and their role in commercial forestry

Eucalypts have a high level of hybridisation with 289 of the 528 species able to hybridise with at least one other species (Griffin *et al*. 1988). In South Africa in the mid-nineties, eucalypts comprised more than 14.8% of trees grown for pulp (Little *et*

al. 2003). During the 1990's, *E. grandis* hybrids were introduced into South Africa onto otherwise limiting sites. The use of hybrids allows selection for disease resistance, uniformity and increased pulp yield and quality (Little *et al*. 2003). Hybrids also provide the potential for introducing eucalypts onto previously unsuitable areas, such as with frost-tolerant qualities into frost prone areas (*E. nitens* x *E. globulus*) (Turnbull 1999) or soils prone to salt-waterlogging (*E. camaldulensis* x *E. globulus*) (Meddings *et al.* 2003).

Hybridisation of susceptible and non-susceptible species may allow greater resistance to environmental conditions or disease such as canker-causing fungi (van Heerden *et al.* 2005). *Chrysoporthe cubensis*, formerly *Cryphonectria cubensis* (Gryzenhout *et al*. 2004) is regarded as an important pathogen of eucalypts in South Africa (van Heerden and Wingfield 2002). Variation in susceptibility exists within eucalypts and screening of material has been used to evaluate resistance between clones in different environments (van Heerden and Wingfield 2002). The study by van Heerden and Wingfield (2002) showed that not only resistance differed significantly between clones, but resistance also was determined by the regions where the clones were planted. They suggest that clonal testing for disease resistance to *C. cubensis* should be conducted where the material is to be grown commercially.

Hybrid resistance to disease in environments where either parent is susceptible to disease has been documented in most parts of the world where hybrids are grown commercially. The hybrid *E. urophylla* x *E. grandis* has shown resistance to *C.*

cubensis in Brazil (Turnbull 1999; Wingfield 2003), where *E. grandis* has been found to be highly susceptible to canker disease (Wingfield 2003). In New Zealand, *E. grandis* x *E. nitens* were observed to be less susceptible to *M. cryptica* and *Teratosphaeria eucalypti* than *E. nitens*, and appeared to have denser and healthier crowns (Shelbourne *et al.* 1999).

In Australia, however, hybrids are seen to be more susceptible to pests and pathogens (Potts and Dungey 2004). In Tasmania, *E. nitens* x *E. globulus* hybrids have been described as being more susceptible to MLD (*M. cryptica* and *M. nubilosa*) than either parent (Dungey *et al*. 1997). *Eucalyptus globulus* is more susceptible to MLD than *E. nitens*, particularly *M. nubilosa* (Carnegie *et al*. 1998; Milgate *et al*. 2001; Carnegie and Ades 2002). Dungey *et al*. (1997) also reported that this hybrid showed higher susceptibility to MLD and mammal browsing than either parent. Carnegie and Ades (2002) describe the lesions on the *E. nitens* x *E. globulus* hybrids as more blighting and larger than those on either *E. nitens* or *E. globulus*. *Mycosphaerella nubilosa* (see Section 1.4.4 for nomenclature) was not isolated from *E. nitens* in this study, however, it was found causing lesions in the hybrid. Carnegie and Ades (2002) speculate that by using the *E. nitens* x *E. globulus* hybrid in such areas as Tasmania, where both parents are grown commercially and one is more resistant to disease than the other, could allow the pathogen to evolve and act as a conduit between the two species.

Potts *et al*. (2003) are also of the opinion that hybrids, through being more susceptible to pests and diseases may act as pathways for new and emerging

epidemics. Coutinho *et al*. (2002) reported that a bacterial blight disease caused by *Pantoea ananatis*, first identified on *E. grandis* x *E. nitens* seedlings in a nursery in South Africa spread to other nurseries and affected other eucalypt species. The disease appears to be driven by environment and possibly spread through propagation techniques (Coutinho *et al.* 2002).

Potts and Dungey (2004) suggest that the success of hybrids overseas may be due to a lack of pests and pathogens that are otherwise present in Australia. The hybrid *E. grandis* x *E. urophylla* is grown successfully overseas, however, Potts and Dungey (2004) surmise that if it were grown commercially in Australia, its success may be compromised by the increased numbers of pests and diseases here.

As mentioned earlier, hybrids can also increase physiological tolerances to the environment. *Eucalyptus nitens* is frost tolerant and is grown on the higher slopes in Tasmania where frosts occur. However, *E. globulus* is grown on the lower slopes, where it is more protected from frost events. The hybrid *E. nitens* x *E. globulus* allows land between the higher and lower sites to be utilised, as the hybrid shows a higher degree of frost tolerance than *E. globulus* (Turnbull 1999).

Hybrids can be planted in areas where other, more easily propagated and deployed eucalypts are not considered to be economically viable. On sites desirable for *E. globulus* in WA, those having high rainfall, low evaporation and preferred soil, Barbour (2003) found *E. globulus* outperformed eucalypt hybrids tested. The hybrids included *E. globulus* x *E. grandis*, *E. globulus* x *E. camaldulensis* and *E. grandis* x *E. camaldulensis*. However, on sites with low rainfall and high evaporation rates, the

hybrids outperformed the *E. globulus*. Water availability reduced the number of stems from pulping stockings to solid wood (Barbour 2003). Moving into less desirable areas has become the only option for tree companies in WA as the cost and availability of land hinders expansion in the more desirable areas. Therefore, land previously not thought of as useful for eucalypt plantations is now being established in areas such as Esperance in the south east of WA.

1.4 Threats to plantation eucalypts in Australia

1.4.1 Environment

The environment is the most influential component in relation to the health of a plantation. The location of plantations is primarily governed by the climate, particularly rainfall and temperature. An ideal climate for an *E. globulus* plantation is one with an annual rainfall of between 550-1500 mm, deep soils and a temperature range of 2-30 °C (Eldridge *et al*. 1994). A combination of low rainfall, shallow soils and high temperatures can result in tree death through drought. Drought is often a problem in Western Australia (Harper *et al*. 1999). Trees must be established on good soils that allow rapid infiltration, drainage and have a sufficient water holding capacity.

Conducive environmental conditions may exacerbate or accelerate the impact of biotic diseases caused by abiotic factors such as an imbalance of nutrients causing stress in the plant, lowering the defence mechanisms (Brown *et al*. 1997). For example, in WA, copper deficiency is commonly encountered, particularly on ex-

pasture sites (Gherardi *et al.* 1999). Copper is an important micronutrient used in many biological activities, such as photosynthesis and also the production of lignin, which is used in defence of fungal infections (Ishaq 1999; Gherardi *et al*. 1999).

1.4.2 Phyllophagous insects

A survey of *E. globulus* plantations in 1998 revealed several important insect species to be present and causing extensive damage in Western Australia (Maxwell *et al*. 1998). Autumn gum moth (*Mnesampela privata*) was found to attack mainly juvenile foliage. The larvae have the capability to consume whole leaves, leaving nothing but the mid rib, causing complete defoliation of the tree. It has also been noted as a significant problem on native stands of *E. globulus* in Tasmania (Hillis and Brown 1984).

An insect that is causing increasing concern in Western Australia is leaf blister saw fly (*Phylacteophaga froggatti*). Often it is initially confused as fungal leaf spots, the insect larva mine under the upper epidermal layer of the leaf surface causing a necrotic blister. The insect has the capability of causing complete defoliation of plantations, which may lead to eventual tree death (Maxwell *et al*. 1998).

Other phyllophagous insects that appear to be increasing in number in Western Australia are chrysomelids and weevils (Maxwell *et al*. 1998). Chrysomelids have been found in eastern Australia where, in the absence of natural predators they have caused extensive defoliation damage to a range of eucalypt plantation species (Hillis and Brown 1984). There has, however, been noticeable intraspecific variation

between provenances in susceptibility to damage caused by the eucalyptus snout beetle (*Gonipterus scutellatus*) in *E. viminalis* and *E. dalrympleana* in Lesotho, Southern Africa (Eldridge *et al*. 1994). This resistance has the potential to become commercially significant where this pest is a major concern.

1.4.3 Stem pathogens

Poor health of a tree may predispose it to pests and pathogens that may cause disease or even death. A canker (or lesion) is an area of dead necrotic tissue caused by either fungi or bacteria (Fraser and Davison 1985; Williams and Woinarski 1997). There are two broad canker categories, annual and perennial, which depend on the area that is infected and also on the host's response to the infection (Fraser and Davison 1985). Annual cankers are characterised by the pathogen tending to infect only the phloem. The plant is able to contain the pathogen to the general area of infection by forming a callus around the diseased area. The canker is often removed when the bark is shed (Tattar 1978). Perennial cankers result when the sapwood, phloem or cambium is invaded by the pathogen before the plant has activated defence mechanisms. Radial growth of the pathogen can cause girdling resulting in distortion or even death of the branch or stem (Davison 1995).

Two fungal pathogens that have the potential to cause perennial cankers of eucalypts in Western Australia are *Holocryphia eucalypti* (formerly *Endothiella*, Gryzenhout *et al.* 2006) and *Neofusicoccum australe* (formerly *Botryosphaeria* Davison and Tay 1983; Crous *et al.* 2006). Both fungi are considered to be opportunistic pathogens with a broad host range, and are thought to enter plants

through wounds caused by wind, animal or insect damage (Morgan 1994). These two pathogens may be found at low levels in a healthy plantation, but they can cause considerable damage to trees that have been stressed by environmental conditions (Davison 1995). In a survey of 26 *E. globulus* plantations in the south-west of WA, Jackson (2003) isolated *Holocryphia eucalypti* from 21 of those plantations. Burgess *et al.* (2006) investigated the movement of *N. australe* between *E. marginata*, *E. diversicolor*, *E. phylacis* and *E. globulus* in the same geographical region. Their study concluded that there was no restriction of *N. australe* between the three endemic eucalypt species and the exotic *E. globulus.*

It is when a plant is stressed that infection by a pathogen causing disease is most likely (Nichol *et al*. 1992; Smith *et al*. 1994). Fungal infection of the stem may affect wood quality, resulting in reduced growth rates, increased mortality and reduced economic value of the timber for industrial use (Davison 1995). Since *N. australe* is so widespread, Burgess *et al.* (2006) suggested that it is native to south-western Australia and is a recent introduction to the plantation estate in that region.

1.4.4 Foliar pathogens

There are many leaf-inhabiting fungi that have been recorded as causing leaf spots or blight in eucalypt plantations within Australia. Most are considered to be endemic in native eucalypt forests, but have been observed to cause epidemics if conditions become conducive (Barber 1998). Fungi associated with eucalypts in Australia, known to cause leaf diseases, include coelomycetes, hyphomycetes and ascomycetes. The modes of nutritional requirements of these fungi are either

biotrophic and/or necrotrophic (Park 1984). Fungi are often categorised according to their ecological and nutritional requirements (Isaac 1992). There are three broad groups of fungi, saprophytes, necrotrophs and biotrophs. However, many species may fit into more than one category at any one point in their life history.

The most important foliar diseases of plantation eucalypts world-wide are eucalypt rust (*Puccinia psidii*), leaf blight associated with *Cylindrocladium* species, corky leaf spot caused by *Aulographina eucalypti* and leaf blotch associated with *Mycosphaerella* (and *Teratosphaeria*) species (Park *et al*. 2000). *Puccinia psidii* is considered to be an important pathogen of shoots and leaves on eucalypts in Central and South America (Park *et al*. 2000; Tommerup *et al.* 2003). It is not a recent record, as it was first isolated from leaves of *Psidium* (guava) species in Brazil in 1884. This fungus has the potential to infect a range of myrtaceous genera (Park *et al*. 2000). In 1944, it was positively identified from eucalypt material. In 2005 it was reported to be infecting *Heteropyxis natensis*, a native deciduous tree from South Africa, in artificial inoculation trials in Brazil (Alfenas *et al*. 2005). This was the first report of this rust fungus infecting a host outside of the Myrtaceae, but within the order Myrtales (Alfenas *et al*. 2005). It poses a serious threat to eucalypt plantations outside South America, particularly Australia, where it has the potential of causing devastating effects on native forests eucalypts and other myrtaceous genera (Coutinho *et al.* 1998; Rayachhetry *et al.* 2001; Langrell *et al*. 2008).

Another rust species described infecting species within the Myrtaceae is *Uredo rangelii*. *Uredo rangelii* was first described by Simpson *et al.* (2006) from *Myrtus*

communis in Argentina and also *Syzygium jambos* in Jamaica. This species was recently isolated from infected *Agonis flexuosa* cv. 'Afterdark' leaves in New South Wales, Australia (Carnegie *et al.* 2010). Sequencing of the ITS r DNA and nested PCR used for specific detection of *P. psidii* (Langrell *et al.* 2008) were synonymous with sequences of *P. psidii* (Carnegie *et al.* 2010) and these two species can only be separated based on morphological features (Simpson *et al.* 2006; Carnegie *et al.* 2010) and are now considered to be part of the *P. psidii* complex (Carnegie and Lidbetter 2012). The incursion of *P. psidii* has increased the host range previously recorded by Simpson *et al.* (2006) and includes *Agonis*, *Callistemon* and *Syncarpia* (Carnegie *et al.* 2010), *Syncarpia, Leptospermum, Tristania, Metrosideros* and *Gossia* (Department of Agriculture, Fisheries and Forestry 2010). There is currently a response plan being implemented for control and possible eradication of this disease in New South Wales, Australia (Department of Agriculture, Fisheries and Forestry 2010; Carnegie and Cooper 2011). An internet system for weather-based mapping of plant pathogens was developed in 2007 to determine areas at risk of exotic incursions in America, and has now been used to map the areas most at risk of *P. psidii* in Australia (Magarey *et al.* 2007). It was concluded that the north and central east coast of Australia are most at risk from an incursion and the likely risk to Western Australia is minimal because climatic conditions are unsuitable (Magarey *et al*. 2007).

Calonectria species have caused major damage in plantations in Brazil, India, South Africa, Vietnam and China (Park *et al*. 2000; Lombard *et al*. 2010). These pathogens are non-specialised and have wide host ranges. *Calonectria reteaudii*

(=Cylindrocladium quinqueseptatum) is the most commonly found species in this genus to be isolated from eucalypts in Asia, India and northern Australia (Park *et al*. 2000). It is considered to be the most serious disease causing death of eucalypt seedlings in Vietnam, where a combination of high rainfall and humid conditions make this an ideal environment for high levels of disease (Booth *et al.* 2000). This pathogen, like *Puccinia psidii,* does not pose a high risk to Western Australia, as conducive climatic conditions are not present (Booth *et al*. 2000).

Aulographina eucalypti is the pathogen that causes target or leafy spot, and is often associated with leaf disease of eucalypts in Australia (Swart 1988). It has also been isolated in New Zealand, South Africa, Brazil, the United Kingdom and Vietnam (Park *et al*. 2000). It has been found to infect species in the subgenera *Symphyomyrtus*, *Monocalyptus* and *Angophora*. It has been isolated from *E. globulus* in Victoria but had not caused significant damage at that point of time (Barber 1998). *Aulographina eucalypti* has, however, reached epidemic proportions in *E. regans* after logging in Victoria and has caused severe spotting in *E. obliqua* also after logging (Park *et al*. 2000). Within plantations in eastern Australia, the damage has been described as minor (Carnegie and Keane 2003). It has also been found on lesions associated with *Mycosphaerella suberosa* on adult foliage of *E. globulus* in Western Australia at very low levels (A Maxwell, pers com).

1.5 Mycosphaerella Leaf Disease

1.5.1 Taxonomic concepts of the *Mycosphaerella* and *Teratosphaeria* genera

The number of *Mycosphaerella* and *Teratosphaeria* species and associated anamorphs described around the world from *Eucalyptus* has increased substantially over the last twenty years (Crous *et al.* 2009a). The anamorph genera in particular have undergone various name changes over that time and the nomenclature remains in a state of flux as technology and a greater understanding of the biology of the organisms increase. Crous *et al.* (2007b) proposed a new family, Teratosphaeriaceae, to accommodate species in *Teratosphaeria*, the type species being *T. fibrillosa* described from *Protea grandiflora* (=*Protea nitida*) (Taylor *et al.* 2003). Taylor *et al.* (2003) attempted to establish phylogenetic differences, however, synonymised the two genera. Later, Crous *et al.* (2007b) re-examined *Mycosphaerella* and similar genera and concluded that Teratosphaeriaceae should be separate to Mycosphaerellaceae based on both molecular phylogenetic evidence and morphological differences. The morphological differences between *Teratosphaeria* and *Mycosphaerella* are not always observed in the smaller-spored species and not all species have all the key taxonomic features (Crous *et al.* 2007b). The main features that differentiate *Teratosphaeria* from *Mycosphaerella* are superficial stroma linking ascomata together, ascospores that are brown within the asci or turn brown soon after release, pseudoparaphyses (uncommon), ascospores covered with mucous sheath, multi-layered endotunica of asci (uncommon) and ostiolar periphyses (Crous *et al.* 2007b).

Both genera and their associated anamorphic genera are currently the subject of re-evaluation and hence published articles refer to either genus (Silva *et al.* 2009; Pérez *et al.* 2010). **Species within this thesis will be referred to as currently listed in MycoBank (Roberts *et al*. 2005), with the exception of *Uwebraunia dekkeri* for which the current name on MycoBank has not yet been updated (Crous pers comm.). MycoBank is being corrected to reflect the taxonomy of Li *et al*. (2012). The species names used herein and their synonyms are listed in Table 1.2.**

Table 1.2 Names used within the current thesis and the synonymous species names.

Current name in MycoBank	Synonymous species	Reference
Mycosphaerella africana Crous & MJ Wingf. 1996	*Teratosphaeria africana*	Robert *et al*. (2005)
M. cryptica (Cooke) Hansf. 1956	*T. cryptica*	Robert *et al*. (2005)
M. flexuosa Crous & MJ Wingf. 1998	*T. flexuosa*	Robert *et al*. (2005)
M. molleriana (Thüm.) Lindau 1897	*T. molleriana, M. ambiphylla, M. vespa*	Robert *et al*. (2005)
M. suberosa Crous, F.A. Ferreira, Alfenas & M.J. Wingf. 1993	*T. suberosa*	Robert *et al*. (2005)
M. suttoniae Crous & MJ Wingf. 1997	*T. suttoniae, Kirramyces epicoccoides, Phaeophleospora epicoccoides*	Robert *et al*. (2005)
Pseudocercospora fori (G.C. Hunter, Crous & M.J. Wingf.) G.C. Hunter, Crous & M.J. Wingf. 2009	*M. fori*	Robert *et al*. (2005)
T. associata (Crous & Carnegie) Crous &	*M. associata*	Robert *et al*. (2005)

Current name in MycoBank	Synonymous species	Reference
U. Braun 2007		
T. eucalypti (Cooke & Massee) Crous 2009	*K. eucalypti, P. eucalypti*	Robert *et al*. (2005)
T. jonkershoekensis (P.S. van Wyk, Marasas & Knox-Dav.) Crous & U. Braun 2007	*M. jonkershoekensis*	Robert *et al*. (2005)
T. mexicana (Crous) Crous & U. Braun 2007	*T. mexicana*	Robert *et al*. (2005)
T. multiseptata (Carnegie) Carnegie 2009	*M. multiseptata*	Robert *et al*. (2005)
T. parva (R.F. Park & Keane) Crous & U. Braun 2007	*M. parva, M. grandis*	Robert *et al*. (2005)
T. ohnowa (Crous & M.J. Wingf.) Crous & U. Braun 2007	*M. ohnowa*	Robert *et al*. (2005)
Readeriella dendritica (Crous & Summerell) Crous & Summerell 2009	*M. dendritica*, *T. dendritica*	Robert *et al*. (2005)
Uwebraunia dekkeri (de Hoog & Hijwegen) Crous 2012	*Dissoconium dekkeri, M. lateralis, U. lateralis*	Li *et al*. (2012)
Zasmidium citri (Whiteside) Crous 2009	*M. citri*	

1.5.2 Recent history of disease in Australia

In Western Australia, the most serious fungal disease of plantation eucalypts is MLD (Maxwell *et al*. 1998). Mycosphaerella leaf disease is caused by various *Mycosphaerella* and *Teratosphaeria* species and their associated anamorphs (Crous 1998; Crous et al. 2009b). The genera *Mycosphaerella* and *Teratosphaeria* belong to the division Ascomycota, with over 1900 named species (Robert et al. 2005). There are at least 100 species that have been isolated from a number of *Eucalyptus*

and *Corymbia* species (Crous *et al.* 2007a). However, there are still *Mycosphaerella* and *Teratosphaeria* species yet to be described that have been isolated from *Eucalyptus* plantations (Carnegie and Keane 1994; Carnegie *et al*. 1997; Maxwell *et al*. 2003). The species occurring on eucalypts have ungone revision in recent times and continues to be revised. A study of Western Australian plantations found *Mycosphaerella* and *Teratosphaeria* as the main fungal genus causing leaf spots, although several other fungi species, such as *Aulographina* and *Harknessia* were also found to cause leaf spots (Maxwell *et al*. 1998). The latter study concluded that MLD occurred in low levels in WA and recommended that the pathogens and the disease they cause should be closely monitored. South-eastern Australia had a similar experience with MLD in 1977 and 1980, when an epidemic emerged on several different *Eucalyptus* spp. (Park and Keane 1982b). It was determined that MLD on juvenile foliage in south-eastern Australia was caused by *M. cryptica* and *M. nubilosa* (Carnegie and Keane 1994). These two species have also been identified as causing the most damage in Western Australia (Maxwell *et al*. 2003).

1.5.3 Impact of MLD on plantation eucalypts

Mycosphaerella leaf disease has the potential, depending on species, to completely defoliate both the juvenile and adult foliage. In New Zealand, MLD was first described in 1971 occurring on *E. delegatensis* and *E. regans*. The causal agent was identified as *M. nubilosa*; however, Park and Keane (1982a) later re-identified it as being caused by *M. cryptica*. *Mycosphaerella cryptica* was also found to cause stem cankering and shoot die-back, causing distortion and loss of apical dominance,

decreasing the economic value of the timber (Beresford 1978). The anamorph stage was also found associated with lesions. It was originally placed into the *Colletogloeum* genus; however, later a new genus was proposed and accepted, *Colletogloeopsis* (Ganapathi and Corbin 1979; Crous and Wingfield 1997). Due to the initial confusion of the identity of *M. cryptica*, the anamorph is known as *Colletogloeopsis nubilosum*.

One of the major effects of defoliation caused by MLD is a reduction in growth (Lunquist and Purnell 1987). A foliar pathogen can cause a reduction in photosynthesis in its host. As this is the most important activity of green plants, it could be assumed that foliar pathogens would affect the overall growth of the host (Lucas 1998). A reduction in photosynthesis alters the pathway of diffusion of carbon dioxide into and within the leaf. This reduction in carbon dioxide reduces the amount of energy available to the plant (Scholes 1992).

In South Africa, MLD was first recorded in 1925 on *E. globulus*. This species was abandoned as a plantation species in South Africa during the 1930's either due to the eucalypt snout beetle (*Gonipterus scutellatus*) or from MLD, however it is generally accepted that MLD was the likely cause (Lundquist and Purnell 1987; Crous 1998).

1.5.4 Strategies to overcome MLD

1.5.4.1 Host/pathogen interactions

The future success of monocultures world-wide relies on the management of diseases. One of the strategies is breeding for disease resistance. In natural stands of mixed species, the diversity of potential pathogens is high, but the individual occurrence is often low. *Mycosphaerella* and *Teratosphaeria* species associated with eucalypts are often host specific; however, there are always exceptions. *M. cryptica*, a foliar pathogen, has been isolated from eucalypt species from both the *Monocalyptus* and *Symphyomyrtus* subgenera, on adult and juvenile leaves (Park and Keane 1982a & b). Carnegie (2007a) reported *M. cryptica* on 20 eucalypt species from New South Wales alone, including nine new host species. In comparison, *M. nubilosa* had only been isolated from species from the *Symphyomyrtus* subgenus, namely *E. bridgesiana*, *E. cypellocarpa* and *E. globulus* (Park and Keane 1982a & b). More recently it has been isolated from *E. nitens*, *E. dunnii* and *E. maidenii*, which are all from the Section *Maidenaria*, Series *Globulares* (Table 1.1) (Carnegie 2007a). It has been recently isolated from adult foliage of *E. globulus* in Western Australia (Maxwell *et al*. 2001). Previously, *M. nubilosa* was known only from juvenile foliage.

A study by Lundquist and Purnell (1987) showed, for the first time, the impact MLD can have on the growth and productivity on *E. nitens*. Trees that had been heavily infected and consequently defoliated by MLD did not grow as rapidly as other less infected trees. Variation between provenances of *E. globulus* to MLD has been

found to exist in Australia. For example, seedlings of various subspecies of *E*. *globulus* inoculated with *M. cryptica* and *M. nubilosa* expressed a range of responses of resistance (Park 1984). Further investigation is needed on the impact of MLD on growth and productivity on *E. globulus* within Australia.

Several authors have recorded intraspecific variation of eucalypts. *Eucalyptus regans* grown in New Zealand has shown provenance variation in susceptibility to *M. cryptica* (Potts and Pederick 2000). A provenance trial in New Zealand showed that Tasmanian provenances were more resistant than Victorian provenances to *M. nubilosa* (Carnegie *et al*. 1994; Dungey *et al*. 1997). *Eucalyptus nitens* provenances in New South Wales have shown more resistance to infection from *M. nubilosa* than Victorian provenances (Lunquist and Purnell 1987). In a study investigating provenance variation of MLD on adult foliage of *E. globulus* at Tostaree in Victoria, Carnegie *et al*. (1994) reported that the most susceptible provenances were from Judbury (Tasmania), Otway National Park (Victoria) and King Island (Tasmania), while the least susceptible was from Wye River (Victoria) (Carnegie *et al*. 1994; Carnegie 2000). Carnegie *et al.* (1994) attempted to explain the differences in susceptibility between provenances of *E*. *globulus* to *M*. *nubilosa* and *M. cryptica*. Those provenances that experience high summer rainfall which had the potential to exacerbate disease (Park 1988) may have undergone a higher degree of natural selection. The seed sources of those provenances that do not experience summer rainfall, that are planted into environments receiving summer rainfall were seen to be more susceptible to infection by *M. nubilosa*. Carnegie (2000) observed at a trial site at Silver Creek in Victoria that *E. globulus* sourced from the Otway ranges, where

there is a relatively high summer rainfall and/or mean maximum temperatures, had little MLD compared to those that were sourced from areas with a low summer rainfall and/or mean maximum temperatures such as Uxbridge, Denison and Pepper Hill in Tasmania.

Dutkowski and Potts (1999) found strong regional differences within provenances of *E. globulus* that led them to be able to divide it into different races. These differences can result from migration, adaptation or genetic drift; however, they also found climatic variation within a geographical region (Dutkowski and Potts 1999). For example, Dutkowski and Potts (1999) found an east-west cline in bark thickness and drought tolerance of *E. globulus* in the Otway ranges in Victoria. This cline coincided with a decline in rainfall and has been surmised to be an adaptation to fire frequency and/or a tolerance to water deficit (Dutkowski and Potts 1999). These studies show that breeding programs could have the potential to manage MLD in the future.

1.5.4.2 Biocontrol and fungicides

Biological control agents are largely being sought after for agricultural pests and pathogens; however, there have been studies where these agents have been aimed at the forestry industry (Shoeman *et al*. 1999). Many of the studies described deal with *Trichoderma* spp., which are used in the control of many Basidiomycetes. In controlling foliar diseases *Trichoderma* spp. could be applied to the seed or soil. By doing this the *Trichoderma* spp. would not control the plant pathogen by producing toxic compounds to the pathogen, but rather they elicit an induced systemic resistance to the pathogen by the host (Harman *et al.* 2008). The systemic response

may be more long lasting (Harman *et al.* 2008). *Ampelomyces quisqualis*, *Bacillus subtilis* and *Trichoderma harzianum* T39 control powdery mildew (*Podosphaera aphanis*) of strawberries (Pertot *et al.* 2008). When applied, the biological control agents did not adequately control the pathogen; however, in conjunction with chemical control, levels of *Podosphaera aphanis* were contained to a manageable level, while reducing the amount of fungicide used (Pertot *et al*. 2008). This regime also had a positive effect on the predatory mite (*Amblyseius andersoni*) used to control the spider mite *Tetranychus urticae* (Pertot *et al.* 2008).

The use of a biological control agent offers the potential benefit of being host specific and self-perpetuating. This means that repeated applications of an agent should not be required once it becomes established in the plantation environment. For a leaf pathogens such as *Mycosphaerella* or *Teratosphaeria*, a mycoparasite could be used to successfully control blight, if a suitable candidate were to be found. A mycoparasite is a fungus that acquires most or all of its nutrients from another fungus (Isaac 1992). Mechanisms associated with a mycoparasite vary from organism to organism and so determining a relationship is often hard, because no direct contact is needed (Elad 1995). The use of biological control agents to reduce the impact and spread of *Mycosphaerella* species in Australia is an attractive method.

Uwebraunia dekkeri (as *M. lateralis*) was found in association with *M. cryptica* and *M. nubilosa* lesions on *E. globulus* (Maxwell *et al*. 2000). Crous *et al*. (1999) also described *U. dekkeri* as being associated on lesions caused by other pathogens on

several eucalypt species in southern Africa. The *Uwebraunia* genus is closely related to *Dissoconium* which has a wide host range, and includes *Dissoconium* species that have been reported as antagonists or mycoparasites on other leaf pathogens (de Hoog *et al*. 1991; Li et al. 2012). De Hoog *et al*. (1991) suggested *U. dekkeri* (as *D. dekkeri*) as being a hyperparasite.

The application of fungicides in a silvicultural context is problematic, as they are expensive and impractical on such a large scale. Furthermore, many fungicides used may harm beneficial micro-organisms such as mycorrhizal fungi and the environment in general. The aerial application of insecticides on plantations in the south-west of WA highlighted the problem of applying chemicals to large areas, with large public outrage and condemnation (Schirmer and Tonts 2003). The plantation industry received significant adverse publicity, which for a developing industry is not sound economics. Therefore, the application of fungicides will most likely receive a similar response.

1.5.5 Presence of MLD on eucalypts in Western Australia

Until recently, *E. globulus* plantations and native forests had been largely ignored in WA with respect to MLD and other foliar pathogens (Figure 1.1a). This is partly due to plantations only being planted on a commercial scale in the last twenty years (Figure 1.1b). However, a survey by Carnegie *et al.* (1997) revealed the presence of three *Mycosphaerella* species associated with *E. globulus*, one of which, *M. suberosa* had not been isolated outside Brazil and Indonesia (Crous *et al*. 1993; Crous and Wingfield 1997; Crous 1998). They also isolated *M. cryptica* from *E.*

globulus, and *E. marginata* and *M. marksii* from *E. globulus*. A more intensive study of *E. globulus* plantations in 1999 confirmed those pathogens previously described by Carnegie *et al.* (1997) and led to the isolation of two new pathogens: *M. nubilosa* and *U. dekkeri* (as *M. lateralis*), the latter being a new record for Australia (Maxwell *et al*. 2000; 2001). Due to the importance of finding *M. nubilosa*, *E. globulus* plantations were regularly sampled over the following year.

From these subsequent surveys one newly discovered *Mycosphaerella* species, *Mycosphaerella aurantia,* was described, two new records for Australia were isolated, *Mycosphaerella molleriana* (as *M. ambiphylla*) and *Teratosphaeria mexicana*, and two new records for Western Australia, *Mycosphaerella gregaria* and *Teratosphaeria parva* were also isolated (Maxwell *et al*. 2003).

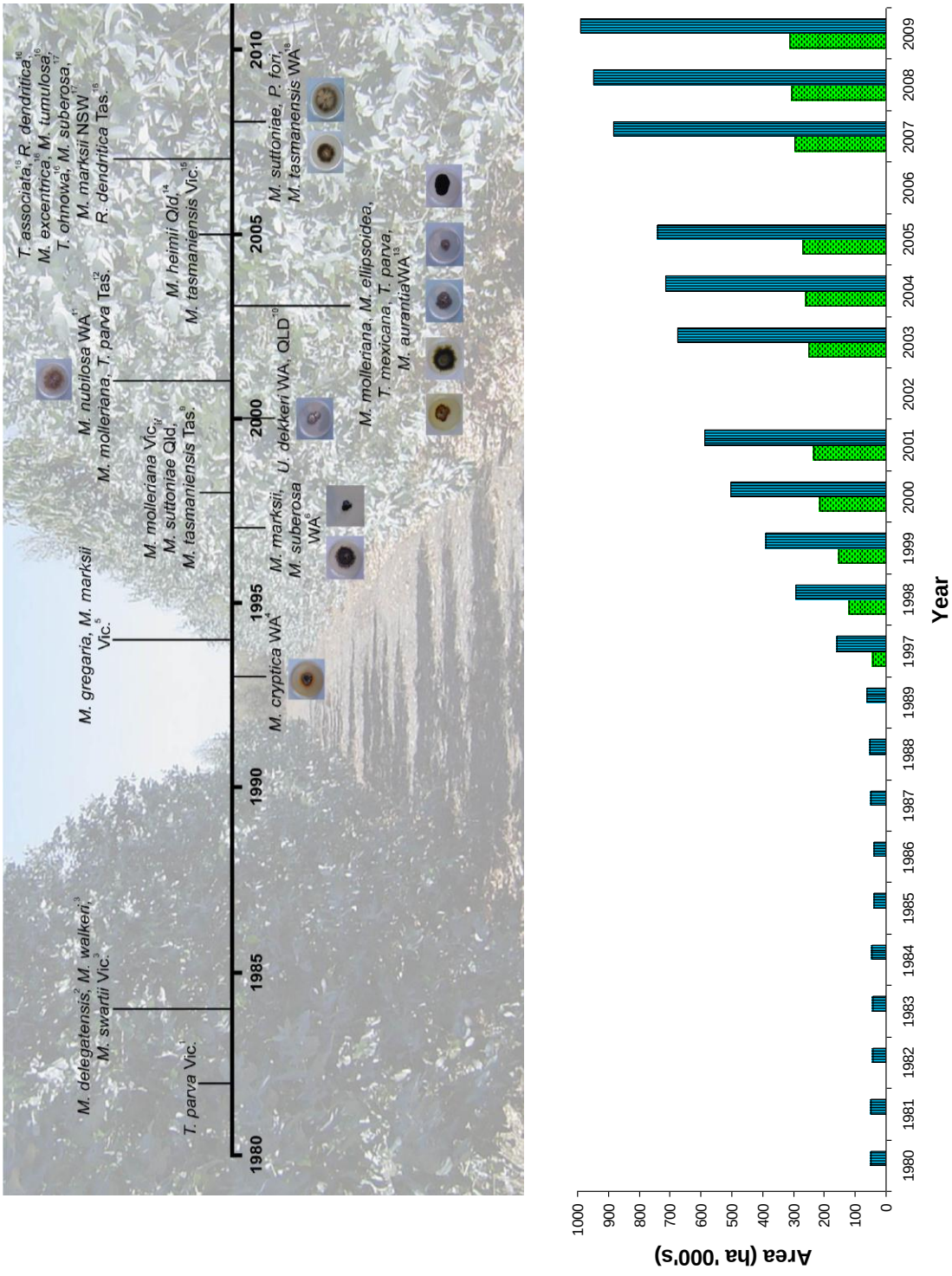

Figure 1.1 Time line depicting the increasing number of Mycosphaerella leaf disease associated species published in Australia since 1980 (A) and the area of eucalypt plantations (hectares) in Western Australia (green/ dots) and Australia (blue/ lines) since 1980. References for Figure 1.1A are listed at the end of this chapter along with corresponding number. References for Figure 1.1B are annotated by an (*) at the end of this chapter.

1.5.6 The biology of *Mycosphaerella* species on eucalypts

The epidemiology and aerobiology of *M. cryptica* on *E. delegatensis* and other *Eucalyptus* species was investigated in New Zealand (Cheah 1977; Beresford 1978) in response to the devastating effects MLD had on the plantations there. Beresford (1978) found that wet periods, followed by a drying period were required for ascospore discharge, and six to eight weeks later lesions appeared on the youngest juvenile leaves. This study also showed that infection and disease development was seasonal. Leaves that had senesced had up to 25–50% of the leaf area infected by *M. cryptica*. The highest period of defoliation occurred in the summer months; however, the extent differed from year to year. Up to 40% of leaves were infected in December 1975 and 80% by February 1976 (Cheah 1977). The study by Cheah (1977) suggested that ascospores of *M. cryptica* were the primary inoculum source, but also that conidia were able to infect leaves. After 24 hours, 50% of ascospores had germinated on a wet slide, while conidia did not germinate until after thirty hours. The relative humidity (RH) appeared to be the key factor for ascospore dispersal, with 98–100% RH needed (Cheah 1977). Park (1988) also studied the epidemiology of *M. cryptica* and *M. nubilosa* in south-eastern Australia. He found *M. nubilosa* to be mono or bicyclic whereas *M. cryptica* was polycyclic. Park (1984) also found that a RH of above 98% was needed for ascospore discharge for both species.

Park (1984) also studied the infection process of *M. cryptica*, *M. nubilosa* and *T. parva* on several eucalypt species including *E. globulus*. He concluded that *M. cryptica* was able to form appressoria and could infect the upper surface of the leaf directly as well as through stomata on the lower surface. He found *M.*

nubilosa to only infect leaves through stomata on the lower surface, and concluded that *T. parva* was able to only act as a saprophyte, as no infection of the leaves was observed. These studies are seen as important, as they provide an overall picture of the biology, pathology and epidemiology of the two major *Mycosphaerella* species causing most damage to plantations in Australia and New Zealand.

The centre of origin is not known for either *M. cryptica* or *M. nubilosa*. Present studies suggest that *M. nubilosa* has a lower genetic diversity in WA than eastern populations in Australia, which possibly indicates that it is most likely a recent introduction to WA (Maxwell 2004). Studies on *M. cryptica* are still being conducted (K Taylor unpublished). In WA, *M. nubilosa* has only been isolated from *E. globulus* (Maxwell *et al*. 2001), even though it has been found to infect other members of the subgenus *Symphyomyrtus* in eastern Australia (Park 1984). As previously mentioned, *M. cryptica* has been repeatedly isolated from *E. marginata* and also *E. diversicolor*. Molecular studies currently being conducted will identify the genetic diversity of *M. cryptica* both within WA and Australia wide (K Taylor unpublished).

There is growing concern that fungal pathogens growing on exotics could spread into native forest and *vice versa*. In theory, it is possible for the native eucalypt species in WA to have a lower resistance to introduced pathogens. For example, it is only recently that *M. cryptica* has been isolated from two of the most important endemic eucalypt species (*E. marginata* and *E. diversicolor*) in WA. Currently, it is not known how *M. cryptica* infects either *E. marginata* or *E. diversicolor*, and there is no information on disease development on these two

species. As previously stated, *M. cryptica* is one of the most severe MLD pathogens on eucalypts world-wide, consequently it is of concern that it has been isolated from these two native WA eucalypts.

Another *Mycosphaerella* species that has been repeatedly isolated in WA from *E. globulus* is *M. marksii*. No biological or pathogenic studies have been conducted on this species anywhere. This may be because it is not seen as a primary pathogen (Carnegie *et al*. 1997). However, the incidence of *M. marksii* appears to be increasing, and isolations have been made without association with *M. nubilosa* or *M. cryptica* (A. Maxwell, pers com). This indicates that *M. marksii* may in fact act as a primary pathogen.

1.6 Aims

The early sections of this chapter have highlighted the knowledge gaps concerning MLD in Australian plantations and the extent to which the composition of MLD species is changing with time in WA. The epidemiology, biology and host/pathogen interactions are fundamental to the understanding of plant disease and their subsequent control. Studies on *Mycosphaerella* species on eucalypts have established the difficulty in working with this fungal genus. However, slow but steady progress is being made on these important aspects. The aims of the current study were to:

- determine the *Mycosphaerella* and *Teratosphaeria* species associated with leaf spots collected from plantation and endemic eucalypts from WA and Queensland (**Chapter 2**);

- assess the number of fungal species that may be contributing to MLD in *E. globulus* plantations in WA and to evaluate changes in species and their incidence since the first surveys were conducted (**Chapter 3**);
- sequence the ITS region of the type material of *T. parva*, *M. grandis* and *M. gregaria* using culture and herbarium specimens and compare them to existing sequences from GenBank (**Chapter 4**);
- determine the use of glycerol as a surfactant and its effect on ascospore viability; and study the infection process by scanning electron microscopy and determine the pathogenicity of *M. marksii* on *E. globulus* (**Chapter 5**);
- determine the occurrence of *Mycosphaerella* and *Teratosphaeria* species involved in MLD over a period of a year from ten trees; to determine the level of defoliation in juvenile foliage in a plantation over a year and to determine the precision of ten assessors for MLD; and develop and test species-specific primers for the less frequently isolated *Mycosphaerella* and *Teratosphaeria* species on *E. globulus* in WA (**Chapter 6**);
- determine if *U. dekkeri* is a hyperparasite of *M. nubilosa* or *M. cryptica*, secondly to determine if *U. dekkeri* is able to infect *E. globulus* leaves and thirdly to investigate the mode of conidiogenesis (**Chapter 7**);
- determine whether regular application of fungicides and insecticides increases the growth and profitability of *E. globulus* plantations aged 1–4 years (**Chapter 8**);
- provide a general overview of the findings presented in this thesis (**Chapter 9**).

These topics were chosen because of their relevance to the *E. globulus* plantation industry in south-western Australia. The threat of MLD in the region is

high and equilibrium has not yet been established, making it difficult for breeding programs for MLD resistance to be implemented for the correct target organisms.

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CHAPTER 2

NEW HOST RECORDS OF *MYCOSPHAERELLA* FROM EUCALYPTS IN WESTERN AUSTRALIA AND QUEENSLAND

2.1 INTRODUCTION

In WA, the most serious foliar disease of eucalypt plantations is MLD (Chapter 1). However, little systematic sampling for MLD has been carried out in WA. Carnegie *et al.* (1997) were the first to reliably associate *Mycosphaerella cryptica* as the causal agent of MLD in WA, on *E. diversicolor*, *E. marginata* and *E. globulus*. Later, Maxwell *et al.* (2003) surveyed *E. globulus* plantations in the south-west corner of the state, from Bunbury in the west to Albany in the south and Esperance to the east, and identified five new records for Australia, which included two previously unidentified species and three new records for WA. Currently, ten species of *Mycosphaerella*/ *Teratosphaeria* have been recorded in WA from *Eucalyptus* (Carnegie *et al.* 1997; Maxwell *et al.* 2000, 2001, 2003). No eucalypt hybrids were included in these surveys in WA. However, since 2000, hybrid eucalypts are being planted in WA in trial plots (S. Collins pers. com) and in commercial plantings in south Queensland. Breeding for disease resistance of MLD in *Eucalyptus* is affordable, sustainable and necessary for plantation companies who are Forest Stewardship Accredited. However, it is important to understand what potential pathogens are present in Australia in order to breed for resistance.

In Queensland, MLD has been largely ignored. The plantation estate in Queensland is comprised of two climatic zones, tropical in far north Queensland and sub-tropical in central and southern Queensland. The eucalypt species grown are largely indigenous to these respective areas. *Eucalyptus pellita* occurs naturally in New Guinea and far north Queensland. Prior to the 1980's, *E. pellita* was not considered as a plantation species in tropical environments as other

species such as *E. camaldulensis*, *E. grandis*, *E. tereticornis* and *E. urophylla* were more commercially viable (Harwood *et al.* 1997). However, provenances of *E. pellita* sourced from Papua New Guinea (PNG) have proved more successful than *E. urophylla* on sites of low fertility (Harwood *et al.* 1997). The PNG *E. pellita* also suffers less from insect and fungal attack in Australia (Harwood *et al.* 1997).

Another important plantation species in southern Queensland is *E. dunnii*, found naturally in a south-eastern pocket of Queensland and north-eastern New South Wales (NSW) (Jovanovic *et al.* 2000). Due to its restricted distribution in natural stands, it is considered endangered and is thought to have limited genetic variation. Despite this, it has been successfully grown in both Brazil and China (Jovanovic *et al.* 2000). This species has proved more successful in frost-prone areas compared to the widely planted *E. grandis*, which is susceptible to frost damage (Jovanovic *et al.* 2000).

To date, few studies have been published concerning MLD on the plantation estate in Queensland. The first record of *U. dekkeri* (as *M. lateralis*) in Australia was isolated from both southern Queensland (*E. maidenii*, *E. grandis* x *tereticornis*) and WA (*E. globulus*) (Maxwell *et al.* 2000; Maxwell *et al.* 2003). More, recently, Whyte *et al.* (2005) isolated *M. heimii* from *E. dunnii*, previously only recorded from Madagascar (*Eucalyptus* sp.) and Indonesia (*E. urophylla*) (Crous 1998). The origin of this species has not been determined (Whyte *et al.* 2005).

The aim of this chapter was to determine the cause of leaf spots collected from plantation and endemic eucalypts from WA and Queensland. The reasons for including Queensland in this Thesis are as follows:

1. Some of the hybrid eucalypts being trialled in WA are also grown in south Queensland.
2. There is movement of planting stock or mother plants between the east and west coast and this may facilitate pathogen dispersal.

2.2 METHODS

2.2.1 Leaf Sampling

In Albany, WA, juvenile and/or adult leaves of the following *Eucalyptus* hybrids: *E. grandis x camaldulensis*, *E. globulus x urophylla* (Figure 2.1), *E. grandis x globulus* and *E. grandis x resinifera* in trial plots established by the plantation sector, were surveyed for MLD in October 2004. The hybrids were 3 years old at sampling. In addition, diseased leaves with leaf spots or general necrosis were opportunistically sampled from the endemic *E. diversicolor* (Denmark, Porongurup Range) and *E. rudis* (Nannup) in late 2004.

Leaves with necrotic spots were opportunistically collected from *E. pellita*, *E. camaldulensis* and *E. tereticornis* plantations in far north Queensland in January 2003 and from *E. globulus*, *E. dunnii* and *E. grandis* plantations in central/ southern Queensland in May 2003.

Figure 2.1 *Eucalyptus globulus* x *urophylla* sampled in the current study for Mycosphaerella leaf disease at a genetic trial in Albany in 2004. Photos courtesy of Sally Collins.

2.2.2 Isolation and identification of *Mycosphaerella* and *Teratosphaeria* spp. from leaf material

The isolation and identification methods for *Mycosphaerella* and *Teratosphaeria* species were carried out according to Crous (1998) and Maxwell *et al*. (2003). Briefly, this involved measuring spore size within asci, ascospore germination patterns on release and cultural morphological characteristics of colonies on 2% Difco® Malt Extract Agar (MEA).

Whole leaf pieces or excised lesions were soaked in water for at least two hours before being dried with a paper towel and attached, using double sided sticky tape to the lid of a Petri plate containing 2% MEA. For whole leaf pieces, the leaf was cut length ways and opposite sides were placed on the lids to face the agar, otherwise lesions with pseudothecia were placed facing the agar. Plates were inverted and left at room temperature for 24 hr. This allowed sufficient time for ascospores to be ‘shot’ upwards on to the agar without allowing sufficient time for colonies from germinating ascospores to overgrow each other. Single spore

isolations were made by transferring spores to new 2% MEA plates and incubated at 20°C in the dark. Slides were made at the same time as isolation. A small piece of agar containing the spores was transferred to a microscope slide with a small amount of lactoglycerol [85% lactic acid, glycerol and DI water, 1:1:1 (v/v)] and gently warmed and a cover-slip placed over the agar and squashed. Spores were then viewed under an Olympus BH-2 microscope at 100–1000X magnification. All slides were made permanent by sealing the cover-slip edges with nail varnish. Germination patterns were compared to those described by Crous (1998) and Maxwell *et al*. (2003).

Pseudothecia associated with released ascospores were squashed to facilitate identification. The pseudothecia were removed from lesions with a needle using a dissecting microscope. The pseudothecia were then mounted on to a microscope slide with lactoglycerol and gently heated and squashed. Up to 30 measurements of length and width of ascospores within asci were made under an Olympus BH-2 microscope (x 1000) for each species isolated.

2.2.3 Photography

Photographs of leaf material were made using a digital Canon PowerShot Pro1 camera. All photographs were edited using Adobe® Photoshop® 7.0 software.

2.2.4 DNA isolation

Mycelia were scraped directly from cultures using a sterile bade, placed into microfuge tubes and frozen at -20°C until required. The mycelia were then ground using an electric pellet mixer (Kontes, Vineland, NJ USA) and 200 µl of extraction buffer (200 µl; 200 mmol Tris HCl pH 8.5, 250 mmol NaCl, 25 mmol

EDTA and 0.5% SDS; Raeder and Broda 1985) was added to each tube. This solution was incubated at 65°C for 2–4 hr. The DNA samples were purified using the Ultrabind DNA purification kit following the manufacturer's instructions (MO BIO Laboratories, Solana Beach, CA USA) with a few variations. The tubes were then microfuged for 10 minutes at 13 200 g (Beckman Microfuge E, Fullerton, CA USA) after which the supernatant was transferred to a new sterile microfuge tube containing 600 µl NaI solution and 10 µl of silica slurry. The solution was gently inverted until well mixed and left at room temperature for 10 min. The tubes were microfuged for 1 min and the supernatant removed. The pellet was washed with 600 µl of Ultrawash, inverted, microfuged for 10 s with the supernatant removed. The pellet was washed again with 100% ethanol, inverted, microfuged for 10 s and the supernatant removed and re-microfuged. Excess supernatant was removed using a sterile pipette tip and samples were left to dry within an enclosed container for 1–2 hr. After drying, 25 µl of sterile water was added to the pellet and mixed using a pipette tip. Samples were left at room temperature for 5 min before being microfuged for 10 min. The supernatant was removed and transferred to sterile microfuge tubes. Diluted (10^{-1} and 10^{-2}) DNA samples were made by adding 5 µl of undiluted DNA to 45 µl of sterile water. A 5 µl sample of the 10^{-1} dilution was then transferred to new microfuge tubes containing 45 µl of sterile water.

2.2.5 DNA amplification

The extracted DNA samples were used to confirm identification by amplifying the ITS1, 5.8S and ITS2 regions of the rDNA operon using the primers ITS1f, ITS1, ITS2, ITS3 and ITS4 (White *et al.* 1990; Gardes and Bruns 1993) or species-

specific primers (Maxwell *et al.* 2005). The DNA was amplified in a 25 µl reaction volume containing; 2.5 mM MgCl2, 1.1 U Taq DNA polymerase (Fisher Biotec Australia), 1x polymerisation buffer (67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg ml^{-1} gelatin, 0.2 mM dNTPs) (Fisher Biotec Australia), 0.4 µM primer, 1–5 ng of DNA and sterile deionised water. The PCR reactions were performed (Applied Biosystems, Foster City, CA USA; Gene Amp 2400 thermocycler) according to the following parameters: Initial denaturing step of 96°C for 2 min; then 30 cycles of 94°C (30 s) denaturing, 55–58°C (30 s) annealing, 72°C (2 min) extension; 7 min extension at 72°C; held at 15°C. The PCR products were stored at -20°C prior to cleaning and sequencing. A water control with no DNA for every primer combination was also included in each reaction. The PCR products were electrophoresed on a 1% agarose gel in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0), at 90 V for 40 min. The products were visualised under UV following gel staining with ethidium bromide (0.5 μg^{-1}) for 20–30 min and de-staining in 1x TAE buffer for 10 min.

The size of the PCR products was determined against a 100 bp molecular weight marker (FN1 Fisher Biotech, Australia). PCR products were cleaned using the Ultrabind purification kit (MO BIO Laboratories) as per the manufacturer's instructions. DNA concentrations were determined by electrophoresing and staining the products as previously described and compared with a 100 bp Promega molecular weight marker. Nested PCR reactions were completed where initial PCR reactions failed, using the internal ITS2 and ITS3 or a specific primer. The primary PCR product was diluted to 10^{-1} and 10^{-2} and used as template. The PCR reaction volumes, thermocycler parameters and visualisation of PCR products were as described above. Gel images were taken using a digital camera

(EDAS 120, Kodak Digital Science™) under UV light and viewed using Kodak Digital Science™ ID (v 3.0.2) software.

2.2.6 DNA sequencing and analysis

Cleaned PCR products were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA USA) using the same primers used in the initial amplification. Standard quarter reactions were performed using; 2 µl ABI PRISM® BigDye Terminator Ready Reaction Cycle Sequencing Kit mix (3.0 or 3.1), 1.6 pmoles primer (3.2 pmoles for version 3.1), 80–160 ng PCR product. Sequencing reactions were performed according to the following parameters: Initial denaturing step of 96°C for 2 min; then 25 cycles of 94°C (30 s) denaturing, 50°C (5 s) annealing, 60°C (4 min) extension; hold at 15°C. Products were then ethanol precipitated as per Applied Biosystems recommendations. The products were separated by polyacrylamide gel electrophoresis on an ABI Prism 377 DNA automated sequencer (PE Applied Biosystems, Foster City, CA USA). Sequence analysis and editing was completed using GeneTool ver. 1.0 (BioTools Inc. 2000) with manual adjustments where necessary. All new records have been lodged with the WA Plant Pathology Culture Collection at the WA Department of Agriculture.

2.3 RESULTS

2.3.1 *Mycosphaerella* and *Teratosphaeria* spp isolated from WA

From the hybrid trial, *M. nubilosa* was isolated from *E. grandis* x *resinifera* and *E. urophylla* x *globulus* (Table 2.1, Figure 6.3 Chapter 6). *Mycosphaerella marksii* was isolated from *E. grandis* x *camaldulensis*, as well as two endemic eucalypt

species, *E. rudis* from the Nannup area and *E. diversicolor* from the Porongurups (Table 2.1, Figure 6.3 Chapter 6). It was not isolated in combination with any other *Mycosphaerella* or *Teratosphaeria* species. *Teratosphaeria parva* was isolated from *E. globulus* x *urophylla* and was the only species isolated from those lesions. Unlike previous descriptions (Park and Keane 1982; Crous 1998; Maxwell *et al.* 2003) several isolates of *T. parva* from *E. grandis* x *camaldulensis* formed a red-brown pigment on 2% MEA after growth at 20°C for 2 months in the dark. This has not been recorded previously for *T. parva.*

Mycosphaerella/ *Teratosphaeria* lesions on each eucalypt were amphigenous and irregular. Often on *E. grandis* x *resinifera*, *E. globulus* x *urophylla, E. globulus* x *grandis* and *E. grandis* x *camaldulensis* distinct red margins were observed, however, this host response was not species specific (Figures 2.2–2.6). Lesions on these hybrids occurred at a moderately high frequency and juvenile leaves were often blighted. Lesions on *E. diversicolor* and *E. rudis* were small, discrete and occurred at low frequency compared to the other eucalypt species surveyed.

Pseudothecia of each *Mycosphaerella* and *Teratosphaeria* species were amphigenous although predominantly epiphyllous for *M. marksii* and hypophyllous for *T. parva* and *M. nubilosa*. Identification was confirmed using species-specific primers. The primers positively amplified products from DNA extracts of their target species. They did not amplify DNA from 15 closely related *Mycosphaerella* species associated with eucalypts, or from non-infected *E. globulus* leaves (Maxwell *et al*. 2005). No cultures were obtained from *E. grandis x globulus* even though lesions were present.

2.3.2 *Mycosphaerella* spp. isolated from Queensland

Mycosphaerella marksii was isolated from *E. globulus*, *E. tereticornis*, *E. dunnii* and *E. pellita* (Table 2.1, Figure 6.3 Chapter 6). *Teratosphaeria parva* was isolated from *E. dunnii*. With the exception of the *E. globulus* record, this extends the host range of *M. marksii* and *T. parva*. Both species are new records for Queensland. Cultural morphology, ascospore germination, as well as ascospore and ascus dimensions were recorded and were consistent with Crous (1998). Lesions on each eucalypt species were amphigenous and irregular. Pseudothecia of *M. marksii* were amphigenous, although they were predominantly epiphyllous on each *Eucalyptus* species from which they were isolated from. Pseudothecia for *T. parva* were hypophyllous. Identification was confirmed as described in section 2.3.1.

Table 2.1 *Mycosphaerella* and *Teratosphaeria* isolates for which ITS rDNA was sequenced and/or tested using species-specific primers. Table first published as Table 1 in Maxwell *et al.* (2005) and amended by the current author.

Fungal species	**Isolate no. (WAC)[a]**	**Host**	**Origin[b]**	**Isolates sequenced (S) and DNA primers tested (T) against[e]**
M. marksii[c]	11436	*E. grandis* x *camaldulensis*	WA, Albany	T
M. marksii[c]	11442	*E. diversicolor*	WA, Porongurups	S, T
M. marksii[c]	11444	*E. rudis*	WA, Nannup	T
M. nubilosa[c]	11445	*E. grandis* x *resinifera*	WA, Albany	T
M. nubilosa[c]	11446	*E. globulus* x *urophylla*	WA, Albany	T
T. parva[c]	12406	*E. grandis* x *camaldulensis*	WA, Albany	S, T
T. parva[c]	12415	*E. globulus* x *urophylla*	WA, Albany	S, T
M. marksii[d]	11438	*E. pellita*	FNQ	T
M. marksii[d]	11440	*E. dunnii*	SEQ	S, T
M. marksii[d]	11443	*E. tereticornis*	FNQ	T
M. marksii[d]	11437	*E. globulus*	SEQ	T
T. parva[d]	11435	*E. dunnii*	Qld	T

[a]WAC, Western Australian Department of Agriculture Plant Pathogen Collection; [b]Origin of isolates: Western Australia (WA), South-east Queensland (SEQ), Far North Queensland (FNQ); [c]New host record; [d]New state record; [e]See Figure 6.3, Chapter 6.

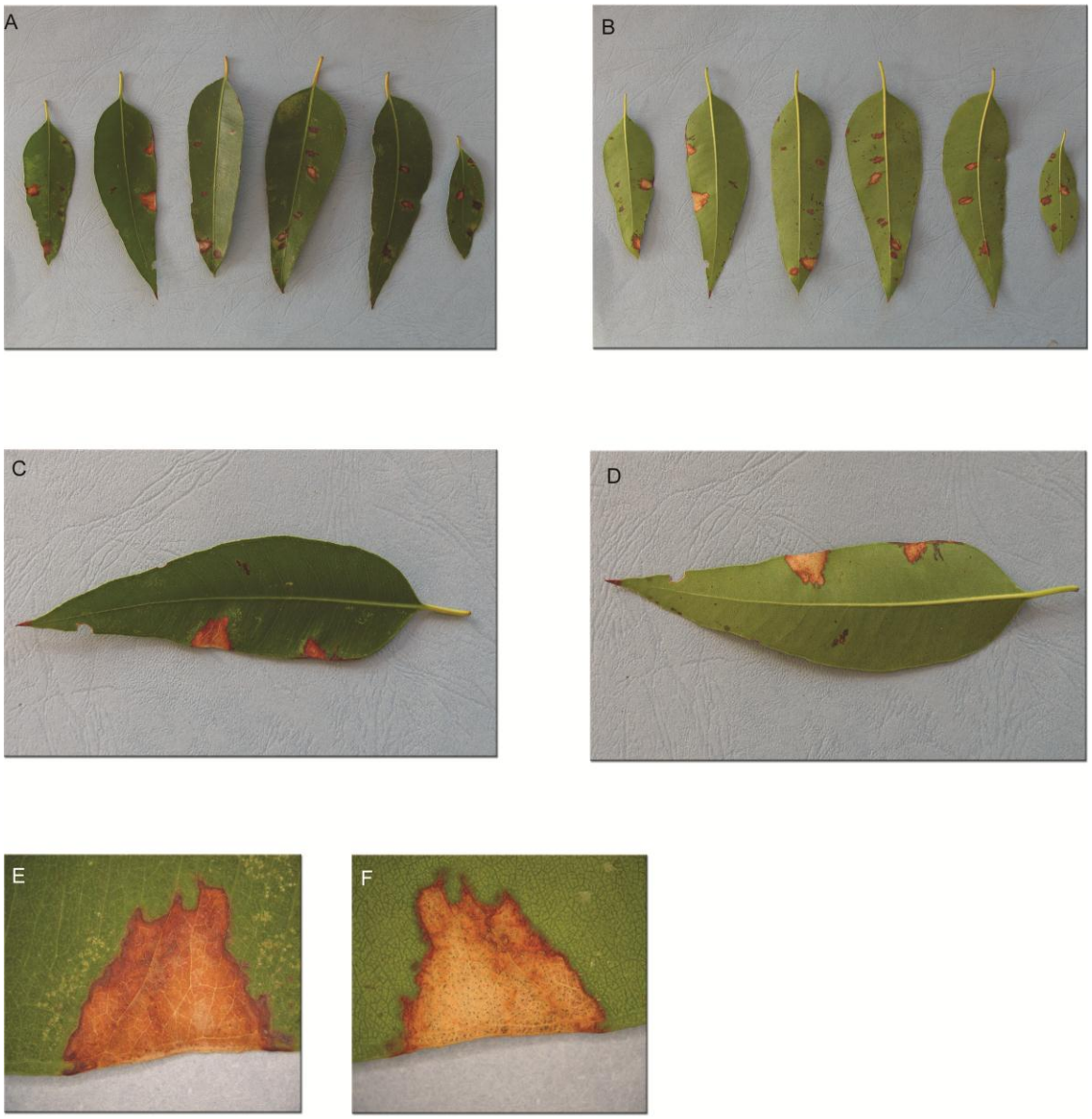

Figure 2.2 Adult leaves, adaxial (**A**, **C**, **E**) and abaxial (**B**, **D**, **F**), of *Eucalyptus grandis* x *resinifera* from Albany, Western Australia with lesions caused by Mycosphaerella leaf disease associated species.

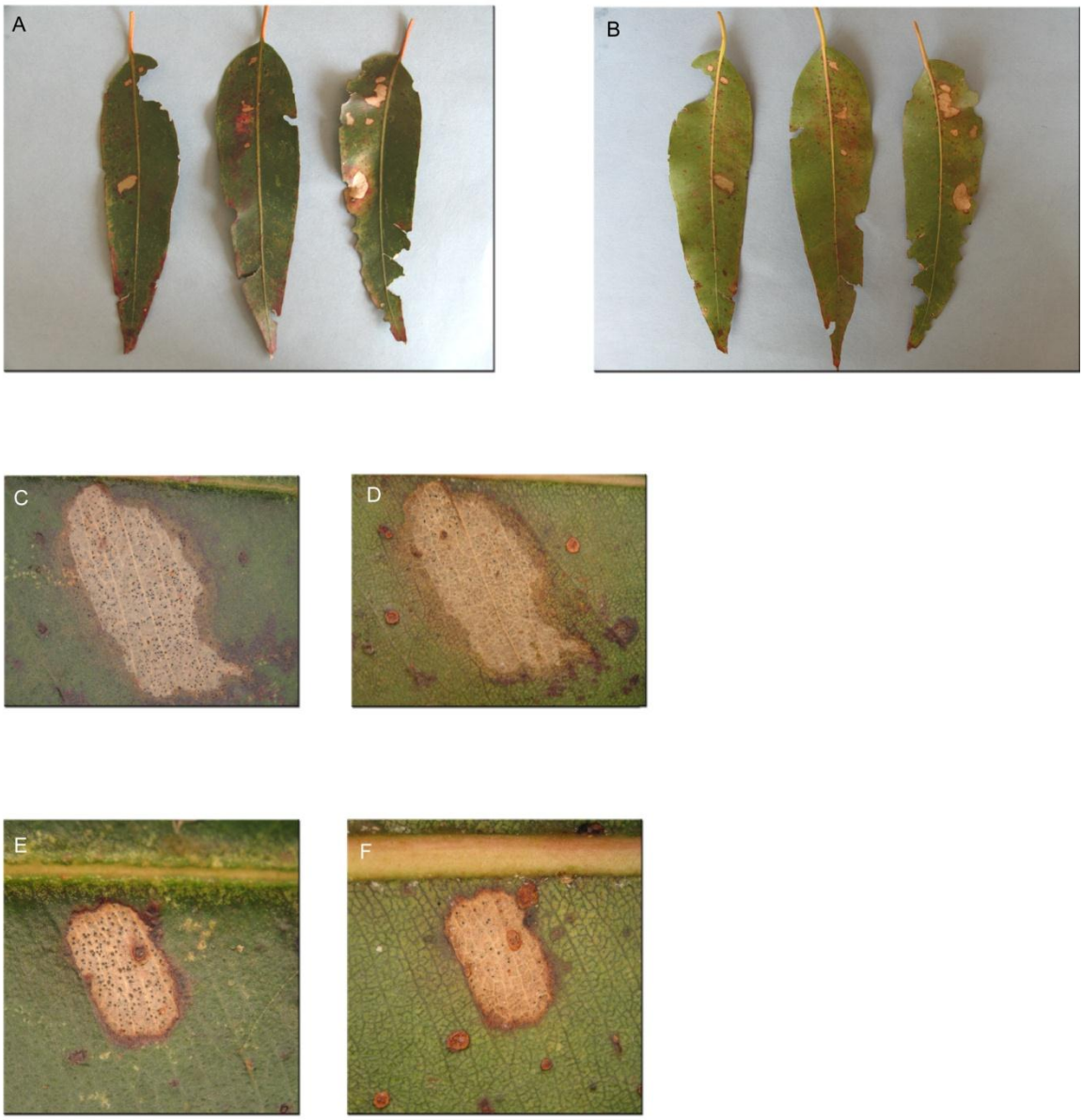

Figure 2.3 Adult leaves, adaxial (**A**, **C**, **E**) and abaxial (**B**, **D**, **F**), of *Eucalyptus grandis* x *resinifera* from Albany, Western Australia with lesions caused by Mycosphaerella leaf disease associated species.

Figure 2.4 Juvenile and intermediate leaves, adaxial (**A**, **C**, **E**) and abaxial (**B**, **D**, **F**), of *Eucalyptus globulus* x *urophylla* from Albany, Western Australia with lesions caused by Mycosphaerella leaf disease associated species.

Figure 2.5 Adult leaves, adaxial (**A**, **C**, **E, G**) and abaxial (**B**, **D**, **F, H**), of *Eucalyptus globulus* x *urophylla* from Albany, Western Australia with lesions caused by Mycosphaerella leaf disease associated species. Lesions on these leaves had a notable red margin around the lesions (**E**, **F**, **G**, **H**).

Figure 2.6 Adult leaves, adaxial (**A**, **C**) and abaxial (**B**, **D**), of *Eucalyptus globulus* x *grandis* from Albany, Western Australia with lesions caused by Mycosphaerella leaf disease associated species. Lesions on these leaves had a notable red margin around the lesions on the abaxial surface (**E**).

2.4 DISCUSSION

This study has identified ten new host records of *Mycosphaerella*/ *Teratosphaeria* species from *Eucalyptus* in WA (Table 2.1). In Queensland, five new host records were described and the range of three species in Queensland has been extended (Table 2.1).

The presence of such a range of *Mycosphaerella*/ *Teratosphaeria* species on eucalypt hybrids is surprising, given that many of these eucalypt species do not occur in large plantation areas, just in small genetic trial blocks. The occurrence of *M. gregaria* and *M. cryptica* on *E. grandis* x *resinifera* in Esperance extends the geographic range for these species that were not isolated on *E. globulus* by Maxwell (2004). More sampling of *E. globulus* is required in this region to appreciate the full suite of *Mycosphaerella*/ *Teratosphaeria* species present in order to identify which species are native and those that have been introduced.

The discovery of *M. nubilosa* on *E. grandis* x *resinifera* and *E. urophylla* x *globulus* is significant. This is the first time *M. nubilosa* has been isolated from hosts within the series Resiniferae, which includes *E. urophylla*, *E. resinifera* and *E. pellita*. The occurrence of *M. nubilosa* on these hosts could reflect an increased susceptibility due to the hybridisation of these otherwise resistant hosts, or it may be due to the planting of these species in areas with high inoculum levels from nearby *E. globulus* plantations. *Mycosphaerella nubilosa* was the most frequently isolated *Mycosphaerella*/ *Teratosphaeria* species from *E. globulus* in WA according to Maxwell *et al.* (2003). Therefore, nearby *E. globulus* plantations are likely to have been a source of abundant inoculum of *M. nubilosa* to infect these hybrids.

During the present study, *M. marksii* was commonly isolated species from one of the hybrid trials in Albany. This species is generally regarded as a minor pathogen (Park *et al.* 2000). However, the frequency of isolations and the wide host range indicated that it could possibly become more of a threat in the future. Further investigations into disease development would demonstrate whether or not this organism is a primary pathogen. This work also needs to be repeated for *T. parva*. Park and Keane (1987) were unable to induce lesions during pathogenicity trials on *E. globulus*.

The extended plantings of hybrid eucalypts in WA raises several important key issues including quarantine regulations. As more exotic eucalypt species and their hybrids are being planted in the south-west of WA, it remains unknown what the effect of potential pathogens will be on these. Conversely, it is unknown what affects the present plant pathogens will have on these new hybrids being evaluated in the region in research trials. It is important that clonal resistance to MLD be understood prior to genetics being selected for large scale plantings. Therefore, monitoring for emerging pathogens and their impact is essential for the long-term success of the hybrid eucalypt plantation industry.

The first survey to accurately identify *Mycosphaerella*/ *Teratosphaeria* as the causal agents of leaf blight in WA found three species associated with *E. globulus*, namely *M. marksii*, *M. cryptica* and *M. suberosa* (Carnegie *et al.* 1997). They also isolated *M. cryptica* from *E. diversicolor* and *E. marginata*, two important endemic species to WA. Following this survey, Maxwell *et al.* (2003) collected diseased leaves from thirty plantations across the south-west of WA. That study described two new species and extended the geographic range of five

other species. In total ten *Mycosphaerella*/ *Teratosphaeria* species were isolated (Maxwell *et al.* 2003). Maxwell (2004) also sampled *E. diversicolor*, *E. marginata*, *E. jacksonii* and *Corymbia calophylla* (=*E. calophylla*) for MLD. Maxwell (2004) found MLD at all sites of *E. globulus* and *E. diversicolor* and 80% of the *E. marginata* sites sampled. No MLD was recorded from *C. calophylla*. *Mycosphaerella cryptica* was the only species isolated from *E. diversicolor*, *E. marginata* and *E. jacksonii* (Maxwell 2004). In the current study, only a small number of plantations were surveyed, and an increased number of *Mycosphaerella*/ *Teratosphaeria* species were isolated extending both the host range and geographical area of those species. It is therefore important that continuous monitoring of the plantation estate and, in particular genetic trials, be continued in this region.

The next chapter will explore in more detail *Mycosphaerella*/ *Teratosphaeria* species in *E. globulus* in south-western Australia, the geographic region which is the main focus for this thesis.

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CHAPTER 3

INCIDENCE AND NEW RECORDS OF *MYCOSPHAERELLA* SPECIES ASSOCIATED WITH *EUCALYPTUS GLOBULUS* PLANTATIONS IN WESTERN AUSTRALIA

3.1 INTRODUCTION

The predominant hardwood plantation species in WA is *Eucalyptus globulus*, a native to south-eastern Australia (Chapter 1). Eastern Australia is separated from Western Australia by deserts, which have been effective barriers to natural gene flow in flora and fauna and associated pests and pathogens since the early Tertiary period (30–40 million years) (Boland *et al*. 1984). There is a high degree of endemism in the flora of WA and few species are sympatric with eastern Australia (Burbidge 1960). Thus, *E. globulus* can be considered an exotic species in WA. Furthermore, many of the plantations on ex-agricultural land in WA are adjacent to remnant vegetation with native eucalypts such as *E. marginata* and *E. diversicolor*. The health of these remnants has generally suffered under agricultural regimes, partly in response to the alteration of hydrologic and nutrient cycles in the past (Grierson and Adams 1999). The movement of pathogens, and the diseases they cause, into these remnants from plantations could contribute further to their decline (Burgess *et al.* 2006b).

The most serious foliar disease of eucalypt plantations in WA is Mycosphaerella leaf disease (MLD) (Maxwell *et al*. 2003). Since the commencement of the plantation industry, several fungal species contributing to MLD, previously known only in eastern Australia or overseas, have been reported on *E. globulus* in WA (See Figure 1.1A for the chronology). Carnegie *et al*. (1997) identified *Mycosphaerella cryptica* from *E. diversicolor*, *E. marginata* and *E. globulus* and *M. marksii* and *M. suberosa* from *E. globulus*. Abbott (1993) had previously noted the occurrence of a *Mycosphaerella* species from native eucalypt forest in WA but did not identify the species. Later, Maxwell *et al*. (2000; 2001) reported *M. nubilosa* and *Uwebraunia dekkeri* (as *M. lateralis*) from *E. globulus*. Two years

later, Maxwell *et al*. (2003) identified five new records for WA, including one previously unidentified species (*M. aurantia*), two species previously known from eastern Australia (*M. gregaria* and *Teratosphaeria parva*) and two new records for Australia, *M. molleriana* (as *M. ambiphylla)* and *T. mexicana*. *Mycosphaerella ambiphylla* has since been synonymised with *M. molleriana* (Hunter *et al*. 2006), but is still a first record for Australia. Currently, ten species of *Mycosphaerella* and *Teratosphaeria* have been recorded in WA from *Eucalyptus* (Carnegie *et al*. 1997, Maxwell *et al*. 2000; 2001; 2003; Figure 3.1).

Many *Mycosphaerella* and *Teratosphaeria* species affecting *Eucalyptus* worldwide have yet to be recorded in Australia (Maxwell 2004; Hunter *et al*. 2004; Crous *et al*. 2006). The centre of origin of *Eucalyptus* is Australia and Papua New Guinea, and it has been hypothesised by Crous *et al*. (2006) and others (Whyte *et al*. 2005) that most *Mycosphaerella* and *Teratosphaeria* species associated with eucalypts in exotic environments will in time, be found in Australia. Recently *M. heimii*, previously found only in Indonesia and Madagascar (Bouriquet 1946; Crous 1998), was isolated from *E. dunnii* in Queensland (Whyte *et al*. 2005). An alternative hypothesis is that some of these associations may be ‘new encounter’ pathogens that have evolved on non-eucalypt hosts outside Australia (Park *et al*. 2000; Crous *et al*. 2006).

The recent establishment of the first *E. globulus* genetics trials in the region provides an opportunity to assess the number of fungi that may be contributing to MLD and to evaluate changes in species and their incidence since the first surveys were conducted by Carnegie *et al*. (1997).

The aim of this chapter was to assess the number of fungi that may be contributing to MLD in *E. globulus* plantations in WA and to evaluate changes in species and their incidence since the first surveys were conducted.

3.2 METHODS

3.2.1 Sampling, isolation and identification of *Mycosphaerella* and *Teratosphaeria* species

In October 2003, a two-year-old genetics trial in Albany, WA, consisting of 60 full-sib *E. globulus* families from multiple provenance heritages, mainly from Portugal, Flinders Is (near Tasmania) or Jeeralang (Victoria), were surveyed for the presence of MLD. In this study, presence was defined as successful isolation of mature ascospores and colony growth in culture. Juvenile and where possible, adult foliage were collected from one to five trees per family. Up to four leaves with lesions and pseudothecia from each sample were selected for isolation of *Mycosphaerella* and *Teratosphaeria* species.

The isolation and initial identification of *Mycosphaerella* and *Teratosphaeria* species were carried out according to Crous (1998) and Maxwell *et al*. (2003), as described in Chapter 2.

Direct isolations were also made from the mitosporic stage of *Mycosphaerella* and *Teratosphaeria* species. These were done by gently teasing conidia from the leaf with a needle and transferring them to 2% MEA plates. After 24 h, germinating spores were transferred again to fresh 2% MEA plates. Leaf sections of diseased lesions were made of the asexual fruiting structures by hand using a razor blade and mounted on a microscope slide in lactoglycerol or aniline blue

(0.5%, w/v) in lactoglycerol. These were examined at x400 magnification using an Olympus BH-2 microscope.

Mycosphaerella and *Teratosphaeria* species were identified by comparing features of the fungi identified with published keys and descriptions (Crous 1998; Maxwell *et al*. 2005). In addition, culture and herbarium material from species previously recorded in Australia were examined. Of particular relevance was isotype material for *M. gregaria* recorded in Victoria (DAR 72368), and culture and herbarium material of *M. gregaria* (CBS110501, WAC10152, WAC10154, WAC10155) recorded in Western Australia. Morphological evidence for assignment of taxa was supported by molecular evidence.

3.2.2 DNA isolation, amplification and sequencing

Molecular identification was carried out according to Maxwell (2004). Briefly, the ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified using the primer pair ITS1-F (Gardes and Bruns, 1993) and ITS4 (White *et al*. 1990). For selected isolates that were recalcitrant to PCR, nested PCR reactions were performed to amplify the ITS1 and ITS2 regions in separate reactions. For nested reactions 1 µl of 10^{-1} and 10^{-2} dilutions of PCR product from the primary reaction were used as templates. The ITS1 region was amplified with primer pair ITS1 and ITS2 (White *et al*. 1990) and the ITS2 region was amplified with primer pair ITS3 and ITS4 (White *et al*. 1990). The PCR reaction volumes, thermocycler parameters and visualisation of PCR products were as described in Maxwell (2004). Gel images were taken using a digital camera (EDAS 120, Kodak Digital Science™) under UV light and viewed using Kodak Digital Science™ ID (v 3.0.2) software.

Cleaned PCR products were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) using the primers listed above. Standard quarter reactions were performed using 2 µl ABI PRISM® BigDye Terminator Ready Reaction Cycle Sequencing Kit mix (3.0 or 3.1), 1.6 pmol primer (3.2 pmol for version 3.1), 80–160 ng PCR product. Sequencing reactions were performed according to the following parameters: initial denaturing step of 96°C for 2 min; then 25 cycles of 94°C (30 s) denaturing, 50°C (5 s) annealing, 60°C (4 min) extension; hold at 15°C. Products were then ethanol precipitated as per Applied Biosystems recommendations. The products were separated by polyacrylamide gel electrophoresis on an ABI Prism 377 DNA automated sequencer (PE Applied Biosystems, Foster City, CA, USA).

3.2.3 Phylogenetic analysis

In order to compare the *Mycosphaerella* and *Teratosphaeria* isolates obtained in this study with other *Mycosphaerella* and *Teratosphaeria* species, ITS rDNA sequences obtained from GenBank, including all *Mycosphaerella* and *Teratosphaeria* spp. previously reported from WA, were used in the phylogenetic analysis (Table 3.1). Sequence data were analysed using Sequence Navigator version 1.0.1™ (PerkinElmer Corp., Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character, all ambiguous characters, and parsimony uninformative characters were excluded prior to analysis. The most parsimonious trees were obtained in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b 10 (Swofford 2003) by using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were

unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis and Huelsenbeck 1992). Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985).

3.3 RESULTS

3.3.1 New *Mycosphaerella* and *Teratosphaeria* species recorded from WA and phylogenetic analysis

Four new records of *Mycosphaerella* species were identified in this study; *M. ellipsoidea*, *Pseudocerospora fori* (=*M. fori*), *M. suttoniae* and *M. tasmaniensis*. *Mycosphaerella ellipsoidea* and *P. fori* are first records for Australia, and *M. suttoniae* and *M. tasmaniensis* are first records for WA.

Isolates isolated in WA and previously described as *M. gregaria* (WAC 10155, WAC 10152, AY509756) were re-examined and found to be morphologically identical to the type description of *M. ellipsoidea* in terms of cultural characteristics on MEA. In addition, images of *M. ellipsoidea* from South Africa (Hunter 2002) were examined and cultural morphology of these was identical to that observed for those same isolates from WA. Australian quarantine laws prohibit the import of exotic plant pathogens therefore it was not possible at the time to make a direct comparison in the laboratory with *M. ellipsoidea* isolates from South Africa. The cultural morphology of an isotype of *M. gregaria* (DAR 72368) from eastern Australia differed in some key respects to isolates from WA previously recorded as *M. gregaria* (Table 3.2). They differed in pigmentation and in texture on MEA after two months growth.

Table 3.1 *Mycosphaerella* and *Teratosphaeria* species and isolates considered in the phylogenetic study with new records in bold.

Culture accession no.[a]	Teleomorph	Anamorph	Host	Location	Collector	GenBank accession no.
STE-U 794	*M. africana*	Unknown	*Eucalyptus* sp.	South Africa	PW Crous	AF173314
CBS 110500	*M. aurantia*	Unknown	*E. globulus*	Western Australia	A Maxwell	AF150331
	M. aurantia	Unknown	*E. globulus*	Western Australia	SL Jackson	AF509742
	M. cryptica	*Colletogloeopsis nubilosum*	*E. globulus*	Western Australia	A Maxwell	AY509753
	M. cryptica	*C. nubilosum*	*E. globulus*	Western Australia	A Maxwell	AY509754
CMW 9099	*M. ellipsoidea*	*Uwebraunia ellipsoidea*	*Eucalyptus* sp.	South Africa	G Hunter	AF468875
WAC 10155	***M. ellipsoidea***	***U. ellipsoidea***	***E. globulus***	**Western Australia**	**A Maxwell**	**AY509755**
	M. ellipsoidea	*U. ellipsoidea*	*E. globulus*	Western Australia	A Maxwell	AY509756
WAC 10152	*M. ellipsoidea*	*U. ellipsoidea*	*E. globulus*	Western Australia	A Maxwell	AY509757
STE-U 1225	*M. ellipsoidea*	*U. ellipsoidea*	*Eucalyptus* sp.	South Africa	MJ Wingfield	AF173303
CMW 9100	*M. ellipsoidea*	*U. ellipsoidea*	*Eucalyptus* sp.	South Africa	G Hunter	AF468876
CMW 9098	*M. ellipsoidea*	*U. ellipsoidea*	*Eucalyptus* sp.	South Africa	G Hunter	AF468874
CMW 9095	*M. fori*	*Pseudocercospora fori*	*Eucalyptus* sp.	South Africa	G Hunter	AF468869
CMW 9094	*M. fori*	*P. fori*	*Eucalyptus* sp.	South Africa	G Hunter	AF468868
WAC 12414	***M. fori***	***P. fori***	***E. globulus***	**Western Australia**	**SL Jackson**	**DQ787325**
STE-U 1084	*M. keniensis*	Unknown	*Eucalyptus* sp.	Kenya	MJ Wingfield	AF173300
WAC 12265	*M. marksii*	Unknown	*E. globulus*	Western Australia	A Maxwell	AF509765
	M. marksii	Unknown	*E. globulus*	Western Australia	A Maxwell	AF509766
CMW 4940	*M. molleriana*	*Colletogloeopsis molleriana*	*Eucalyptus* sp.	Portugal	MJ Wingfield	DQ239969
CBS 110499	*M. molleriana*	*C. molleriana*	*E. globulus*	Western Australia	A Maxwell	AY150675
CMW 6210	*M. molleriana*	*C. molleriana*	*E. globulus*	NSW	MJ Wingfield	AF449095
	M. molleriana	*C. molleriana*	*E. globulus*	Western Australia	A Maxwell	AY553586

Culture accession no.[a]	Teleomorph	Anamorph	Host	Location	Collector	GenBank accession no.
	M. nubilosa	Unknown	*E. globulus*	Western Australia	A Maxwell	AY509777
	M. nubilosa	Unknown	*E. globulus*	Western Australia	A Maxwell	AY509778
CBS 113265	*M. punctiformis*	*Ramularia endophylla*	*Quercus robor*	Netherlands		AY490763
	M. suberosa	Unknown			A Milgate	AY045503
CBS 436.92	*M. suberosa*	Unknown	*E. dunnii*	Brazil	MJ Wingfield	AY626985
CPC 515	*M. suberosa*	Unknown				AY725579
	M. suberosa	Unknown	*E. globulus*	Western Australia	SL Jackson	DQ787327
MUCC 424	*M. suttoniae*	*Kirramyces epicoccoides*	*E. camaldulensis* × *grandis*	Queensland	G Hardy	DQ632703
MUCC 428	*M. suttoniae*	*K. epicoccoides*	*E. camaldulensis* × *grandis*	Queensland	TI Burgess	DQ632707
WAC 11452	***M. suttoniae***	***K. epicoccoides***	***E. globulus***	**Western Australia**	**SL Jackson**	**DQ787326**
STE-U 1346	*M. suttoniae*	*K. epicoccoides*	*Eucalyptus* sp.	Indonesia		AF173306
MUCC 426	*M. suttoniae*	*K. epicoccoides*	*E. globulus*	Western Australia	SL Jackson	DQ632704
	M. suttoniae	*K. epicoccoides*		Queensland	A Milgate	AF045519
CMW 5348	*M. suttoniae*	*K. epicoccoides*	*Eucalyptus* sp.	Indonesia	MJ Wingfield	AF309621
	M. tasmaniensis	*Passalora tasmaniensis*				AY045515
STEU 1555	*M. tasmaniensis*	*Pa. tasmaniensis*	*E. nitens*	Tasmania	MJ Wingfield	AY667578
	M. tasmaniensis	*Pa. tasmaniensis*				AY045511
WAC 11451	***M. tasmaniensis***	***Pa. tasmaniensis***	***E. globulus***	**Western Australia**	**SL Jackson**	**DQ784689**
	M. tasmaniensis	*Pa. tasmaniensis*	*E. globulus*	Victoria	PA Barber	AY534228
STE-U 1458	unknown	*P. paraguayensis*	*Eucalyptus* sp.	Brazil	MJ Wingfield	AF309596
STE-U 1266	unknown	*P. basiramifera*	*Eucalyptus* sp.	Thailand	MJ Wingfield	AF309595
	T. parva	Unknown	*E. globulus*	Western Australia	A Maxwell	AF509779
CBS 110503	*T. parva*	Unknown	*E. globulus*	Western Australia	A Maxwell	AF509782

Culture accession no.[a]	Teleomorph	Anamorph	Host	Location	Collector	GenBank accession no.
	T. mexicana	Unknown	*E. globulus*	Western Australia	SL Jackson	AY509771
WAC 10163	*T. mexicana*	Unknown	*E. globulus*	Western Australia	A Maxwell	AY509769
	M. lateralis/ unkown	*U. dekkeri*	*E. maidenii*	Queensland	A Maxwell	AY509758
	M. lateralis/ unkown	*U. dekkeri*	*E. globulus*	Western Australia	A Maxwell	AY509762
CMW 7773	*Neofusicoccum ribis*		*Ribes* sp.	New York, USA	B Slippers	AY236936

[a] Designation of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; STE-U = Stellenbosch University, South Africa; WAC = Plant Pathogen Collection Western Australian Department of Agriculture; MUCC, Murdoch University Culture Collection.

Table 3.2 Comparison of culture morphology, asci and ascospore dimensions of *Mycosphaerella gregaria* and *M. ellipsoidea.*

Species	Asci (µm)	Ascospores (µm)	Description of culture from current study on 2% MEA
M. ellipsoidea	30–45 × 6–8	(8–) 10–11 × (2–) 2.5–3	Aerial mycelia profuse, white with pink patches, reverse olivaceous-black[a]
M. gregaria (DAR 72368)	37.5–47.5 × 6.5–8.5	10.5–15.5 × 2.5–3.5	Pale olivaceous-grey with apricot hue, dark grey reverse[b]
M. gregaria (CBS110501)	28–32 × 5.5–7	9.5–11 × 2–2.5	Aerial mycelia profuse, white with pink patches, reverse olivaceous-black[c]

[a]Crous and Wingfield (1996); Hunter (2002); [b]Carnegie and Keane (1997); [c]Maxwell *et al.* (2003)

Although a comparison of ascus and ascospore dimensions (Table 3.2) showed considerable overlap between *M. gregaria* and *M. ellipsoidea*, the isolates previously described as *M. gregaria* in WA were in the smaller size range and therefore more similar to the type of *M. ellipsoidea* than to the type of *M. gregaria.* In addition to this, molecular phylogeny (Figure 3.1) placed all isolates of *M. gregaria* recorded in WA in the same clade as *M. ellipsoidea*. Therefore, it is concluded that the previous records for *M. gregaria* in WA were incorrect and that the taxon recorded here is actually *M. ellipsoidea*. The *Uwebraunia* anamorph associated with *M. ellipsoidea* was not observed in the current study.

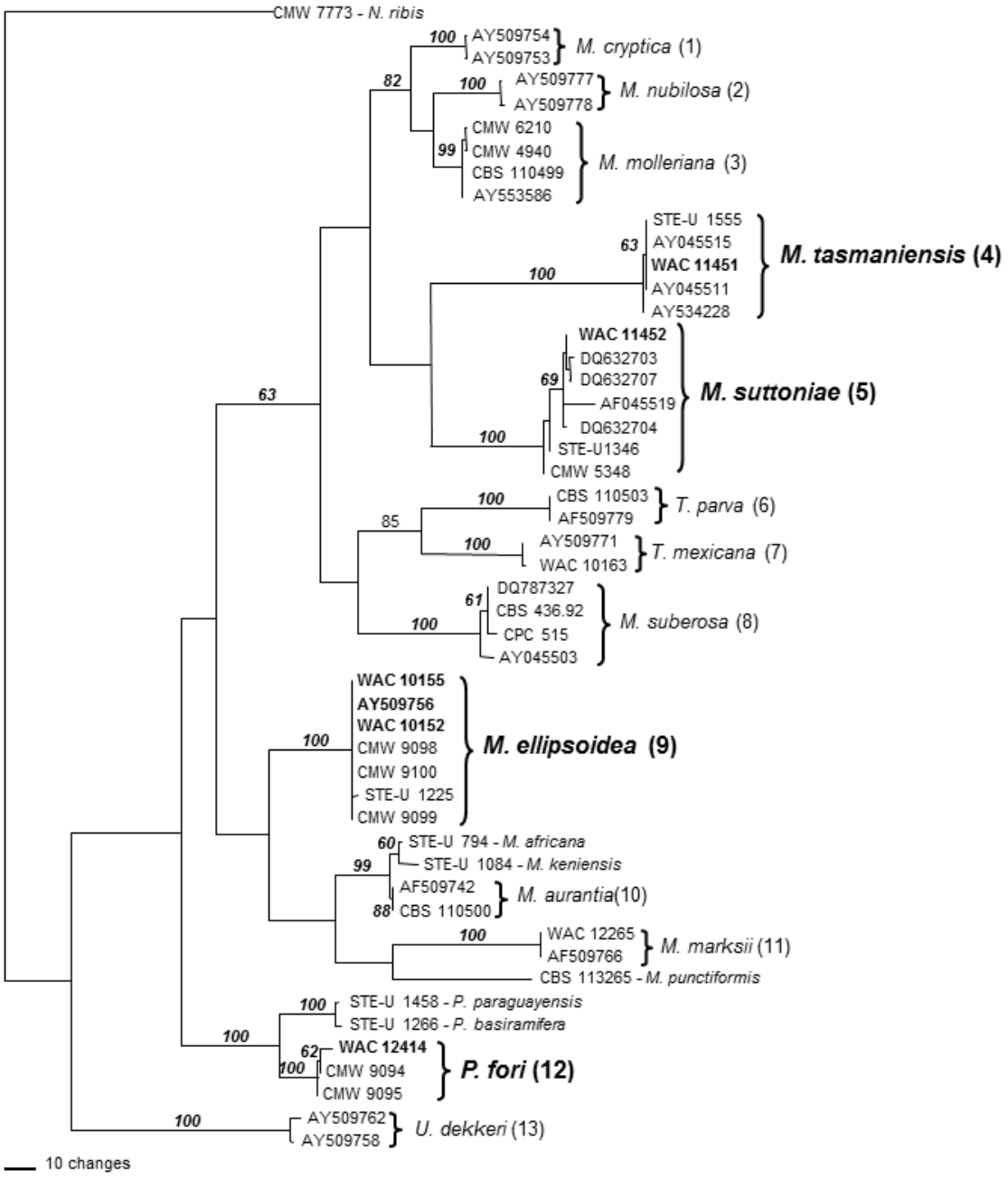

Figure 3.1 A phylogram of one of the nine most-parsimonious trees obtained from the ITS sequence data of Mycosphaerella leaf disease associated species. Bootstrap values are given above the branch. The tree is rooted to *Neofusicoccum ribis.* Numbers in parenthesis represent the species currently reported in Western Australia. Isolates from Western Australia are in bold.

Pseudocercospora fori was isolated from *E. globulus* and this is the first report of this species in Australia. The asexual stage of *M. suttoniae* (*Kirramyces epicoccoides*) and *M. tasmaniensis* (*Passalora tasmaniensis*) were isolated from *E. globulus*, being the first report of these species in WA.

Morphological characteristics and ITS sequence data for these three species match published descriptions (Crous 1998; Hunter *et al*. 2004 and Barber *et al*. 2005). No conidia of *P. fori* were observed on 2% MEA or water agar. However, anamorphs of *M. suttoniae* and *M. tasmaniensis* were observed on both water agar and 2% MEA after two months incubation at 20°C in the dark.

A phylogenetic tree was constructed including species identified in the current study and other known *Mycosphaerella* and *Teratosphaeria* species from *Eucalyptus* in WA (Maxwell *et al*. 2003; 2005) and where required, the most closely related species based on blast searches on GenBank. The aligned data set consisted of 907 characters of which two large indels from *M. ellipsoidea* (WA population; WAC 10155, WAC 10152, AY509756) and *M. suttoniae* (WA population; WAC11452, DQ632704) respectively, were excluded leaving 531 characters of which 248 were parsimony informative. The data set contained significant (P <0.001, g1=−0.61) phylogenetic signal compared to 1000 random trees. Initial heuristic searches in PAUP resulted in 9 most parsimonious trees of 911 steps (CI=0.58, RI=0.86). The resultant tree separated the 13 *Mycosphaerella* and *Teratosphaeria* species from WA into well-supported terminal clades (Figure 3.1, TreeBASE=SN 2945). The identification of the four new records were confirmed as *M. ellipsoidea* (WAC 10155,

WAC 10152, AY509756), *P. fori* (WAC 14288), *M. suttoniae* (WAC 11452, DQ632704) and *M. tasmaniensis* (WAC 11451). The isolates of *M. ellipsoidea* from WA fell into a strongly supported terminal clade with the sequences of *M. ellipsoidea* from South Africa (Figure 3.1). The type sequence was not available for comparison on GenBank. Two isolates of *M. suttoniae* from WA were sequenced and WAC 11452 contained a large 200 bp indel as did two isolates from Queensland (DQ632703 and AF045519). However, other isolates of *M. suttoniae* from WA (DQ632704) and Queensland (DQ632707) did not contain the indel.

3.3.2 Disease incidence of *Mycosphaerella* and *Teratosphaeria* species in a genetic trial near Albany, WA

Eight species of the sexual stage of *Mycosphaerella or Teratosphaeria* species were identified from the *E. globulus* family trial in Albany. Several species were frequently isolated from the same leaves and/ or lesions. The most frequently isolated species from juvenile foliage was *M. marksii* (77% from leaves sampled) followed by *M. nubilosa* (33%), *M. ellipsoidea* (11%) and *T. parva* (7.7%). Also, *M. aurantia* was isolated from both juvenile (1.1%) and adult (0.8%) foliage. *M. nubilosa* was most frequently isolated from adult leaves (88%) followed by *T. parva* (7.5%), *M. molleriana* (3.3%), *U. dekkeri* (0.8%) and *M. cryptica* (0.8%). Three species, *M. molleriana*, *U. dekkeri* and *M. cryptica*, were only isolated from adult leaves while *M. ellipsoidea* was only isolated from juvenile leaves. Data were not collected from the juvenile leaves of four of the 60 families, because the trees were dead, or they lacked diseased leaves. Data were not collected for adult leaves of 20 families, as

the trees had not produced adult foliage at the time of sampling. No specificity was observed for any of the *Mycosphaerella*/ *Teratosphaeria* species on any of the *E. globulus* families and occurrence was evenly distributed across families in the plantation (Figure 3.4).

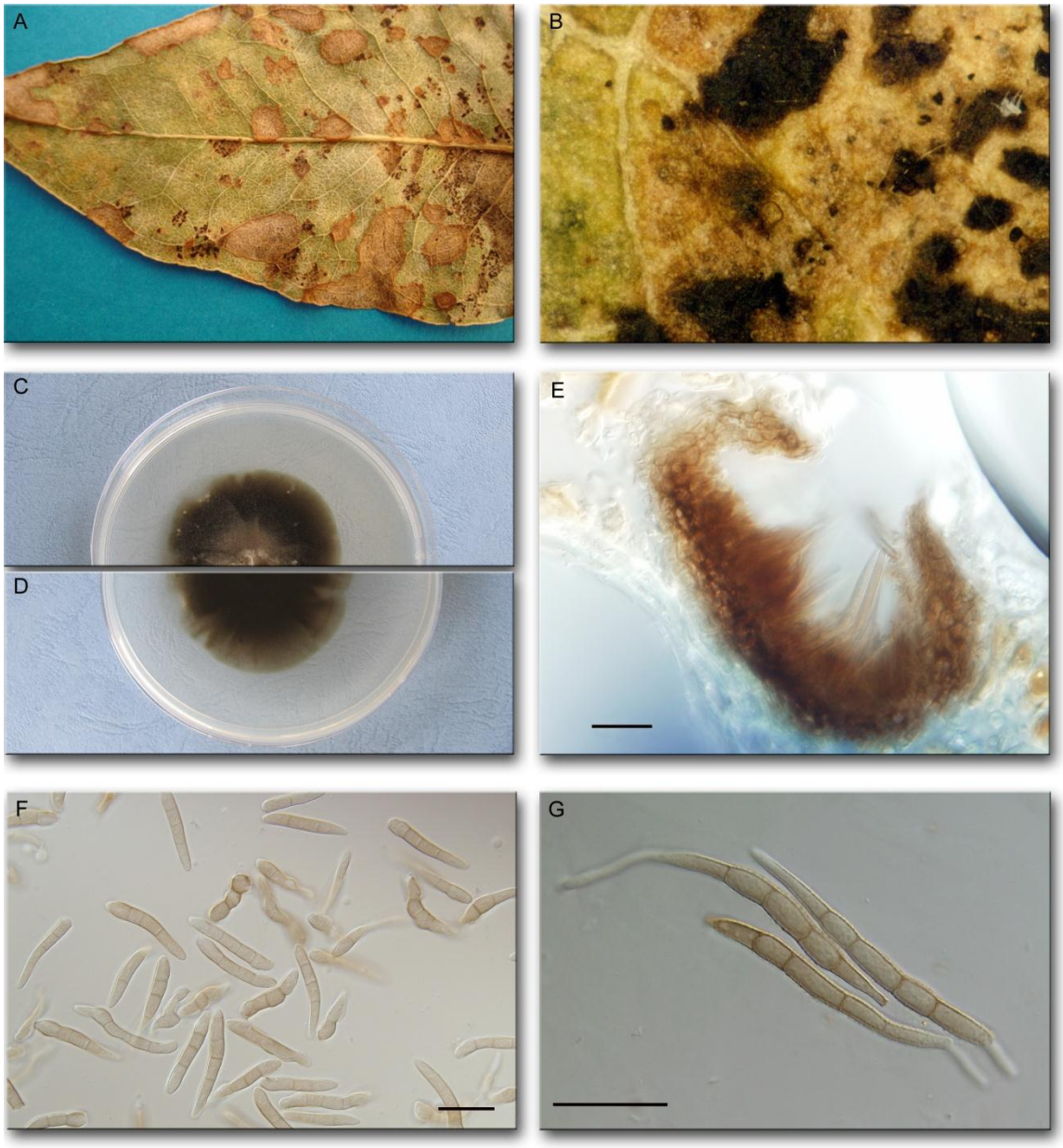

Figure 3.2 Leaf symptoms, conidia and culture of *Mycosphaerella suttoniae* from *Eucalyptus globulus* in Western Australia. Sooty appearance of *M. suttoniae* conidia on the abaxial leaf surface of *E. globulus* (**A** and **B**); Culture of *M. suttoniae* on 2% MEA after 4 months at 20°C, upper (**C**) and lower (**D**). Free-hand section of a pycnidium (**E**); Germinating conidia on 2% Malt Extract Agar (MEA) (**F**) and conidia produced *in vitro* on 2% MEA (**G**). Bars = 20 µm.

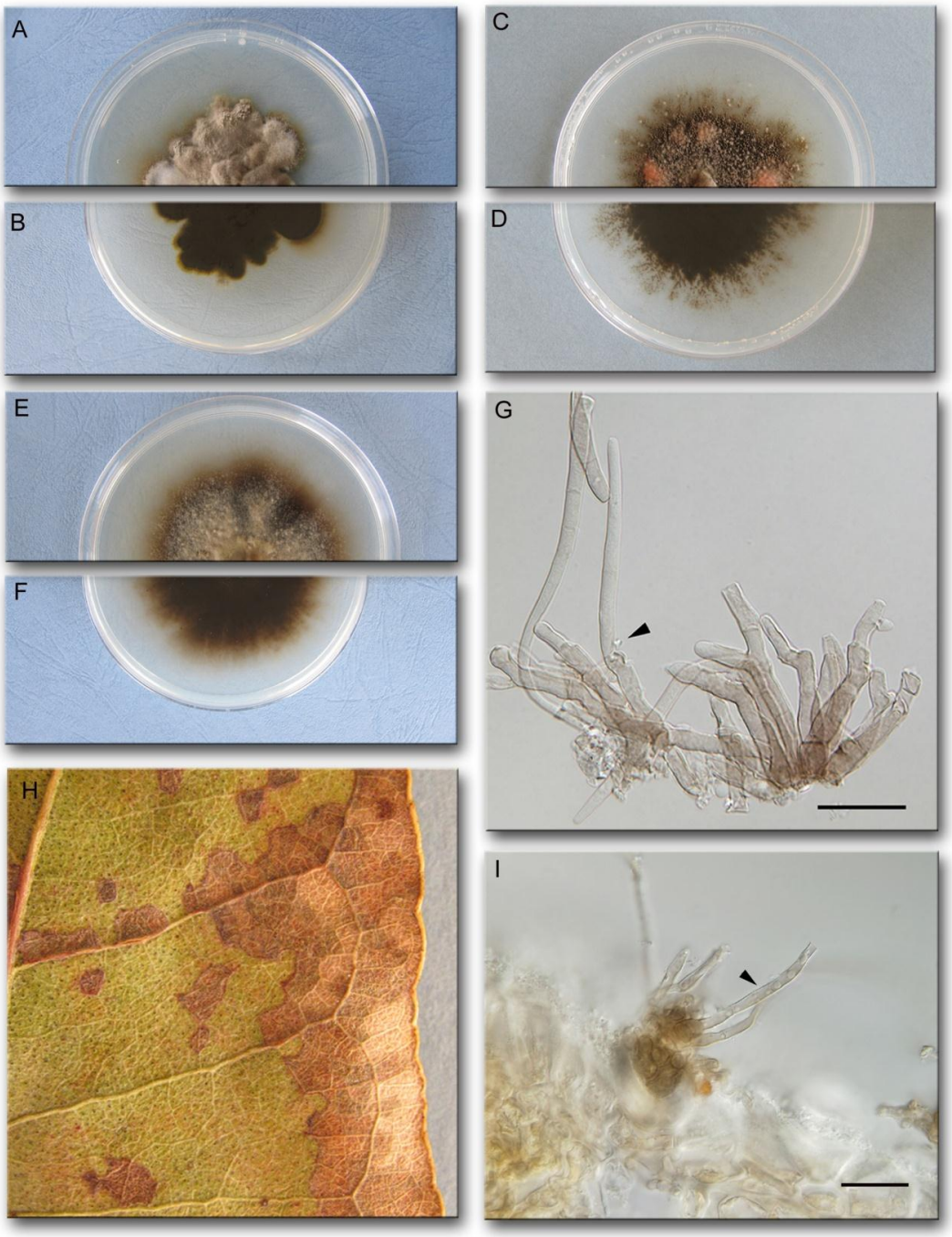

Figure 3.3 New *Mycosphaerella* records from Western Australia and Australia. Culture of *P. fori*, upper (A) and lower (B); *M. ellipsoidea*, upper (C) and lower (D); *M. tasmaniensis* upper (E) and lower (F) on 2% MEA after four months at 20°C; Fascicle of conidiophores of *P. fori* with attached conidium (G, arrow); Leaf symptoms on the abaxial surface of *P. fori* on juvenile *Eucalyptus globulus* leaves (H); Fascicle of conidiophores of *P. fori* (arrow) imbedded in the leaf surface (I). Bars = 20 µm.

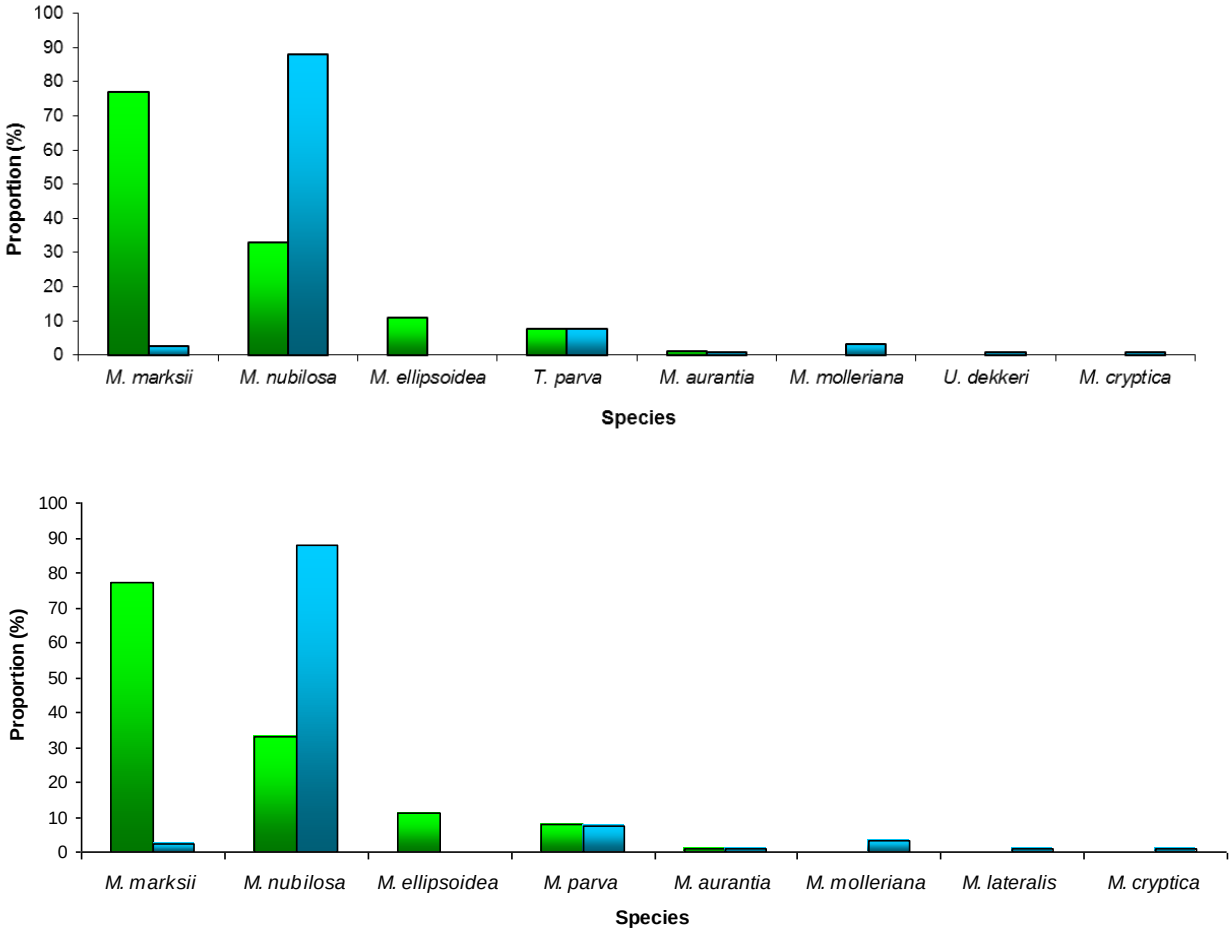

Figure 3.4 Proportion of Mycosphaerella leaf disease associated species isolated from juvenile (green) and adult (blue) leaves from 60 *Eucalyptus globulus* families in a genetic trial in Albany, Western Australia.

3.4 DISCUSSION

The current study documents an increase in the number of *Mycosphaerella* and *Teratosphaeria* species associated with *E. globulus* plantations in WA from 10 in 2003 to 13 in 2008. *P. fori* and *M. ellipsoidea* are new records for Australia, while *M. tasmaniensis* and *M. suttoniae* are new records for WA. Of the present species recorded in WA, 11 were isolated from a single genetics trial in Albany. There are a

number of important implications that arise from these detections including the potential impact on plantations in WA; biosecurity implications of the origin and spread of eucalypt diseases; and the ecological function of the diverse *Mycosphaerella/ Teratosphaeria* assemblage that is associated with *Eucalyptus* forests and plantations.

The current study detected a diverse array of *Mycosphaerella* and *Teratosphaeria* species in one plantation, with a total of 10 *Mycosphaerella* and one *Teratosphaeria* species present. Previous studies in the region have detected much fewer numbers of *Mycosphaerella* and *Teratosphaeria* species associated with eucalypt forests. Three species, *M. marksii*, *M. suberosa* and *M. cryptica* were the first accurately identified species associated with MLD on *E. globulus* in WA (Carnegie *et al*. 1997). A more intensive survey of plantations in WA recorded two new species and extended the recorded geographic range of five other species (Maxwell *et al*. 2003). Neither study found more than four *Mycosphaerella* or *Teratosphaeria* species present at a single plantation. The ecological role of many of these fungi remains to be understood as does the reason for the high diversity at this site.

It is postulated that the large number of *Mycosphaerella/ Teratosphaeria* species detected is due to the genetic diversity of the *E. globulus* families present at the site. There are two competing hypotheses, as to how this host genetic diversity could account for the high level of *Mycosphaerella/ Teratosphaeria* diversity. Firstly, there is the “host-movement” hypothesis where the pathogens have arrived with host material that has been transported from diverse origins to this site. The majority of

the plant material was sourced from Portugal landraces, Flinders Is (off Tasmania) or Jeeralang (Victoria) provenances. Therefore, it is possible the *Mycosphaerella/ Teratosphaeria* species were moved with asymptomatic nursery stock. The second hypothesis to explain the high *Mycosphaerella/ Teratosphaeria* diversity is the “new encounter” hypothesis that they may be moving from native eucalypts onto the exotic plantation species. The diverse host genetics may provide more opportunities for colonisation from a more diverse array of indigenous fungi than the limited genotypes often present in commercial plantations.

The ecological role of the *Mycosphaerella/ Teratosphaeria* species present may be considered in terms of whether they exist as pathogens or as endophytes; and in terms of the niche that they occupy, for example whether they colonise juvenile or adult leaves and whether more than one species can co-occur on the same lesion. The occurrence on adult and juvenile foliage varied amongst the 11 *Mycosphaerella/ Teratosphaeria* species detected in the current study. *Mycosphaerella marksii* was the dominant species on juvenile foliage (77%) and was also a minor coloniser of adult foliage (2.5%). This is in contrast to Maxwell (2004) who found that *M. nubilosa* was the dominant species on juvenile foliage. In the current study, *Mycosphaerella nubilosa* was the most commonly isolated species from diseased adult foliage (88%). By comparison, Maxwell (2004) observed *M. nubilosa* on less than 1% of adult foliage sampled. One explanation for these varying results is that there are changes in the dominance of particular species over time or with season as the leaf cohort ages or as environmental conditions conducive to maturation and sporulation of each *Mycosphaerella* species changes. *M. nubilosa* is an effective early coloniser

of young expanding leaves, as the leaf ages other species may begin to dominate, mature and sporulate. A similar change in the species dominance over time was observed in South African plantations. In the 1990's, *M. juvenis* was the dominant species contributing to outbreaks of MLB (Crous and Wingfield 1996), whilst in 2003, the dominant species was *M. nubilosa* (Hunter *et al*. 2004).

The dominance of *M. nubilosa* on adult foliage is a cause for concern. Previous studies have either not detected *M. nubilosa* on adult foliage or have found it as a minor component of diseased adult foliage. This pathogen causes severe defoliation events in juvenile foliage, which if replicated on adult foliage would devastate susceptible *Eucalyptus* species in forests and plantations. In addition to the change in frequency of isolation there has been an observed increase in the extent of MLD on adult foliage. If the severity of damage to adult leaves continues to increase then breeding for resistance will need to be explored. This is because *E. globulus* develop adult phase leaves by 18 months of age, after which juvenile foliage is of decreasing importance in terms of contributing to growth.

Mycosphaerella marksii has been generally regarded as a minor pathogen (Park *et al*. 2000). However, its frequency of isolation in the present study was much higher than previously observed and it could become more of a threat. Recently, use of a specific primer and/or ITS sequencing (unpublished) showed that it was also isolated from two endemic eucalypt species in WA, *E. diversicolor* and *E. rudis* (Jackson *et al*. 2005a; Maxwell *et al*. 2005) and also from eucalypt species in Queensland, namely *E. dunnii*, *E. pellita* and *E. tereticornis* (Jackson *et al*. 2005b; Maxwell *et al*.

2005). *Mycosphaerella marksii* was one of the most frequently isolated species of *Mycosphaerella* in eucalypt plantations in NSW (Carnegie 2007a), and caused significant foliar damage (Carnegie 2007b). Further investigations into host specificity and disease development would demonstrate whether or not this organism is a primary pathogen.

The emergence of *P. fori* and *M. suttoniae* in WA is of particular concern because they have caused severe defoliation of eucalypt plantations outside of Australia (Crous 1998; Hunter *et al*. 2004; Burgess *et al*. 2006a), and *M. suttoniae* is a serious pathogen of *E. grandis* plantations in eastern Australia (Carnegie 2007a; Carnegie 2007b). The centres of origin of *P. fori* and *M. suttoniae* remain unknown. Some *M. suttoniae* isolates from WA had a large indel matching 100% to isolates collected from Queensland. The large indel represents a single evolutionary event and when excluded the sequence differs by only 1 bp from the other WA isolates, which in turn match the sequence of isolates from other locations (China, Indonesia, South Africa and New South Wales) (V Andjic pers comm). However, within the *M. suttoniae* clade there is a lot of sequence variation (*e.g.* CMW5348 from Indonesia) suggesting a species complex. More gene regions would need to be sequenced to resolve this issue. *Pseudocercospora fori* has only been previously recorded in South Africa from *E. grandis* (Hunter *et al*. 2004). *Mycosphaerella tasmaniensis* was originally described from Tasmania (Crous *et al*. 1998) and has recently been found on mainland Australia in Victoria, where it was found to occur at low levels (Barber *et al*. 2005). Interestingly, only the asexual stages of *P. fori*, *M. suttoniae* and *M. tasmaniensis* were isolated from WA.

There have been several instances of misidentification of *Mycosphaerella*/ *Teratosphaeria* species occurring on *Eucalyptus*. The most notable is *M. nubilosa* in New Zealand, where *Colletogloeopsis nubilosum* was described as the anamorph for this species (Ganapathi and Corbin 1979), which was later correctly identified as *M. cryptica* (Park and Keane 1982). *Mycosphaerella nubilosa* and *M. molleriana* were both described over 100 years ago, and were synonymised by Crous *et al*. (1991); however, after taxonomic review they were subsequently considered separate species (Crous and Wingfield 1996). The descriptions of *M. gregaria* (Carnegie and Keane 1997) and *M. ellipsoidea* (Crous and Wingfield 1996) are not dissimilar (Table 3.2). However, the germination patterns vary, as *M. ellipsoidea* develops lateral branches 24–48 h after spore release (Crous and Wingfield 1996), while *M. gregaria* germinates from both ends parallel to the long axis of the spore (Carnegie and Keane 1997). Isolates from WA previously described as *M. gregaria,* isolates from South Africa identified as *M. ellipsoidea* (anamorph: *Uwebraunia ellipsoidea*) and isolates from the current study all form a single taxonomic clade with 100% bootstrap support. These results for the ITS region concur with multi-gene analyses published by Hunter *et al*. (2006), in that isolates from WA form a single clade with South African isolates of *M. ellipsoidea*. All isolates from WA were characterised by the presence of a large indel, not present in the South African isolates and also morphologically, by the absence of an *Uwebraunia* anamorph state. However, as the morphological characteristics of the teleomorph overlap and the sequence data (minus the indel) are identical, it is concluded that the species present in WA is *M. ellipsoidea* and not *M. gregaria* as previously published

(Maxwell *et al*. 2003). Therefore, *M. gregaria* appears to be restricted to south-eastern Australia only.

In the present study, although ITS sequences of *M. aurantia* matched those of *M. africana* and *M. keniensis*, morphologically the isolates were most similar to the description of *M. aurantia* (Maxwell *et al*. 2003). Multi-gene phylogenetic studies have shown that *M. aurantia*, *M. keniensis* and *M. africana* form a single clade (Hunter *et al*. 2006). However, the authors stopped short of combining these species because of discrepancies between the morphological descriptions. Therefore, there remains a need to examine the type cultures of *M. aurantia*, *M. keniensis* and *M. africana* simultaneously under the same conditions. There are a number of other *Mycosphaerella*/ *Teratosphaeria* taxa on eucalypts described on different continents that require similar examination for the same reason. However, the current quarantine laws of Australia prohibit the import of plant pathogens into the country, limiting what can be achieved here.

The current work shows an increase in the number of *Mycosphaerella*/ *Teratosphaeria* species associated with plantation eucalypts in WA and Australia. With the exception of *M. cryptica*, none of these species were known in WA prior to the commencement of large-scale *E. globulus* plantations, and with *M. cryptica* as the exception, none have a known impact on the major native eucalypts in the region. The increase in number of taxa raises two important questions with respect to the management of eucalypt forests and plantations. Firstly, are these newly isolated *Mycosphaerella*/ *Teratosphaeria* species moving from endemic eucalypts

into the plantations? Secondly, are these species being moved on symptomatic and/or asymptomatic nursery stock between the east and west coasts of Australia? It is therefore important that continuous monitoring for MLD in the plantation estate and associated native eucalypt remnants be continued in this region. It is also critical that strict quarantine and hygiene methods are used and maintained when transporting nursery stock between states. Until temporal and spatial surveys are conducted on a regular basis many important questions regarding the biology, genetics and pathology of *Mycosphaerella*/ *Teratosphaeria* species in plantation eucalypts in WA will remain unresolved.

It is clear from this study that the taxonomy of *Mycosphaerella*/ *Teratosphaeria* species associated with eucalypts is incomplete and the discipline is likely to be in a state of flux for some considerable time. The next chapter will explore in more detail some of the nomenclature and biology of taxa referred to above.

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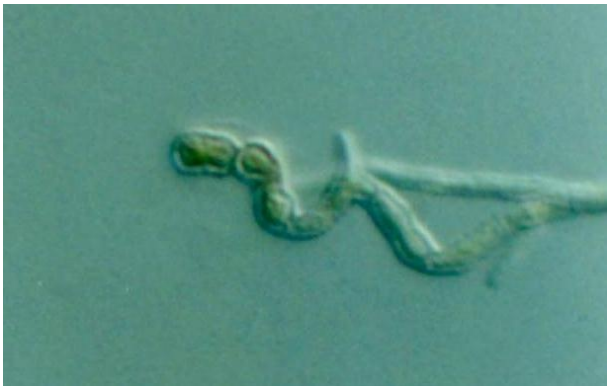
CHAPTER 4

USING MOLECULAR DATA TO RESOLVE THE TAXONOMY OF LESSER KNOWN *MYCOSPHAERELLA* SPECIES—NEW COMBINATIONS/ SYNONYMY



4.1 INTRODUCTION

There are many leaf-inhabiting fungi that have been recorded as causing leaf spots or blight in eucalypt plantations within Australia (Chapter 1). Many are considered to be endemic to native eucalypt forests, but have been seen to cause epidemics if conditions are conducive to disease (Carnegie 2007).

The Ascomycota is the largest and most diverse group of fungi with more than 28 000 species (Isaac 1992). Traditional taxonomy of the ascomycetes has relied on the morphological descriptions of the ascoma or ascus type (Liu *et al.* 1999; Lumbsch 2000), often made even more difficult by the absence of fruiting bodies, of which there are more than 20 000 species (Lumbsch 2000). *Mycosphaerella* and allied genera, including *Teratosphaeria*, have the greatest number of decribed species within the Acomycota (Corlett 1991; Crous *et al.* 2006). The taxonomy is predominantly based on characters of the sexual state, such as measurements of the pseudothecia, asci and ascospores. Other characters such as asexual traits and host are also used to differentiate between species.

The identification of *Mycosphaerella*/ *Teratosphaeria* species from eucalypts has had a short history in Australia. This is most likely due to plantation eucalypts not being of economic importance here. However, since the expansion of the plantation estate throughout Australia in the last thirty years, there has been an increase in the interest of this group of fungi.

It is important to expand our knowledge of the biology, ecology and pathology of the *Mycosphaerella* and *Teratosphaeria* genera occurring on *Eucalyptus* in Australia, especially in relation to plantation forestry. One aspect of the biology of *Mycosphaerella*/ *Teratosphaeria* species on eucalypts that has lacked consistent study is the infection process and subsequent disease development. This has been due to the difficulty in obtaining a spore suspension with which to inoculate plants, as many *Mycosphaerella*/ *Teratosphaeria* species do not produce conidia or ascospores in culture on agar (Crous 1998; Maxwell *et al.* 2003). Consequently, ascospores have to be collected from naturally infected material; however, this is further exacerbated since more than one *Mycosphaerella* or *Teratosphaeria* species can be isolated from a single lesion (Maxwell *et al.* 2003; Crous *et al.* 2009). Those species where little is known of their biology, pathogenicity or their role in the MLD complex are often described as minor species.

Park (1984) was the first intensive study of the biology of *Mycosphaerella* and *Teratosphaeria* species isolated from eucalypt species in Australia. His work was based on four species, two of which were described and identified during the course of that study, *T. parva* (Park and Keane 1982a) and *M. delegatensis* (Park and Keane 1984). Park and Keane (1982a) were the first to introduce the use of germination patterns on water agar to determine different *Mycosphaerella*/ *Teratosphaeria* species. Crous (1998) furthered this concept by using 2% malt extract agar (MEA) for all of his germination pattern descriptions, and grouped the different germination patterns. This advancement allowed the most common

Mycosphaerella/ *Teratosphaeria* species to be identified quickly without having to wait 1–2 months for cultures to grow.

The sequencing of the internal transcribed spacer (ITS) region has had a marked impact on the taxonomy of *Mycosphaerella* and related genera. It has allowed taxonomists to re-examine the phylogeny of these genera and correct misidentifications. The use of this molecular technology has reduced the value of some previously used morphological characters such as measurements of teleomorphic characters and the use of germination patterns.

Teratosphaeria parva and *M. grandis* are morphologically similar species recorded on eucalypt hosts in Australia and elsewhere. The former is thought to be a saprophyte and the latter a pathogen (Park and Keane 1982a; Carnegie and Keane 1994). However, isolates of *T. parva* and *M. grandis*, are recorded on GenBank with identical ITS sequences. *Teratosphaeria parva* (as *M. parva*) was first described from Victoria by Park and Keane (1982a) from diseased juvenile *E. globulus* leaves. Based on the germination pattern and morphological measurements, it was easily distinguishable from *M. nubilosa* and *M. cryptica*. Due to it being only found in association with these two species and the inability to induce disease, it was considered a saprophyte (Park and Keane 1982b). A second species, *M. grandis*, was described by Carnegie and Keane (1994) from diseased *E. grandis* leaves, also from Victoria. It was described as a pathogen often in association with *M. gregaria*. Although it was morphologically similar to *T. parva* it was considered different from

T. parva because it was often the sole species isolated from a lesion and therefore considered a pathogen (Carnegie and Keane 1994).

Mycosphaerella aurantia was first described from *E. globulus* plantations in WA in 2003 (Maxwell *et al.* 2003). More recently, it has been isolated from infected leaves on *E. grandis* in Uruguay (Pérez *et al*. 2009). Maxwell *et al*. (2003) also described *M. gregaria* as a new record for WA; however, during a review of *Mycosphaerella* species in WA (Chapter 3; Jackson *et al*. 2008), it was found that *M. gregaria* was actually *M. ellipsoidea*. It was therefore necessary to sequence the type species of *M. gregaria* to confirm identification.

The aims of this chapter were to use morphological characters and DNA sequences to determine whether *T. parva* and *M. grandis* are synonymous; and whether *M. gregaria* and *M. aurantia* are synonymous. The type material and DNA sequences of *T. parva*, *M. grandis, M. aurantia* and *M. gregaria* were compared along with a range of sequences from Australia, Ethiopia, South Africa, Portugal and Spain.

4.2 METHODS

4.2.1 Herbarium and culture specimens

Herbarium material of *T. parva* (isotype) was obtained from the New South Wales Agriculture Plant Pathology Herbarium—DAR 41956 a. Culture material of *T. parva* (AJC 86), *M. grandis* (AJC 165) and *M. gregaria* (isotype) (DAR 72368) and herbarium samples of *M. grandis* (isotype) (AJC 60) were provided by Angus Carnegie, New South Wales (NSW) Industry and Investment (Tables 4.1 and 4.2).

Additional cultures (AJC 395; AJC 399; AJC 410; AJC 444; AJC 466; AJC 468; AJC 541) provided putative identity based on morphology, were not included in Table 4.1 due to lack of host information.

4.2.2 DNA isolation, amplification and sequencing

Molecular identification was carried out according to Chapter 3 (section 3.2.2) with the exception of recalcitrant isolates of *T. parva* or samples obtained from leaf material, where the PCR reactions were nested with a species-specific primer (Maxwell *et al.* 2005) with either ITS2 or ITS3 (White *et al.* 1990).

4.2.3 Sequence comparison

Selected sequences of the ITS rDNA region of *T. parva* and *M. grandis* isolates (Table 4.1) were obtained from GenBank and aligned in BioEdit Sequence Alignment Editor© (Hall 1999) including partial sequences. As the ITS rDNA sequences on GenBank of *M. gregaria* matched those of *M. ellipsoidea* (Jackson *et al.* 2008), only selected sequences of *M. aurantia* and several closely matching species were used for the alignment of *M. aurantia* (Table 4.2). The alignment was completed as described for *T. parva/ M. grandis*.

4.2.4 Photography

Photos of leaf material and cultures were made using a digital Canon PowerShot Pro1 camera. All photos were edited using Adobe® Photoshop® 7.0 software.

4.3 RESULTS

4.3.1 Alignment and sequences of the ITS region of *Teratosphaeria parva* and *Mycosphaerella grandis*

All sequences obtained in the current study from cultures and leaf material matched the sequence (ITS1 and ITS4) of the isotype of *T. parva* (RF Park & Keane) Crous & Braun 2007 and all isolates of *T. parva* and *M. grandis* on GenBank. Repeated sequences of all samples sequenced in the current study had three polymorphisms at 304, 433 and 447 bp of either a C or T (Table 4.3). Those polymorphisms were also present in the sequences obtained from GenBank. Other polymorphisms were present among the isolates; however, they were generally unique to that isolate (Table A4.1 Appendix).

Table 4.1 List of *Mycosphaerella* and *Teratosphaeria* isolates used for comparison against *T. parva.* Isolates in bold denotes isotype.

GenBank accession no.	Teleomorph	Host	Location	Collector	Culture accession no.[a]	Study sequenced
	M. grandis	*E. grandis*	Victoria	AJ Carnegie	AJC60[b]	This study
AY626986	*M. grandis*	*E. saligna*	Victoria		AJC165	Glen *et al.* (2007)
	M. grandis	***E. saligna***	**Victoria**	**AJ Carnegie**	**AJC165**	**This study**
AY244407	*M. grandis*	*E. globulus*	Ethiopia		CMW101989	
AY244408	*M. grandis*	*E. globulus*	Ethiopia		CMW10376	
AY045514	*M. grandis*	*E. nitens*	Tasmania		Q/1/1/1	
FJ515722	*M. grandis*	*E. globulus*	Portugal	H Machado	EFNX21D	Silva *et al.* (2009)
AY509779	*T. parva*	*E. globulus*	Western Australia	A Maxwell	AM248	Maxwell (2004)
AY244405	*T. parva*	*E. globulus*	Ethiopia	A Gezahgne/ J Roux	CMW10186	Gezahgne *et al.* (2006)
AY244406	*T. parva*	*E. globulus*	Ethiopia	A Gezahgne/ J Roux	CMW10187	Gezahgne *et al.* (2006)
AY725576	*T. parva*	*Eucalyptus* sp.	South Africa	P Crous	CBS116289	Crous *et al.* (2004)
AY509780	*T. parva*	*E. globulus*	Western Australia	A Maxwell	AM249	Maxwell (2004)
AY509781	*T. parva*	*E. globulus*	Western Australia	A Maxwell	AM250	Maxwell (2004)

GenBank accession no.	Teleomorph	Host	Location	Collector	Culture accession no.[a]	Study sequenced
AY509782	*T. parva*	*E. globulus*	Western Australia	A Maxwell	CBS110503	Maxwell *et al.* (2003)
AY939527	*T. parva*	*E. globulus*	Western Australia	A Maxwell	MURU033	Maxwell *et al*. (2005)
AY939533	*T. parva*	*E. globulus*	Western Australia	A Maxwell	MURU037	Maxwell *et al*. (2005)
FJ515725	*T. parva*	*E. globulus*	Portugal	H Machado	EFNX40D7	Silva *et al.* (2009)
FJ515717	*T. parva*	*E. globulus*	Portugal	H Machado	EFNX15A	Silva *et al.* (2009)
FJ515711	*T. parva*	*E. globulus*	Portugal	H Machado	EFNNX7B	Silva *et al.* (2009)
FJ515713	*T. parva*	*E. globulus*	Portugal	H Machado	EFNY27A	Silva *et al.* (2009)
	T. parva	*E. globulus*	Victoria	AJ Carnegie	AJC 86	This study
	T. parva	***E. globulus***	**NSW**	**R Park**	**DAR41956a[b]**	**This study**
	T. parva-like	*E. amplifolia*	NSW	AJ Carnegie	AJC 395	This study
	T. parva-like	*Eucalyptus sp.*	NSW	AJ Carnegie	AJC 399	This study
	T. parva-like	*C. variegata,*	NSW	AJ Carnegie	AJC 410	This study
	T. parva-like	*Eucalyptus sp.*	NSW	AJ Carnegie	AJC 444	This study
	T. parva-like	*E. biturbinata*	NSW	AJ Carnegie	AJC 466	This study

GenBank accession no.	Teleomorph	Host	Location	Collector	Culture accession no.[a]	Study sequenced
	T. parva-like	*E. saligna*	NSW	AJ Carnegie	AJC 468	This study
	T. parva-like	*E. globulus*	Vic.	D. Smith	AJC 541	This study

[a] Designation of isolates and culture collections: CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW, Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa.

Table 4.2 List of *Mycosphaerella* isolates used for comparison against the *M. gregaria* isotype. Isolates in bold denotes isotype.

GenBank accession no.	Teleomorph	Host	Location	Collector	Culture accession no.[a]	Study sequenced
AY509742	*M. aurantia*	*E. globulus*	Western Australia	S Jackson	AM152	Maxwell *et al.* (2003)
	M. gregaria	***E. grandis***	**Victoria**	**A Carnegie**	**DAR72368**	**This study**
AY150331	***M. aurantia***	***E. globulus***	**Western Australia**	**A Maxwell**	**CBS110500**	**Maxwell *et al*. (2003)**
AY509743	*M. aurantia*	*E. globulus*	Western Australia	A Maxwell	AM221	Maxwell *et al.* (2003)
AY509744	*M. aurantia*	*E. globulus*	Western Australia	A Maxwell	AM222	Maxwell *et al*. (2003)
EU042175	*M. aurantia*	*E. globulus*	Western Australia	S Jackson	SJ30	Maxwell *et al*. (2005)
	M. aurantia	*E. globulus*	Western Australia	S Jackson	SJ31	This study
	M. aurantia	*E. globulus*	Western Australia	S Jackson	SJ100	This study
EU255896	*M. aurantia*		Spain			Otero *et al*. (2007)
DQ123604	*M. aurantia*	*Coffea arabica*				Sette *et al*. (2006)
EU707856	*M. buckinghamiae*	*Buckinghamia* sp.			CBS112175	Crous *et a*l. (2008)
AY626981	*M. africana*	*E. viminalis*	South Africa	P. Crous	CBS680.95	Glen *et al*. (2007)

[a] Designation of isolates and culture collections: CBS, Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; DAR, New South Wales Agriculture Plant Pathology Herbarium.

Table 4.3 Positions of polymorphic nucleotides from aligned sequence data of the ITS gene region sequenced from *Teratospharia parva* and *Mycosphaerella grandis* isolates. For comparison purposes polymorphisms not shared with the first isolate are in bold and no base (*) indicates it was not sequenced. Duplicate sequences are noted in brackets.

***Mycosphaerella* species**	**GenBank accession no.**	**304 bp**	**433 bp**	**447 bp**
T. parva	AY509780	T	T	C
T. parva	AY509779	**C**	T	**T**
T. parva	AY244406	T	T	**T**
T. parva	AY725576	**C**	T	**T**
T. parva	AY244405	T	T	C
T. parva	AY509781	T	T	C
T. parva	AY509782	T	**C**	C
T. parva	AY939527	T	T	C
T. parva	AY939533	T	T	C
T. parva	FJ515725	T	**C**	C
T. parva	FJ515717	T	T	**T**
T. parva	FJ515711	T	T	C
T. parva	FJ515713	T	T	C
T. parva AJC86		**C**	T	*
T. parva AJC86 tpr[a]		T	*	*
T. parva AJC86 tpf[b]		T	*	*
T. parva AJC86 ITS3		T	T	C
T. parva DAR41956a		T	T	C
T. parva DAR41956a (2)		**C**	T	C
T. parva DAR41956a tpr		T	T	C
T. parva DAR41956a ITS2		T	*	*
T. parva DAR41956a ITS2 (2)		T	*	*
T. parva DAR41956a ITS3		T	T	C
T. parva DAR41956a tpf		T	*	*
T. parva DAR41956a tpf (2)		T	*	*
T. parva DAR41956a tpr (2)		T	*	*
M. grandis AJC60		T	T	C
M. grandis AJC60 (2)		T	T	C
M. grandis AJC60 ITS2		T	*	*
M. grandis AJC60 ITS2 (2)		T	*	*
M. grandis AJC60 ITS3		T	T	C
M. grandis AJC60 tpf		T	*	*
M. grandis AJC60 tpr		T	*	*
M. grandis AJC60 tpr (2)		T	T	C
M. grandis AJC60 tpr (3)		T	*	*
M. grandis	AY626986	**C**	T	**T**
M. grandis AJC 165		**C**	T	**T**
M. grandis AJC 165 (2)		T	T	C
M. grandis AJC 165 ITS2		T	*	*
M. grandis AJC 165 ITS3		**C**	T	*
M. grandis	AY244407	**C**	T	**T**
M. grandis	AY244408	**C**	T	**T**
M. grandis	AY045514	**C**	T	**T**
M. grandis	FJ515722	**C**	T	**T**

[a] tpr = *T. parva* specific primer reverse; [b] tpf = *T. parva* specific primer forward.

4.3.2 Morphological comparison of *Teratosphaeria parva* and *Mycosphaerella grandis*

The descriptions of the type specimens of *T. parva* and *M. grandis* share many similarities. *Teratosphaeria parva* was isolated from *E. globulus* and *E. grandis*, while *M. grandis* was isolated from only *E. grandis* (Table 4.4). Ascospores of both species darken, become pigmented on germination with germ tubes being parallel to the long axis of the ascospore, with the ascospores becoming constricted at the septa. Carnegie and Keane (1994) described *M. grandis* ascospores as distorting and often having multiple germ tubes, which was not mentioned by Park and Keane (1982a) (Table 4.4, Figure 4.1). Carnegie and Keane (1994) also described *M. grandis* pseudothecia were often associated with *M. gregaria* and was considered to be pathogenic; however, *T. parva* was often isolated with *M. nubilosa* and *M. cryptica* and, after pathogenicity studies were inconclusive, was considered to be saprophytic (Park and Keane 1982a). No description of the culture morphology was given for either *T. parva* (Park and Keane 1982a) or *M. grandis* (Carnegie and Keane 1994). However, cultures are similar in appearance when compared to each other (Figure 4.2).

No early germination pattern was published for *T. parva* (Park and Keane 1982), nor do the drawings of germination pattern of *M. grandis* reflect the written description (Carnegie and Keane 1994) (Table 4.4, Figure 4.1). Very few pseudothecia were observed on either side of the *E. grandis* leaf specimen from which the *M. grandis* isotype was collected (Figure 4.2). On the *E. globulus* leaf sample from which the *T.*

parva type was first collected, numerous pseudothecia were apparent, particularly on the abaxial surface of the leaf (Figure 4.2).

Table 4.4 Summary of taxonomic characters of *Teratosphaeria parva* and *Mycosphaerella grandis* published in the literature

	***T. parva*[a]**	***M. grandis*[b]**
Host	*E. globulus, E. grandis*	*E. grandis*
Pseudothecia	hypophyllous, immersed, stromatic, black, punctiform, globose, glabrous 42–91	amphigenous, immersed, predominantly hypophyllous 60–70
Asci (µm)	ellipsoidal or obclavate, straight or incurved 29–48 x 6–13	obclavate, straight to slightly curved 35–37.5 x 10
Ascospores (µm)	straight, ellipsoidal tapering to each end, guttulate, constricted at septa, hyaline but darkening after germination 7–10 x 1–3	hyaline, predominantly constricted 10.5–14.5 x 3–4.5
Germination pattern	ascospores become pigmented on germination. Straight germ tubes with constrictions at septa	initially germinating parallel from one cell, gross distortion and darkening of the spore, multiple germ tubes
Associated species	*M. nubilosa, M. cryptica*	*M. gregaria*
Trophic status	Saprotroph	Hemibiotroph/ Necrotroph

[a] Park and Keane (1982a)

[b] Carnegie and Keane (1994)

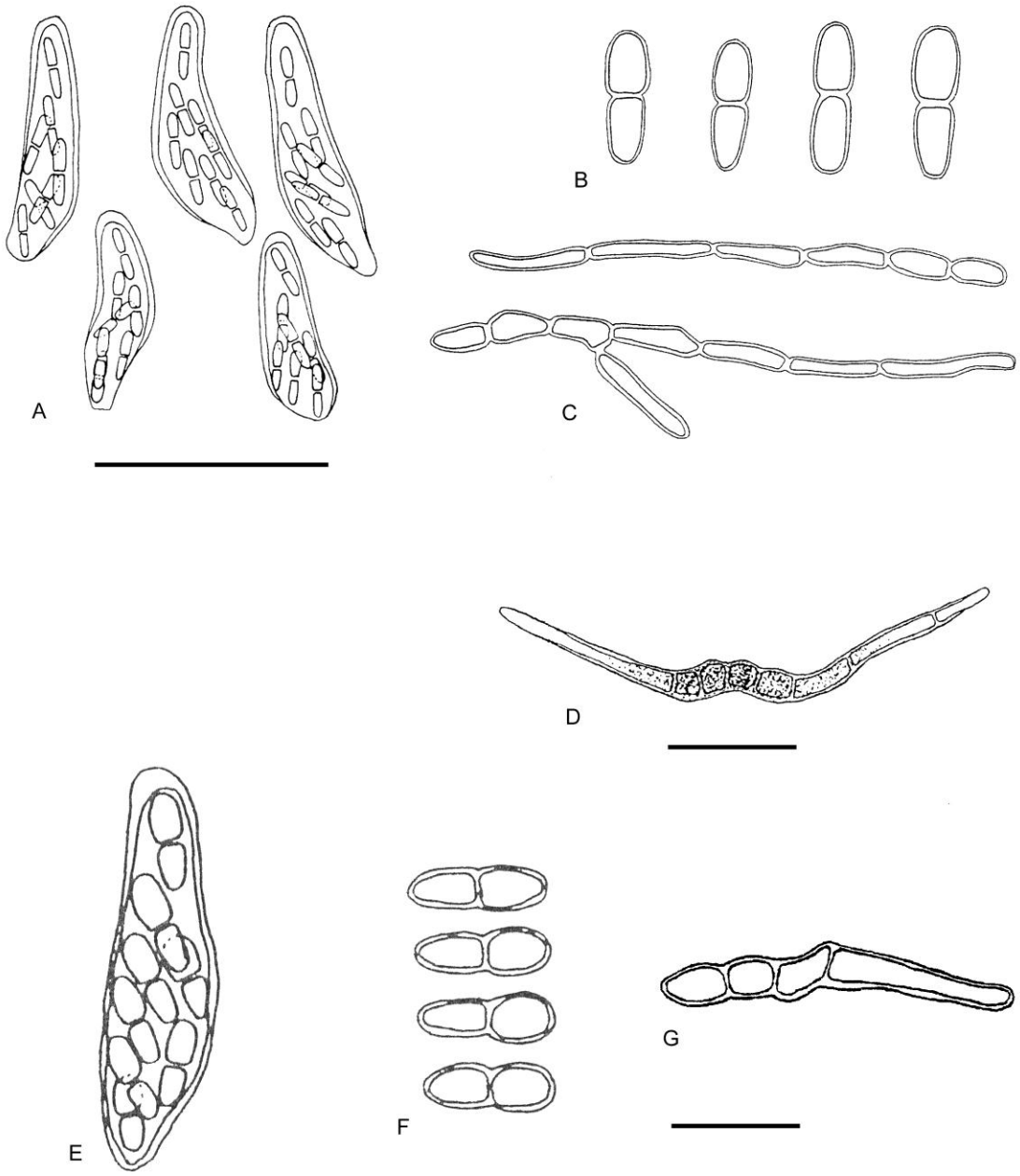


Figure 4.1 Comparison of asci, ascospores and ascospore germination of *Teratosphaeria parva* **A**–**D** and *Mycosphaerella grandis* **E**–**G**. **A**–**C** taken from Park (1984); **D**–**G** Carnegie (2000). Bars = 10µm.

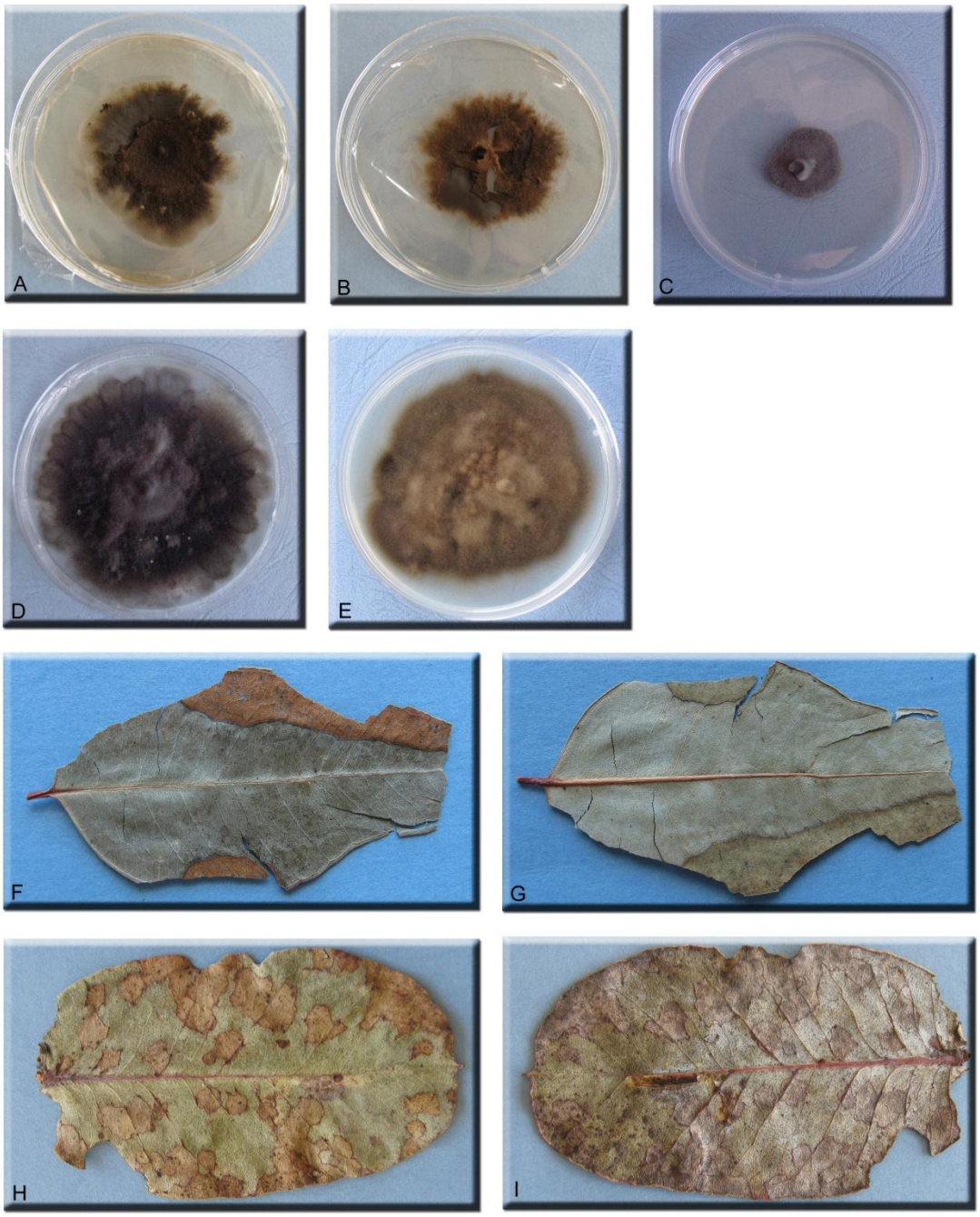


Figure 4.2 Cultures (**A**–**E**) and lesions from *Eucalyptus grandis* (**F** and **G**) and *E. globulus* (**H** and **I**) of *Teratosphaeria parva* (**A** = AJC 86; **C** = AM 250 at 1 month; **D** = AM 250 at 4 months; **H** = adaxial leaf surface; **I** = abaxial leaf surface); *Mycosphaerella grandis* (**B** = AJC 165; **F** = adaxial leaf surface; **G** = abaxial leaf surface); **E** = *T. parva* - like (AJC 410).

4.3.3 Alignment and sequences of the ITS region of *Mycosphaerella gregaria*

The sequence from the isotype culture of *M. gregaria* (DAR72368) matched 100% to those on GenBank of *M. aurantia*. It also matched closely to *M. africana* and *M. buckinghamiae* (Table 4.5, Table A4.2 Appendix). One isolate (SJ100) from WA had three base changes compared to the isotype sequence, while an isolate from Spain (EU255896) had four base changes compared to the isotype sequence (Table 4.5). *Mycosphaerella buckinghamiae* matched 100% with the type sequence of *M. gregaria*.

Table 4.5 Positions of polymorphic nucleotides from aligned sequence data of the ITS gene region sequenced from *Mycosphaerella aurantia* isolates. For comparison purposes polymorphisms not shared with the first isolate are in bold, indels indicated by (-) and no base (*) indicates it was not sequenced.

Isolate	**GenBank accession no.**	**105**	**109**	**110**	**131**	**132**	**133**	**134**	**449**	**474**	**480**
M. aurantia	AY509742	-	G	G	A	T	C	A	C	T	T
M. gregaria DAR72368		-	G	G	A	T	C	A	C	T	T
M. aurantia	AY150331	-	G	G	A	T	C	A	C	T	**-**
M. aurantia	AY509743	-	G	G	A	T	C	A	C	T	T
M. aurantia	AY509744	-	G	G	A	T	C	A	C	T	T
M. aurantia	EU042175	-	G	G	A	T	C	A	C	T	T
M. aurantia SJ31		*	*	*	A	T	C	A	C	T	T
M. aurantia SJ100		-	**A**	**T**	**A**	T	C	A	C	T	**-**
M. aurantia	EU255896	**G**	G	G	C	**A**	**T**	**C**	C	**-**	**-**
M. aurantia	DQ123604	-	G	G	A	T	C	A	**T**	T	T
M. buckinghamiae	EU707856	-	G	G	A	T	C	A	C	T	T
M. africana	AY626981	-	G	G	A	T	C	A	C	T	T

4.3.3 Morphological comparison of *Mycosphaerella gregaria* and *M. aurantia*

The descriptions of the germination pattern for *M. aurantia* and *M. gregaria* are very similar with both species having germ tubes occurring from the long axis of the spore (Carnegie and Keane 1994; Carnegie 2000; Maxwell *et al.* 2003). Maxwell *et al.* (2003) then goes on to describe *M. aurantia* ascospores remaining hyaline but becoming verruculose at 24 hours, while at 36 hours lateral branches were observed (Figure 4.3).

From the type description of *M. gregaria* cultures after eight weeks, the aerial hyphae in the centre was whitish grey, slightly raised, becoming flat, light grey to dark grey at the irregular outer edge (Carnegie and Keane 1994). Hyphae became pink and a pinkish brown pigment developed in the medium after two months (Carnegie and Keane 1994) (Table 4.6, Figure 4.4). Maxwell *et al.* (2003) described *M. aurantia* cultures after two months in the dark as becoming brownish orange with red crystals developing in the agar (Table 4.6, Figure 4.4). However, isolates of *M. aurantia* that were collected in the current study ranged in colour from grey-pink to orange and did not form red crystals in 2% MEA agar. Although no isolates of *M. buckinghamiae* were examined in the current study, Crous *et al.* (2000) described the culture as being erumpent, with aerial mycelium sparse, the margins smooth, lobed, and the surface of inner region off-white. The intermediate region was rose, while the outer region was pale olivaceous grey (Table 4.6). No isolates of *M. africana* have been collected in Australia; therefore, cultures could not be examined in the current study. However, Crous and Wingfield (1996) first described the culture

of this species from South Africa as having aerial mycelium as grey olivaceous with erect hyphal tufts and frequently with aerial white-grey mycelium. The colonies were described as black and producing a diffuse brown pigment on MEA (Crous 1998).

Table 4.6 Summary of taxonomic characters of *Mycosphaerella gregaria* and similar *Mycosphaerella* species.

	M. gregaria[a]	***M. aurantia***[b]	***M. africana***[c]	***M. buckinghamiae***[d]
Host	*E. grandis*	*E. globulus*	*E. viminalis*	*Buckinghamia* sp.
Culture morphology	On 2 % MEA after 8wk at 25°C aerial hyphae in the centre was whitish grey slightly raised, becoming flat, light grey to dark grey at the irregular outer edge. Hyphae becomes pink and pinkish brown pigment develops in media after 2 months	On 2 % MEA after 8wk at 25°C in the dark, surface brownish orange. Red crystals form in agar	Grey olivaceous, erect hyphal tufts white-grey mycelium, produce diffuse brown pigment in agar cells cluster in agar	Erumpent, aerial mycelium sparse, margins smooth, lobed, surface of inner region off-white, intermediate region rose outer region pale olivaceous grey
Pseudothecia (µm)	Amphigenous, scattered clumps, superficial, black, globose, glabrous 60–75	Amphigenous, sparse, black, globose, 87–105 x 83–102 µm	Amphigenous single black subepidermal globose 50-65 x 50-70	Predominantly epiphyllous, black, subepidermal, becoming erumpent, globose, 100–200
Asci (µm)	Cylindrical to clavate, straight or slightly curved 37.5–47.4 x 6.3–7.5	Obovoid to ellipsoid, straight to incurved, 22–85 x 8–16	Obovoid to broadly ellipsoidal, straight or incurved 28–45 x 8–11	Aparaphysate, fasciculate, bitunicate, sub-sessile, narrowly ellipsoidal to obovoid, straight to slightly curved, 30–40 x 6–8
Ascospores (µm)	Hyaline, guttulate, straight, oval to fusiform, widest at midpoint of apical cell, prominent constriction at septum 12.5–15 x 2.5–3.7	Hyaline, guttulate, fusoid-ellipsoid, ends rounded, 1-septate not constricted, tapering toward basal end, 9–15 x 2–3	Colourless guttulate thick walled straight fusoid-ellipsoidal with obtuse ends widest in middle of apical cells constricted at septa tapering toward both ends but more toward the base 7–11 x 2–3	Hyaline, guttulate, thin-walled, straight, fusoid-ellipsoidal with obtuse ends, widest just above the septum, tapering towards both ends, slightly more prominently towards the lower end 9–13 x 2.5–3.5
Germination pattern	Germ tubes parallel to the long axis of the ascospore	From both ends parallel to the long axis of the spore, remaining hyaline but becoming slightly constricted and finely verruculose at 24 h then form lateral branches after 36 h	Irregular from both ends or from different positions in cells with two or more germ tubes darkening and distorting	Germinate from both ends, with germ tubes growing parallel to the long axis of the spore, constricted at the septum
Associated species	*M. grandis*	*M. cryptica*, *M. nubilosa*, *T. parva* or *M. gregaria* (as *M. ellipsoidea*) on the same lesion		

[a] Carnegie and Keane (1994); Carnegie (2000)

[b] Maxwell *et al.* (2003)

[c] Crous and Wingfield (1996)

[d] Crous *et al*. (2000)

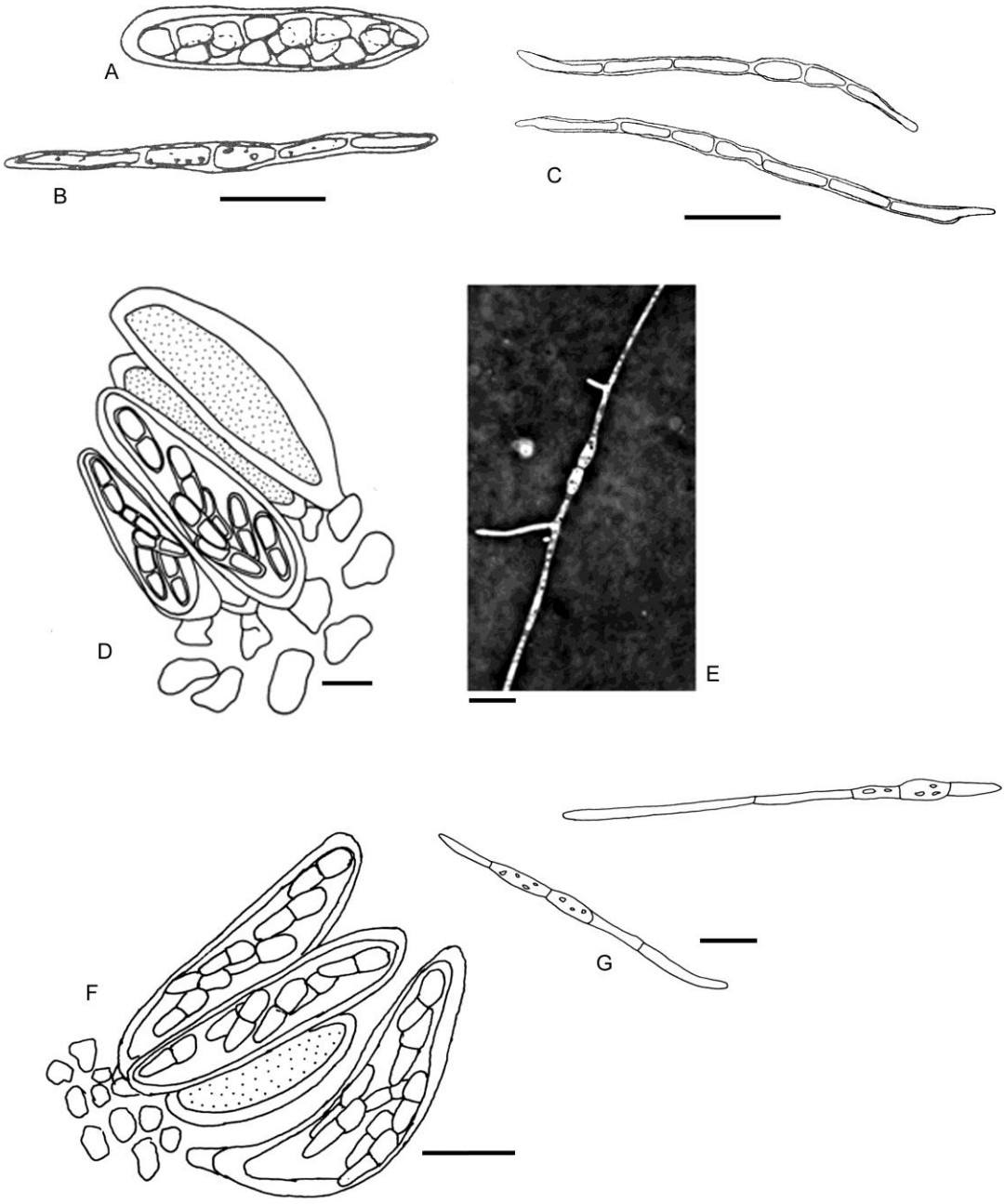


Figure 4.3 Comparison of asci, ascospores and ascospore germination of *Mycosphaerella gregaria* (**A**–**C**); *M. aurantia* (**D**, **E**); and *M. ellipsoidea* (**F**, **G**). **A** and **B** taken from Carnegie and Keane (1994); **C** Carnegie (2000); **D** and **E** Maxwell *et al.* (2003); **F** and **G** Maxwell (2004). Bars = 10µm.

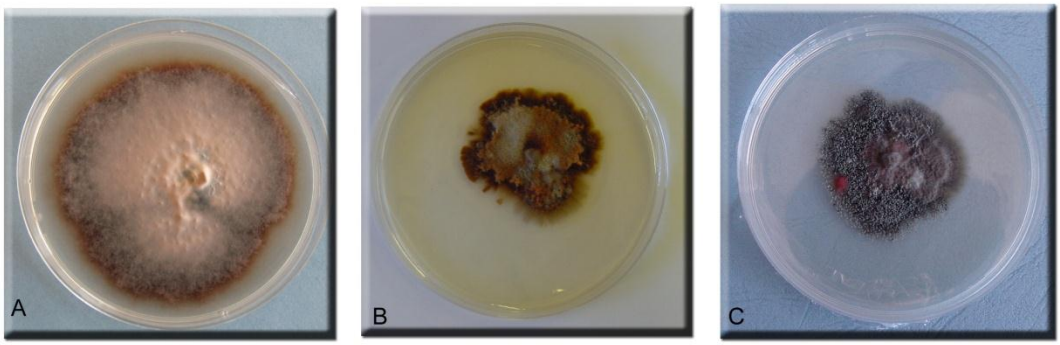


Figure 4.4 Cultures of *Mycosphaerella gregaria* (**A** = DAR72368); *M. aurantia* (**B** = AM152) and *M. ellipsoidea* (**C** = AM237) in the current study.

4.4 DISCUSSION

This is the first study to examine and sequence the type material of *M. grandis*, *T. parva* and *M. gregaria*. As the sequences of the ITS region of *M. grandis* and *T. parva* are identical it is concluded that *M. grandis* is reduced to synonymy with *T. parva*. *Mycosphaerella aurantia*, *M. buckinghamiae* and *M. africana* also match the type sequence of *M. gregaria*. Therefore, these should all be synonymised to *M. gregaria*. Also, this study is the first to describe ITS sequence variation within the same *Mycosphaerella* isolate.

Crous (1998) reviewed *T. parva* and *M. grandis* and synonymised the two species; however, in Australia *M. grandis* is still considered to be a separate species (Park *et al.* 2000; Glen *et al.* 2007). Park *et al.* (2000) suggested that further studies were required as *M. grandis* was considered to be pathogenic and *T. parva* to be saprophytic, despite no pathogenicity studies having been conducted on *M. grandis*. Carnegie (2000) distinguished between *T. parva* and *M. grandis*, as *T. parva* ascospores were not as distorted after germination and germ tubes were not as

branched. Isolates matching the description of *M. grandis* from the eastern states (Australia) were sequenced and lodged on GenBank (Milgate *et al.* 2001; Kularatne *et al.* 2004). Maxwell *et al.* (2003) isolated *T. parva* from *E. globulus* in Western Australia (WA); however, it varied from the type description of Park and Keane (1982a) by having narrower ascomata and smaller asci. Also unlike the type description, it was isolated in WA alone from older lesioned leaves or in combination with *M. cryptica*, *M. ellipsoidea*, *M. marksii*, *T. mexicana* or *M. nubilosa* (Maxwell *et al.* 2003).

The taxonomic characters of the genus *Mycosphaerella* are well conserved, and therefore differences between species are often based on other factors such as biology for identification. The taxonomy of *Mycosphaerella* is generally host based. However, as more researchers lodge DNA sequences on accessible databases evidence is emerging for *Mycosphaerella* species to occur on diverse alternative hosts (Crous *et al.* 2006). The type *M. gregaria* sequenced in the current study also matched the ITS of two other *Mycosphaerella* species. *Mycosphaerella buckinghamiae* was first recorded and described from a *Buckinghamia* sp. in Australia (Crous *et al.* 2000). Crous *et al.* (2008) does not mention the similarities between *M. buckinghamiae* and *M. aurantia* in his review; however, they distinguish it from *M. africana* as it does not have ascospores that darken upon germination, nor does it produce a pigment in agar. *Mycosphaerella buckinghamiae* also has larger ascospores (9–13 x 2.5–3.5 µm), and colonies of *M. buckinghamiae* contain rose and off-white sectors that has not been observed in *M. africana*. The ascospore size range of *M. buckinghamiae* overlaps published ascospore measurements of *M.*

aurantia where some variation was observed (Maxwell *et al*. 2003); therefore, ascospore size should not be considered a useful distinguishing feature for these taxa.

Hunter *et al.* (2006) sequenced multiple gene regions of several *Mycosphaerella* spp. and *M. africana* and *M. aurantia* consistently grouped together. He separated them based on *M. africana* having constricted ascospores compared to no constrictions in ascospores of *M. aurantia*. However, Maxwell *et al*. (2003) does state that constriction does occur upon germination; therefore, this should not be considered as a taxonomic character used to distinguish these two species. Currently, only a few isolates of *M. africana* have sequences lodged in GenBank. More isolates of *M. africana* need to be described and sequenced to determine its validity as a species. One isolate of *M. aurantia* from GenBank was isolated from *Coffea* sp.; however, although the sequence matched 99% to *M. gregaria*, no morphological characteristics have been published (Sette *et al.* 2006).

Based on the sequence and morphological analysis, it is suggested that *M. aurantia* A Maxwell and *M. buckinghamiae* Crous and Summerell should be synonymised with *M. gregaria* Carnegie and Keane.

Instances of past misidentification of *Mycosphaerella* species occurring on eucalypts are becoming more regular as molecular technologies improve. Recent studies have concluded that several recently isolated species from Australia have actually been previously described. Hunter *et al.* (2006) synonymised *M. ambiphylla* A Maxwell and *M. vespa* Carnegie and Keane with *M. molleriana* (Thüm.) Lindau and also *M.*

intermedia MA Dick and Dobbie with *M. marksii* Carnegie and Keane. A recent review of isolates of *M. gregaria* from WA found that the species reported from there is actually *M. ellipsoidea* (Chapter 3; Jackson *et al.* 2008). Without the use of ITS sequencing, it is easy to see how this misidentification could be made (Figures 4.3 and 4.4). A comparison of biological drawings of *M. gregaria* (Carnegie and Keane 1994) and *M. gregaria* (Maxwell *et al.* 2003) show that germination patterns and asci are very similar. It was not until a comparison of cultures and DNA sequences could be made that it was concluded that *M. gregaria* (Maxwell *et al.* 2003) was *M. ellipsoidea* (Jackson *et al.* 2008).

Anamorphs are also used in the identification of fungal species; however, there are several problems to using them as an absolute taxonomic character. If the anamorph has been taken directly from leaf material, it is not guaranteed to be connected to the teleomorph. Also many *Mycosphaerella* species have not been linked to an anamorphic state. There is also variation amongst isolates on the development of the anamorph in culture. Other taxonomic characteristics include measurements of the fruiting structures including ascospores, the asci and the pseudothecia. These structures are small in size and measurements can be easily influenced by stains that are used, as some stains do not stain the cell wall (Carnegie and Keane 1994). The size of these structures can also vary within a species, making it hard to differentiate to the species level. Previously, the biology of the *Mycosphaerella* genus has also aided in the identification to species. For example, although the genus is one of the largest Ascomycete groups, the individual species were thought to be host specific. However, as more species are being

described from multiple hosts, this attribute can no longer be used (Crous *et al.* 2009). Within those species occurring on eucalypts, it has been regarded in the past that host-pathogen interactions are highly conserved. The most reliable method of identification of *Mycosphaerella* species has been DNA sequencing. This has aided in differentiating between species that were morphologically similar.

The sequencing of the internal transcribed spacer region (ITS) has also raised questions of the identification of *Mycosphaerella*/ *Teratosphaeria* species occurring on eucalypts that have been previously considered separate species. One such situation has been the similarities in the ITS of *T. parva* and *M. grandis*. *Teratosphaeria parva* was first described by Park and Keane (1982) infecting juvenile *E. globulus* leaves from Victoria. Based on germination pattern and morphological measurements it was easily distinguished from *M. nubilosa* and *M. cryptica*, although considered a saprophyte in association with lesions of these two species. When first described, *T. parva* was also found on *E. grandis*. Infection studies showed that it did not infect juvenile *E. globulus* leaves via stomata; however, ascospores did germinate and grow on the leaf surface.

Mycosphaerella grandis was described by Carnegie and Keane (1994) infecting *E. grandis* leaves, also in Victoria. It was described as a pathogen often in association with *M. gregaria*. The measurements of pseudothecia and asci fall within the range of *T. parva*; however, the ascospore measurements are slightly larger (Table 4.1). Both Park and Keane (1982) and Carnegie and Keane (1994) used water agar to

view ascospores germination. Germination patterns of *M. grandis* are very similar to *T. parva* (Table 4.1).

Carnegie and Keane (1994) described the main difference between *T. parva* and *M. grandis* was that *M. grandis* was considered to be pathogenic as it was isolated from lesions that were not associated with other *Mycosphaerella*/ *Teratosphaeria* species unlike *T. parva*.

During the current study, several *T. parva*-like isolates from the east coast of Australia were examined (AJ Carnegie pers com). The ITS rDNA of seven isolates were sequenced (data not shown) and compared to other *Mycosphaerella* species on GenBank. Two of the isolates matched 100% with *T. ohnowa* that has only recently been described from Australia (Crous *et al.* 2007). This may have been only recently isolated due to the morphological characteristics being so close to those of other *T. parva* cultures examined in the present study, even though the published descriptions are quite different, with *T. ohnowa* cultures resembling *M. nubilosa* (Crous *et al.* 2004). Another four of the isolates examined and sequenced had a 100% match with *Teratosphaeria associata* and *T. jonkershoekensis*. *Teratosphaeria jonkershoekensis* was first isolated from Australia on several *Protea* spp. (Crous *et al.* 2000). Crous *et al.* (2007) described *T. associata* from *Lembosina* sp., and it was also isolated from *Corymbia henryii*, *C. variegata*, *Lembosina* sp. and *E. dunnii*. The ITS rDNA sequence of *T. associata* differs from *T. jonkershoekensis* by one nucleotide in each of ITS1 and ITS2. From the data obtained in the present study, it may be possible that these species are the same, as it has been shown that

there are variable copies of the ITS region within the nucleus. Also, as *T. associata* was isolated from different hosts, this can now not be used as a taxonomic character as previously mentioned (Chapter 2). It is therefore suggested that *T. associata* be synonymised with *T. jonkershoekensis*.

Maxwell (2004) also described intra-specific variation with the ITS region of *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa* as well as *T. parva*. Goodwin *et al*. (2001) found an average of 1.38 nt differences within the ITS sequences within a species, with slightly more differences within ITS1 than ITS2. They suggest that taxa with ITS sequences that differ by two or more nucleotides may be distinct species; however, only eight *Mycosphaerella* species were examined, with *M. fragariae* having seven nt differences between two isolate sequences and *M. fijiensis* having six nt from five isolate sequences. Recently, a new *Uwebraunis* (as *Dissoconium*) species was described from *Musa* that differed by two nt (one in each of ITS1 and ITS2) from *U. dekkeri* isolated from WA on *E. globulus* (C = T) (Maxwell *et al*. 2000; Arzanlou *et al.* 2008). Despite the closeness in the ITS sequences, it was considered to be different based on growth rate as it grew slower in culture than the WA isolates. It was not mentioned if the isolates were grown at the same time under the same conditions or if the rate was compared to earlier published rates (Arzanlou *et al.* 2008). Growth of a species in culture is dependent on medium composition, temperature, quantity of medium, light regime, pH and time (Shih *et al.* 2007; Kim *et al.* 2010). The *U. dekkeri* isolates from WA grew at a rate of 15–25 mm month^{-1} Maxwell *et al.* (2000) compared to *U. musae* which had a growth rate of 10 mm month^{-1}.

Simon and Wei (2008) cloned the ITS gene region of *M. punctiformis* and found that polymorphisms did occur and that the intragenic polymorphisms were single nucleotide polymorphisms. It was also reported that transitions from A to G and T to C were more common that transitions from G to A or from C to T in *M. punctiformis* (Simon and Wei 2008). Therefore, cloning in the current study may have reduced the number of polymorphisms occurring, but that cloning may not have reduced the number to zero. The differences in the sequences within one isolate of *T. parva* may also be due to Taq polymerase mis-readings, however, Lloyd-Macgilp *et al.* (1996) referred to taq polymerase as introducing base substitutions errors into the sequence at a low but detectable rate, but that the probability that two sequences have independently acquired the same error at the same site, and therefore contribute false information to the phylogenetic analysis, is negligible. Therefore, the sequences of *T. parva* in the current study that had a single base change should be considered as real and not a sequencing error. This is evidence that isolates contain multiple copies of the ITS region and differentiating species on one or two base changes should be done with care.

This is the first study to examine the isotypes of *T. parva*, *M. grandis* and *M. gregaria*. From sequencing the ITS region, *M. grandis* should be synonymised with *T. parva* and *M. aurantia* with *M. gregaria*. The newly described *T. associata* should be synonymised with *T. jonkershoekensis*. Through the examination of *Mycosphaerella* isolates, it is recommended that sequencing of the ITS region should be the minimal molecular diagnostic used for identification, as morphological characteristics can be easily misinterpreted.

4.5 REFERENCES

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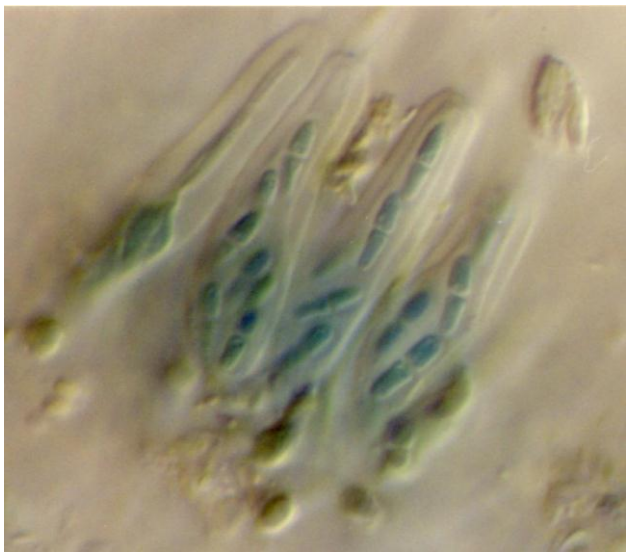
CHAPTER 5

INFECTION AND PATHOGENICITY OF *MYCOSPHAERELLA MARKSII* ON *EUCALYPTUS GLOBULUS*



5.1 INTRODUCTION

As mentioned in earlier chapters, it is important to expand our knowledge of the biology, ecology and pathology of the fungi occurring on *Eucalyptus* in Australia, especially in relation to plantation forestry. One aspect of the biologies of *Mycosphaerella* and *Teratosphaeria* species on eucalypts that has lacked consistent study is the infection process and subsequent disease development. This has been due to the difficulty in obtaining a spore suspension with which to inoculate plants, as many *Mycosphaerella*/ *Teratosphaeria* species do not produce conidia or ascospores aseptically on agar (Crous 1998; Maxwell *et al.* 2003). Consequently, ascospores have to be collected from naturally infected material; however, this is further exacerbated since more than one species can co-exist on a single lesion (Maxwell *et al.* 2003; Crous *et al.* 2009).

Park (1984) completed an intensive study on the infection process and disease development of *M. cryptica*, *M. nubilosa* and *T. parva*. Spore suspensions were made using Tween 20 as a surfactant, to assist adhesion of the spore suspension to the leaf surface. Park (1984) had limited success using macerated hyphae to inoculate plants compared with an ascospore suspension. The results of that study showed that *M. cryptica* was able to infect both surfaces of juvenile leaves of *E. globulus*. On the adaxial surface, *M. cryptica* ascospores directly penetrated the epidermal layer through the formation of appressoria. On the abaxial surface, *M. cryptica* ascospores entered leaves through stomata. Lesions began to develop 3–4 weeks after inoculation. *Mycosphaerella nubilosa* ascospores, when sprayed on

juvenile *E. globulus* leaves were found to infect via stomata only. Infection occurred more frequently on the lower surface, due to the greater number of stoma. Again, lesions appeared 3–4 weeks after inoculation and were pale green regions on the leaf which became necrotic with time. *Teratosphaeria parva* germinated on the leaf surface but failed to infect (Park 1984). Through these infection studies using *M. cryptica*, *M. nubilosa* and *T. parva* on *E. globulus* in eastern Australia, Park (1984) concluded that *M. cryptica* and *M. nubilosa* were primary pathogens, whilst *T. parva* was a saprophyte, since it was only able to invade lesions caused by *M. nubilosa.*

Jackson (2001) investigated the infection and disease development of *M. nubilosa* and *M. cryptica* on *E. globulus* and three eucalypt species endemic to WA; *E. marginata, E. diversicolor* and *C. calophylla*. *Mycosphaerella nubilosa* ascospores germinated after three days on all eucalypt species except *E. marginata*. Ascospores penetrated stoma of *E. globulus* and *E. diversicolor* after six days. Hyphal swellings, like those observed by Park (1984), were observed on the abaxial surface of *E. globulus*, but they could not be confirmed to be appressoria (Jackson 2001). Lesions were evident on *E. globulus* seedlings after thirteen weeks (Jackson 2001). Ascospores of *M. cryptica* germinated and infected leaves of *E. globulus*, *E. marginata* and *E. diversicolor*, known hosts of *M. cryptica* after 3–6 days (Jackson 2001). Infection occurred direct via stoma openings and directly through appressoria. Lesions were only observed on *E. globulus* 12 weeks after inoculation. Jackson (2001) also investigated the infection of *M. marksii* and *Uwebraunia dekkeri* (as *Dissoconium dekkeri*) on *E. globulus*. Although *U. dekkeri* conidia germinated,

they did not infect leaves, however, *M. marksii* ascospores were observed to infect leaves via stoma only.

Park (1984) also investigated several factors that may have affected infection of *M. nubilosa* and *M. cryptica*, including the concentration of ascospores in suspension. He found that the ascospore concentration of the inoculum suspension had an effect on disease development. The higher concentrations of *M. nubilosa* caused lesions followed by defoliation while lower concentrations only produced lesions. At the lower concentrations, the onset of the disease was slower than at the higher concentrations. At the higher concentrations of *M. cryptica*, similar results were obtained for that of *M. nubilosa*; however, lower concentrations were unsuccessful. Park (1984) believed this was due to poor viability of ascospores. Jackson (2001) found that disease development of *M. nubilosa* took 12 weeks before lesions appeared on *E. globulus*. This may have been due to the low concentration of the ascospore suspension. Therefore, the use of other surfactants to increase ascospore numbers should be investigated.

A *Mycosphaerella* species of increasing importance in Western Australia is *M. marksii* (Maxwell 2004; Jackson et al. 2008; Chapter 3). Jackson (2001) conducted a small trial to test infection and pathogenicity of *M. marksii* on *E. globulus*. That study concluded *M. marksii* ascospores could infect leaves via stomata, however, no lesions were observed after 13 weeks.

The aim of Chapter 5 was to identify the infection pathway at the leaf aurface using scanning electron microscopy and to determine the pathogenicity of *M. marksii* on *E. globulus*.

5.2 METHODS

5.2.1 Inoculation of *Eucalyptus globulus* leaves

Three excised healthy, fully formed juvenile leaves of *E. globulus* were placed abaxial surface up in the lid of a 9 cm Petri-dish on top of damp paper towel. Lesions from naturally infected *E. globulus* leaves with pseudothecia on the adaxial surface that had been soaked for a minimum of 1 h and dried on the adaxial surface were placed on the bottom of the Petri-dish. The dish was placed in the dark at 20°C. This was replicated three times. One leaf of each treatment was harvested for clearing and staining 3 and 6 days after inoculation. Lesions used in this experiment were then attached to the lid of a Petri-dish with the bottom containing 2% MEA inverted over the lesions in order to confirm the *Mycosphaerella* and *Teratosphaeria* species present.

5.2.2 The use of glycerol for the preparation *Mycosphaerella marksii* ascospore suspension

Six concentrations of glycerol mixed with sterile water were made up (0%, 10%, 25%, 50%, 75% and 100%). Infected *E. globulus* leaves with necrotic lesions with pseudothecia characteristic of *M. marksii*, were excised and soaked for at least 1 hr in water, dried with paper towel and placed onto Petri-dish lids. An empty base of a

Petri dish was inverted over the leaf segments and left at room temperature for 24 hr to allow for ascospore discharge. A total of 30 plates were prepared and examined for discharged ascospores. Plate bases that contained ascospores were treated with a 10 µl aliquot of a particular concentration of the glycerol solution. Each aliquot was agitated with a pipette tip and then placed into a PCR tube for storage. A 1 µl sub-sample was then placed on a glass microscope slide and ascospores were counted at x200 magnification using a BH-Olympus Model microscope. Five 1 µl sub-samples from each tube were placed on fresh 2% MEA plates and incubated at 20°C in the dark to determine ascospore viability.

5.2.3 Clearing and staining

Harvested leaves were placed in clearing solution [1:3 lactic acid (80%) to absolute ethanol] at 60°C for one to six hr. Cleared leaves were rinsed in tap water and stained with aniline blue CI 42755 (0.05% w/v) at 60°C for one hr. Leaves were mounted onto microscope slides with lactoglycerol and examined at x400 magnification using an Olympus BH2 light microscope. Leaf pieces on which ascospores were observed were dissected from the leaf and prepared for SEM.

5.2.4 SEM specimen preparation

Leaf segments (50 mm^2) with germinating ascospores were rinsed in tap water to remove the lactoglycerol before being placed onto a microscope slide and air dried in a hot air drying cupboard for three days. Each segment was mounted onto an

aluminium stub using carbon glue tabs, and sputter coated with gold and examined under a Philips XL 20 SEM at 15 kv.

5.2.5 Uncontrolled pathogenicity testing of *Mycosphaerella marksii* on *Eucalyptus globulus* seedlings

Lesions characteristic of *M. nubilosa*, *M. cryptica* or *M. marksii* were cut out of naturally infected *E. globulus* leaves, soaked for at least one hour, patted dry with paper towel and attached using double sided adhesive in order to cover ten Petri-dish lids. The base of the plate containing 2% MEA was inverted over lesions and left at room temperature over night. Single spore isolations were made to confirm species that had discharged ascospores. From each plate (ten in total), five single ascospores were removed and placed on fresh 2% MEA, giving a total of 50 isolations for the experiment. Plate lids containing either *M. nubilosa*, *M. cryptica* or *M. marksii* lesions were then transferred to one of four large white opaque plastic containers (32 x 42 x 30 cm) and placed with double sided adhesive to the interior of the container: four plates on the top, two each length ways and one at each end. Plates were misted with water.

Eucalyptus globulus seedlings were sourced from an open-air commercial tree nursery and had not been treated with fungicide. They were ca. 6 months old and 30 cm in height and ten each of three different genetics (FM 001, FM 004, FM 009), were tagged and placed randomly in a seedling tray, a total of 30 seedlings per tray, three test trays in total. Each tray was then placed into a container with enough water to just cover the bottom (Fig. 5.1). The control seedlings were set up in the

same manner, with three seedlings from each family, i.e. a total of nine control seedlings. Plants were sprayed until dripping with water. The container lids with the plates with the lesioned leaves were then placed over the plants and placed at 20°C in an incubator in the dark. The controls were treated in the same manner, except no lesioned plant material was placed in the container. The plants were removed for two hours the following day and sprayed twice with water until run-off then they were returned to the incubator for a further 24 hrs. The plants were then removed from the incubator and the lid removed from each container and left at room temperature for the remainder of the experiment.

Figure 5.1 *Eucalyptus globulus* seedlings tagged and placed in container **(A)**; Petri dish lids with *Mycosphaerella marksii*-like lesions attached to the inside of the container **(B)** and the fully enclosed container containing both the seedlings and the lesions **(C)**.

5.3 RESULTS

5.3.1 The use of glycerol as a solution for a *Mycosphaerella marksii* ascospore suspension

The use of glycerol as a medium to wash ascospores from the Petri-dish surface was deemed inadequate. Although the number of ascospores increased with the increasing concentration of glycerol with 75% being the optimum level (Table 5.1),

the viability of the ascospores was compromised, with no germination observed at 75% or 100% glycerol concentration (Figure 5.2). This technique was therefore discontinued for the pathogenicity component of the chapter.

Table 5.1 The number of *Mycosphaerella marksii* ascospores from a 1 µl aliquot visualised at x200 magnification at six different concentrations of glycerol from five replicate plates

Glycerol concentration (%)	**No. of observed ascospores**				
	Plate 1	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
0	0	0	0	0	0
10	0	0	0	0	0
25	0	0	2	0	0
50	0	0	0	0	0
75	74	26	41	10	382
100	0	37	9	2	1

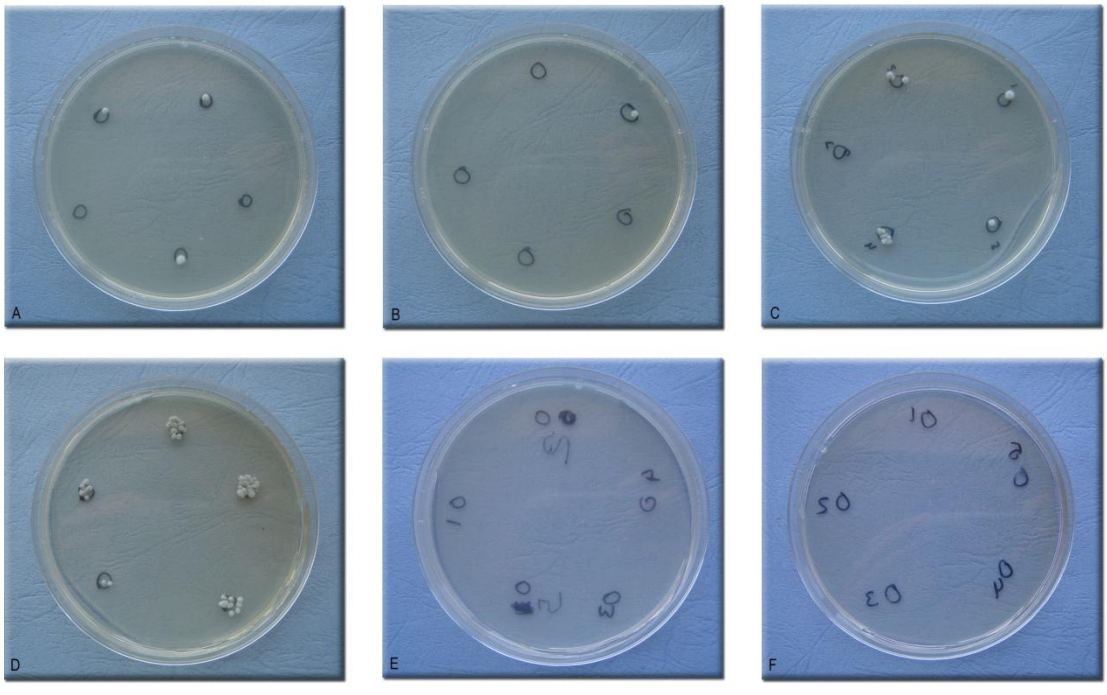


Figure 5.2 *Mycosphaerella marksii* ascospore viability test using six concentrations of glycerol; 0% **(A)**; 10% **(B)**; 25% **(C)**; 50% **(D)**; 75% **(E)** and 100% **(F)**. Colonies were visible four days after germination.

5.3.2 Infection of *Mycosphaerella marksii* on *Eucalyptus globulus* leaves

Mycosphaerella marksii ascospores were observed germinating on the surface of *E. globulus* leaves 3–6 days after inoculation (Figure 5.3). Hyphal swellings were also observed that may have allowed for direct penetration of the leaf surface (Figure 5.4). *Mycosphaerella marksii* was observed to infect leaves via stoma (Figure 5.5).

5.3.3 Uncontrolled pathogenicity testing of *Mycosphaerella marksii* on *Eucalyptus globulus* seedlings

Identification of *Mycosphaerella* species from the lesioned *E. globulus* leaves was based on ascospore germination and culture morphology after one month incubation at 20°C. From the 50 isolations made from *M. nubilosa*-like lesions, 33 were *M. nubilosa*, 15 were unidentifiable *Mycosphaerella* species and 2 were of another genus. All 50 isolations made from *M. marksii*-type lesions or *M. cryptica*-like lesions were *M. marksii* and *M. cryptica*, respectively.

There was no effect of host genetics on lesion formation by *M. cryptica* or *M. nubilosa*. Lesions and pseudothecia formation were apparent on the younger foliage four weeks and eight weeks after initial infection, respectively (Figure 5.6). Lesions were observed on both the control and *M. marksii* inoculated plants; however, they were only present on the older juvenile leaves, indicating that they were not caused

by the inoculum source. Pseudothecia were only observed on the lesion on the abaxial surface of the leaf. No pseudothecia were observed on the adaxial surface at anytime throughout the 16 weeks trial period.

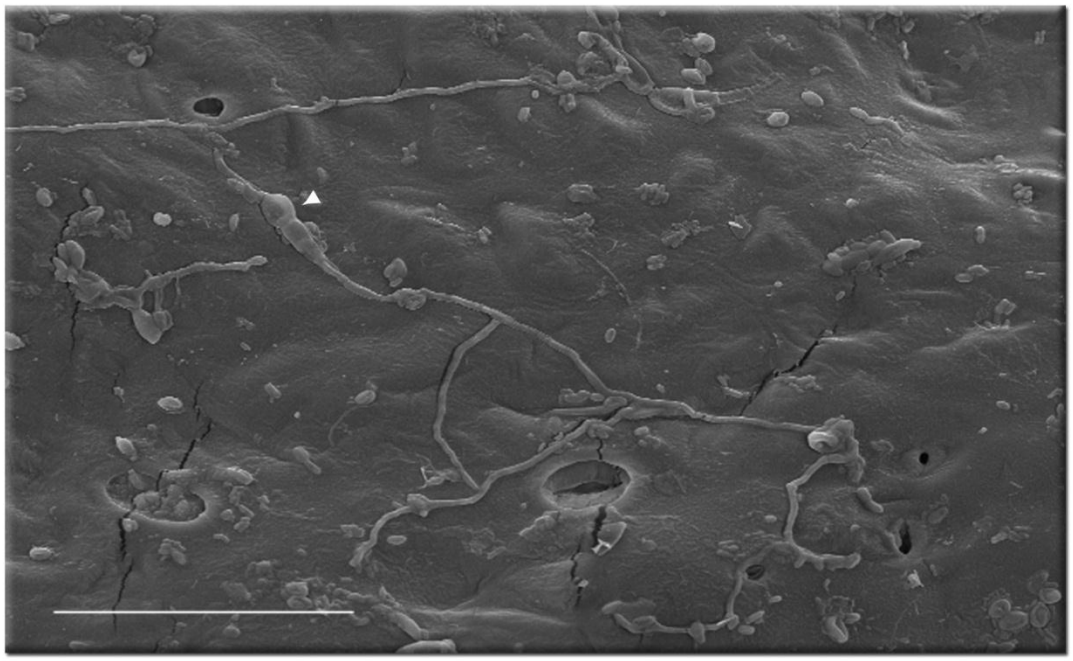


Figure 5.3 A *Mycosphaerella marksii* ascospore (arrow head) germinating on the abaxial side of a *Eucalyptus globulus* leaf surface. Bar = 50 µm.

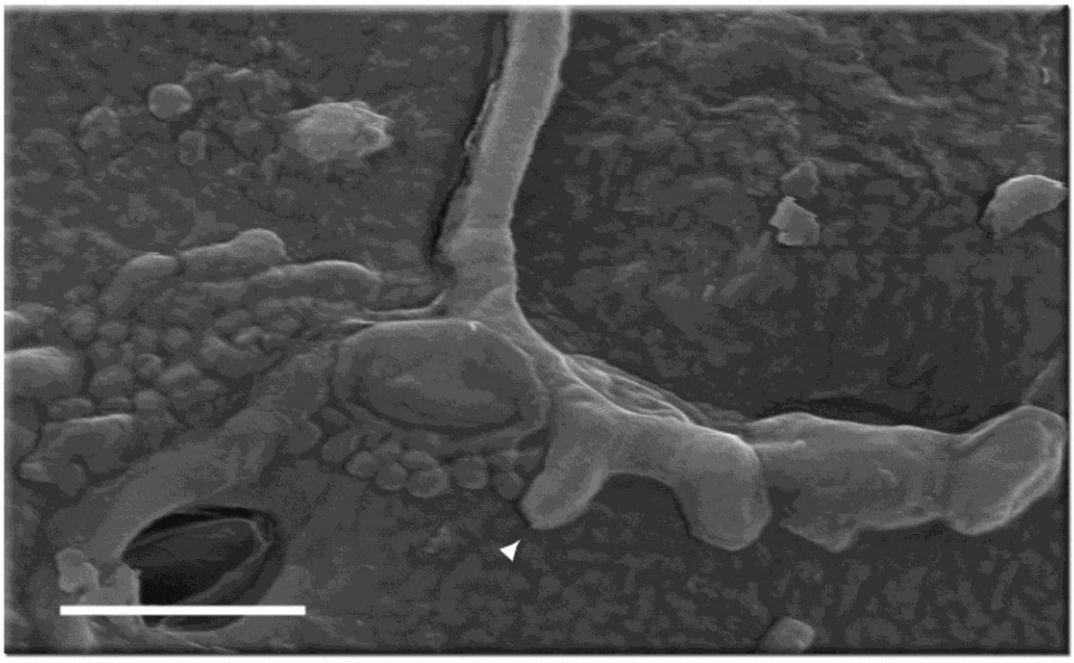
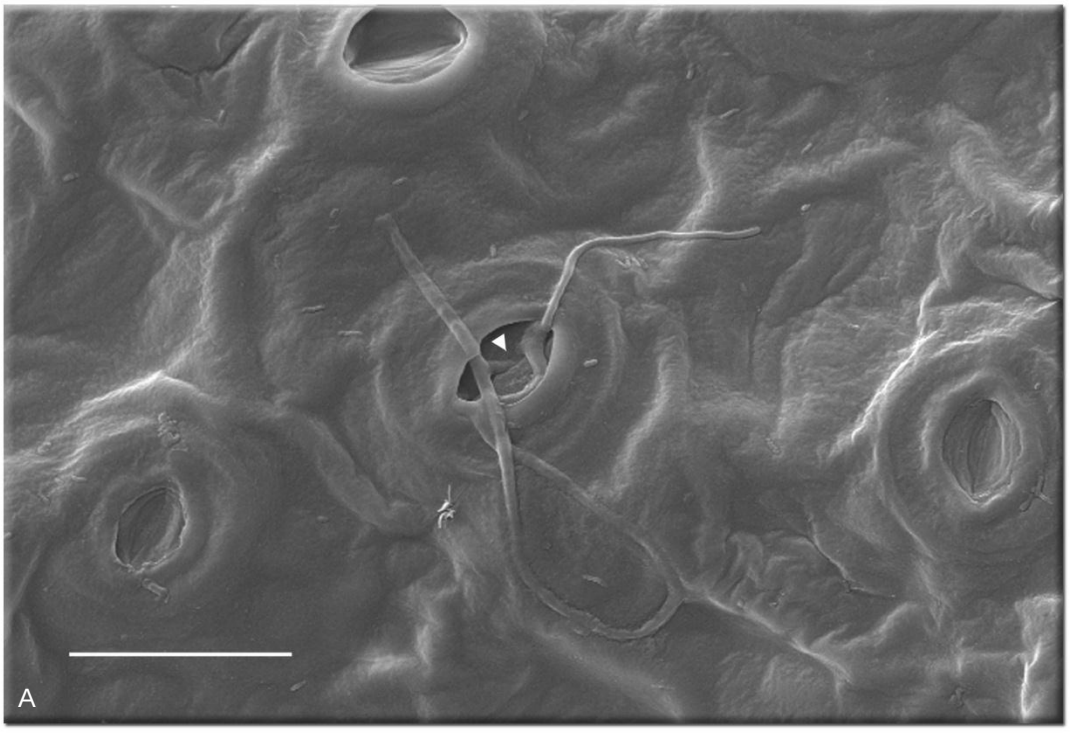


Figure 5.4 Magnified section of leaf above with possible hyphal swelling (arrow) of *Mycosphaerella marksii* on a *Eucalyptus globulus* leaf surface. Bar = 5 µm.



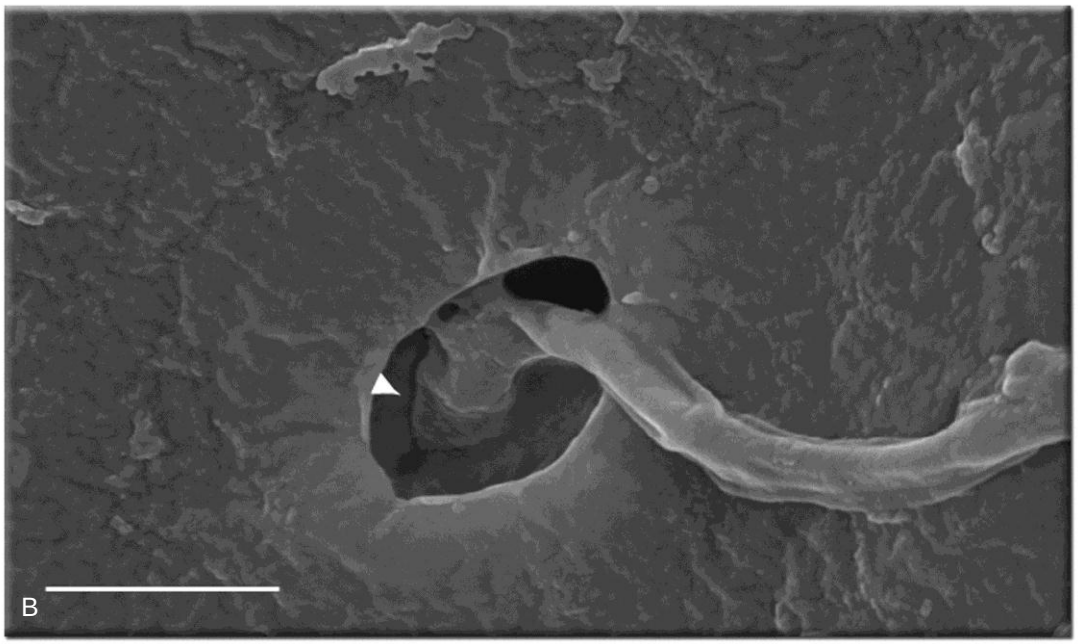


Figure 5.5 *Mycosphaerella marksii* hyphae infecting a stoma (arrow) on the abaxial surface of a *Eucalyptus globulus* leaf. A. Bar = 20 µm B. Bar = 5 µm.

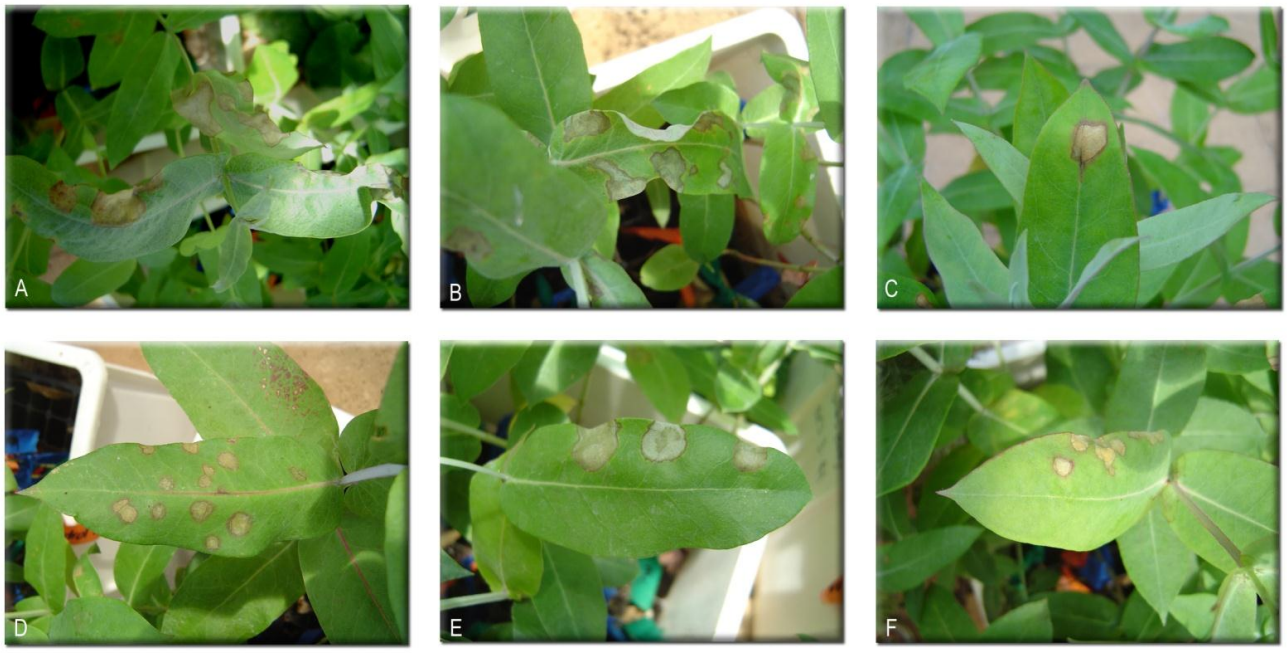


Figure 5.6 Lesion developed on *Eucalyptus globulus* six weeks after uncontrolled infection of *Mycosphaerella cryptica* (**A** and **B**) and *M. nubilosa* (**C–E**). Lesions of unknown *Mycosphaerella* species were also observed on mature leaves of control plants (**F**).

5.4 DISCUSSION

This study was unable to confirm pathogenicity of *M. marksii* on *E. globulus* seedlings under laboratory conditions. However, *M. marksii* ascospores were able to germinate and enter *E. globulus* stoma 3–6 days after initial infection. The use of glycerol as a surfactant in an ascospore suspension increased ascospore retrieval from Petri-dish lids, but compromised the ascospore viability at high concentrations. Therefore, the use of glycerol in the current study did not yield a reliable ascospore suspension. The amount of lesions required to suspend enough ascospores in 1 µl of suspension to inoculate leaves was deemed too time consuming.

Jackson (2001) used Tween 80 as a surfactant for infection studies and found that using Tween 80 a smaller proportion of ascospores of *M. nubilosa* germinated than with water alone. The SEM confirmed infection of *M. marksii* via stoma seen by Jackson (2001). There also appeared to be hyphal swellings, possibly indicating a direct method of penetration of the leaf surface. This requires further investigation.

Lesions with pseudothecia of *M. marksii* did not develop after 16 weeks. Jackson (2001) also did not observe any lesion development on plants infected with *M. marksii* after 13 weeks. Lesions of *M. nubilosa* with pseudothecia were seen on plants infected with *M. marksii* ascospores, on older juvenile leaves and also on older juvenile leaves of several control plants. This may have been due to leaves

being infected at the start of the experiment as the seedlings were sourced from an open-aired nursery and had not been treated with fungicides. After 8 weeks, defoliation of the lower foliage was evident and coincided with an increase in temperature and/or nutrient depletion.

Mycosphaerella marksii lesions with pseudothecia on the adaxial surface were often observed in *E. globulus* plantations in WA on older pre-senescing juvenile leaves and often in association with other *Mycosphaerellal Teratosphaeria* species (Maxwell 2004). It should be noted that Carnegie et al. (1997) first isolated *M. marksii* in WA on *E. globulus* and *E. botryoides*; however, its impact was considered minor. The only other *Mycosphaerella* species isolated during that survey were *M. suberosa* and *M. cryptica*. Later, Maxwell (2005) isolated *M. marksii* from 9% of juvenile *E. globulus* foliage surveyed, while Jackson *et al.* (2008) isolated *M. marksii* from 77% of juvenile *E. globulus* foliage.

Hewison (2006) investigated the infection and pathogenicity of *M. cryptica* and *M. nubilosa* ascospores on *E. gomphocephala* seedlings and used a spore suspension containing Tween 80, together with the placement of lesions directly to uninfected leaves on intact plants or on excised leaves attached to a Petri-dish lid over lesioned leaves. That study found that shedding ascospores directly onto leaf material from a distance gave a higher proportion of infection compared to the spore suspension or lesioned leaf tissues placed directly onto leaves. Smith (2006) sprayed resistant and non-resistant *E. globulus* seedlings with an inoculant of homogenised lesions. Lesions were observed 74 days after inoculation and this delay to lesion

development was attributed to cold weather. However, Park (1984) suggested that a minimum number of spores may be needed for infection to occur, therefore the delayed infection in his trial may have been due to insuffiecient inoculum. The inoculum used by Smith (2006) was not a pure spore load; therefore the delayed infection may also have been due to the inoculum source.

In the current study, infection of *M. marksii* was not observed. This may have been due to low inoculum levels, non optimal infection conditions, or as *M. marksii* lesions are most often seen on older juvenile leaves, the experiment may not have been conducted for long enough. Nevertheless, it would appear that *M. marksii* would not be considered an important nursery pathogen of young *E. globulus* seedlings. However, further evaluation and experimentation is required. This line of research was not pursued further in the thesis as it was felt that more emphasis should be given to MLD epdidemiology in the field (Chapter 6).

5.5 REFERENCES

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CHAPTER 6

SEASONAL DEVELOPMENT OF MYCOSPHAERELLA LEAF DISEASE IN *EUCALYPTUS GLOBULUS* NEAR MT BARKER, WESTERN AUSTRALIA

6.1 INTRODUCTION

Very few field-based epidemiological studies have been conducted under field conditions on Mycosphaerella leaf disease (MLD). One of the reasons for this is the difficulty involved with the number of species contributing to the disease complex. With the exception of *M. cryptica* and *M. nubilosa* (Park 1984), the pathogenicity of most eucalypt-associated *Mycosphaerella* and *Teratosphaeria* species is unknown. Many *Mycosphaerella*/ *Teratosphaeria* species have been isolated in association with each other and how they interact remains unknown. It is possible that some species sporulate on necrotic tissue and are not primary pathogens, or that they can co-exist within the same niche for a period of time before competition exhausts nutritional resources or the environment changes in some way (Fitt *et al.* 2006). Many studies on MLD provide only a snapshot of what is occurring within a plantation (Jackson *et al.* 2008) and continual temporal systematic sampling is not undertaken. This is mainly due to the large plantation estate in Australia and the time constraints in conducting eucalypt plantation health surveys.

The Crown damage index (CDI) (Stone *et al.* 2003) can be used to monitor the health of a plantation using a standardised method of assessment. The CDI is based on the most commonly encountered types of crown damage, defoliation, necrosis and discolouration (Stone *et al*. 2003). It is not used to identify the cause of the damage, nor the impact on long-term growth (Stone *et al.* 2003) both of which can be difficult to determine. The CDI has been tested for accuracy and precision of pest and disease damage in *Eucalyptus* plantations in Tasmania. Smith *et al*. (2005)

reported that incidence of foliar necrosis was the most accurate parameter estimated by assessors, with 75% of estimates within +/- 10% of the overall mean of all assessors. The greatest factor affecting assessment accuracy was the experience of the assessors with differences between morning and afternoon assessments decreasing with increased experience (Smith *et al.* 2005). Other factors that may affect the accuracy of the assessor using the CDI are described by Stone *et al.* (2003), and include environmental factors such as light quality, tree genetics and the interaction of the tree with site conditions.

Morphological taxonomy of *Mycosphaerella* and *Teratosphaeria* species is often regarded as difficult (Chapter 3, 4). This is because there are very few distinguishable characters, particularly with *Mycosphaerella* and *Teratosphaeria* species isolated from eucalypts. They have traditionally been differentiated based on ascospore size, shape, germination pattern (Crous 1998) and host. However, recently several *Mycosphaerella* species first described on *Eucalyptus* have been found on different plant genera, such as *M. marksii* on *Leucadendron tinctum* (Crous *et al*. 2006). Likewise, there are a number of species listed on GenBank that have the same ITS sequence, but have been described as different taxa primarily based on the host (Chapter 1). An example is *M. buckinghamiae*, isolated from a *Buckinghamia* sp., which matches 100% with *M. aurantia* from *E. globulus* (Crous *et al*. 2000; Maxwell *et al*. 2003) (Chapter 4). Other difficulties in defining species within this genus are that morphological characteristics often overlap with other species and there can also be variation within a species (Maxwell *et al*. 2005). As mentioned in Chapter 1 the anamorphic stage can also be used for differentiation, however, it

may take weeks or months for an anamorph to be produced in culture, or specific media may be required (Maxwell *et al*. 2005). Therefore, the design of primers that are specific to a particular species can be a useful tool for fast identification or for use in epidemiological studies (Maxwell *et al*. 2005; Glen *et al*. 2007).

Following on from Chapter 4, this chapter aims to document the seasonal development of MLD at one site in WA. To achieve this, a number of methods were developed and applied. Specific objectives were to:

- develop and test species-specific primers for the less frequently isolated *Mycosphaerella*/ *Teratosphaeria* species on *E. globulus* in WA;
- test the reliability and reproducibility of specific primers to detect and identify *Mycosphaerella* infection in latent, early and advanced stages of disease expression;
- determine the occurrence and succession of *Mycosphaerella*/ *Teratosphaeria* species involved in MLD over a period of a year from ten 1 year-old plantation trees;
- determine the level of defoliation in juvenile foliage in a plantation over a year;
- and determine the level of defoliation in juvenile foliage in a plantation over a year and to compare the accuracy of the CDI assessment method as applied by 13 assessors against ASSESS (Lamari 2002) an image software based system for measuring MLD.

6.2 METHODS

6.2.1 Development of a molecular test for Mycosphaerella leaf disease causing species from leaf material

6.2.1.1 Species-specific primer design and testing

Specific primers were designed according to Maxwell *et al.* (2005). Briefly, the consensus sequences for the ITS1f/ ITS4 rDNA region for ten *Mycosphaerella* and/or *Teratosphaeria* species were imported, at that time, *U. dekkeri* was *M. lateralis* (Maxwell et al. 2005) along with all other available *Mycosphaerella*/ *Teratosphaeria* species on the National Centre for Biotechnology Information (NCBI) GenBank database, into GeneTool (ver. 1.01 BioTools Inc Applications, 1998). Forward and reverse primers were designed in the 'sequence editor' module of GeneTool for *M. marksii*, *T. parva* and *U. dekkeri*. Primer sites were chosen from the variable (ITS-1 or ITS-2) regions of the rDNA that were within the 17–23 nt size range and with a Tm of 54–61°C that were free of structural impediments to annealing, and that would amplify a product of 300–400 nt. These were screened against all available sequences of each species worldwide in order to ensure their activity against their known populations. The sequences of all other *Mycosphaerella*/ *Teratosphaeria* species on the NCBI database, were downloaded, aligned and searched for matches to those primer sites to ensure species specificity. The basic local alignment search tool (BLAST) software available on the NCBI database was used to ensure that the primer sites were not present on other fungal species associated with eucalypts, or in the host plant DNA. Forward and reverse primers

specific for *U. dekkeri* (UD1F and UD1R), *M. marksii* (MM1F and MM1R) and *T. parva* (TP1F and TP1R) (Table 6.1) were tested against DNA extracts from each of the species listed in Table 6.2.

Table 6.1 List of species-specific primers developed for three species causing Mycosphaerella leaf disease on *Eucalyptus globulus* foliage in Western Australia. Table first published as Table 2 in Maxwell *et al.* (2005) and amended by the current author.

Species	Primer name	Direction	Sequence (5 k–3 k)	Length (nt)	Tm (°C)
M. marksii	MM1F	Forward	cggcccgacctccaacc	17	57
M. marksii	MM1R	Reverse	gatgccacaacgctcggaga	20	55
T. parva	TP1F	Forward	cctccgggctcgacctcca	19	60
T. parva	TP1R	Reverse	tctcgcaagcggatgattaaacc	23	55
U. dekkeri	UD1F	Forward	aaacgccggggccttcg	17	54
U. dekkeri	UD1R	Reverse	cgacgtctccgccgatgttttcc	23	61

6.2.1.2 Species-specific primer sensitivity on fungal DNA

The sensitivity of the primer pairs was tested in PCR reactions against known amounts of DNA for each target species in a dilution series. The PCR reactions were conducted with the following amounts of DNA template: 10 ng, 1 ng, 100 pg, 10 pg and 1 pg for three isolates of each species: *U. dekkeri* (MURU 253–255), *M. marksii* (MURU 234, 242, 243) and *T. parva* (MURU 012, 013, 250) in 25 µl reactions as described in Maxwell *et al.* (2005). PCR products were electrophoresed on a 1% agarose gel in Tris-acetate (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) which had 0.5 µg/ml ethidium bromide added to it during preparation, run at 90V for 20 min. The products were visualised under UV.

6.2.1.4 Testing of species-specific primers against leaf material

Non-diseased leaves and leaves with lesions typical of *M. marksii* and multiple species infection were collected from three *E. globulus* plantations near Albany in Western Australia. Lesion pieces ranging from 2–50 mm^2 were dissected from leaves that had been washed in a solution of sodium hypochlorite (2.5% w/v) for 30 sec and rinsed three times in deionised water in order to remove surface spores. DNA was extracted and quantified and adjusted as described in Maxwell *et al.* (2005). Maxwell *et al.* (2005) previously tested the sensitivity of the PCR-based technique using a *M. nubilosa*-specific primer-pair against DNA extracts from three replicate *E. globulus* leaves. PCR reactions (25 ml with 1 ng template DNA) were conducted to test the effectiveness of the three species-specific primers on DNA extracted from ten lesions from the following five categories:

- non-infected leaves at the growing tip of the branch;
- *M. cryptica* lesions characterised by ascomata densely arranged on both surfaces of young juvenile leaves with a waxy bloom;
- *M. marksii* lesions characterised by ascomata only on the adaxial leaf surface of young juvenile leaves ;
- *M. nubilosa* lesions characterised by lesions only on the abaxial leaf surface of young leaves; and

- mixed species lesions characterised by lesions on older juvenile leaves without a waxy bloom with ascomata densely arranged on the abaxial surface and more sparsely arranged on the adaxial surface.

A 100 mm^2 piece of each lesion was dissected and single ascospore isolations made according to Crous (1998) in order to identify the species associated with each lesion using conventional means (Chapter 2). Briefly, this involved measuring spore size within asci (if applicable), ascospore or conidia germination patterns on release and cultural morphological characteristics of colonies on 2% MEA. Whole leaf pieces or excised lesions were soaked in water for at least 2 hr before being dried with paper towel and attached using double sided adhesive tape, to the lid of a Petri-plate containing 2% MEA. Plates were inverted and left at room temperature for 24 hr (Maxwell *et al*. 2003). Single spore isolations were made at this time by transferring spores to new 2% MEA plates and incubated at 20 C in the dark. Slides were made at the same time as isolation. A small piece of agar containing the spores was transferred to a microscope slide with a drop of lactoglycerol [85% lactic acid, glycerol and DI water, 1:1:1 (v/v)], gently warmed and a coverslip placed over the agar and gently squashed. Spores were then viewed under an Olympus BH-2 microscope at ×100, 400, and 1000 magnification. All slides were made permanent by sealing the cover slip with nail varnish. Germination patterns were compared to those described by Crous (1998) and Maxwell *et al*. (2003).

The products from the species-specific PCR reactions using DNA extracted from leaves were separated on agarose gels and visualised as previously described in

section 6.2.1.2. PCR products were purified and sequenced in 10 µl reactions using their respective specific primers as described by Maxwell *et al.* (2005). Each sequence was used in a BLAST search to identify those sequences that were most homologous on the database.

Table 6.2 Mycosphaerella leaf disease causing isolates for which ITS rDNA sequences were screened and tested for species-specific primer development for *U. dekkeri*, *M. marksii* and *T. parva*. Table first published as Table 1 in Maxwell *et al.* (2005) and amended by the current author.

Species	**Isolate no. (MURU)[a]**	**GenBank accession no.**	**Host (Eucalyptus)**	**Origin[b]**	**Isolates sequenced (S) and DNA primers tested (T) against**
Mycosphaerella aurantia	1	AY509743	*E. globulus*	WA	S
M. aurantia	2	AY509744	*E. globulus*	WA	S
M. aurantia	151	AY150331	*E. globulus*	WA	S, T
M. aurantia	152	AY509742	*E. globulus*	WA	S
M. aurantia	340	AY509742	*E. globulus*	WA	T
M. colombiensis	352		*E. camaldulensis*	VTN	T
M. cruenta	353		*E. camaldulensis*	VTN	T
M. cryptica	089	AY509747	*E. globulus*	WA	S, T
M. cryptica	090	AY509748	*E. globulus*	WA	S, T
M. cryptica	091	AY509749	*E. globulus*	WA	S, T
M. cryptica	101	AY509750	*E. globulus*	WA	S, T
M. cryptica	102		*E. globulus*	WA	S, T
M. cryptica	110	AY509751	*E. globulus*	WA	S, T
M. cryptica	114	AY509752	*E. diversicolor*	WA	S, T

Species	Isolate no. (MURU)[a]	GenBank accession no.	Host (Eucalyptus)	Origin[b]	Isolates sequenced (S) and DNA primers tested (T) against
M. cryptica	115	AY509753	*E. globulus*	WA	S, T
M. cryptica	117		*E. grandis x camaldulensis*	Qld	S, T
M. cryptica	118	AY509754	*E. delegatensis*	Vic	S, T
M. cryptica	120		*E. globulus*	Vic	S, T
M. cryptica	145		*E. diversicolor*	WA	S
M. ellipsoidea	237	AY509755	*E. globulus*	WA	S, T
M. ellipsoidea	240	AY509757	*E. globulus*	WA	S
M. ellipsoidea	246	AY509756	*E. globulus*	WA	S
M. marksii	234	AY509764	*E. globulus*	WA	S, T
M. marksii	242	AY509767	*E. globulus*	WA	S, T
M. marksii	243	AY509766	*E. globulus*	WA	S, T
M. marksii	247	AY509765	*E. globulus*	WA	S
M. marksii	178		*E. globulus*	WA	T
M. marksii	179		*E. globulus*	WA	T
M. marksii	180		*E. globulus*	WA	T
M. marksii	181		*E. grandis x camaldulensis*	WA	T
M. marksii	182		*E. grandis x camaldulensis*	WA	T
M. marksii	183		*E. grandis x camaldulensis*	WA	T

Species	Isolate no. (MURU)[a]	GenBank accession no.	Host (Eucalyptus)	Origin[b]	Isolates sequenced (S) and DNA primers tested (T) against
M. marksii	184		*E. grandis x camaldulensis*	WA	T
M. marksii	185		*E. grandis x camaldulensis*	WA	T
M. marksii	186		*E. globulus*	Qld	T
M. marksii	187		*E. globulus*	Qld	T
M. marksii	188		*E. pellita*	Qld	T
M. marksii	189		*E. grandis*	Qld	T
M. marksii	190		*E. dunnii*	Qld	T
M. marksii	191		*E. dunnii*	Qld	T
M. marksii	192		*E. dunnii*	Qld	T
M. marksii	193		*E. tereticornis*	Qld	T
M. marksii	194		*E. diversicolor*	WA	T
M. marksii	195		*E. tereticornis*	Qld	T
M. marksii	196		*E. rudis*	WA	T
M. molleriana	3		*E. globulus*	WA	S, T
M. molleriana	211	AY150675	*E. globulus*	WA	S, T
M. molleriana	200		*E. globulus*	Tas	T
M. nubilosa	301		*E. globulus*	WA	S, T
M. nubilosa	302	AY509775	*E. globulus*	WA	S, T

Species	Isolate no. (MURU)[a]	GenBank accession no.	Host (Eucalyptus)	Origin[b]	Isolates sequenced (S) and DNA primers tested (T) against
M. nubilosa	304	AY509776	*E. globulus*	WA	S, T
M. nubilosa	051	AY509777	*E. globulus*	Vic	S, T
M. nubilosa	052		*E. globulus*	Vic	T
M. nubilosa	055		*E. globulus*	Vic	T
M. nubilosa	056		*E. globulus*	Vic	T
M. nubilosa	057	AY509778	*E. globulus*	Vic	S, T
M. nubilosa	025	AY509772	*E. globulus*	WA	S, T
M. nubilosa	026	AY509773	*E. globulus*	WA	S, T
M. nubilosa	328		*E. grandis x resinifera*	WA	T
M. nubilosa	317		*E. grandis x resinifera*	WA	T
M. nubilosa	329		*E. grandis x resinifera*	WA	T
M. nubilosa	346		*E. globulus x urophylla*	WA	T
M. suberosa	263		*E. globulus*	WA	S, T
M. suberosa	245		*E. globulus*	WA	S, T
M. suttoniae	327		*E. globulus*	WA	T
M. tasmaniensis	323		*E. globulus*	WA	T
Pseudocercospora fori	324		*E. globulus*	WA	T

Species	Isolate no. (MURU)[a]	GenBank accession no.	Host (Eucalyptus)	Origin[b]	Isolates sequenced (S) and DNA primers tested (T) against
T. mexicana	006	AY509768	*E. globulus*	WA	S, T
T. mexicana	007	AY509769	*E. globulus E.*	WA	S, T
T. mexicana	008	AY509770	*E. globulus*	WA	S
T. mexicana	197	AY509771	*E. globulus*	WA	S
T. parva	248	AY509779	*E. globulus*	WA	S, T
T. parva	012	AY509780	*E. globulus*	WA	S, T
T. parva	250	AY509781	*E. globulus*	WA	S, T
T. parva	013	AY509782	*E. globulus*	WA	S, T
T. parva	170		*E. globulus*	WA	T
T. parva	171		*E. globulus*	WA	T
T. parva	172		*E. globulus*	WA	T
T. parva	173		*E. grandis x camaldulensis*	WA	T
T. parva	174		*E. grandis x camaldulensis*	WA	T
T. parva	175		*E. grandis x camaldulensis*	WA	T
T. parva	176		*E. dunnii*	Qld	T
T. parva	213		*E. globulus*	WA	S, T
T. parva	337		*E. grandis x urophylla*	WA	T
T. parva[C]	204		*E. grandis*	Tas	T

Species	Isolate no. (MURU)[a]	GenBank accession no.	Host (Eucalyptus)	Origin[b]	Isolates sequenced (S) and DNA primers tested (T) against
U. dekkeri	252	AY509758	*E. maidenii*	Qld	S, T
U. dekkeri	253	AY509761	*E. maidenii*	Qld	S, T
U. dekkeri	254	AY509760	*E. globulus*	WA	S, T
U. dekkeri	255	AY509759	*E. globulus*	WA	S, T
U. dekkeri	256		*E. globulus*	WA	T
U. dekkeri	257	AY509762	*E. globulus*	WA	S, T
U. dekkeri	258	AY509763	*E. globulus*	WA	S, T
U. dekkeri	177		*E. globulus*	WA	T
Zasmidium citri	251		*E. camaldulensis*	VTN	T

[a] MURU, culture collection of Murdoch University.

[b] Origin of isolates: Vietnam (VTN), Western Australia (WA), Queensland, Australia (Qld), Victoria, Australia (Vic) and Tasmania, Australia (Tas).

[c] As *M. grandis*.

6.2.2 Field study of MLD over twelve months

6.2.2.1 Sampling leaves and observations

Ten one-year-old trees were selected at a genetics trial 15 km west of Mt Barker (34 59.39S, 117 61.25E) and rated for MLD on a monthly basis for 12 months, from June 2004–May 2005. One tree from two full sibling families was chosen from each of the five replicated plots. Therefore, there were five trees for each family, and ten trees in total. For each tree, six branches were tagged at chest height and labelled 1–6. Every month, each tree was assessed by eye for total tree MLD and defoliation, branch MLD, defoliation and insect feeding damage, and all observations were recorded as a percentage.

Leaves were removed from one side of the branch. These were numbered from closest to the main stem to the branch tip, bagged, scanned (Epson Perfection 610) and stored at -18°C until further analysis. After six months, the original branches were revisited consecutively each month and the remaining leaves were removed, numbered and tagged as described above. In the laboratory each leaf was assessed for three stages of MLD development in terms of absence, waxless, anthocyanic or necrotic lesions (Figure 6.1) and presence/absence of pseudothecia.

6.2.2.2 Disease assessment

Each image of the collected leaves from each branch was assessed for disease necrosis using ASSESS© (Figure 6.2). The percentage of total necrosis associated

with *Mycosphaerella* was calculated for each leaf and then a total obtained for each branch.

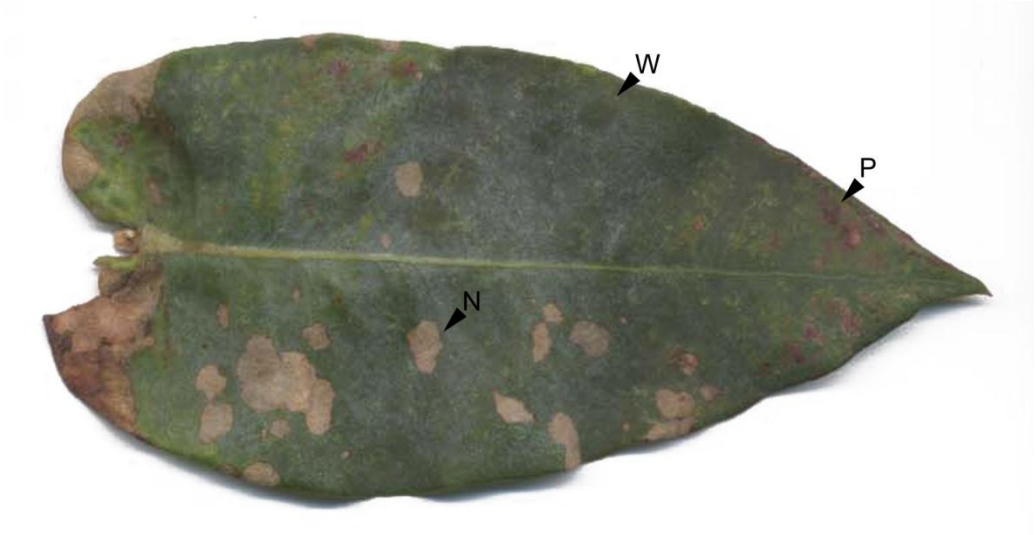

Figure 6.1 Leaf symptoms of Mycosphaerella leaf disease assessed during the field trial showing waxless appearance (W); anthocyanic (P) and necrotic lesions (N).

Weather data were collected from the Department of Agriculture and Food Western Australia's Mt Barker site (34 38.02S, 117 32.00E), the nearest weather station to the sampled plantation. Variables of temperature, rainfall and relative humidity were compared to rating data of MLD.

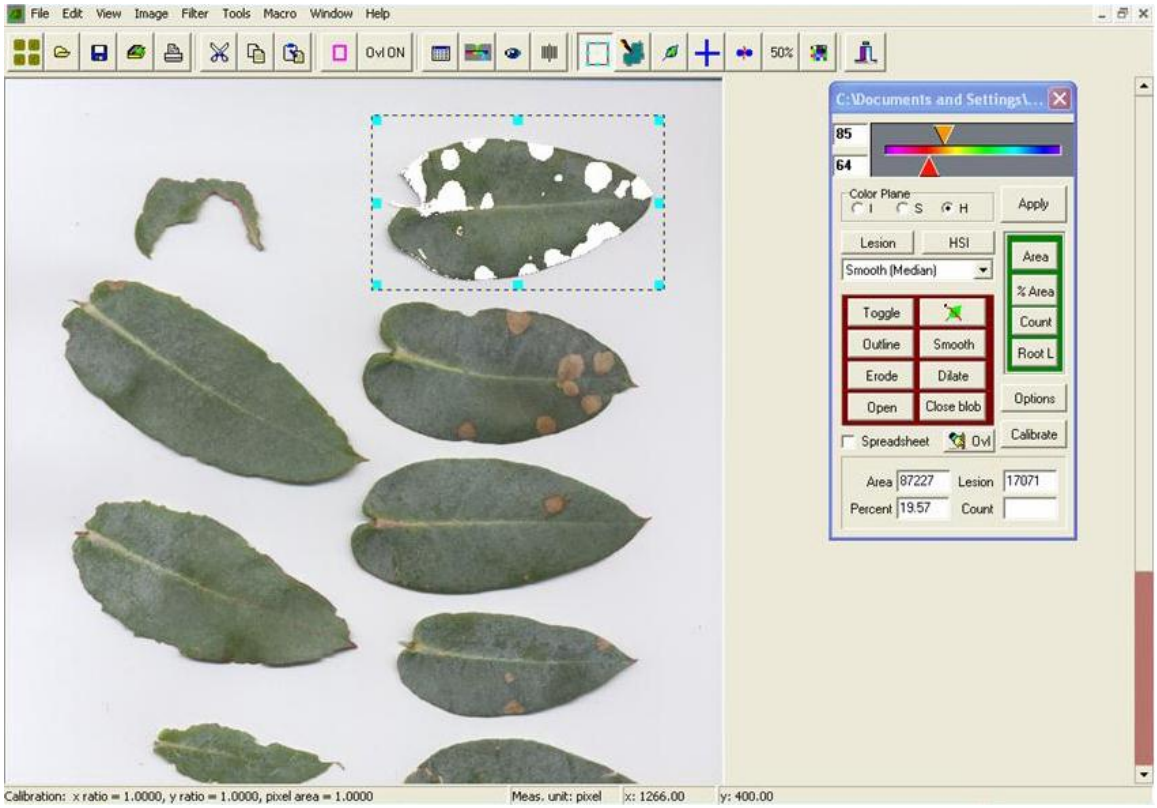

Figure 6.2 Assessing Mycosphaerella leaf disease leaf necrosis from leaves collected and scan from the field trial using the program ASSESS©.

6.2.2.3 Isolation and identification of Mycosphaerella leaf disease species

The isolation and initial identification of MLD species were carried out according to Crous (1998) and Maxwell *et al*. (2003) as described in Section 6.2.1.4. However, instead of using leaf pieces, the entire leaf was cut length ways and the adaxial surface of one half and the abaxial surface of the other half was secured facing the agar.

To facilitate identification, the pseudothecia were removed from lesions with a needle under a dissecting microscope. The pseudothecia were then mounted on a microscope slide with lactoglycerol and gently heated and squashed. Up to 30

measurements of length and width of ascospores within asci were made under an Olympus BH-2 microscope (x1000) for each species isolated.

Molecular identification was carried out according to Maxwell (2004). Briefly, the ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified using the primer pair ITS1-F (Gardes and Bruns 1993) and ITS4 (White *et al*. 1990). For selected isolates that were recalcitrant to PCR, nested PCR reactions were performed to amplify the ITS1 region and the ITS2 regions in separate reactions. For nested reactions 1 µl of 10^{-1} and 10^{-2} dilutions of PCR product from the primary reaction was used as template. The ITS1 region was amplified with primer pair ITS1 and ITS2 (White *et al*. 1990) and the ITS2 region amplified with primer pair ITS3 and ITS4 (White *et al*. 1990). The PCR reaction volumes, thermocycler parameters and visualisation of PCR products were as described in Maxwell (2004). Gel images were taken using a digital camera (EDAS 120, Kodak Digital Science™) under UV light and viewed using Kodak Digital Science™ ID (v 3.0.2) software.

Cleaned PCR products were sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA) using the primers listed above. Standard quarter reactions were performed using 2 µl ABI PRISM® BigDye Terminator Ready Reaction Cycle Sequencing Kit mix (3.0 or 3.1), 1.6 pmol primer (3.2 pmol for version 3.1), 80–160 ng PCR product. Sequencing reactions were performed according to the following parameters: initial denaturing step of 96°C for 2 min; then 25 cycles of 94°C (30 sec) denaturing, 50 C (5 sec) annealing, 60°C (4 min) extension; hold at 15°C. Products were then ethanol precipitated as per

Applied Biosystems recommendations. The products were separated by polyacrylamide gel electrophoresis on an ABI Prism 377 DNA automated sequencer (PE Applied Biosystems, Foster City, CA, USA).

6.2.3 Detection of *Mycosphaerella nubilosa* from leaf material over a six month period using species-specific primers

6.2.3.1 Mycosphaerella nubilosa *DNA extraction from leaf material using bulked lesions*

One 3 mm diameter disc was removed from each leaf from one side of a branch of each tree. Where possible, *Mycosphaerella* lesions were selected within the disc. For each branch, the discs were bulked together, placed in a microfuge tube, and stored at -20°C until required for DNA extraction. Discs were immersed in 300 µL of extraction buffer (200 µl; 200 mmol Tris HCl pH 8.5, 250 mmol NaCl, 25 mmol EDTA and 0.5% SDS; Raeder and Broda 1985) and incubated for 24 hr at 65°C. Samples were then centrifuged at 13 200 *g* (Beckman Microfuge E, Fullerton, CA USA) for 10 min. The DNA was purified using the Ultrabind DNA purification kit following the manufacturer's instructions (MO BIO Laboratories, Solana Beach, CA USA) with a few variations. Briefly, the supernatant was transferred into a microfuge tube containing 600 µL of NAI solution and 10 µL of silica slurry and incubated for 5 min at room temperature. This was centrifuged for 10 sec and the supernatant removed. The pellet was washed with 600 µL of Ultra Wash, centrifuged for 5 sec and the supernatant removed, followed by a 100% ethanol wash. The samples were centrifuged for 5 sec and the supernatant removed. The pellet was aspirated with a

pipette tip and air-dried until all moisture had evaporated. The pellet was resuspended in 25 µL of sterile water and incubated for 5 min at room temperature. The samples were then centrifuged for 1 min and the supernatant transferred to sterile 0.5 mL microfuge tubes and stored at -20°C.

6.2.3.2 DNA amplification from leaf material using Mycosphaerella nubilosa *species-specific primers*

The presence or absence of *M. nubilosa* was confirmed using a species-specific primer developed for this species (Maxwell *et al.* 2005). This species was investigated because of the availability of a highly specific primer (Maxwell 2004; Maxwell *et al.* 2005) and the high prevalence of the pathogen on juvenile leaves in *E. globulus* plantations in WA.

Each DNA sample was diluted 1:10 and 1:100 using sterile PCR water. The ITS1 and 2 regions of the rDNA were amplified using *M. nubilosa* specific primer linked with ITS1. DNA was amplified in a 25 µl reaction volume containing 1x polymerization buffer (67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg mL^{-1} gelatin, 0.2 mM dNTPS), 2.0 mM $MgCl_2$, sterile PCR grade water, 0.4 µM primer, 1–5 ng of DNA and 1.1 U Taq DNA polymerase (Fisher Biotec Australia). The PCR reactions were performed (Applied Biosystems, Foster City, CA USA; GeneAmp 2400 thermocycler) according to the following parameters: Initial denaturing step of 96°C for 2 min; then 35 cycles of 96°C (30 sec) denaturing, 60°C (30 sec) annealing, 72°C (2 min) extension; 7 min extension at 72°C; hold at 15°C. The PCR products were stored at -20°C cold room. All PCR assays contained a

sample without the template DNA as the negative control and a sample of 1:10 diluted fungal DNA targeted by each specific primer pair as the positive control. PCR amplified products (5 µl) were electrophoresed on a 1% agarose gel containing 0.5 µg ml^{-1} ethidium bromide at 90 V for 20–30 min with Tris-acetate (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0) as the running buffer. The size of PCR products were determined against either a 100bp (FN1 Fisher Biotech Australia) or a 1kb (Promega) molecular weight marker. DNA bands were visualised under UV, photographed with GelDoc 2000 and viewed with Quantity One software. The above protocol was repeated using GoTaq® Green Master Mix (Promega).

6.2.3.3 Testing for inhibition of Mycosphaerella nubilosa *DNA in bulked leaf samples*

This was performed as a positive control to ensure the visualisation of DNA under UV was not inhibited by components inhibiting DNA polymerisation in the bulked leaf samples (Goller *et al.* 1998). Undiluted, 1:10 and 1:100 dilutions of two bulked leaf samples; samples 35 and 37 were randomly selected from the available samples. The six samples at the different dilutions were amplified with or without the addition of 1:10 diluted *M. nubilosa* DNA using the PCR conditions, and with the primer pair as described in Section 6.2.3.1. The initial PCR products were electrophoresed on 1% agarose gel, visualised under UV and photographed as previously described in Section 6.2.1.2.

When the reactions of DNA from bulked leaf samples with the primer pair were too faint for UV visualization, a nested PCR was performed to amplify DNA from bulked leaf samples in the first PCR round. A 1:10 dilution of the products obtained in the first PCR round was used as the template for the second PCR round with *M. nubilosa* specific primer and ITS3. This PCR was performed and electrophoresed on 1% agarose gels, visualised and photographed as described in Section 6.2.2.3.

6.2.3.4 Disease progression of Mycosphaerella nubilosa *for six months using nested PCR with species-specific primers*

From the initial PCR, the 1:100 diluted DNA from each of the 60 samples (two samples from 30 trees taken 6 months apart) were used in a nested PCR. The PCR samples were diluted (1:10) with sterile PCR grade water and used as the template for the second round PCR. The second round PCR was performed as described in Section 6.2.2.3. However, a *M. nubilosa* specific primer (Maxwell *et al.* 2005) and ITS3 primer were used. The products were electrophoresed on 1% agarose gels, visualised and photographed as previously described. The data obtained from the agarose gel images were compared with the data on disease assessment based on the presence or absence of necrosis on the collected leaf samples.

6.2.4 Correlation between field ratings and ASSESS results for MLD

A comparison was made of the effectiveness of the CDI (Stone *et al*. 2003) visual rating system of leaf damage with actual values measured by the computer program ASSESS. In order to obtain actual damage values using ASSESS all leaves from ten

branches (one per tree) were scanned (Epson Perfection 610) into the computer and the damage values recorded. Those same leaf images were saved as a PowerPoint slide presentation that was provided along with instructions to rate that damage using the reference diagrams for the CDI visual rating system (Stone *et al*. 2003) to 13 people. The subjects then recorded their ratings for each of those branches following the instructions provided. Each assessor rated the level of disease as a value between 0 and 100% damage for each branch based on a visual comparison with the reference diagrams. Each assessor was also ranked in terms of their previous experience in rating leaf disease in order to compare whether this experience influenced their ability to estimate actual damage as measured by ASSESS.

6.2.5 Statistical analysis

6.2.5.1 Genetics and disease development

Data analyses were conducted using the General Linear Models in STATISTICA 6.0 (StatSoft Inc. 2002). The data were analysed as a repeated measures ANOVA with genetics as the predictor variable and time as the independent repeat measures factor and ‘tree damage’ (MLD branch; MLD tree; Defoliation branch; Defoliation tree; Insect feeding damage) as the repeat measures dependent variable. Defoliation relates to whole leaf removal. To protect against violations of the sphericity assumption the Greenhouse-Geisser correction (Greenhouse and Geisser, 1958, 1959) was applied.

6.2.5.2 Correlation between field ratings and ASSESS results for MLD

To determine the robustness of the field observations, a basic correlation coefficient was calculated between field observation of MLD and results obtained by ASSESS at a branch level.

For statistical analysis the assessors experience was ranked as either high or low. A non parametric analysis using Friedman ANOVA and Kendall Coefficient of Concordance was used in STATISTICA 6.0 (StatSoft Inc. 2002). A Spearman rank coefficient was calculated for each assessor against the results from the ASSESS program.

6.3 RESULTS

6.3.1 Development of a molecular test for species causing Mycosphaerella leaf disease from leaf material

6.3.1.1 Species-specific primer design and testing

A BLAST search for the sequence sites for each of the primer pairs UD1F and UD1R; MM1F and MM1R found no 100% matches of both sets of primers in a primer pair with non-target fungal or plant DNA. The primer pair TP1F and TP1R did not match any DNA sequences from the non-target *T. parva*, except for *M. grandis*, which had identical or almost identical ITS sequences to *T. parva* and is believed to be conspecific with *T. parva* based on morphological and molecular data (Crous 1998, Maxwell 2004) (Chapter 4). The PCR reactions resulted in products of 432, 306 and 407 bp by the ML1, MM1 and MP1 primer pairs, respectively (Figure 6.3). Primer pairs ML1F and ML1R; MM1F and MM1R; and TP1F and TP1R amplified a product for DNA extracted from all target species isolates, these being *U. dekkeri*, *M. marksii*, and *T. parva*, respectively. There were no false positive amplifications of the 16 non-target species that these three primer pairs were tested against (Figure 6.3).

6.3.1.2 Species-specific primer sensitivity on fungal DNA

Primer pairs MM1F/ MM1R detected DNA at 1 pg per 25 mL reaction, the lowest concentration tested. The lowest detection limit of the remaining primer pairs was 10 pg of DNA per 25 ml reaction. This result was consistent across the three isolates tested for each of the 16 species. The intensity of the banding pattern for the primers is illustrated for the DNA template amounts 1 pg to 1 ng in Figure 6.4. The PCR product band intensity generally decreased with decreasing amount of DNA template.

6.3.1.3 Testing of species-specific primers against leaf material

All three primer pairs were able to detect each of their target species from DNA extracts of the lesions (Figure 6.5). Sequencing of the PCR products from each of the primer pairs confirmed their identity as belonging to each of their respective target species on the basis that they shared more than 99% sequence homology (Table 6.3). Frequently, more than one species was associated with a particular lesion type. For example, specimen MURU031 gave products for *U. dekkeri* and *M. marksii* (Figure 6.5). Conventional isolation techniques from these 10 specimens only yielded three species, *M. marksii*, *M. nubilosa* and *M. cryptica*, for specimens MURU031, MURU037 and MURU094, respectively (Table 6.3).

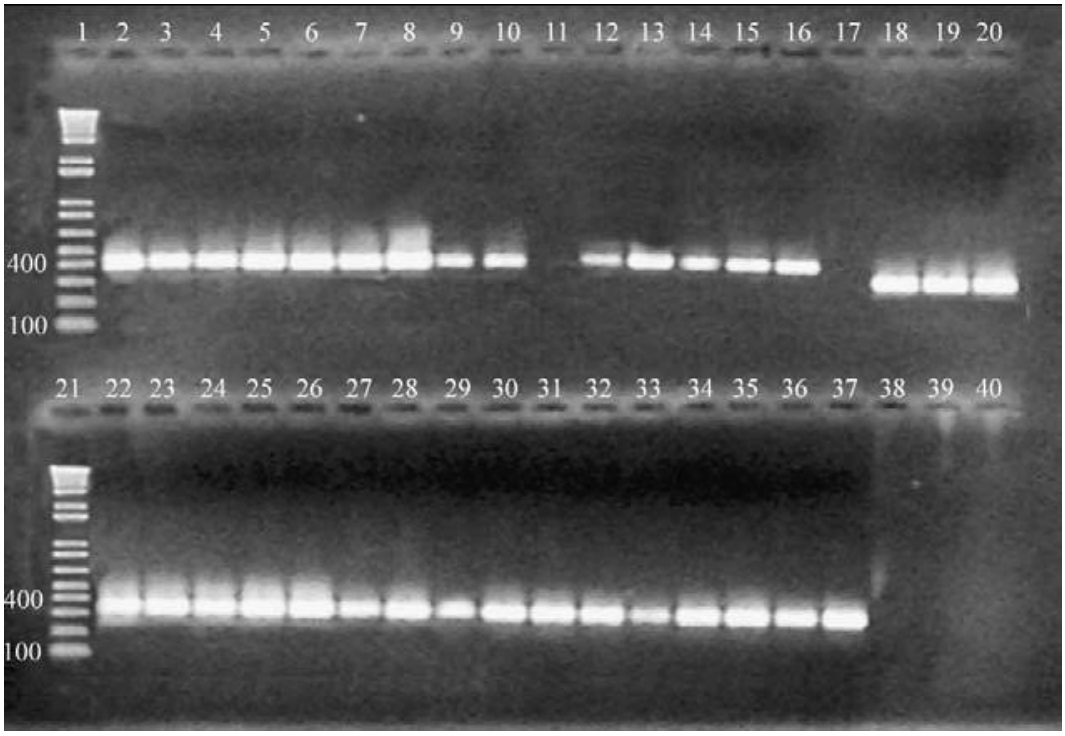

Figure 6.3 Agarose gel (1%) of purified PCR product from DNA using primers specific for *Teratosphaeria parva*, *Uwebraunia dekkeri* or *M. marksii*. Lanes 1 and 21, 1 Kb+DNA ladder, bands at 100 and 400 bp indicated. Lanes 2–10 ***T. parva*** (MURU170–176, MURU204, MURU213) amplified with MP1F & MP1R. Lane 11 negative control of *M. molleriana* (as *M. ambiphylla*), *M. aurantia*, *M. citri*, *M. colombiensis*, *M. cruenta*, *M. cryptica*, *M. ellipsoidea*, *M. lateralis*, *M. marksii*, *T. mexicana*, *M. nubilosa*, *M. suberosa*, *M. suttoniae* and *M. tasmaniensis* amplified with TP1F & TP1R; Lanes 12–16 ***U. dekkeri*** (MURU177, MURU253, MURU256–258) amplified with UD1F & UD1R. Lane 17 negative control of *M. molleriana* (as *M. ambiphylla*), *M. aurantia*, *Z. citri*, *M. colombiensis*, *M. cruenta*, *M. cryptica*, *M. ellipsoidea*, *M. marksii*, *T. mexicana*, *M. nubilosa*, *T. parva*, *M. suberosa*, *M. suttoniae* and *M. tasmaniensis* amplified with UD1F & UD1R. Lanes 18–37 ***M. marksii*** (MURU178–196) amplified with MM1F & MM1R. Lane 38 negative control of *M. molleriana* (as *M. ambiphylla*), *M. aurantia*, *M. citri*, *M. colombiensis*, *M. cruenta*, *M. cryptica*, *M. ellipsoidea*, *M. marksii*, *T. mexicana*, *M. nubilosa*, *T. parva*, *M. suberosa*, *M. Suttoniae* and *M. tasmaniensis* amplified with MM1F & MM1R. Lanes 39–40 are blank. Isolates used as negative controls are listed as tested (T) in Table 6.1. Figure first published as Figure 3 in Maxwell *et al.* (2005). Species in bold indicate from present study.

In this study species were identified from lesions using two methods; firstly only a single species was isolated from each of these lesions using conventional isolation techniques. Secondly, species were also identified according to PCR products generated from all of the lesions tested. PCR products were not generated from any non-infected leaf tips by any of the three species-specific primer pairs.

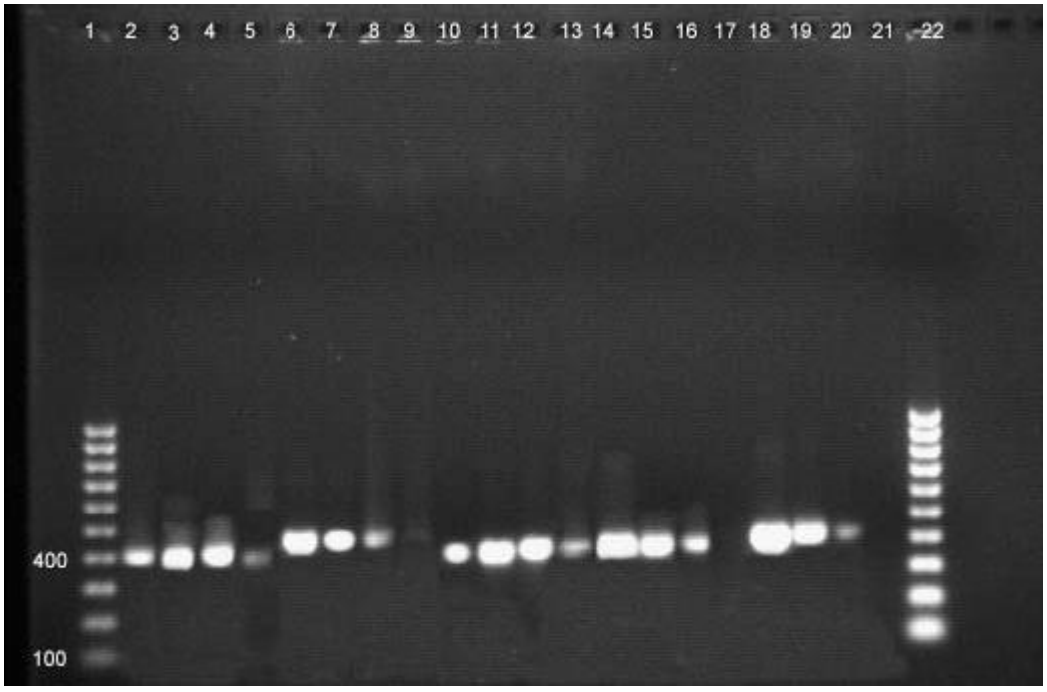

Figure 6.4 Agarose gel (1%) indicating the sensitivity of PCR primer pairs MC2F/MC2R, UD1/UD1R, MM1/MM1R, MN1/MN1R, TP1/TP1R specific for *Mycosphaerella cryptica* (L2–5), ***Uwebraunia dekkeri*** (L6–9), ***M. marksii*** (L10–13), *M. nubilosa* (L14–17) and ***Teratosphaeria parva*** (L18–21), respectively. DNA template amounts were 1ng (L2, L6, L10, L14 and L18), 100 pg (L3, L7, L11, L15, L19), 10 pg (L4, L8, L12, L16 and L20) and 1 pg (L5, L9, L13, L17 and L21). Lanes 1 and 22 a 100 bp DNA ladder with the 400 and 100 bp fragments indicated on the left of the gel. Figure first published as Figure 5 in Maxwell *et al.* (2005). Species in bold indicate from present study.

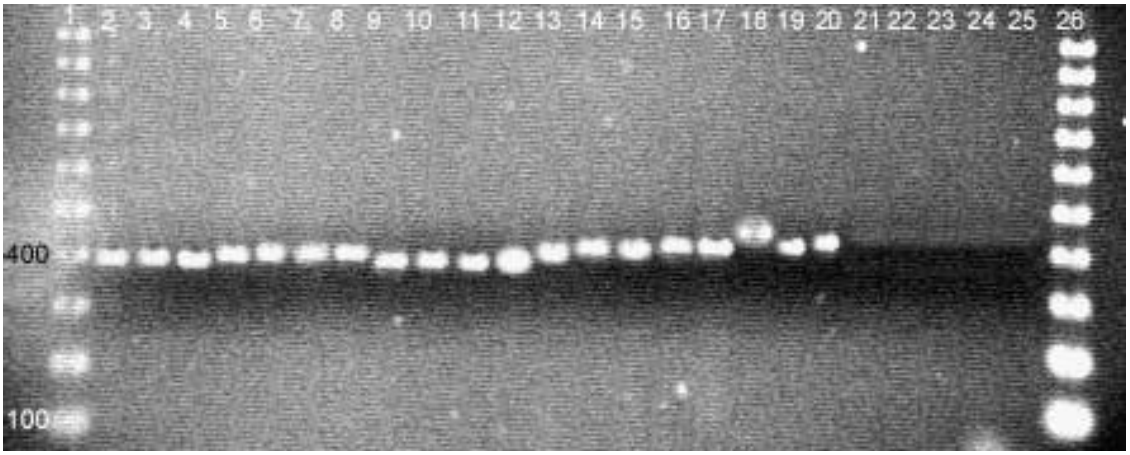

Figure 6.5 Agarose gel (1%) indicating the activity of PCR primer pairs MC2F/MC2R (*Mycosphaerella cryptica*), UD1F/UD1R (***Uwenraunia dekkeri***), MM1F/MM1R (***M. marksii***), MN1F/MN1R (*M. nubilosa*), TP1F/TP1R (***Teratosphaeria parva***) on DNA extracted from diseased *Eucalyptus globulus* leaves. Primer pairs MC2F/MC2R products amplified from sample leaves MURU 035, 037, 039, 094, 100, 149, 168 (L2–8); MM1F/MM1R amplified products from sample leaves MURU 031, 033, 035, 100 (L9–12); MN1F/MN1R amplified products from sample leaves MURU 035, 037, 039, 097 and 168 (L13–17); UD1F/UD1R amplified products from sample leaf MURU 031 (L18); TP1F/TP1R amplified products from sample leaf MURU 033 and 035 (L19–20); MC2F/MC2R, UD1F/UD1R, MM1F/MM1R, MN1F/MN1R, TP1F/TP1R on DNA extracted from non-diseased *E. globulus* leaves, respectively (L21–25). Lanes 1 and 26 100 bp ladder. Figure first published as Figure 6 in Maxwell *et al.* (2005). Species in bold indicate from present study.

Table 6.3 Identification of Mycosphaerella leaf disease species (*Mycosphaerella*, *Teratosphaeria* or *Uwebraunia*) by amplification and sequencing with species-specific primers from DNA extracts of *Eucalyptus globulus* leaves. Table first published as Table 4 in Maxwell *et al.* (2005).

Voucher specimen (MURU)	**Symptom class**[a]	**Specific primers that generated products**	**NCBI Sequence reference**	**Species sequence homology**[b]
031	Marksii	ML1	AY939544	*U. dekkeri*
031		MM1	AY939529	*M. marksii*
033	Marksii	MM1	AY939526	*M. marksii*
033		TP1	AY939527	*T. parva*
035	Marksii	MC2[c]	AY939528	*M. nubilosa*
035		MM1	AY939525	*M. marksii*
035		MN1	AY939530	*M. nubilosa*
035	Marksii	TP1	NS[e]	NS
037	Nubilosa	MC2[c]	AY939531	*M. nubilosa*
037		MN1	AY939532	*M. nubilosa*
037		TP1	AY939533	*T. parva*
039	Nubilosa	MC2[c]	AY939534	*M. nubilosa*
039		MN1	AY939535	*M. nubilosa*
094	Cryptica	MC2[d]	AY939536	*M. cryptica*
097	Nubilosa	MC2[c]	AY939537	*M. nubilosa*

Voucher specimen (MURU)	Symptom class[a]	Specific primers that generated products	NCBI Sequence reference	Species sequence homology[b]
097		MN1	AY939538	*nubilosa*
100	Mixed	MC2[c]	AY939539	*M. nubilosa*
100		MM1	AY939540	*M. marksii*
100		TP1	NS	NS
149	Mixed	MC2[c]	AY939541	*M. nubilosa*
149		MN1	NS	*M. nubilosa*
168	Mixed	MC2[c]	AY939542	*M. nubilosa*
168		MN1	AY939543	*M. nubilosa*

[a] The symptom class 'cryptica' were lesions characterised by pseudothecia densely arranged on both surfaces of young leaves with a waxy bloom; 'marksii' by pseudothecia only on the adaxial leaf surface of young leaves; 'nubilosa' by lesions only on the abaxial leaf surface of young leaves; mixed species by lesions on older leaves without a waxy bloom with pseudothecia densely arranged on the abaxial surface and more sparsely arranged on the adaxial surface.

[b] identities based on greater than 99% sequence homology.

[c] Restriction digest of PCR product with Sacc II generated 2 bands characteristic of *Mycosphaerella nubilosa*.

[d] Restriction digest of PCR product with Sacc II generated one band consistent with *Mycosphaerella cryptica.*

[e] NS, Not sequenced.

6.3.2 Field study of MLD over twelve months

6.3.2.1 Observations and disease assessment of MLD in the field and compared to ASSESS

Field observations of disease (MLD %) of 10 branches from ten trees combined and those calculated using the ASSESS program for the first five months (Figure 6.6) were not different ($p > 0.05$). In November, the level of MLD was much higher ($p < 0.05$) according to the field observations than measured by ASSESS. The remaining six months showed a comparable trend (Figure 6.6).

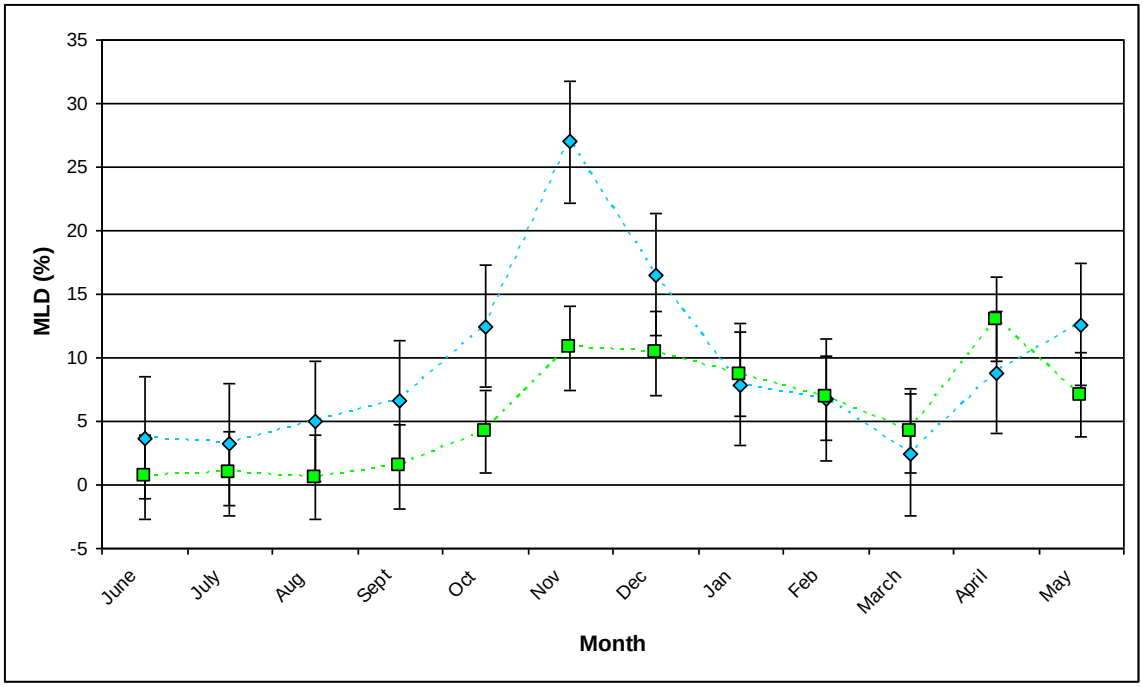

Figure 6.6 Comparison of field observations of ten branches combined from ten trees (blue diamond) of Mycosphaerella leaf disease (%) and calculated using ASSESS (green square) over twelve months. Error bars represent 95% confidence.

The field observations of MLD (%) and those calculated using ASSESS had a positive correlation coefficient (R^2 = 0.6147). The field observations tended to vary more as the level of disease increased (Figure 6.7).

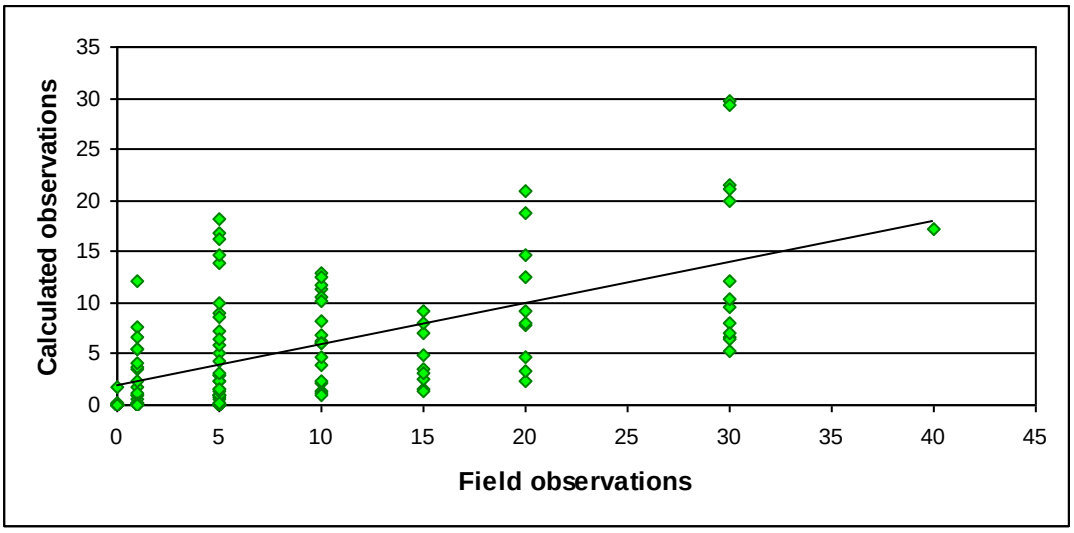

Figure 6.7 Comparison of field observations and percentage of Mycosphaerella leaf disease using ASSESS of 60 branches over twelve months. R^2 = 0.6147

Repeated measures ANOVA of MLD at a tree level showed that the main effect of 'time' and the 2-way interaction of 'time*family' was significant ($p < 0.05$) (Table 6.4), however, after Greenhouse-Geisser correction was applied this interaction was no longer significant ($p < 0.05$) (Table 6.5). Measured tree MLD peaked in November for both families and decreased thereafter, until March when diseased levels began to increase until the end of the study period in May 2005 (Figure 6.8).

Table 6.4 Repeated measures ANOVA of Mycosphaerella leaf disease at a tree level of two *Eucalyptus globulus* families over twelve months before Greenhouse-Geisser correction was applied.

	df Effect	**F**	**p-level**	**G-G epsilon**	**Adjusted p-level**
Family	1, 8	0.00483	0.946278		
Time	11, 88	16.58570	**< 0.0001**	0.19277	**0.00008**
Family x time	11, 88	2.54250	**0.007738**		

Table 6.5 Repeated measures ANOVA of Mycosphaerella leaf disease at a branch level of two *Eucalyptus globulus* families over twelve months after Greenhouse-Geisser was applied.

	df Effect	**F**	**p-level**	**G-G epsilon**	**Adjusted p-level**
Family	1, 8	0.631097	0.449875		
Time	11, 88	9.283010	**< 0.0001**	0.31635	**< 0.0001**
Family x time	11, 88	0.552594	0.861564		

Repeated measures ANOVA of defoliation at a tree level showed that the main effect of ‘time’ and the 2-way interaction of ‘time*family’ was significant ($p < 0.05$) both before and after the Greenhouse-Geisser correction was applied (Tables 6.6 and 6.7). Measured tree defoliation peaked in November for both families and plateaued at that level for the remainder of the study period (Figure 6.9).

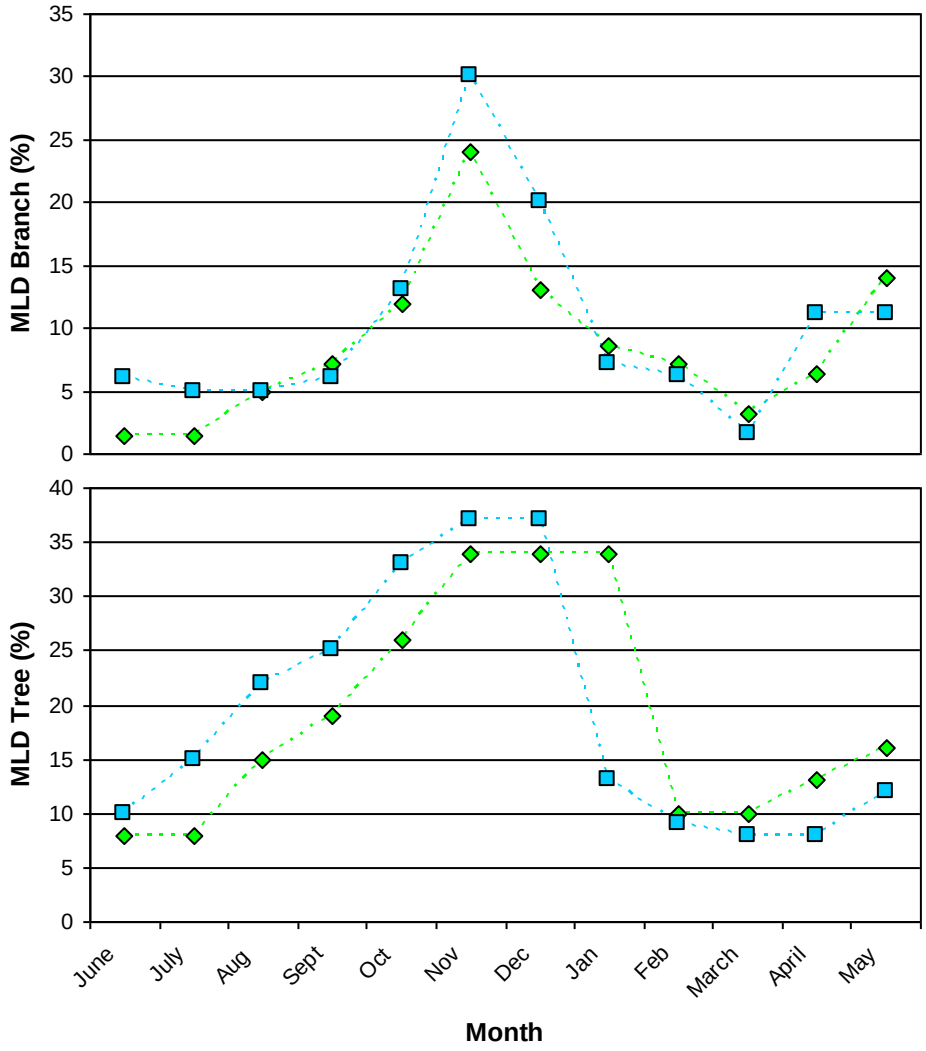

Figure 6.8 Percentage (%) of Mycosphaerella leaf disease at a tree and branch level for two families of *Eucalyptus globulus* over twelve months.

Table 6.6 Repeated measures ANOVA of the defoliation at a branch level of two *Eucalyptus globulus* families over 12 months before Greenhouse-Geisser correction was applied.

	df Effect	**F**	**p-level**	**G-G epsilon**	**Adjusted p-level**
Family	1, 8	0.00019	0.989418		
Time	11, 88	33.29786	**< 0.0001**	0.30741	**< 0.0001**
Family x time	11, 88	0.33017	0.976959		

Table 6.7 Repeated measures ANOVA of the defoliation at a tree level of two *Eucalyptus globulus* families (green and blue) over 12 months after Greenhouse-Geisser was applied.

	df Effect	**F**	**p-level**	**G-G epsilon**	**Adjusted p-level**
Family	1, 8	2.2744	0.169957		
Time	11, 88	102.3532	**< 0.0001**	0.34754	**< 0.0001**
Family x time	11, 88	2.9652	**0.002183**	0.34754	**0.03695**

6.3.2.2 Comparison of weather traits with disease and defoliation

Rainfall and temperature appeared to have had an effect on the level of MLD at both the branch and tree level. Rainfall during the months of May 2004 and August 2004 was between 50–100 mm, dropping to between 5–25 mm from September 2004 to March 2005 (Figure 6.10). There was a 'once in 100 year' rainfall event in April 2005 (exceeding 225 mm over 24 hours); however, the effect of that rainfall event was not able to be determined as the study period ended one month later. The MLD at a branch level remained substantially higher than the tree level until February 2005. This would indicate that the MLD was concentrated on the lower half of the tree, or that sampling of the branch was more accurate using the ASSESS program. However, MLD sharply declined from November 2004 to March 2005, after which the percentage began to increase at both the tree and branch level (Figure 6.10). The decline in MLD corresponded with an increase in the level of defoliation. Defoliation began to increase as temperatures rose and rainfall fell. Defoliation levels were higher at a tree level until December 2004. This was most likely due to the defoliation occurring below the sampled branches which were at breast height (1–2 m). After this time, defoliation remained higher at a branch level, indicating that the new flush of

growth in March 2005 did not replace the previously senesced leaves (Figure 6.11).

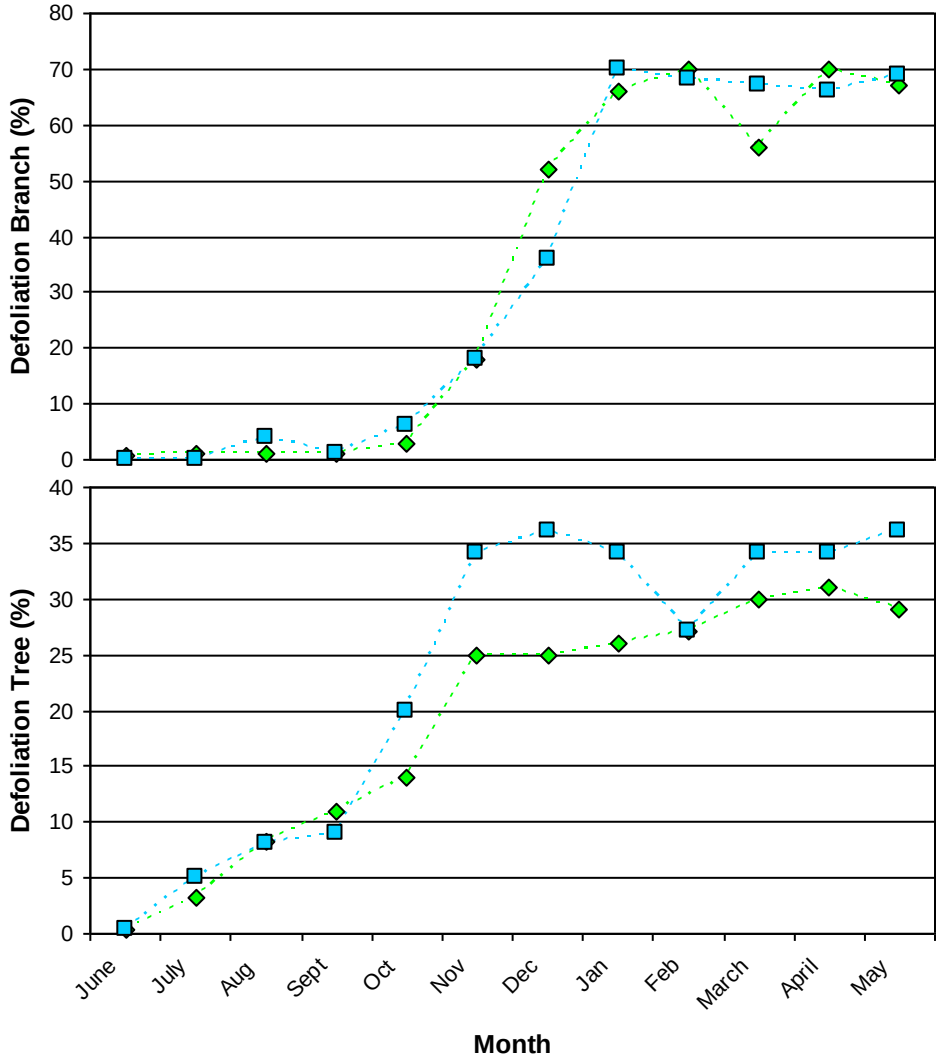

Figure 6.9 Percentage (%) of defoliation at a tree and branch level for two families of *Eucalyptus globulus* over twelve months.

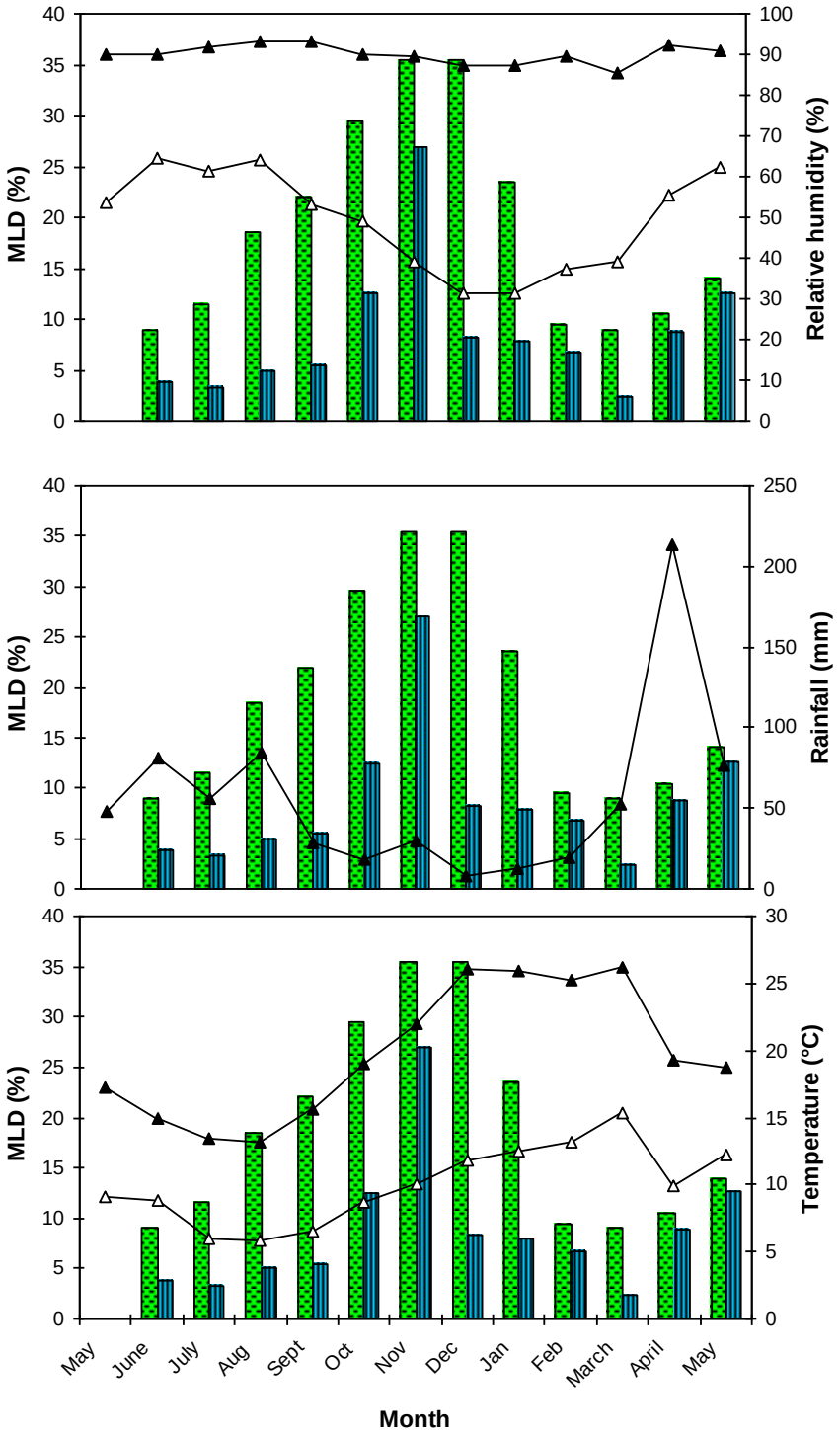

Figure 6.10 Monthly average maximum (bold triangle) and minimum (white triangle) temperatures (°C), monthly average rainfall (mm) and monthly relative humidity (%) from Mt Barker weather station over thirteen months compared to Mycosphaerella leaf disease occurrence (%) at a branch (green/ dots) and tree level (blue/ lines) of ten trees.

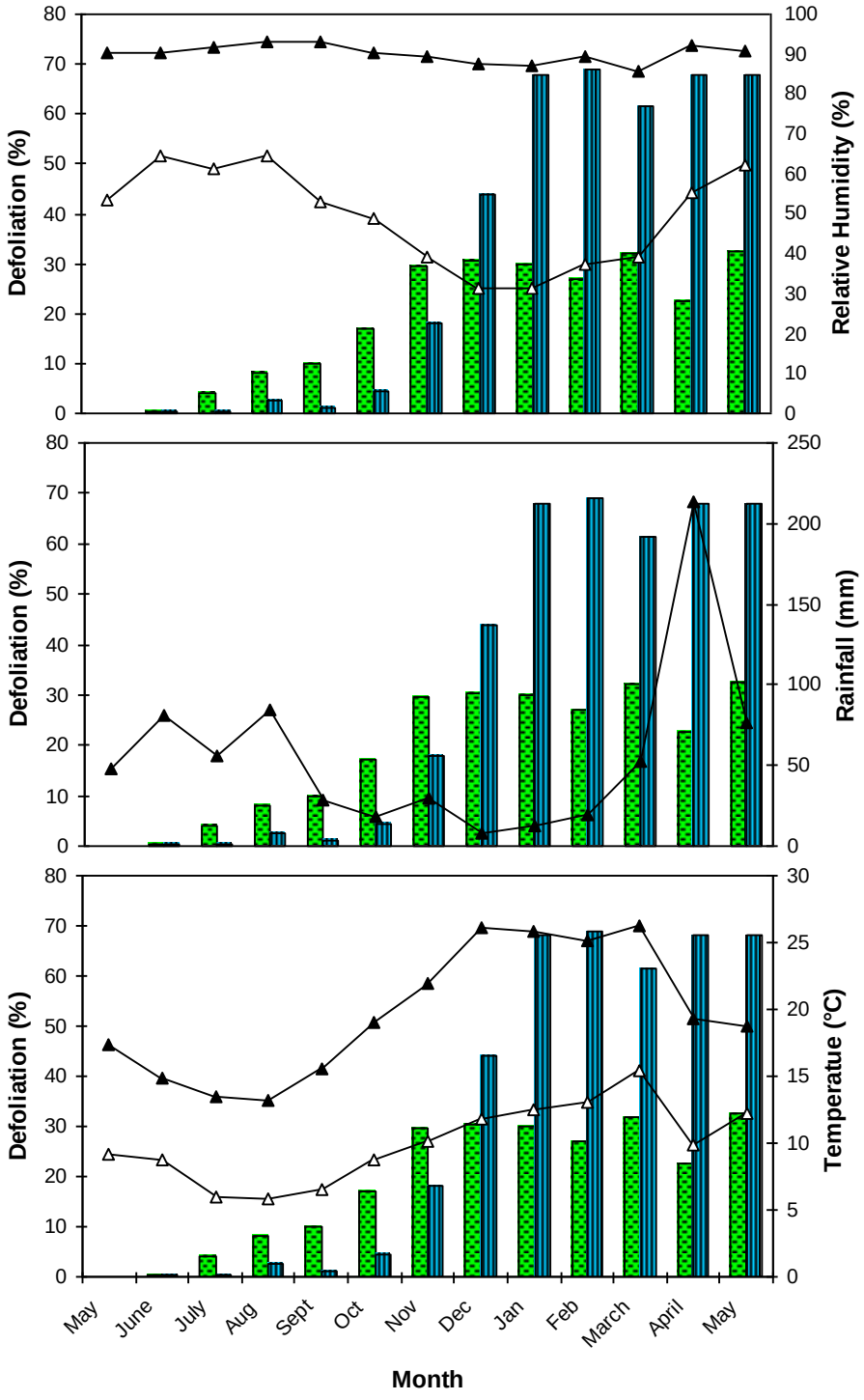

Figure 6.11 Monthly average maximum (bold triangle) and minimum (white triangle) temperatures (°C) monthly average rainfall (mm) and monthly relative humidity (%) at Mt Barker over thirteen months compared to defoliation (%) at a branch (green/ dots) and tree level (blue/ lines) of ten trees.

6.3.2.3 Isolation and identification of MLD species using traditional methods

Periodic assessments of leaf samples for the presence of *Mycosphaerella* species were conducted throughout the year that trees were visually assessed. The most common species isolated from juvenile foliage was *M. nubilosa*. Other species isolated included *T. parva*, *M. marksii*, *M. molleriana*, *M. aurantia* and a *Mycosphaerella* species not previously described that did not match any species on GenBank using the BLAST element on the NCBI website.

6.3.3 Detection of *Mycosphaerella nubilosa* from leaf material over six months using species-specific primers

6.3.3.1 Testing DNA amplification and inhibition of Mycosphaerella nubilosa *DNA in bulked leaf samples of* Eucalyptus globulus

Two bulked leaf samples (samples 35 and 37) were randomly selected from the available samples taken from Mt Barker and were amplified in a single round of PCR. The selected samples were amplified in undiluted, 1:10 and 1:100 dilutions on its own and mixed with *M. nubilosa* DNA. The results showed that with the standard PCR procedure, there was no recovery of *M. nubilosa* DNA fragments from the bulked leaf samples amplified on its own (Figure 6.12a; lanes 1–6), while with the addition of *M. nubilosa* DNA, there was also a low recovery of *M. nubilosa* DNA with only one band present (Figure 6.12a; lanes 9, F). The expected DNA band of *M. nubilosa* was between 250 to 500 bp.

When the nested PCRs were performed using the first round PCR products as template, no band was observed using the undiluted of both samples (Figure 6.12b; lanes 1, 4, 7, 10). Furthermore, no bands were observed for 1:10 diluted

samples except for the 1:10 diluted sample 37 that was amplified with *M. nubilosa* DNA (Figure 6.12b; lane 11). However, there was a consistent recovery of DNA from 1:100 diluted samples that were similar in size to the expected DNA band in the *M. nubilosa* sample (Figure 6.11b, lanes 3, 6, 9, 12).

The PCRs were repeated using GoTaq® Master Mix (Promega) in order to test the reliability and reproducibility of the specific primers. The repeated tests yielded different results. Bands that did not appear in the first PCRs were present in the subsequent PCRs. However, bands that were present in the first PCRs did not appear in the subsequent PCRs (data not shown). Due to time and resource constraints, this could not be resolved. Therefore, the testing of the other *Mycosphaerella* species-specific primers to determine the reliability and reproducibility of specific primers to detect and identify *Mycosphaerella* infection in latent, early and advanced stages of disease expression was abandoned.

6.3.3.2 Disease progression of Mycosphaerella nubilosa *on* Eucalyptus globulus *over a six month period using nested PCR with species-specific primers*

From the June samples, DNA bands corresponding to the expected band in the *M. nubilosa* sample were observed in six of the ten samples (Figure 6.13a; lanes 1–10). However, in some samples where lesions were observed, there was no band in the gel which indicates an absence of *M. nubilosa* (Figure 6.13a; sample 3). Besides that, a band was observed in the sample where no necrosis was observed on the collected leaves (Figure 6.13a; sample 4). In the July samples, a band was observed in seven of the ten samples (Figure 6.13b; lanes 12, 13, 15–19). From the band intensities, samples 15, 17, and 19 had less DNA than samples 12, 13, 16 and 18 (Figure 6.13b; lanes 11–20). As the observed bands

were from the samples in which necrosis was observed on the collected leaves, this showed the presence of *M. nubilosa*. Although necrosis was observed, *M. nubilosa* was not present in sample 20 because no band was present in its lane on the agarose gel (Figure 6.13b; sample 20).

From the August collection, presence of *M. nubilosa* was indicated by eight of the ten samples although there were only five records of necrosis present on the samples and bands were mostly observed in samples where necrosis was not present (Figure 6.13c; lanes 21, 22, 24, 26, 27). Based on the intensities of the observed bands, these samples had approximately equal amounts of DNA. Samples 29 and 30 were the only samples with lesions that did not generate a PCR product (Figure 6.13c).

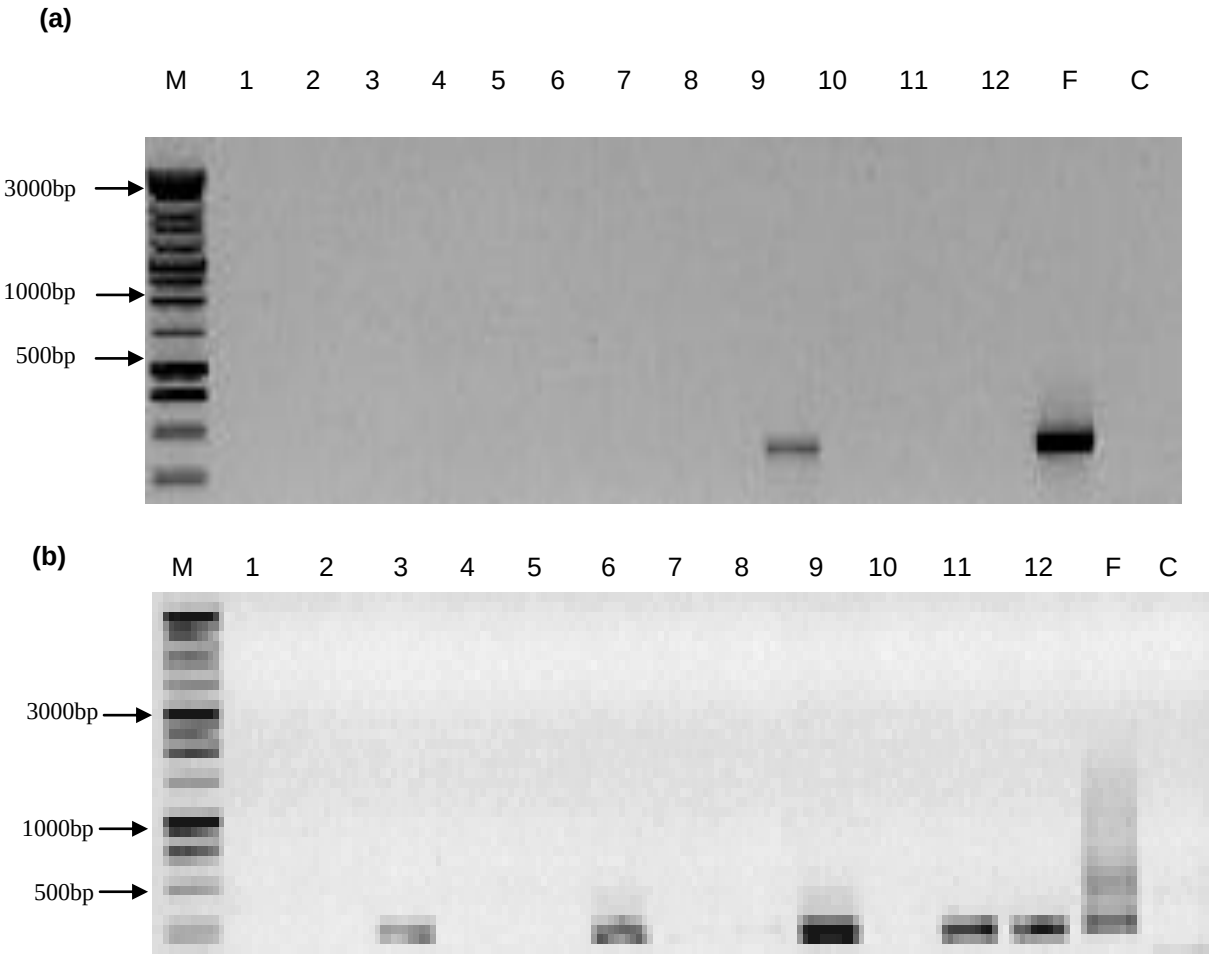

Figure 6.12 (a) PCR products of *E. globulus* leaf DNA (samples 35 and 37) at undiluted, 1:10 and 1:100 dilutions; with and without the addition of *M. nubilosa* DNA extract at 1:10 dilution, using *M. nubilosa* specific primer; and ITS1 **(b)** nested PCR products of *E. globulus* leaf DNA (samples 35 and 37) at undiluted, 1:10 and 1:100 dilutions; with and without the addition of *M. nubilosa* DNA extract at 1:10 dilution, using *M. nubilosa* specific primer and ITS3. Lane M: Promega 1kB molecular marker; lane 1: undiluted sample 35; lane 2: 1:10 diluted sample 35; lane 3, 1:100 diluted sample 35; lane 4, undiluted sample 37; lane 5, 1:10 diluted sample 37; lane 6: 1:100 diluted sample 37; lane 7: undiluted sample 35 with *M. nubilosa* DNA extract; lane 8: 1:10 diluted sample 35 with *M. nubilosa* DNA extract; lane 9: 1:100 diluted sample 35 with *M. nubilosa* DNA extract; lane 10: undiluted sample 37 with *M. nubilosa* DNA extract; lane 11: 1:10 diluted sample 37 with *M. nubilosa* DNA extract; lane 12: 1:100 diluted sample 37 with *M. nubilosa* DNA extract; lane F: 1:10 diluted DNA sample of *M. nubilosa* as a positive control; lane C: PCR master mix as a negative control.

Some DNA was amplified from eight of the ten samples for the September collection (Figure 6.14a; lanes 31–40). Thus, the presence of *M. nubilosa* in *E. globulus* was relatively high in that month. Necrosis was present in nine samples; samples 34 and 36 did not indicate the presence of *M. nubilosa* because there was no band observed on the agarose gel. Comparing the intensities of the bands with the expected band for the pure fungal DNA sample, most samples, but not sample 40, had more DNA in the samples than the pure fungal DNA sample (Figure 6.14a; lanes 31–40, F).

Similar results were obtained in the October and November samples as all leaf samples showed the presence of necrosis, however, only three out of the ten samples each month indicated the presence of *M. nubilosa*. Therefore, there was a low infection level of *M. nubilosa* in these two months. Based on the intensities of the observed bands, there was less *Mycosphaerella* DNA in the October collection than in the November collection (Figure 6.14b, c).

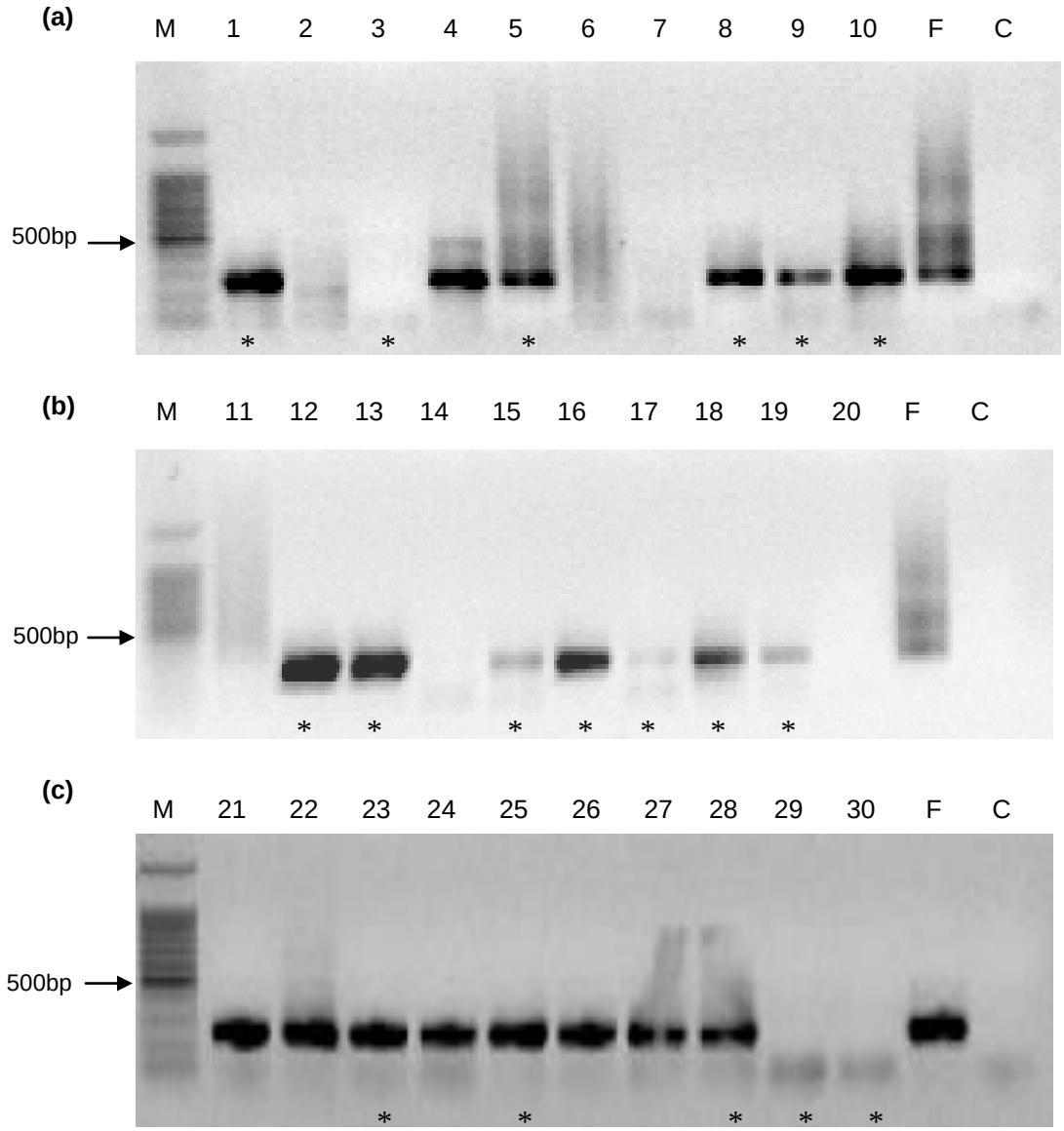

Figure 6.13 Nested PCR products of *Mycosphaerella nubilosa* DNA from *Eucalyptus globulus* leaf samples from **(a)** June, **(b)** July, and **(c)** August collections at 1:100 dilutions using a *M. nubilosa* specific primer. Lane M: 100bp molecular marker; lanes 1–30 represent samples 1–30, respectively; lane F: 1:10 diluted DNA sample of *M. nubilosa* as a positive control; lane C: PCR master mix as a negative control. * denotes that leaf necrosis was visible.

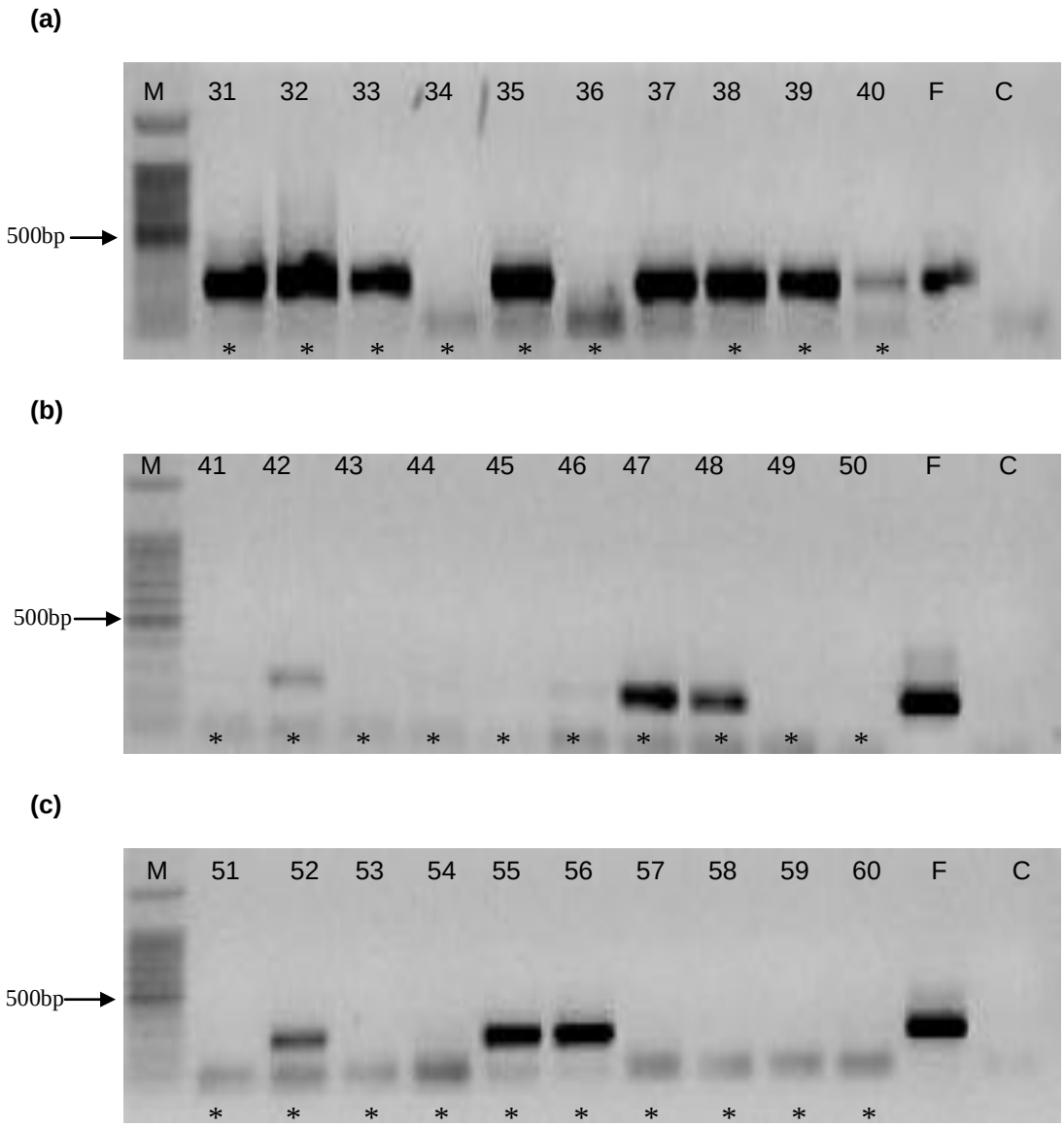

Figure 6.14 Nested PCR products of *Mycosphaerella nubilosa* DNA from *Eucalyptus globulus* leaf samples from **(a)** September, **(b)** October, and **(c)** November collections, at 1:100 dilutions using *M. nubilosa* specific primer. Lane M: 100bp molecular marker; lanes 31–60 represent samples 31–60, respectively; lane F: 1:10 diluted DNA sample of *M. nubilosa* as a positive control; lane C: PCR master mix as a negative control. * denotes that leaf necrosis was visible.

6.3.4 Correlation between field ratings and ASSESS results for Mycosphaerella leaf disease

According to the Friedman two-way analysis of variance by ranks there was a highly significant ($p < 0.000$) difference in damage amongst the branches. Therefore, there was sufficient difference between the branches for the assessors to be evaluated. The Kendall Coefficient of Concordance (0.628) showed a moderate correlation between assessors and results calculated using ASSESS. The influence of level of experience was compared using the Spearman rank coefficient. The person with the most experience had the highest Spearman rank coefficient (Table 6.8). Of the 13 people assessed, six had a significant ($p < 0.05$) Spearman rank, while eight were not significant ($p > 0.05$). Typically people over estimated the level of disease, with the largest variation occurring when there was a higher incidence of disease (Figure 6.15).

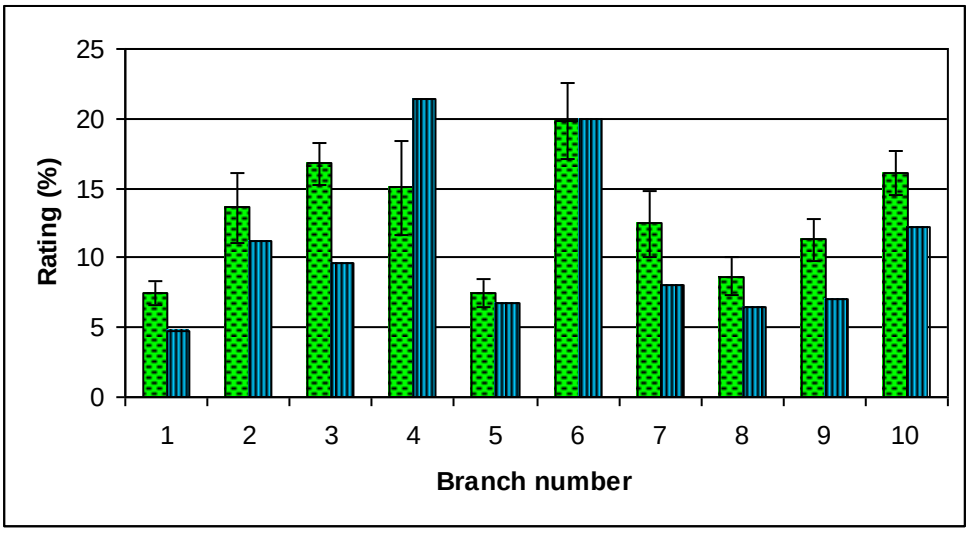

Figure 6.15 The average rating (%) of 13 assessors (green/ dots) compared to the actual level of disease calculated using ASSESS (blue/ lines) of 10 branches. Error bars represent 95% confidence.

Table 6.8 The Spearman rank coefficient for each assessor compared to the values calculated by ASSESS of ten branches, and the level of experience of each assessor.

Assessor	Spearman rank coefficient (bold indicates significant at p <0.05)	Level of Experience (L= Low, H= High)
A	**0.887425**	H
B	0.624973	L
C	**0.632222**	L
D	0.355335	L
E	0.406202	L
F	0.495434	L
G	0.301120	L
H	0.349603	L
I	0.610498	L
J	**0.791471**	L
K	**0.729259**	L
L	**0.823186**	H
M	**0.914179**	H

6.4 DISCUSSION

Species-specific primers were successfully designed and tested for three MLD casuing species that occur on *E. globulus* in WA. Primer pairs MM1F/ MM1R (*M. marksii*) detected DNA at 1 pg per reaction, the lowest concentration tested. The lowest detection limit of the remaining primer pairs was 10 pg of DNA per 25 ml reaction. The development of species-specific primers would be a useful aid to quickly identify cultures where the taxonomy is unknown or questionable. It allows for a much quicker and cheaper result compared to sequencing. The successful testing of *M. nubilosa* species-specific primers on leaf tissue indicated that the species-specific primers designed in this chapter could also be used to

determine *Mycosphaerella*-like species directly from leaf tissue without the need for culturing. Due to time constraints these could not be tested. However, the use of species-specific primers could be used to detect species where pathogenicity has yet to be determined, or where ascocarp development is delayed on lesions that have multiple species present. Specific primers also have the ability to detect DNA from species that are not able to be cultured.

Although the development of species-specific primers are very useful for distinguishing species that are morphologically similar, such as *Teratosphaeria eucalypti* and *T. destructans* (Andjic 2008), there are several aspects of this technology that limit its use and effectiveness. Often the standards that are published are not reproducible between laboratories, due to variation in PCR thermocyclers, efficiencies of DNA polymerases and the presence of PCR inhibitors (Hoorfar *et al*. 2003). The use of an internal amplification control (IAC) in a PCR reaction where there is no amplification of target DNA band but amplification of the IAC could indicate a false negative. If the amplification of both the target DNA and the IAC fail, the PCR reaction has failed (Hoorfar *et al.* 2003). Schoder *et al*. (2003) tested six new thermocyclers for performance and reproducibility. They found a difference between the thermocyclers, and suggest that those that did not perform well may not have reached an adequate denaturation temperature. They concluded that a false negative result may be caused by the template DNA not being sufficiently melted. A false negative result could have implications when testing for an exotic pathogen such as *Puccinia psidii* (eucalypt rust), in allowing infected material into the country that had previously tested negative (Chapter 1; 1.4.4).

The main disadvantage of using species-specific primers is that unless all species are known in a region, the positive band should still be periodically sequenced to ensure specificity. Other gene regions may have to be used if a primer within the ITS region cannot be found that is specific enough such as those designed for *M. cryptica* (Kularatne *et al.* 2004; Maxwell *et al.* 2005). The disadvantage of using other gene regions, however, is that they have yet to be sequenced for many of the species that cause MLD listed on GenBank.

Theoretically, amplification of DNA through PCR should give a million-fold increase of the original amount of DNA (Goller *et al.* 1998). However, sometimes the yield of DNA might be too low to detect during visualisation under UV (Goller *et al.* 1998). When the *M. nubilosa* DNA was amplified from the randomly selected bulked leaf samples, a low recovery of DNA was observed on the agarose gel, whether the samples were amplified on their own or mixed with additional purified *M. nubilosa* DNA. This may have been due to the presence of compounds inhibiting DNA polymerisation that required the original DNA sample to be diluted to a very low level (Goller *et al.* 1998). The presence of compounds inhibiting DNA polymerisation may have been confirmed when the presence of added *M. nubilosa* DNA was not observed on the agarose gel. However, the use of an IAC in the PCR reaction would have established this beyond doubt (Hoorfar *et al.* 2003), and should be explored in the future.

This study has developed species-specific primers to enable research into understanding disease progression of MLD. In this study, *M. nubilosa* was already causing lesions on *E. globulus* when the samples were collected in June. This was indicated when six out of ten samples showed a band that

corresponded to the expected band for *M. nubilosa*. Infection by *M. nubilosa* increased in July and the highest levels were recorded in August and September, there were seven out of ten samples from the July collection and eight samples each from the August and September collections that indicated the presence of *M. nubilosa*. However, the infection of MLD by *M. nubilosa* decreased considerably in October and November when only three out of ten samples indicated the presence of this species. This is more than likely due to the sudden increase in defoliation of the juvenile foliage at this time.

When necrotic lesions were observed on the collected leaf samples, a DNA band would be expected indicating the presence of a fungal pathogen from the PCR amplified bulked leaf sample. However, when no band was observed for leaf samples with necrosis, such as in the October and November collections, this may have been due to the necrotic lesions being caused by other *Mycosphaerella*-like species, another fungus from a different genus, a false negative, a complete PCR failure, or a combination of these. Once again, the use of an IAC would have indicated why no band was present.

Bulked leaf samples that did not have any necrosis indicated the presence of *M. nubilosa* after the samples were amplified with the species-specific primer developed for this species as observed for the samples from the August collection. From the ten samples collected in August, necrotic lesions were observed in five of the leaf samples but presence of *M. nubilosa* was found in eight of the samples (Figure 4.13c, samples 21–30). An earlier study showed that a *M. nubilosa* specific primer was able to detect this *Mycosphaerella* species in lesions that were not clearly visible (Maxwell *et al.* 2005). Therefore, as indicated

by the results of amplification of the August collection samples, *M. nubilosa* was detected before the symptoms developed.

The problem of low yields of DNA from a standard PCR can be overcome by using nested PCR where a second round of PCR is performed by using the first round PCR products as template with primers that anneal within the firstly amplified products (Goller *et al.* 1998). Nested PCR was found to be able to increase detection sensitivity in molecular assays by a factor of 1000 when compared to standard PCR procedures (Zhang *et al.* 2005). With nested PCR, *M. nubilosa* DNA was recovered in 1:100 diluted samples only, whether the samples were amplified on their own or mixed with *M. nubilosa* DNA. This means that when the original bulked leaf samples were diluted to 1:100, there were less compounds inhibiting DNA polymerisation compared to undiluted samples. Moreover, with two rounds of PCR through nested PCR, there is a higher yield of DNA in the samples compared to first round PCR products. Thus, the DNA was more easily detected. Therefore, the results suggest that the collected bulked leaf samples must be diluted to 1:100 and amplified in two rounds of PCR with species-specific primers to enable successful record of disease progression of MLD. Therefore, amplification of *Mycosphaerella*-like DNA from bulked leaf samples should be done using nested PCR with species-specific primers in 1:100 diluted samples.

Meteorological conditions appeared to determine the defoliation of juvenile foliage and not MLD as levels of MLD remained relatively low throughout the trial period. The MLD levels increased throughout spring as warm wet conditions favoured the development of disease especially on the flush of new juvenile

foliage. Also, new foliage emerged after late summer rainfall. As disease pressure mounted, the trees responded through defoliation. Also, as temperatures increased and the juvenile foliage aged, there is likely to have been an increase in the defoliation of leaves. Therefore, by mid-summer defoliation levels reached a similar level to disease and insect damage. Following leaf defoliation and the emergence of new juvenile and adult leaves, the relative amount of disease on the trees decreased. This is because most of the disease was present on the older juvenile foliage that had been shed.

Field observations can be a reliable indication of disease progression. Although field observations at a branch level over-estimated levels of MLD when there was a higher level of foliage, there was still a similar trend in the amount of disease when compared to the ASSESS program. Some experience in disease monitoring would indicate a more accurate assessment of MLD. It is interesting to note that the assessors tended to overestimate disease when MLD was at a higher level, and this also included the author (Figures 6.15).

The use of species-specific primers to determine presence or absence of particular species under field conditions should be conducted using an IAC and also traditional techniques should still be employed as large levels of leaf material can actually inhibit the PCR reaction.

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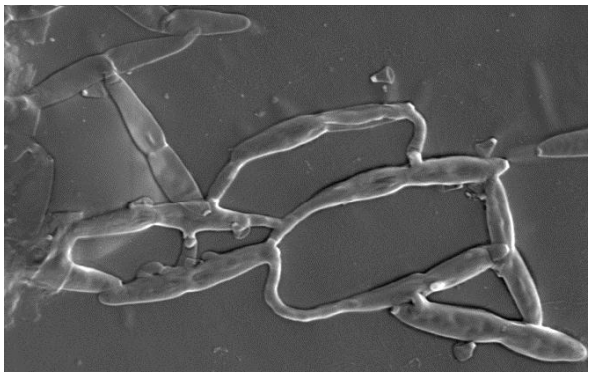
CHAPTER 7

INFECTION, HYPERPARASITISM, CONIDIOGENESIS OF *UWEBRAUNIA DEKKERI* ON *EUCALYPTUS GLOBULUS* IN WESTERN AUSTRALIA



7.1 INTRODUCTION

Frequently more than one *Mycosphaerella* or *Teratosphaeria* species are isolated from a lesion (Chapter 6). This can cause confusion as to which species is the pathogen and which is a secondary pathogen or saprophyte. However, it is possible that some species are hyperparasites. *Uwebraunia* is one genus that has been postulated as a mycoparasite under the name *Dissoconium dekkeri* (de Hoog *et al*. 1991). Other synonyms for *U. dekkeri* include *M. lateralis* and *D. lateralis*. *Uwebraunia dekkeri* was isolated in association with *M. cryptica* and *M. nubilosa* from both diseased juvenile and adult leaves of *E. globulus* in WA (Maxwell *et al*. 2000).

The genus *Dissoconium* was first described by de Hoog *et al*. (1983) to accommodate *Dissoconium aciculare*, which was isolated as a hyperparasite from *Erysiphe* on *Medicago lupulina* and which forcibly discharged conidia in pairs. De Hoog *et al*. (1991) isolated *D. dekkeri* from *Juniperus chinensis* and observed that the fungus was antagonistic towards *Tilletiopsis* on water agar. It also discharged both single-celled microconidia and two-celled macroconidia (de Hoog *et al*. 1991). They speculated that *D. dekkeri*, like *D. aciculare*, could be a hyperparasite. However, Crous (1998) and Maxwell *et al*. (2000) isolated what they believed to be the teleomorph of *U. dekkeri* (as *M. lateralis*), from diseased eucalypt foliage in association with other known pathogenic *Mycosphaerella* species and indicated that *U. dekkeri* might be parasitic on eucalypt leaves. Although the asexual state of *U. dekkeri* occurs readily in culture and has been isolated from a range of hosts (de Hoog *et al*. 1991), it has not been observed on a eucalypt host.

It is not yet known whether conidia of *U. dekkeri* are able to infect *E. globulus* leaves or whether it is a hyperparasite of other *Mycosphaerella* species causing MLD. The aims of the current study were to:

- determine if *U. dekkeri* is a hyperparasite of *M. nubilosa* or *M. cryptica in vitro*;
- determine if conidia of *U. dekkeri* are able to infect *E. globulus* leaves and;
- investigate the mode of conidiogenesis.

7.2 METHODS

7.2.1 Leaf infection

7.2.1.1 Production of conidial suspension of Uwebraunia dekkeri

Conidia of *U. dekkeri* (MURU0014, MURU0015) were obtained from single-spore isolates of *U. dekkeri* grown on water agar over-laid with sterile cellophane. The cellophane was sterilised according to a method modified from Howard (2001). Briefly, the cellophane discs (80-mm-diameter) were boiled for 2 hr in 5 L of deionised water (DIW) and 0.2 g of EDTA, then rinsed in DIW and boiled for a further 2 hr in DIW. The discs were then autoclaved at 121°C for 20 min on three consecutive days to ensure sterility. After inoculation, the cultures were kept at 20°C in the dark for 6 weeks in order to induce conidial production. Conidial suspensions of *U. dekkeri* were made by agitating cultures with 1 mL of Tween 80 solution (0.1 mL/L sterile water), a surface tension depressant. The concentration of conidia in the suspension was determined with a haemocytometer.

7.2.1.2 Inoculation of Eucalyptus globulus *leaves*

Three recently expanded juvenile leaves of *E. globulus* seedlings grown in a tunnel house, were excised and placed abaxial surface up in the lid of a 90-mm-diameter Petri-dish on top of a damp paper towel. Six, 20 µL drops of conidial suspension (5×10^3 conidia/ mL) were placed on each leaf. The bottom of the Petri-dish was placed over the leaves to create a humid chamber and incubated at 20°C in the dark. This was replicated three times and repeated with leaves on the adaxial surface. Two plates of controls, inoculated with the dilute Tween 80 solution only on either the abaxial or adaxial leaf surface, were also included. One leaf of each treatment was harvested for clearing and staining on days 3 and 6 after inoculation.

7.2.1.3 Clearing and staining

Harvested leaves were placed in clearing solution [1:3 lactic acid (80%): absolute ethanol] at 60°C for 1–6 hr. Cleared leaves were rinsed in tap water and stained with aniline blue CI 42755 (0.5 g/L) at 60°C for 1 hr. Leaves were mounted in lactoglycerol on microscope slides and examined at ×400 magnification using an Olympus BH2 light microscope. Pieces on which conidia were observed were dissected from the leaf and prepared for SEM.

7.2.1.4 SEM specimen preparation

Leaf segments (50 mm^2) with germinating conidia were rinsed in tap water to remove the lactoglycerol before being placed onto a microscope slide and air dried in a drying cupboard for three days. Each segment was mounted onto an

aluminium stub using carbon glue tabs, and sputter coated with gold and examined under a Philips XL 20 SEM at 15 kV.

7.2.2 Hyperparasitism

7.2.2.1 Fungal isolates

Single-spore isolates of *M. cryptica* (MURU0018), *M. nubilosa* (MURU0027) and *U. dekkeri* (MURU0014, MURU0015) were obtained from diseased *E. globulus* leaves as per Crous (1998). Briefly, lesions were excised from diseased leaves, soaked in sterile water for 2 hr, and then attached to the lid of a Petri-dish, with fruiting bodies facing downwards. They were then inverted over the base containing 2% MEA. They were incubated in the dark at 20°C for 24–48 hr in order to stimulate spore discharge. Single spores were then aseptically transferred to 2% MEA plates and maintained at 25°C in the dark.

7.2.2.2 Media

Interactions of *U. dekkeri* with *M. nubilosa* or *M. cryptica* were investigated using three types of media: 2% MEA (20 g Difco malt extract, 20 g Difco agarose/L tap water), 0.2% MEA (2 g malt extract, 20 g agarose/L tap water) or water agar (20 g agarose/L tap water). Cultures were grown on the respective media over-laid with sterile cellophane discs (80-mm-diameter).

7.2.2.3 Experimental design

An isolate each of *M. cryptica* and *M. nubilosa* was challenged *in vitro* with two isolates of the putative hyperparasite, *U. dekkeri*. Along the equator of each plate three, 9 mm^2 mycelial colonies of a *U. dekkeri* isolate were placed at a distance

of 5 mm from 9 mm^2 mycelial colonies of either *M. nubilosa* or *M. cryptica*. Three replicate plates were established for each interaction. The plates were incubated at 25°C in the dark. As soon as the colonies came into contact with each other, a small cellophane piece (5 × 10 mm) containing mycelia of the two interacting fungi was removed with a sterile scalpel and mounted onto a microscope slide with lactoglycerol [50% acidified (0.1% lactic acid) glycerol] and stained with aniline blue (0.5 g/L). The interactions between the *U. dekkeri* isolates and *M. cryptica* or *M. nubilosa* on the cellophane were examined under oil at 1000× magnification with an Olympus BH2 compound light microscope.

7.2.3 Conidiogenesis of *Uwebraunia dekkeri*

Scanning electron microscopy was used to investigate the conidiogenesis of *U. dekkeri*. Cultures were grown in Petri-dishes on 1.5% water agar overlaid with sterile cellophane. After three weeks growth, 9 mm^2 pieces of cellophane covered with mycelia were cut from the agar. These were air-dried on microscope slides for three days in a drying cupboard or fixed and critical point dried. The latter material was fixed in 2.5% glutaraldehyde in phosphate buffer (0.025 M, pH 7.0) for 2 hr, rinsed in buffer then post fixed with 1% aqueous osmium tetroxide for 2 hr. Samples were dehydrated in an ethanol series of two changes of 30%, 50%, 70%, 80%, 90% and 100% for 15–30 min each. Ethanol was removed in two changes of amyl acetate for 15 min each and the specimens were critical point dried. Air-dried and critical point dried material were mounted onto aluminium stubs, sputter coated with gold and examined under a Philips XL 20 SEM.

7.3 RESULTS

7.3.1 Infection

Numerous *U. dekkeri* conidia had germinated on both leaf surfaces of *E. globulus* 3 days after inoculation. Hyphae were frequently observed penetrating via abaxial stomata at days 3 and 6 (Figure 7.1). No infection structures such as appressoria were observed. Anastomosis of *U. dekkeri* hyphae was frequently observed on the abaxial surface by day 6. Germinated conidia did not penetrate the adaxial surface.

7.3.2 Hyperparasitism

Mycelia of *U. dekkeri* grew together with both *M. cryptica* and *M. nubilosa* on all three types of media. There was no evidence of zones of inhibition or hyphal coiling, typical of hyperparasitism. Similarly, despite the growth of *U. dekkeri* alongside, and in apparent contact with both *M. cryptica* and *M. nubilosa* hyphae, pores or channels were not observed at ×1000 magnification. There was also no evidence of infection or collapse of *M. cryptica* conidia in the presence of *U. dekkeri* hyphae. In addition, there was no reduction in growth of either *M. cryptica* or *M. nubilosa* when grown in association with *U. dekkeri*.

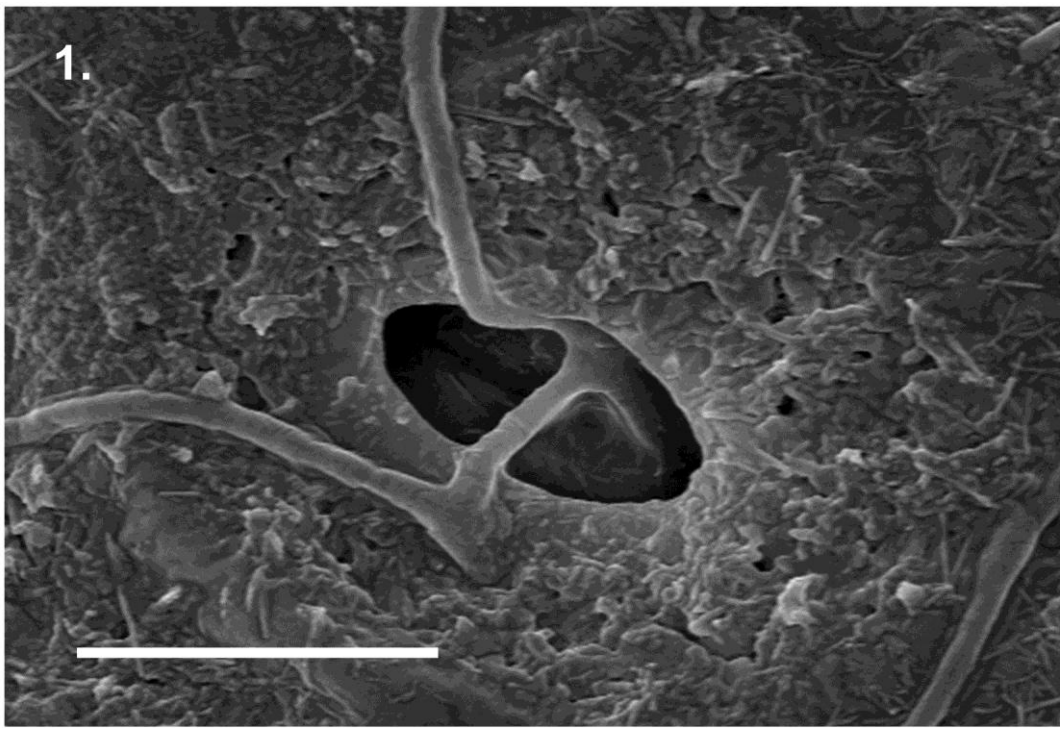


Figure 7.1 *Uwebraunia dekkeri* hyphae infecting via a stoma on the abaxial surface of a *Eucalyptus globulus* leaf. Scale bar = 10 µm.

7.3.3 Discharge and conidiogenesis of *Uwebraunia dekkeri* conidia

Uwebraunia dekkeri macro and microconidia conidia formed on *E. globulus* leaves that had been processed to induce ascospore discharge (Figure 7.2). These conidia were actively discharged from the lesion surface onto 2% MEA plates. Macroconidia were discharged with, and without, microconidia attached. Also, microconidia were actively discharged separately from the macroconidia. In some instances, non-attached microconidia anastomosed with macroconidia or neighbouring hyphae to form a hyphal bridge (Figure 7.3). Conidiogenesis was both sympodial and percurrent (Figures 7.4a–c).

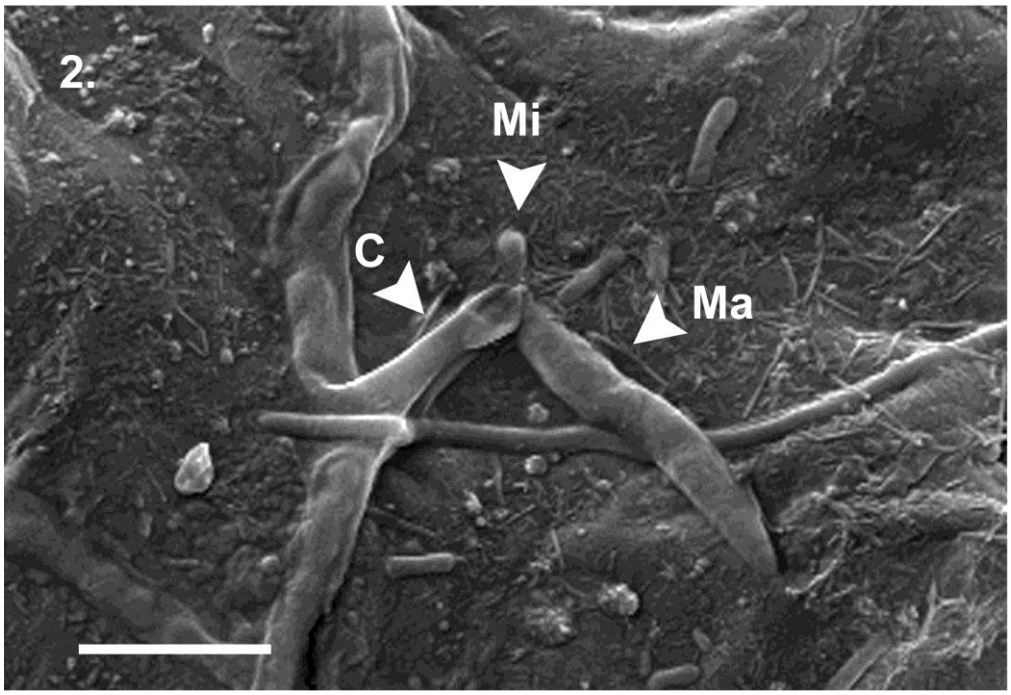


Figure 7.2 Conidiogenous cell (C) forming microconidia (Mi) and macroconidia (Ma) of *Uwebraunia dekkeri* on a *Eucalyptus globulus* leaf. Scale bar = 10 µm.

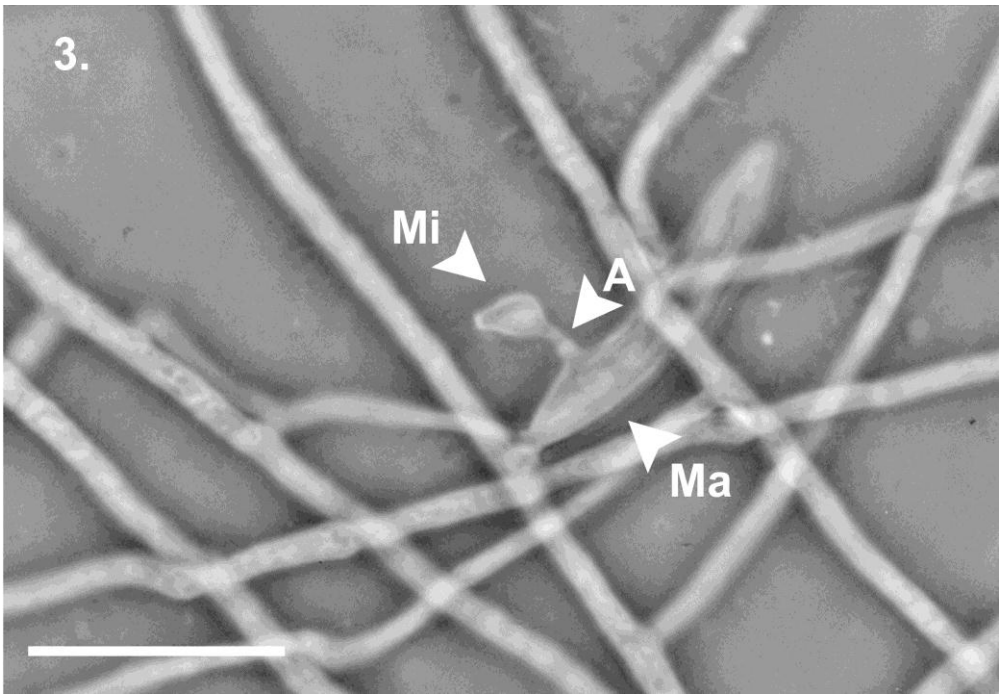


Figure 7.3 Anastomosis (A) of microconidia (Mi) and macroconidia (Ma) of *Uwebraunia dekkeri in vitro*. Scale bar = 10 µm.

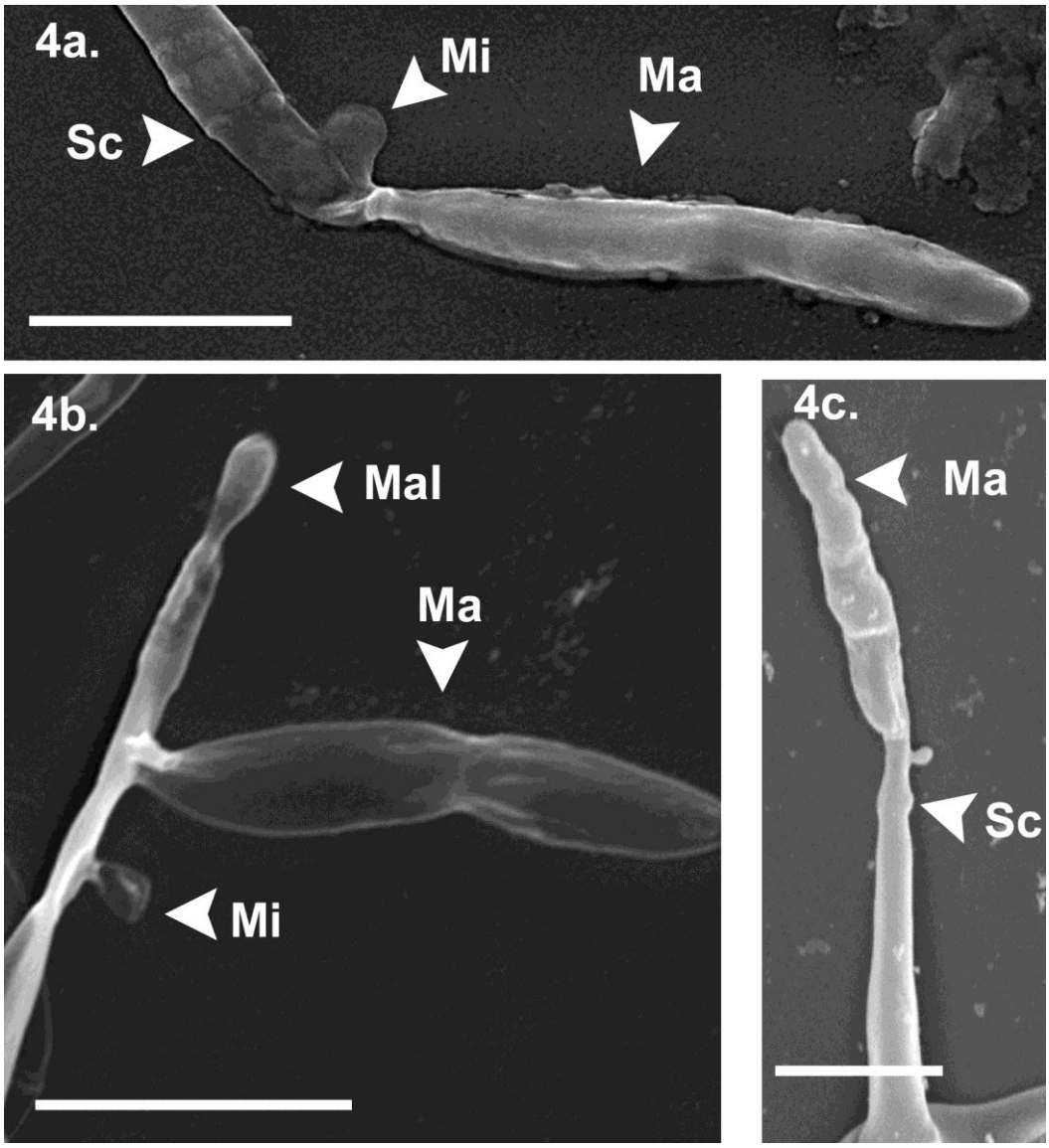


Figure 7.4 Scanning electron micrographs of macroconidia and microconidia formed from sympodial conidiogenesis of *Uwebraunia dekkeri*. (*a*) Macroconidia (Ma) and microconidia (Mi) form adjacent to each other at the apex of the condiogenous cell. The conidia move laterally and are then forcibly discharged. The coniophore proliferates apically between, rather than through, the scars (Sc). (*b*) Macroconidia (Ma) and microconidia (Mi) remain attached to the coniophore and a new macroconidium initial (MaI) forms at the apex of the conidiophore. (*c*) A macroconidium (Ma) forms at the apex of the conidiophore and a scar (Sc) is present where a conidium has been previously formed. Scale bar = 10 µm.

7.4 DISCUSSION

This study demonstrated that conidia of *U. dekkeri* can infect *E. globulus* leaves and that it is not a hyperparasite of *M. cryptica* or *M. nubilosa*. Conidiogenesis was both percurrent and sympodial and the phenomenon of anastomosis was observed for the first time on the leaf surface. In the present study, *U. dekkeri* conidia were isolated directly from *E. globulus* leaves with, and without, microconidia attached.

Germ tubes of *U. dekkeri* conidia were observed entering *E. globulus* leaves via stomatal openings on the abaxial surface. This occurred within three days of the initial inoculation. Although germination was observed on the adaxial surface, penetration was absent, possibly due to the lack of stomatal openings. This mode of penetration is similar to that observed for *M. nubilosa*, which is able to penetrate eucalypt leaves only via stomata (Park and Keane 1982; Park 1988). In the current study, however, it remains unclear whether *U. dekkeri* is deriving any nutrients directly from the plant. Hyphal anastomosis on the leaf surface may be in response to low nutrient availability, as de Hoog and Takeo (1991) have shown that anastomosis of *U. dekkeri* (synonym = *D. dekkeri*) occurs in response to a lack of nutrients *in vitro*. Anastomosis may indicate that the fungus is not deriving any nutrients from the plant. In addition to facilitating nutrient exchange, anastomosis could lead to the transfer of nuclear material.

This study has shown that *U. dekkeri* is not a hyperparasite of the two most common causes of MLD, *M. cryptica* and *M. nubilosa* in Western Australia. It does not cause hyphal lysis or infect their hyphae or conidia *in vitro*. It is unlikely

to be an antagonist to these fungi, as no zone of inhibition occurred and hyphae of *U. dekkeri* frequently grew alongside those of *M. nubilosa* and *M. cryptica*. Also, there was no evidence of a reduction of mycelial growth in either *M. cryptica* or *M. nubilosa*. This is in contrast with de Hoog *et al*. (1991) who found that, *U. dekkeri* (as *D. dekkeri*), was antagonistic to a *Tilletiopsis* sp. However, from their study it is unclear what the mechanism of antagonism was. They do not mention whether antagonism resulted in the death of the *Tilletiopsis* sp., or whether *U. dekkeri* simply out-competed this fungus on water agar. De Hoog *et al*. (1991) also state that *U. dekkeri* may be a hyperparasite of phyllosphere fungal pathogens. However, there is no supporting evidence for this in their paper. Although de Hoog *et al*. (1991) have shown *U. dekkeri* to be an antagonist on agar, the role it plays on a leaf surface needs to be further investigated.

Conidiogenesis of *U. dekkeri* occurred both sympodially and percurrently. Although sympodial conidiogenesis has been reported for *D. aciculare* (de Hoog *et al*. 1983; de Hoog and Takeo 1991), percurrent conidiogenesis has only been described for *U. dekkeri* (de Hoog *et al*. 1991). These observations, along with those relating to the ecology of *U. dekkeri,* have taxonomic implications. In the most recent review of these genera, Li et al. (2012) described proliferation as ‘sympodial but also appearing percurrent’ for both Dissoconium and for Uwebraunia. This is in contradiction to the original descriptions that separated these two genera on this feature, however, is in agreement with the current study published as Jackson *et al*. (2004).

Dissoconium was first erected as a separate genus from *Cordana*, based on the forcible discharge of macroconidia and microconidia. Crous *et al*. (1999) later

erected the genus *Uwebraunia*, which accommodates fungi morphologically similar to *Dissoconium*. These two genera were separated on two criteria: firstly, that *Uwebraunia* species are pathogens of eucalypts, whereas *Dissoconium* species are hyperparasites; secondly, that conidiogenesis in the type specimen for *Uwebraunia, Uwebraunia juvenis,* is percurrent, whereas it is sympodial for the type specimen, *D. aciculare,* in *Dissoconium.* These two distinctions are no longer valid and the separation of these two anamorph genera needs to be reviewed. It is clear from the present study that *U. dekkeri* may be a pathogen or an endophyte of eucalypts and not hyperparasitic as previously suggested by de Hoog *et al*. (1991). Further research is required to determine if *U. dekkeri* is an endophyte or whether it becomes a pathogen under certain conditions, such as when a leaf is approaching senescence or the plant is stressed.

Although de Hoog *et al*. (1983) first described the active discharge of spores from *D. aciculare* in a slime droplet and from cultures of *D. dekkeri* (de Hoog *et al*. 1991), there has been no previous description of active discharge of *U. dekkeri* conidia from *Eucalyptus* plant material. Crous *et al*. (1999) described the simultaneous discharge of microconidia and macroconidia from *U. dekkeri*, but this occurred only in culture and not from leaf material. The role of the microconidia is still not fully understood and requires further study. De Hoog and Takeo (1991) believe microconidia may be involved in exchange of nuclear material, but they were unable to show this. The microconidia were not seen to germinate independently (de Hoog and Takeo 1991). In the present study, it is unclear as to whether they are able to germinate or they are anastomosing with the macroconidia. The bridge hyphae were only observed in close association with a macroconidium or hypha on agar overlaid with cellophane. In the

description of *D. aciculare*, the microconidia were observed germinating soon after release from the conidiogenous cells (de Hoog *et al*. 1983).

In conclusion, studies on the molecular taxonomy of this group indicate that the anamorph *Uwebraunia* has arisen separately a number of times within the teleomorph genus *Mycosphaerella* (Crous *et al*. 2001). Also, according to the sequence homology of the large subunit (28s) of the rDNA, *U. dekkeri* is more closely aligned with *Uwebraunia ellipsoidea* than *U. ellipsoidea* is with *U. juvenis* (Crous *et al*. 2001). Further morphological and molecular studies on *Mycosphaerella* and related anamorphic genera are required to clarify the differentiation of these taxa. Following the publication of the current chapter a number of revisions of *Dissoconium*, *Uwebrania* and related taxa have occurred and in the most recent analysis Li *et al.* (2012) have resurrected *Uwebraunia* to accommodate a number of species including *U. dekkeri* based largely on morphological differences that include small pyriform conidia, absence of sclerotia in culture and lack of yellow pigment in culture. They also state that there are no clear ecological differences between these genera. Studies into pathogenicity and the role that *U. dekkeri* may have in causing MLD are continuing.

7.5 REFERENCES

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CHAPTER 8

THE IMPACT OF FUNGICIDES AND INSECTICIDES ON EARLY PERFORMANCE OF *EUCALYPTUS GLOBULUS* IN TWO PLANTATIONS IN SOUTH-WESTERN AUSTRALIA

8.1 INTRODUCTION

Previous chapters in this thesis have focussed on the causal agents of MLD and aspects of their biology with particular reference to *E. globulus* as a plantation species in southern Australia. This chapter brings the focus back to the plantation scale by considering the management of plantations for diseases and pests.

Maxwell (2004) reported that insect pests and fungal pathogens were the biggest threats to young *E. globulus* plantations in WA. The visual incidence of nutrient deficiency, crown decline and stem distortion, was much lower (Maxwell 2004). The most common disease observed was MLD (Maxwell 2004); however, prior to the studies of Carnegie *et al.* (1997) and Maxwell (2004), little was known of the impact and species involved in MLD on eucalypt plantations in south-western Australia. The general symptoms of MLD can be caused by a number of *Mycosphaerella*-like species. Often the impact of each species cannot be separated as they occur as a disease complex, with one or a number of *Mycosphaerella*-like species involved (Maxwell *et al.* 2005). Mycosphaerella leaf disease has been attributed to cause a loss in photosynthesis, even in asymptomatic leaf tissue of *E. globulus* in Tasmania (Pinkard and Mohammed 2006), and often leads to defoliation of juvenile foliage (Park and Keane 1982b). It is therefore likely that MLD impacts on early tree growth and wood volume at harvest. Defoliation of 25% of *E. nitens* infected with *Mycosphaerella* in South Africa was attributed to a reduction in growth rate (Lundquist and Purnell 1987). In Victoria, Australia, Carnegie *et al.* (1994) showed a significant negative correlation between MLD severity and height and diameter of *E. globulus*, and

more recently Carnegie and Ades (2002a) reported that levels of diseased leaf area as low as 10% resulted in up to a 17% reduction in height of *E. globulus* in plantations.

The International Organisation for Standard (ISO) has developed guidelines which enable organisations to identify processes within their company to limit the impact of their activities on the environment. It is a generic set of guidelines that can be used for any organisation worldwide. The Forest Stewardship Council (FSC) has more specific regulations that not only include environmental impact of plantation wood production, but also protects old growth forest and indigenous communities. Under the FSC accreditation process, companies must agree to use an integrated approach to forestry management including minimising the use of pesticides and how the pesticides are delivered, such as by aerial or ground application (FSC Pesticide Policy 2005).

Companies in Australia have obligations to their investors through managed investment schemes and so must balance the economic cost against environmental and social impacts. As yet it is not known if the costs of applying chemicals to control pests and disease exceed the prospected gain in pulp yield. The profit of growing *E. globulus* is very sensitive to the costs of growing, managing and harvesting (Battaglia *et al.* 2002). The growth rate is the most important determinant of profit; therefore, site selection and management costs can be offset by an increase in production (Battaglia *et al.* 2002).

The aims of this study were to determine: whether the regular application of fungicides and insecticides in plantations aged 1–4 years increases the growth and yield of *E. globulus* at two plantations and if two different plantations

geographically close to each other respond the same from those applications and if the effect is financially beneficial.

8.2 METHODS

8.2.1 Trial design

The experiment was conducted on two one-year-old commercial *E. globulus* plantations and consisted of four spray treatments (fungicide [F], insecticide [I], fungicide plus insecticide [F/I] and non-treated controls [C]), replicated five times with 50 trees per replicate. This regime was designed to determine whether controlling pest and fungal diseases for 2–3 years increases above ground biomass at 2 and 5 years (Figure 8.1). Operational constraints, such as accessibility of machines to the site, the time taken to apply chemicals and ease of application of chemicals by the contractor, required treatments to be in close proximity within the rows, and the replicate treatments had to be located within the same area (Figure 8.1). When the sites were selected, ITC was undertaking routine monitoring of their bluegum estate for foliar nutrients as described by Dell *et al.* (2001). This work was contracted to a private company which provided confidential reports to ITC. ITC advised that on the basis of foliar analysis that nutrient concentrations in the compartments where the trials were established were within the adequate concentration range defined for *E. globulus* by Dell *et al.* (2001).

The trials were established in July 2000, 12 months after commercial planting. The four treatments were randomly allocated to five rows. Treatment plots were separated by three rows, which acted as buffer rows minimising spray drift

between treatments. Within each of the five rows, five plots were randomly selected for height and diameter measurements and leaf damage (insect and pathogen) assessments each consisting of 50 trees. The number of trees per plot for disease incidence was later reduced to 15 per plot after initial power analysis (Microsoft Excel™) had indicated that 15 trees was an adequate sample size for each replicate plot. These plots were located in the centre of each five-row treatment, again, to minimize the chance of any spray drift occurring. The number of trees per treatment at each site was 250, a total of 1000 trees at each plantation (Figure 8.1).

8.2.2 Plantations

The Bills Tree Farm plantation, was located north-east of the Porongurup National Park, approximately 50 km from Albany, WA (Table 8.1). The area has an average annual rainfall of 650 mm (Figure 8.2). The soil was classified as a gravelly duplex, consisting of a fertile top soil of loamy sand to 0.1–0.2 m with a lighter coloured, gravelly loamy fine sand to 0.4–0.5 m, below which was a structured yellow fine sandy clay found in the profile as well as an occasional broken laterite layer at the interface of the sands and clay to a depth of 0.8 m. The vegetation prior to plantation establishment was predominantly rain-fed pasture used for beef cattle grazing. The previous land users had applied fertilizer (NPK) since the 1970's up until the mid-late nineties. The remnant native vegetation on the nearby slopes and ridge tops was dominated by *Allocasuarina fraseriana*, *Corymbia calophylla*, *E. marginata* and *E. staeri*.

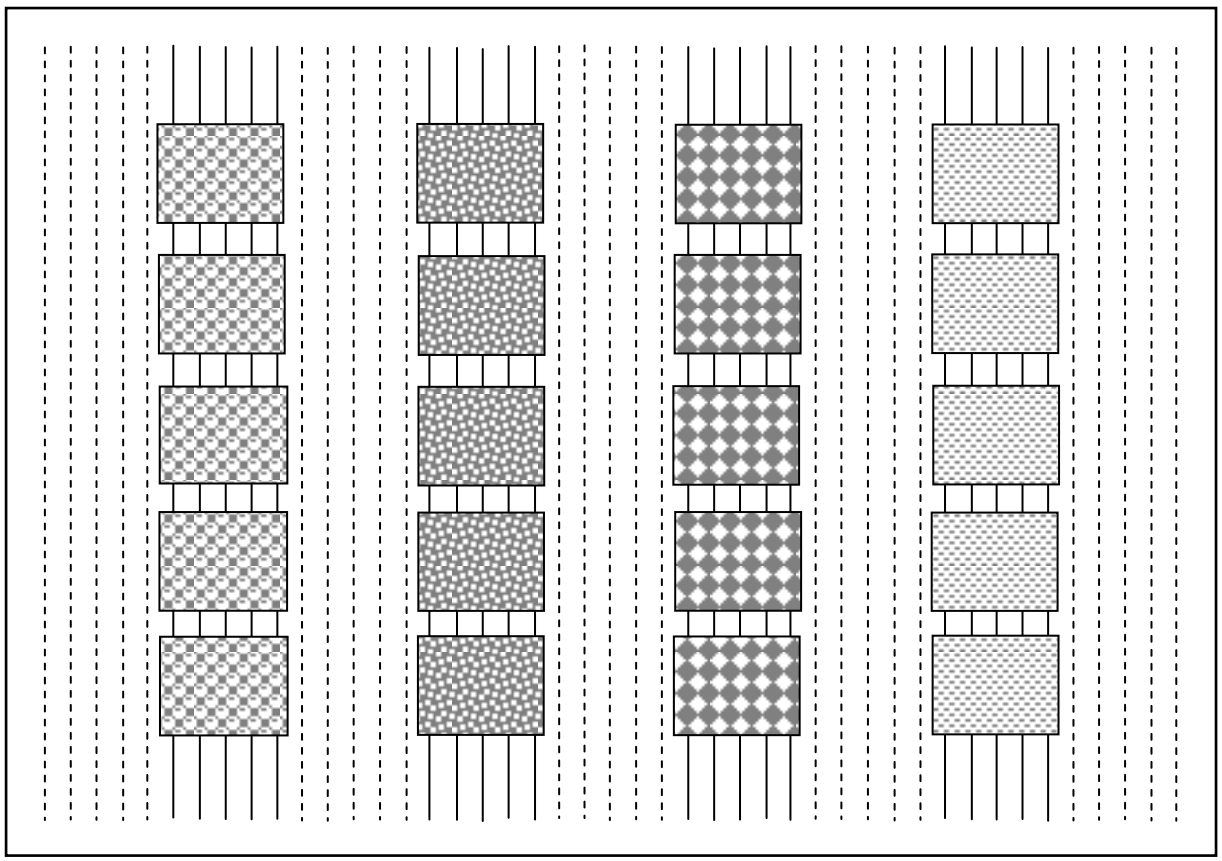

Figure 8.1 Diagrammatic representation of the four treatments at two plantations in Albany WA. From left to right; Control, fungicide, insecticide and fungicide/ insecticide combined. Each square consists of 5 x 10 rows of trees, a total of 250 trees per treatment. Note that due to operational constraints the replicate plots had to be located in the same rows.

The Sixpenny Tree Farm was at the southern edge of the Porongurup National Park (Table 8.2). The district has a mean annual rainfall of 730 mm (Figure 8.3). The soil type was a yellow gravelly duplex or sandy duplex. This property has been progressively cleared since 1965 for sheep production and NPK fertiliser was regularly applied over that time. The remnant native vegetation on the nearby slopes and ridge top was dominated by *C. calophylla*, *E. diversicolor* and *E. marginata*.

Table 8.1 Summary of plantation site characteristics used in the current study.

Site details	Bills Plantation	Sixpenny Plantation
Location reference	118°04’ S, 34°42’ E	117°55’ S, 34 42” E
Average annual rainfall (mm)	650	730
Soil type	Gravelly duplex	Yellow gravelly duplex
Stocking (stems/ha)	1000	800
Total plantable area (ha)	373	58
Mean DBHOB (cm) August 2000	6.1	6.1
Mean height (m) August 2000	2.7	2.5
Establishment	July 1999	July 1999

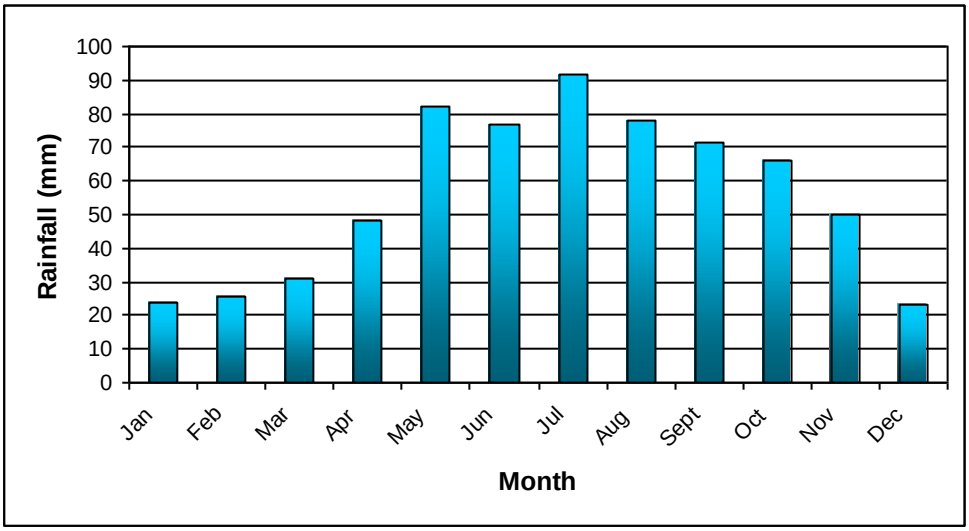

Figure 8.2 Monthly average rainfall (1959–1998) recorded at Windrush rain station (009848), approximately 9 km from Bills plantation. Data courtesy Bureau of Meteorology.

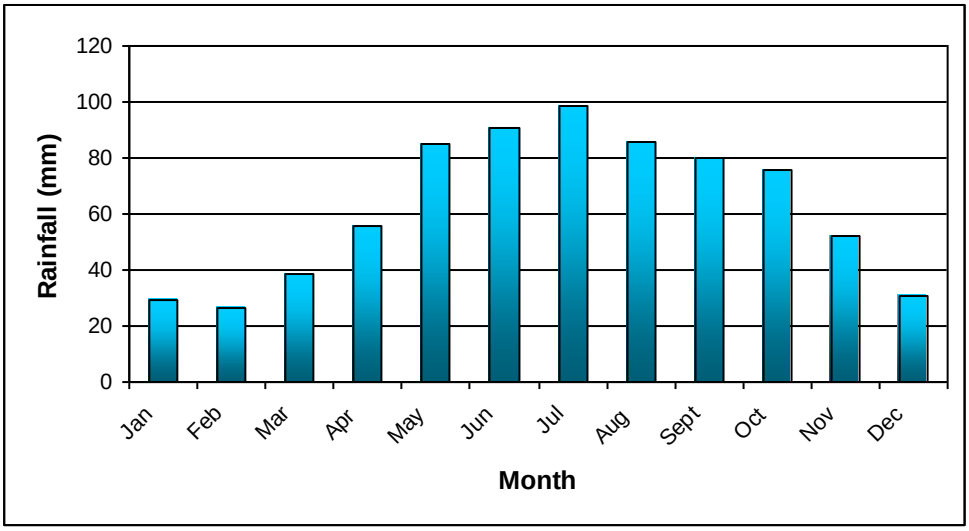

Figure 8.3 Monthly average rainfall (1928–1998) recorded at Yellanup weather station (009265) located approximately 1.72 km from Sixpenny plantation. Data courtesy Bureau of Meteorology.

Prior to establishment in 1999, both sites were ripped to a depth of 0.7 m with a winged tyne and the soil mounded to a height of 0.2 m. A herbicide treatment consisting of glyphosate (0.9 L/ha), simazine 500 (4 L/ha), sulfometuron methyl (30 g/ha) was applied to the rows prior to planting. Commercial *E. globulus* stock seedlings from Flinders Island provenance, approximately 30 cm in height were planted at 800 (5m x 2.5m) or 1000 (5m x 2m) stems per hectare. At planting, 100 g of diammonium phosphate (DAP) was applied at 0.20 cm from each seedling along with a complete trace element mix. Second year weed control was achieved by spraying the inter-rows with knock down and residual herbicides consisting of 250 g/L amitrole 220 g/L ammonium thiocyanate (2 L/ha), sulfometuron methyl (50 g/ha), and simazine granules (1.1 kg/ha).

8.2.3 Spray regime

The trials were initially sprayed (July 2000) with a commercial spray mister (Hardi LE SPV Vineyard Mister) that had a lift mounted mist blower with centrifugal

blower (Figure 8.4). From December 2002 until the end of spraying period (July 2003), a Croplands Big Gun Mister, a cannon type blower unit able to reach up into the canopy, was used. Both devices were towed by a tractor. The first and last row of each treatment (rows 1 and 8) were sprayed using only one side of the mister, switching off the side towards the buffer zone. All other rows of the trial were sprayed using both sides of the mister. The fungicide was applied first followed by the insecticide and then the combination of both. The tank was rinsed with water between the fungicide and insecticide applications. The control treatment was left unsprayed. The dates and spray treatments are listed in Table 8.2.

8.2.4 Chemical treatments

8.2.4.1 Fungicides

The systemic fungicides benomyl (Benlate®, DU PONT Australia Ltd), and chlorothalonil (Bravo® 500 DU PONT Australia Ltd) or chlorothalonil/ ethylene glycol (Rover® 500 Flowable, NUFARM Australia Ltd), were used alternately to ensure fungicide resistance would not occur (Table 8.2).

Figure 8.4 The spraying rig used to deliver the fungicides and insecticide to the treated *Eucalyptus globulus* trees. (Photo by H. Neumeister-Kemp)

Table 8.2 Fungicides and insecticide applied at the two plantations, including rate, application rate and cost involved

Date	Fungicide	Rate	Appl. rate	Insecticide	Rate	Appl. rate	Cost	Plantation
14/08/2000	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	2.5 L/ha	$792.00	Both
4/09/2000	Benomyl	100 g/100L	850 L/ha				$445.50	Both
25/09/2000	Benomyl	100 g/100L	850 L/ha				$445.50	Both
16/10/2000	Chlorothalonil	500 g/L	2 L/ha				$445.50	Both
8/11/2000	Benomyl	100 g/100L	850 L/ha				$445.50	Both
28/11/2000	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
3/01/2001	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
24/01/2001	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
14/02/2001	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
11/03/2001	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
9/04/2001	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
1/05/2001	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
25/05/2001	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
18/06/2001	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
17/07/2001	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
5/08/2001	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
22/08/2001	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
18/09/2001	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$841.50	Both
9/06/2002	Chlorothalonil	500 g/L	2 L/ha	Alphacypermethrin	100 g/L	2.5 L/ha	$1,039.50	Both
16/07/2002	Chlorothalonil	500 g/L	3 L/ha	Alphacypermethrin	100 g/L	2.5 L/ha	$1,039.50	Both
19/12/2002	Chlorothalonil	500 g/L	3 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$594.00	Bills
28/01/2003	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Sixpenny
6/02/2003	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Bills
24/02/2003	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Bills
25/02/2003	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Sixpenny
24/03/2003	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Bills
25/03/2003	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Sixpenny
1/05/2003	Benomyl	100 g/100L	850 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$1221	Both
24/06/2003	Chlorothalonil	720 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Bills
1/07/2003	Chlorothalonil	720 g/L	2 L/ha	Alphacypermethrin	100 g/L	0.3 L/ha	$610.50	Sixpenny
						Total cost	**$21,846.00**	

8.2.4.2 Insecticides

Alphacypermethrin a non-systemic synthetic pyrethroid pesticide (Dominex® 100, Crop Care Australasia Pty Ltd), was applied regularly to ensure minimal defoliation (Table 8.2). It was particularly necessary to control *Mnesampela privata* (autumn gum moth), *Phylacteophaga froggati* (leaf blister sawfly), and other leaf-eating insects such as *Gonipterus scutellatus* (*Eucalyptus* weevils), *Chrysomelinae* spp. (Chrysomelid beetles) and *Ardozyga stratifera* (leaf-tier moth).

8.2.5 Tree measurements

Tree height and stem diameter were measured prior to the experiment (1 year) and twice thereafter (3 years and 5 years). Tree heights where measured using Suunto clinometers and measuring tape. Stem diameter was initially measured using electronic callipers at 30 cm above the ground immediately before the treatments and thereafter at 1.3 m (diameter at breast height over bark, DBHOB). The equation used to calculate volume (m^3) was 0.03739 x DBHOB $(cm)^{1.81507}$ x height $(m)^{1.1455}$ /1000.

8.2.7 Ratings

The trees were assessed for pest and disease incidence pre-spray in August 2000, three months later (November 2000), after six spray applications and three years later (July 2003). All 250 trees in each treatment were assessed in 2000 for MLD and insect damage as a percentage. Thereafter, in July 2003 only 15 trees per replicate plot, using the first five trees in the 2^{nd}, 3^{rd} and 4^{th} row of each plot, were measured after a power analysis showed that it was statistically not

necessary to measure all trees. The rating was initially conducted by walking through the trials and recording the disease and pest incidence on a marked branch at breast height on one side of row. However, as the trees grew, rating assessments from July 2003 were made from a 4.5 m stand mounted on the back of a utility.

The following six point rating scale was used: 0 = 0%, 1 = 1.5%, 2 = 3%, 3 = 6.25%, 4 = 12.5%, 5 = 25% and 6 = 50% where % is the amount of leaf area damaged by the causal agents of either MLD or insect damage (leaf-chew). The total number for each rating category was multiplied by the category percentage. This number was then divided by the number of trees per plot. A total damage score for each treatment plot was then obtained and called the Mycosphaerella damage index (MDI) or Leaf-chew damage lndex (LDI). Rated juvenile leaves in August 2000 and November 2000 were compared. Rated adult leaves in August 2000 and July 2003 were compared for analysis. Only juvenile leaves at Sixpenny from August 2000 and July 2003 were used for analysis as the control juvenile leaves at Bills had senesced.

8.2.8 Statistical analysis

The volume data were analysed using Statistica ver. 5 (Statsoft, 1995), as a repeated measures ANOVA with factors of Plantation, Plot, Treatment and Year (the repeated measures factor). Plot was nested inside site because of the design constraint (Figure 8.1). Tree volumes were standardised by dividing the volume calculated in 2002 or 2004 over the volume in 2000 and these standardised measures were used as the dependent variable in the analysis, after conversion to the 4^{th} root to correct for correlations between means and

variances across the cells of the design. Using the standardised figures there were only two levels of the repeated measures factor (2002 and 2004), so there was no need to adjust the degrees of freedom with the relevant Greenhouse-Geisser epsilon to protect against possible violations of the sphericity assumption.

Rating data were analysed using a multivariate analysis of variance (MANOVA) with factors of Site (Sixpenny and Bills) and Treatment (Control, Fungicide, Insecticide and Fungicide/ Insecticide) and dependent variables of Mycosphaerella damage index and Leaf-chew damage index. The MANOVA was used to overcome problems with dependence between the two variables measured on the same subjects and presents a single test statistic (Wilks' lambda) using both variables for each main effect and for the interaction. If the Wilks' lambda was significant, then the univariate effects were examined to determine the significance of each variable separately.

Data were log-transformed to correct for heteroscedasticity and correlations between means and variances across the cells of the design. In subsequent tables and figures the analyses were based on log-transformed data, while the means reported in figures and tables were untransformed for ease of interpretation.

8.3 RESULTS

8.3.1 Impact of treatment on volume

There was a visual difference between treatments, whereby the trees retained their juvenile foliage much longer than the control and the canopy was much

denser and closed over quicker than the control (Figures 8.5 and 8.6). Standardised tree volumes were higher at Bills than at Sixpenny in each year and the rate of increase between years was also greater at Bills (Figure 8.7). Repeated measures ANOVA of these data showed that all main effects and interactions were significant (P value < 0.05) (Table A8.1, Appendix).

The fungicide/ insecticide treatment at Bills had the greatest improvement at that site with a 10% increase in standardised volume (Figure 8.7). The fungicide treatment had the least amount of improvement of 4.1% (Figure 8.7) while the control had the least amount of volume overall. The combination of fungicide/ insecticide had the greatest improvement in volume at Sixpenny of 13.5%, while the fungicide alone treatment had the lowest of 2.9% (Figure 8.8). The trees at Sixpenny put on more height than girth in the fungicide treatment, while the fungicide/ insecticide and insecticide treatments had similar increases in both height and DBHOB (Figures 8.9, 8.10). The trees at Bills had a greater increase in DBHOB rather than height for all treatments (Figures 8.9, 8.10). The greatest difference between treatments occurred at Sixpenny (Figure 8.7).

Figure 8.5 Comparison between the control (**A**) and insecticide (**B**) treatments after one year of spraying at Bills tree plantation. Note the loss of lower canopy in A.

Figure 8.6 Comparison between the control (**A** and **B**) and fungicide (**C** and **D**) treatments at Bills tree plantation. Trees sprayed with the fungicide treatments retained their juvenile foliage (**C**) much longer and canopy was much denser (**D**) compared to the control (**A** and **B**).

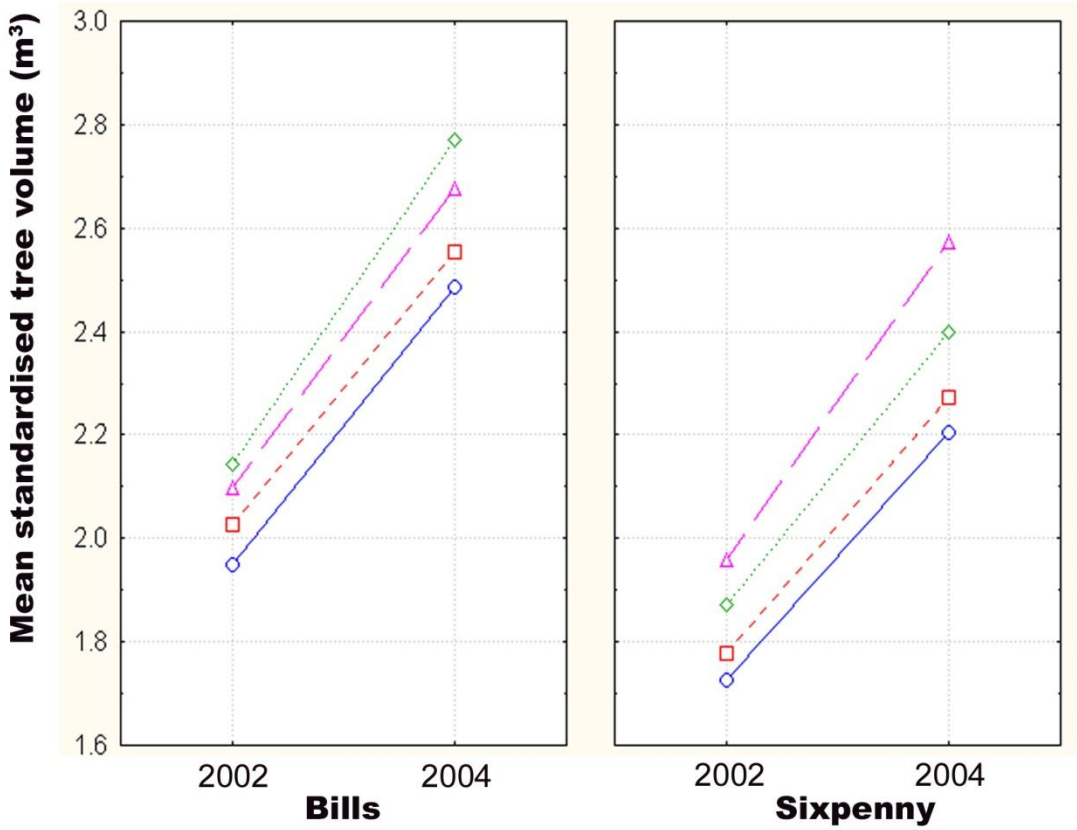

Figure 8.7 Standardised tree volumes at Bills and Sixpenny in 2002 and 2004 for each treatment (blue/ large diamond = control; red/ square = fungicide; green/ small diamond = fungicide and insecticide; pink/ triangle = insecticide).

Post hoc LSD tests showed that at Bills the standardised tree volumes were greater when both insecticide and fungicide were applied (Figure 8.11). The differences in tree volume at Bills as a result of fungicide and insecticide applications by 2004 were comparatively minor, but still significant ($P<0.05$). At Sixpenny, the greatest improvement in volume occurred with the use of insecticide alone (Figure 8.12). The Control treatment at both sites had the lowest standardised tree volume (Figures 8.11, 8.12).

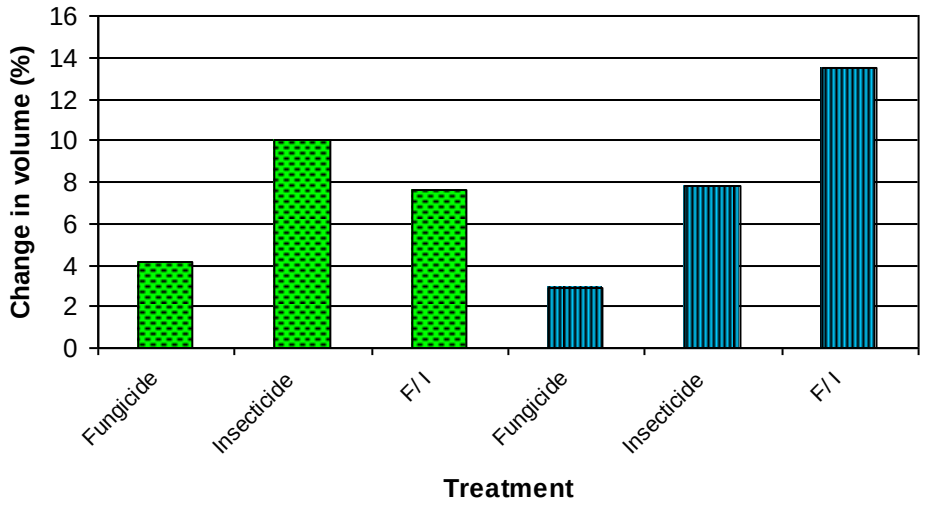

Figure 8.8 Standardised difference in tree volume (%) for three treatments comparing measurements from 2000 and 2004 at Bills (green/ dash) and Sixpenny (blue/ lines). F/I = fungicide and insecticide combined.

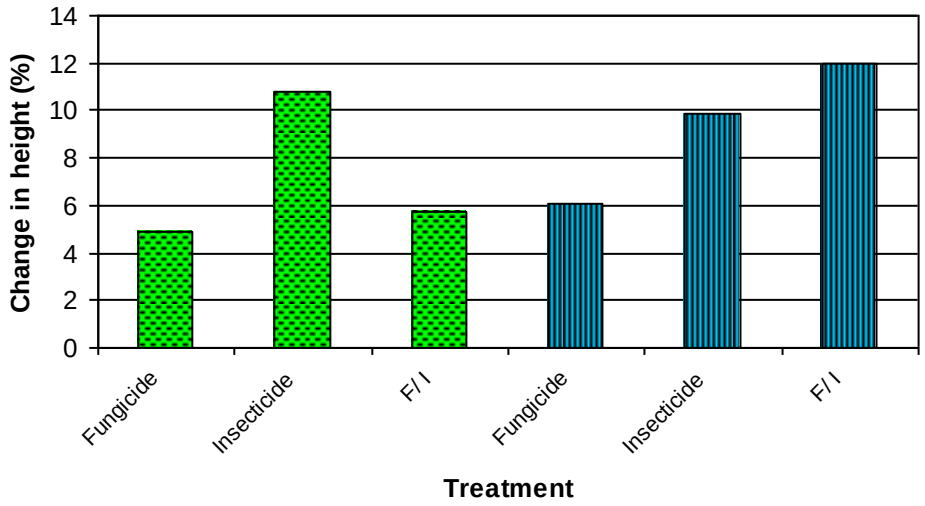

Figure 8.9 Standardised difference in tree height (%) for three treatments comparing measurements from 2000 and 2004 at Bills (green/ dash) and Sixpenny (blue/ lines). F/I = fungicide and insecticide combined.

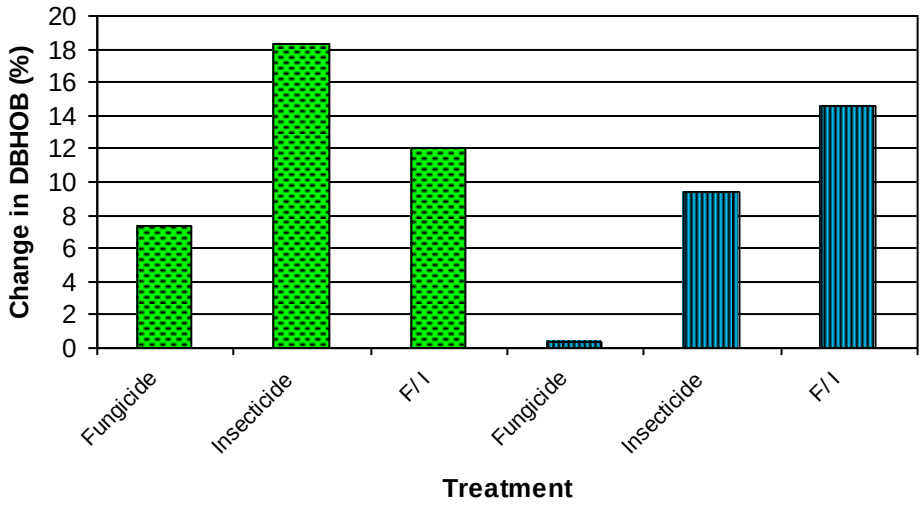

Figure 8.10 Standardised difference in tree diameter at breast height (DBHOB) (%) for three treatments comparing measurements from 2000 and 2004 at Bills (green/ dash) and Sixpenny (blue/ lines). F/I = fungicide and insecticide combined.

A comparison of each treatment in 2000 showed that the trees height and diameter were equally distributed at both Sixpenny and Bills (Figures 8.13, 8.14). After three years of spray treatments, the insecticide treatment at Sixpenny showed less variation between height and DBHOB compared to the control in 2004 (Figure 8.13). At Bills in 2004, the fungicide/insecticide and insecticide treatments had more variation than the insecticide treatment at Sixpenny, however, less than the control at Bills (Figure 8.14).

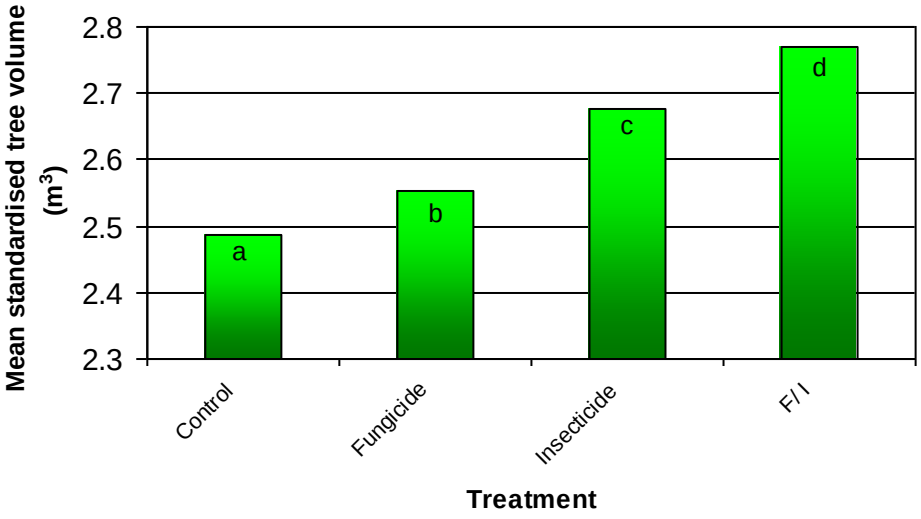

Figure 8.11 Posthoc LSD tests for effects of treatments on standardised tree volumes in 2004 at Bills. Significantly different means are indicated by different letters (P< 0.05). Note the y axis starts at 2.3.

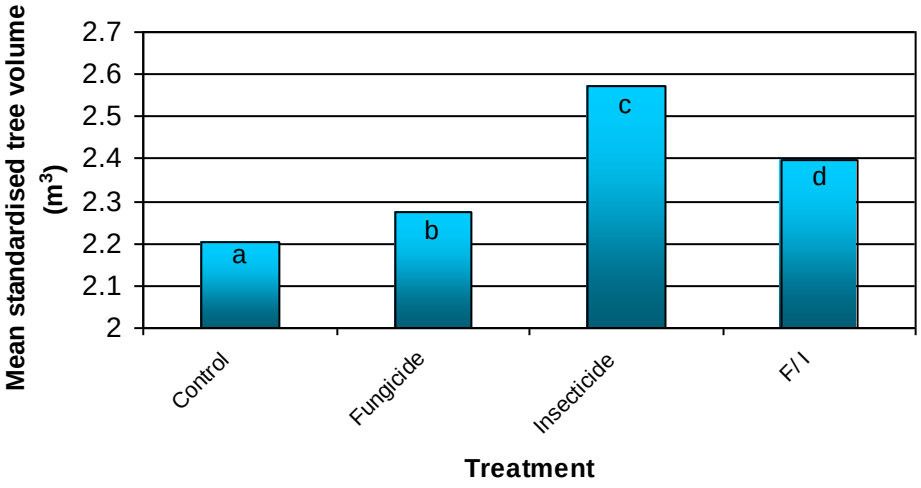

Figure 8.12 Posthoc LSD tests for effects of treatments on standardised tree volumes in 2004 at Sixpenny. Significantly different means are indicated by different letters (P< 0.05). Note the y axis starts at 2.

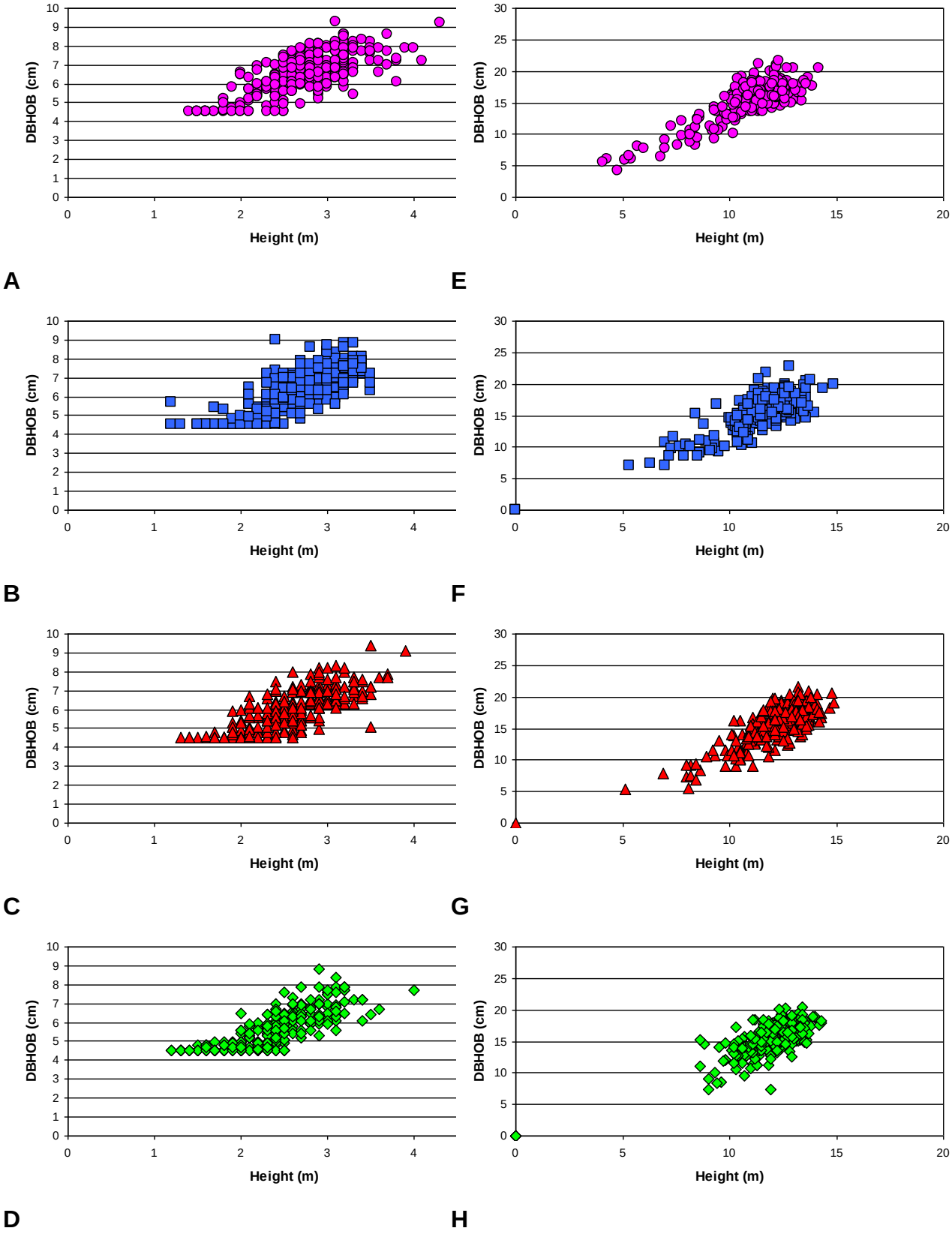

Figure 8.13 The height (m) and diameter (cm) at breast height (DBHOB) for all trees at Sixpenny plantation in 2000 (**A**–**D**) and 2004 (**E**–**H**) for four treatments; Control (Pink/ circle), Fungicide (Blue/ square), Fungicide/Insecticide (Red/ triangle) and Insecticide (Green/ diamond).

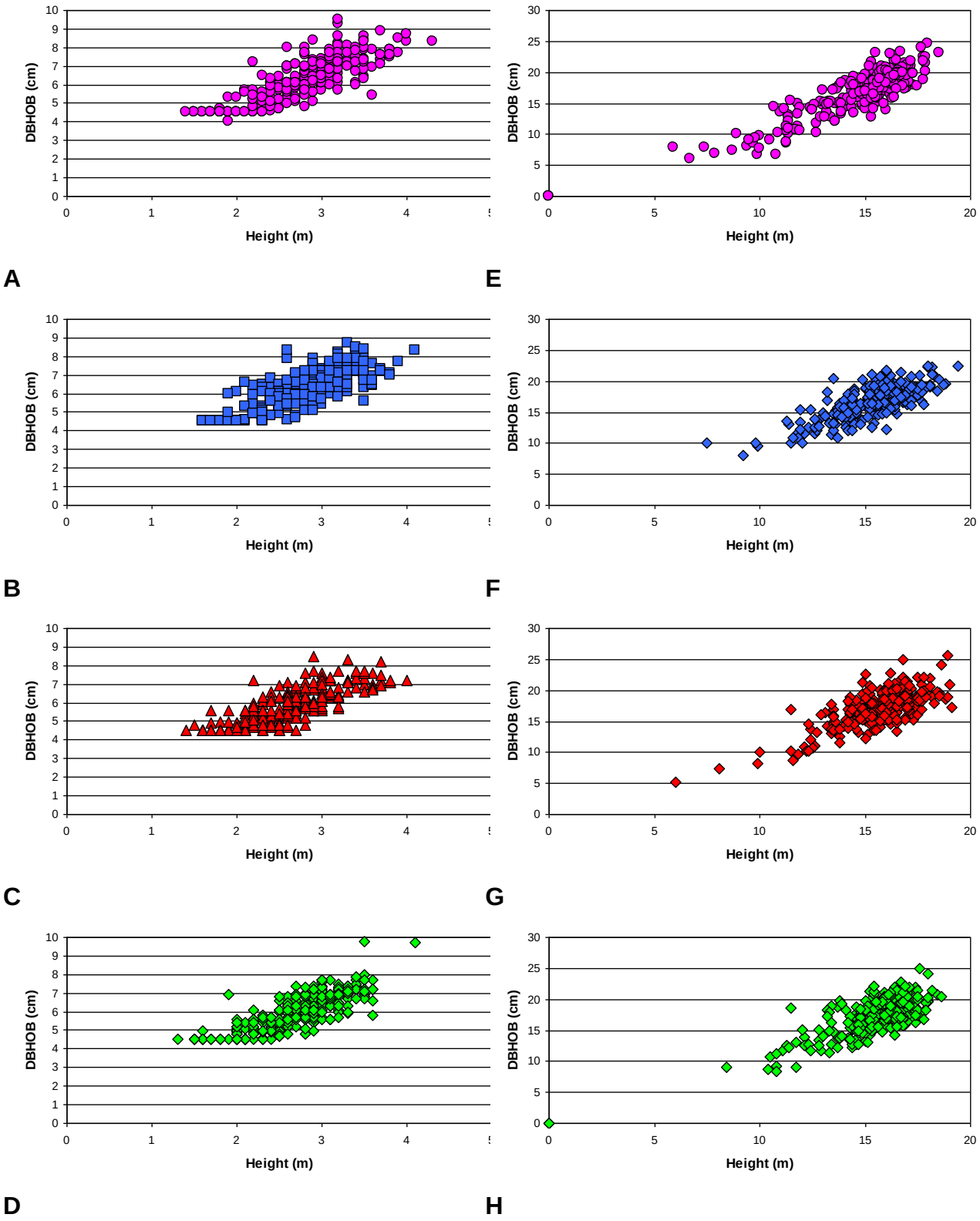

Figure 8.14 The height (m) and diameter (cm) at breast height (DBHOB) for all trees at Bills plantation in 2000 (**A**–**D**) and 2004 (**E**–**H**) for four treatments; Control (Pink/ circle), Fungicide (Blue/ square), Fungicide/Insecticide (Red/ triangle) and Insecticide (Green/ diamond).

8.3.2 Impact of treatments on Mycosphaerella leaf disease and insect chew

8.3.2.1 Comparison of ratings of juvenile leaves from August 2000 and November 2000 from Bills and Sixpenny

Juvenile leaves in 2000 had a highly varied pattern of MLD infection that was not consistently related to site or treatment (evidenced by 3-way interactions in log linear analysis). The insecticide treatment at Bills had the greatest increase in occurrence of MLD in juvenile leaves after six spray treatments compared to the initial assessments at this plantation. The control and fungicide treatments at Bills showed the least amount of increase in MLD (Figure 8.15).

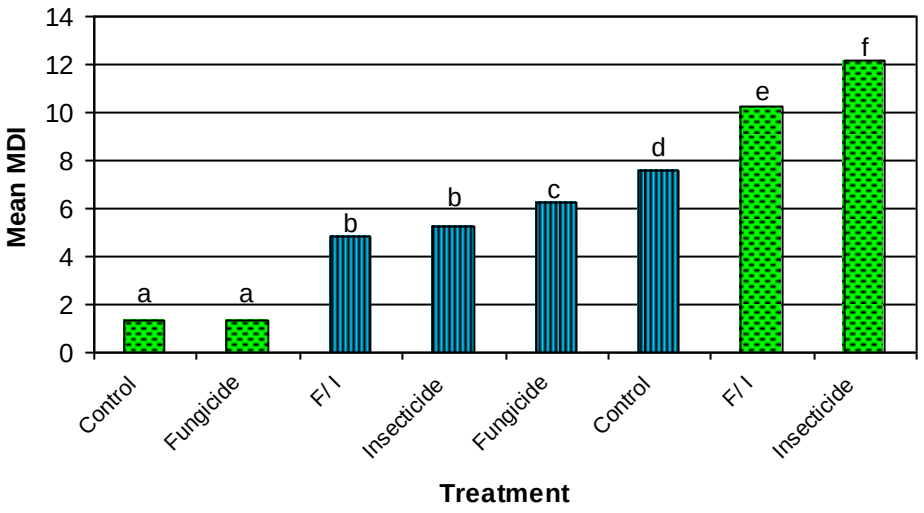

Figure 8.15 Posthoc LSD test of Mycosphaerella damage index (MDI) occurrence comparing incidence from August 2000 (pre-spray) and November 2000 (post six sprays) at both Bills (green/ dash) and Sixpenny (blue/ lines) plantations. Significantly different means are indicated by different letters ($P < 0.05$). F/I = fungicide and insecticide.

The insecticide treatment at Bills had the greatest impact on leaf-chew, followed by the combination of fungicide/insecticide. The fungicide treatment was the most affected by leaf-chew at Sixpenny, while the control treatment had the least occurrence of leaf-chew (Figure 8.16).

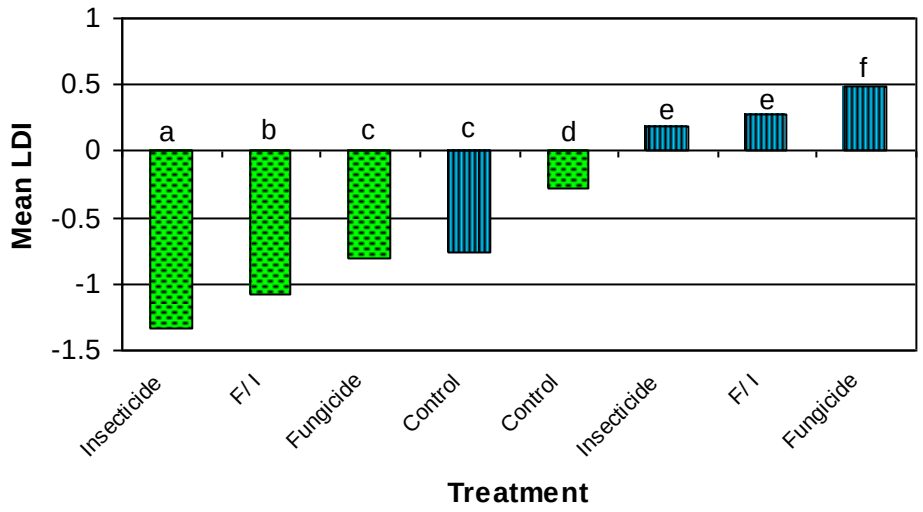

Figure 8.16 Posthoc LSD test of Leaf-chew damage index (LDI) comparing incidence from August 2000 (pre-spray) and November 2000 (post six sprays) at both Bills (green/ dash) and Sixpenny (blue/ lines) plantations. Significantly (P< 0.05) different means are indicated by different letters. F/I = fungicide and insecticide combined.

8.3.2.2 Comparison of ratings of adult leaves at both sites with all treatments based on ratings from 2003 only

Initial MANOVA showed significant (P< 0.05) results for plantation, treatment and the plantation x treatment interaction. Univariate tests with site showed that the MDI was significantly (P< 0.05) higher at Sixpenny but that the LDI did not differ between plantations. Univariate analysis for treatment showed significant (P< 0.05) differences between all treatments for both MDI and LDI (Tables A8.2–8.3 Appendix). Posthoc LSD tests showed that at Bills the control was significantly

(P< 0.05) different to the three treatments combined and this was also true at Sixpenny.

The MDI x LDI interaction was also significant (P< 0.05). Univariate analyses showed that both the (MDI) and (LDI) were significant within the interaction. Posthoc LSD tests showed that the control at Sixpenny was the worst affected of all treatments across both sites, while the insecticide treatment at Bills was the least affected (Figure 8.17). Leaf-chew in the control treatment at both sites was significantly (P< 0.05) more damaging than the other treatments. The insecticide and fungicide/insecticide treatments at Sixpenny had the least leaf-chew damage (Figure 8.18).

At both Sixpenny and Bills level 1 (rated as 1.5%) MLD infection was not reduced significantly by spraying adult leaves and even increased after spraying at Sixpenny. At Sixpenny all spraying treatments at MDI ranks 2 (3%) and 3 (6%) led to a similar reduction in MLD incidence. At Bills, MLD incidence was low at ratings 2 (3%) – 4 (12.5%).

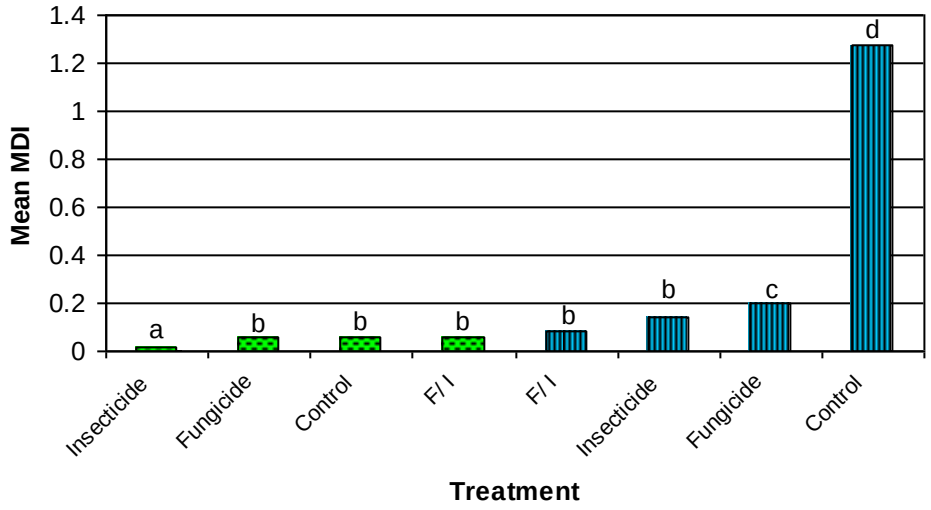

Figure 8.17 Posthoc LSD test of Mycosphaerella damage index (MDI) on adult only leaves at both sites in 2003. Bills (green/ dash) and Sixpenny (blue/ lines). Significantly (P< 0.05) different means are indicated by different letters.

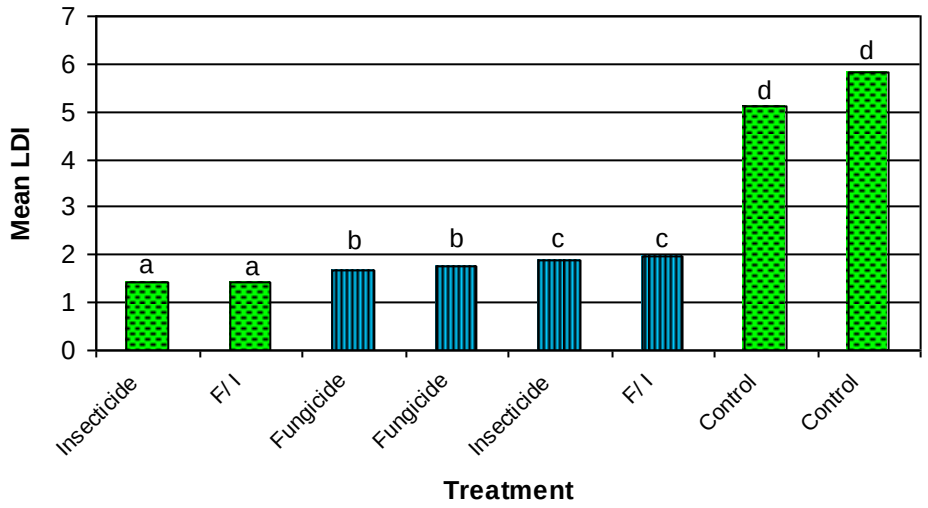

Figure 8.18 Posthoc LSD test for Leaf-chew damage index (LDI) ratings on adult only leaves at both sites in 2003. Bills (green/ dash) and Sixpenny (blue/ lines). Significantly different means are indicated by different letters (P< 0.05). F/I = fungicide and insecticide combined.

8.4 DISCUSSION

The standardised tree volumes were higher at Bills than at Sixpenny, and the rate of increase was highest at Bills than Sixpenny. The insecticide treatment at Bills showed the greatest standardised tree volume increase, with an improvement of 10% compared to the control. The insecticide only treatment at Sixpenny improved wood volumes by 13.5% compared to the control. At both plantations the fungicide only treatments significantly increased tree volumes (4.1% at Bills and 2.9% at Sixpenny) compared to the controls, but were still only comparatively minor. The distribution of height versus DHBOB by 2004 was higher in the controls at both plantations, while the insecticide treatments at Sixpenny resulted in the most uniform trees when comparing height versus DBHOB in 2004. These results may have been influenced by the trial design as the treatments were not randomly placed through the experimental area due to the over-riding operational contraints. The lack of proper randomisation constrained the statistical analysis and the the findings regarding efficacy of particular treatments should be validated in the future with more robust field trials.

Insecticide treatments at Bills had the highest incidence of MLD but the greatest improvement in leaf-chew in juvenile foliage in 2000 after six spray treatments compared to the initial assessments pre-spray. The control treatments at Sixpenny had the highest incidence of MLD but the greatest improvement in leaf-chew control in the juvenile foliage in 2000 after 6 spray treatments. In the adult foliage at Bills in 2003, there was no significant ($P>0.05$) difference of MLD occurrence between the fungicide, insecticide/fungicide and control treatments.

Controls at each site had the highest incidence of insect chew compared to the other treatments.

While site differences had the greatest effect on standardised tree volumes of *E. globulus* between 2002 and 2004, there were also significant treatment effects. The critical question from a management viewpoint is whether the demonstrated increases in standardised tree volume were sufficient to warrant the cost of fungicide and insecticide treatments of the trees?

It is important to point out that overall the plantations experienced a very low incidence of disease and pest attack during the trial period. Even so, the results clearly showed a significant difference between treatment types and disease outcome. This suggests that the use of chemical treatments may be useful in controlling severe disease outbreaks. However, the treatments most likely would have to be ongoing as the initial ratings pre-spray and subsequently after six spray treatments over three months, the incidence of MLD increased, particularly with the use of the insecticide at Bills. There was also no significant ($P< 0.05$) difference between the control and fungicide only treatment for MLD incidence in 2000 at Bills. The highest incidence occurred in the insecticide treatment. At Sixpenny the worst effected treatment was the control followed by the fungicide treatment. The remaining treatments had the lowest incidence of MLD. These results suggest that spraying for MLD will not necessarily reduce the incidence of disease and that the impact of the treatment may be site dependent.

Adult leaves in 2003 did show a clearer pattern. At both Sixpenny and Bills the MDI of level 1 MLD infection was not reduced significantly by spraying, and even appeared to increase after spraying at Sixpenny. At Sixpenny all spraying

treatments at MDI level 2 and 3 led to a reduction in MLD incidence and there was never an MDI of level 3 after any treatments. At Bills, MLD incidence was very low with MDI levels of 2–4. Mean DBHOB at both sites pre-spray was 6.1 cm and the mean height was 2.7 m at Bills and 2.5 m at Sixpenny. In 2004, the mean height at Bills in the control was 14.6 m and the DBHOB was 16.5 cm, while at Sixpenny, the mean height in the controls was 10.9 m and the DBHOB was 15.3 cm.

All chemicals used in the current study have been subsequently banned for use in plantations in Australia under the FSC. Currently, there is an application being sought by the FSC certified Plantations Group of Australia for the derogation of alphacypermethrin for the use against chrysomelids, eucalyptus weevils, autumn gum moth and other detrimental insects.

The use of fungicides on a long-term basis has resistance implications. Benomyl and chlorothalonil have been used for the control of fungal pathogens on several important horticultural crops including peanuts and bananas (Culbreath *et al*. 2002; Cañas-Gutiérrez *et al*. 2006). Benomyl resistance in *Cercospora arachidicola* and *Cercosporidium personatum* foliar pathogens of peanuts occurred in the 1970's and use was reduced in the south-eastern parts of the USA (Cañas-Gutiérrez *et al*. 2006). Cañas-Gutiérrez *et al*. (2006) reported that benomyl-resistant isolates of *C. personatum* still persisted during their studies, and that control of *C. personatum* would be limited after one season of benomyl use. The use of a mixture of chlorothalonil and benomyl or alternating treatments had greater control of disease compared to benomyl alone or alternating blocks

of benomyl and chlorothalonil (Cañas-Gutiérrez *et a*l. 2006). Registration of benomyl has since been withdrawn by the manufacturer.

The application of the insecticides used in the current study had a significant ($P< 0.05$) effect on the visual appearance of leaf-chew. It must also be noted, however, that the fungicide only treatments at both plantations had a similar effect on leaf-chew as the insecticides.

There are several factors that affect investor return at harvest in plantation timber. These include price received for the pulpwood produce, together with harvesting and processing costs. Other factors include exchange rates, interest rates and inflation. Based on a figure of $40/green metric tonne (GMT), the highest return at Sixpenny (using the 13.6% increase in wood volume) would be an extra $758/ha, while at Bills it would be $451/ha extra. The total cost of spraying the three treatments over the three years was $10 929 for each plantation. Therefore, the increase in wood volume would not cover the costs of such intensive management. Although the spraying was for a period of three years, if this had continued until harvesting of the plantation, it is predicted that the costs involved would not be recouped by the volume increase. This has yet to be investigated fully, however, based on the results of the current research, continued spray treatments would have limited usefulness.

Profits are dependent on costs associated with growing, managing and harvesting (Battaglia *et al.* 2002). Through growth modelling, however, tree growth rate is the most important factor in determining profit, and site selection and spatial distribution have the greatest impact on growth (Battaglia *et al.* 2002). This suggests that post planting management costs are subject to tree health,

and intervention in rectifying limiting factors such as nutrient and water availability are costly and reduce profit. Second rotation crops may need additional management due to an increase in pests and pathogens; costs probably would still exceed profit.

The impact of a one-off fungicidal spray, or from the current study, six fungicidal spray treatments, appears to have limited use in suppressing MLD. Pinkard and Mohammed (2006) showed that the presence of MLD reduced light-saturated photosynthesis and the same trend occurred at several plantation sites. They also suggest that light-saturated photosynthesis also affected asymptomatic tissue. This provides problems from a management perspective, as infection level would be hard to predict. The first three months of the current study also supports this issue. Even after six spray treatments, the presence of MLD was higher than at pre-spray levels. This also shows the effectiveness of the treatments was not as good as expected from systemic fungicides. This suggests that leaves were already infected but were asymptomatic.

Pinkard *et al*. (2006b) found that when nitrogen was limiting, defoliation resulted in a decrease in both height and diameter and that, on sites where nitrogen was not limiting, added nitrogen may help maintain stem growth following defoliation. Defoliation of 38%, in the absence of nitrogen application, resulted in a 17% reduction in height after 20 months; however, the diameter of the trees was not as great (Pinkard *et al.* 2006b). The pattern of defoliation was also examined and removal of the upper canopy rather than the lower crown increased the effect of defoliation on stem growth. The lack of response to a bottom up defoliation event

was described as being due to the rapid growth rates observed over the period of the experiment (Pinkard *et al.* 2006b).

Pinkard *et al.* (2006a) also found that application of nitrogen or nitrogen plus phosphorus increased stem diameter and height. The height and diameter was reduced 12 months after application of fertiliser and 10% defoliation. They showed that application of fertiliser can assist to counteract the effect of insect defoliation and that although there were no signs of nutrient stress that site nutrient availability was a limiting factor on growth (Pinkard *et al.* 2006a). The addition of nitrogen also reduced the proportion of juvenile leaves, which could assist in preventing subsequent attacked from insects that prefer juvenile foliage.

Both the incidence of leaf-chew and MLD at Bills and Sixpenny remained low throughout the experiment; however, the control at Sixpenny showed a significantly higher incidence than the other treatments in the adult foliage, followed by the fungicide treatment. The fungicide/ insecticide treatment had the highest incidence of MLD at Bills followed by the control. The insecticide treatment at both sites resulted in the lowest incidence of MLD at both sites. The insecticide and fungicide/insecticide treatments at Sixpenny had the least significant amount of leaf-chew across the sites, whereas the fungicide treatment at Bills was the lowest at that site in adult foliage.

Defoliation in the current study was not adequately recorded. In 2003, for both the control and insecticide treatments, leaves at both sites had senesced; consequently analysis of juvenile foliage could not be undertaken. In contrast, trees treated with the fungicide and fungicide/insecticide had retained their juvenile foliage at both sites. By 2004, the fungicide/insecticide treatment at Bills

had the most significant growth increase, while the insecticide treatment at Sixpenny had the highest growth compared to the other treatments. This possibly indicates a site or treatment effect rather than a defoliation effect. Both sites were located in areas within the marginal rainfall isohyets for productive *E. globulus* plantations (Table 8.1). Also, although the plantations are relatively close to each other, they have different soil types which may also affect the environmental stress and consequently growth input of each site. The provenance used at each sites was the same; however, often a high intra-provenance variation can occur (Milgate *et al*. 2005). Therefore, genetics can also affect the incidence of both MLD and insect herbivory. Milgate *et al*. (2005) found both a significant difference between provenances and within provanences to *M. nubilosa* infection in Tasmania.

Jackson (2003) showed that trees defoliated above 80% for prolonged periods of time compromised them to infection by the canker fungus *Endothellia eucalypti*, and that a defoliation level of 100% was needed to induce significantly larger lesions. Trees also regenerated more rapidly after being defoliated at 90 or 100%, compared to lower defoliation levels and that the higher the defoliation level the higher the photosynthetic rate (Jackson 2003). Jackson (2003) suggests that this adaptation could explain that even at high levels of defoliation, trees were not significantly susceptible to canker fungi. This also supports other studies that have shown that a single MLD event did not have any impact on the growth of *E. globulus* (Collett and Neumann 2002).

In the current study, there was an increase in volume at Sixpenny of 13.5% in the fungicide/insecticide treatment, while at Bills there was an increase in volume of

10% in the insecticide treatment. In the fungicide only treatments there was an increase in volume of 4.1% at Bills and 2.9% at Sixpenny. Smith (2006) found a 17% reduction in volume at 21 months of age between trees treated with fungicides and those left untreated. Trees treated with fungicide (flusilazole) kept the MLD damage threshold to below 13%, while untreated trees with a defoliation level of 20% resulted in a 17% reduction in growth (Smith 2006). It in unknown if this trend continued during leaf phase change into adult foliage. Milgate *et al.* (2005) investigated the effect on growth at both a genetic and phenotypic level. They showed that for every percentage increase in defoliation attributed to MLD there was a potential loss of 1.22 cm in height over four years, or alternatively, a decrease of 10% in disease severity may result in a 3.1% increase in growth. They also observed genetic variability to MLD, suggesting that MLD resistance could be selected.

Carnegie and Ades (2002) showed that a combination of alternating chlorothalonil and benomyl effectively controlled MLD in a field trial of *E. globulus* in Victoria. They observed significantly less disease on both juvenile and adult foliage and less defoliation of juvenile foliage in those treated with the fungicides. In the untreated trees, height and diameter were reduced at low levels of MLD, reducing height by 13% in trees with less than 10% MLD. They also suggest that aerial spraying of plantations at the juvenile leaf stage would not be as effective as ground application as the underside of the leaves would not be adequately covered with the fungicide. As one of the most notable pathogenic *Mycosphaerella* species, *M. nubilosa* is known to infect leaves via stomata on the abaxial side of leaves (Park and Keane 1982b). This was also noticed by Washington *et al.* (1998), who commented that aerial application only covered

the adaxial surface of banana leaves and as *M. fijiensis* infects the abaxial surface may limit the effectiveness of fungicide treatments. After testing the application of chlorothalonil on both surfaces of banana leaves for the control of *M. fijiensis* (black sigatoka) they found that they could control between 76–100% compared to 0–13% when applied to the adaxial leaf surface (Washington *et al.* 1998). This could possibly account for the lack of suppression of MLD in both plantations in the current study after six spray treatments. Although the fungicide was sprayed using a mister, it may not have been adequate to cover both leaf surfaces. However, Carnegie and Ades (2002a) also observed a lag period at the beginning of the fungicide spraying regime. The first results to show a significant difference between sprayed and unsprayed treatment to MLD on juvenile foliage were observed three months after spray commencement. In the current study, after three months and six spray (months later) treatments, there appeared to be no discernable pattern of MLD control between plantations or treatments. The insecticide treatment at Bills had the highest incidence of MLD and Bills control had the lowest from the two sites. With regard to spraying of the adult foliage, low levels of infection (1.5%) did not appear to respond to fungicide treatment at either Bills or Sixpenny. This again could be due to the lack of effectiveness of the fungicide due to the application method.

Insects are also responsible for high levels of defoliation in eucalypt plantations. Loch and Floyd (2001) list the following insects as important post establishment pests: *Gonipterus scutellatus* (eucalypt weevil); *Chrysophtharta* spp*., Cadmus excrementarius* (chrysomelids); *Mnesampela privata* (autumn gum moth) and *Phylacteophaga froggatti* (leaf blister saw fly). Collett and Neumann (2002) reported that total removal of foliage from the lower crown increased height and

that only repeated severe to total crown defoliation during summer negatively impacted on growth rates. From their study, they recommended that pruning of the lower canopy prior to summer would induce premature canopy closure, thus, reducing the impact of light-seeking insect pests that are present before canopy closure (Collett and Neumann, 2002). Jordan *et al.* (2002) also reported that mild and severe damage from sawflies (*Perga affinia* ssp. *insularis*) in Tasmania on *E. globulus*, resulted in a 16 and 31% reduction in basal area of surviving trees. In the current study, the treatment that had the most uniform trees was the insecticide treatment at Sixpenny, indicating that spraying insecticide had a positive effect tree growth. This treatment also had the greatest improvement in volume change at Bills with a 10% increase over the controls. The combination of fungicide/insecticide had the greatest improvement at Sixpenny with a 13.5% increase in volume. It is important to note that at Sixpenny the fungicide/insecticide and insecticide treatment also had the lowest level of insect attack in adult foliage compared to Bills.

The demand for FSC accredited plantation timber from Australia has resulted in several pesticides being banned for use. The fungicides and insecticide used in the current study are either banned or under derogation in Australia. Benomyl is no longer produced by DuPont and in 2001 its registration was cancelled. Alternatives for control of both insects and fungi will need to be studied further. The addition of nitrogen or phosphorus has been investigated for the control of MLD in the eastern states of Australia. Carnegie and Ades (2002b) suggest that low levels of phosphorus increase the susceptibility of *E. globulus* to *M. cryptica* compared to trees given high levels of phosphorus. They surmise this to be either because additional phosphorus increases resistance to infection or by

accelerating transition from juvenile to adult foliage by increasing growth (Carnegie and Ades, 2002b). They also examined the effect of nitrogen; however, did not find any significant correlations between nitrogen content in the leaves and disease severity (Carnegie and Ades, 2002b).

While site differences had the greatest effect on standardised tree volumes of *E. globulus* between 2000 and 2004, there were also significant treatment effects. The critical question from a management viewpoint is whether the demonstrated increases in standardised tree volume are sufficient to warrant the costs of fungicide and insecticide treatments of the trees? From the current study this would not appear so.

This study demonstrates that monitoring for pests and disease is more effective than spraying of chemical treatments for the first three years. The regular use of chemical treatments is expensive to maintain and is proving to be environmentally unacceptable by some communities. This study also showed that spraying for low levels of MLD had little effect on disease incidence and/ or volume increase in *E. globulus* plantations in WA. The most important factors for a healthy plantation appear to be site selection, preparation and tree genetics.

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CHAPTER 9

GENERAL DISCUSSION

9.1 Introduction

This study was the first to investigate the impact of MLD on the growth of *Eucalyptus globulus* plantations in WA. As part of this study, the biology, taxonomy and pathogenicity of the main species present in WA were investigated. The key findings were: **i**) the number, abundance and distribution of *Mycosphaerella* and *Teratosphaeria* species in WA is not static and plantations should be continually monitored for the presence of new potentially threatening species; **ii**) spraying for MLD, although effective in reducing the prevalence and impact on growth, was not economically viable; and **iii**) intragenomic variation of the ribosomal genome may explain sequence variation observed in single spore isolates of *Mycosphaerella* and *Teratosphaeria* and this has taxonomic implications. Aspects of these and some related matters are discussed below.

9.2 Industry needs to manage foliar pathogen threats

During the initial stages of the current study, there was a strong interest from the industry partner in determining their needs with regard to disease management for future planning and operations. In order to do this, issues were raised as to: 1) whether the industry should intervene in trying to manage disease and disease outbreaks; 2) the priority the industry should place on determining taxa causing diseases in plantations and measuring their relative impact; 3) how plantation managers and silviculturalists can know what diseases are present in plantations; and 4) the risks of pathogen introduction when sourcing eucalypt genetics from many places in order to better match genetics with site and environment. Also, there was an opportunity for the industry to be proactive and

to plan for screening of host material for resistance to pathogens that threatened yield forecasts.

During the course of the current study, *M. suttoniae* (≡*Kirramyces epicoccoides*) a key pathogen of tropical eucalypts was isolated near Albany on *E. globulus*. Surveys of trial plots of both eucalypt hybrids and *E. globulus* full-sib family trees were conducted. Also native endemic *Eucalyptus* species were opportunistically sampled for leaf pathogens. The industry was keen to take a pro-active approach and further surveys were conducted at the sister genetic trial site in Esperance (WA) and also surrounding plantations to the infected site in Albany. However, no further incidence was recorded. Therefore, as this was largely a tropical pathogen, found at very low levels in Albany and not found outside the genetic trials, the industry took a 'monitor and wait and see approach' before taking any further action. Only plantations belonging to ITC were surveyed, as unfortunately there was no co-ordinating pathological response group at the time - the Integrated Pest Management Group (IPMG) at that time was solely focussed on insects. Fortunately, it does not appear to have become a major issue. However, as there remains uncertainty as to the likelihood of increased summer rainfall events, especially in the Esperance region continued monitoring is highly recommended in case conditions favour disease outbreak.

Chemicals are routinely used in Australia to control pests and pathogens, especially in agriculture and horticulture. When the current research was planned, application of chemicals in forestry was an acceptable course of action in Australia. The timing of spraying is crucial if control is to be established, as was shown by Park (1984) for *M. cryptica* and *M. nubilosa*. For MLD, the time

between leaf infection and visible signs of necrosis and pseudothecial or conidial production is approximately one month, by which time the spore load would have increased, and spraying of fungicides when necrosis is evident may not prove beneficial to reduce disease impact. For this reason, early detection methods could prove useful. One approach explored in the current study was to test the effectiveness of using species-specific primers to detect the presence of *Mycosphaerella* and *Teratosphaeria* in asymptomatic or early phase symptoms. This approach proved problematic and could not be developed to be sufficiently reliable or cost effective, to be used as an early detection method for disease management.

Real-time PCR techniques could be developed using the species-specific primers that were developed during the course of this study. This would give a quantitative figure to the amount of DNA present in a sample. A practical use of this could be the use of spore-traps, then assessing the amount of DNA that is trapped on the tape and then comparing it to the level of disease present. If developed diagnostically, it may be able to give a prediction of future disease levels. Where this would not be practical is with species that proliferate predominantly with conidia that are not air-borne, such as several *Teratosphaeria* species.

The plantation sector is concerned with the possibility of fungicide resistance with repeated applications of the same chemicals. The disease cycle and control of MLD is complex, as was indicated by the results of the exclusion trial (Chapter 8). Not only are the environment and the host mitigating factors in the level of disease expression, but there also appeared to be a relationship between insects

and MLD. This relationship requires further investigation. Possibilities include insects acting as vectors of pathogens at the branch or canopy level, or that chewed leaves may be more susceptible to infection than undamaged leaves. The application of the insecticides used in the current study had a significant effect on the visual appearance of leaf-chew (Chapter 8). It must also be noted, however, that the fungicide only treatments at both plantations had a similar effect on leaf-chew as the insecticides. It is unclear why this was so.

The demand for FSC accredited plantation timber from Australia has resulted in several pesticides being banned for use. The fungicides and insecticide used in the current study are now either banned or under derogation in Australia. Benomyl is no longer produced by DuPont and in 2001 its registration was cancelled. Alternatives for control of both insects and fungi will need to be studied further. The addition of nitrogen or phosphorus as fertilisers has been investigated for the control of MLD in the eastern states of Australia. Carnegie and Ades (2002) suggest that low levels of phosphorus increase the susceptibility of *E. globulus* to *M. cryptica* compared to trees given high levels of phosphorus. They surmised this to be either because additional phosphorus increases resistance to infection or by accelerating transition from juvenile to adult foliage by increasing growth (Carnegie and Ades, 2002). They also examined the effect of nitrogen; however, did not find any significant correlation between nitrogen content in the leaves and disease severity (Carnegie and Ades, 2002).

Knowing your enemy is proving to be harder and harder with regard to MLD in eucalypt plantations. It is not a simple case of identification of causal species, as

the biology of the majority of species that have been isolated from plantation eucalypts remains unknown, and therefore, treatment or breeding for resistance become problematic. Traditional methods to test for pathogenicity remain unworkable, as many of the MLD species do not grow well in culture and/ or do not produce conidia and a molecular approach for testing for pathogenicity genes is very limited. The questions remain, do foresters need to know what species are causing disease, and would the methods for controlling MLD be any different?

The recent removal of *Eucalyptus grandis* x *camaldulensis* hybrids in Queensland, after a severe incursion of *Kirramyces* (≡ *Teratosphaeria*) (Collins pers comm.), is a good example of where a sound knowledge of the host, environment (climatic conditions and site) and potential pathogens were not well considered. The pathogen was first noticed in 2006, however, disease was at low levels and the trees recovered. By 2009, the level of disease had escalated and the trees were deemed not viable for pulpwood and subsequently removed. However, until techniques used to test pathogenicity of key *Mycosphaerella* and *Teratosphaeria* species are developed, research into disease resistance is still dependent on field-based data of the MLD complex and not individual species. The financial cost of doing this can be significant.

Eucalypt plantations are often planted in close proximity to other eucalypt species (Maxwell 2004) in Australia, and this contrasts with other parts of the world. In WA, many of the plantations are on ex-agricultural/pasture lands that lie adjacent to remnant vegetation with native eucalypts such as *Eucalyptus marginata* and *E. diversicolor.* The impact and implications of pests and diseases

moving from native eucalypt stands into exotic eucalypt plantations and vice versa is poorly understood. There is a need to understand how these movements might impact on native and exotic stands, especially as the latter tend to be even-aged monocultures which could potentially allow massive build up of pests and pathogens which subsequently move into native stands, or alternatively, from native stands to plantation eucalypts. For example, it not that long ago that *M. cryptica* was been isolated from two of the most important endemic eucalypt species (*E. marginata* and *E. diversicolor*) (Carnegie *et al*. 1997) in Western Australia and *M. marksii* from *E. rudis* (Jackson et al. 2005). The incidence of *M. marksii* appears to be increasing, and isolations have been made without association with *M*. *nubilosa* or *M. cryptica*. This indicates that *M. marksii* may in fact act as a primary pathogen, however, until Koch's postulates are confirmed, it should not be called a pathogen. There are also remnant stands of native vegetation within eucalypt plantations and their health and management is also a consideration for plantation managers.

South-western Australia is a region where climate change (reduced rainfall, increased temperature) is already apparent and of concern to the plantation sector (Hughes 2003). A recent study used climate modelling to predict the impact of defoliation caused by MLD on wood volume *of E. globulus* at 5 sites, 2 of which were in WA (Pinkard *et al*. 2010). A changing climate had no effect on severity ratings of MLD at the WA sites, more than likely because the length of favourable conditions remained unchanged in WA compared to the other sites in south-eastern Australia. The amount of rainfall appeared to have the greatest influence on volume. Reductions in stem volume occurred soon after defoliation caused by MLD, and were not recovered during the rotation length. The effect of

MLD on volume was most noticeable in WA with volume reductions under current climate models as high as 12.6% with the average being 6.3% and four of the six sites averaging just 3%. Site conditions including fertility, irrespective of rainfall, had the greatest impact on MLD. Pinkard *et al.* (2010) suggest that the impact of MLD is likely to increase in the future with changes in nitrogen supply, rainfall and temperature, thus, factors associated with climate change are likely to influence the capacity for trees to recover after an incursion of MLD. A review of the possible effects of climate change on forests in Australia suggests that temperature will have no strong positive affect in the rise of pathogens, but that it is strongly host species dependent (Medlyn *et al*. 2011).

9.3 Taxonomy

Part of the aims of the current study was to clarify the taxonomy of *T. parva*, *M. grandis*, *M. aurantia* and *M. gregaria*. This was challenging as the type cultures of *T. parva* and *M. gregaria* were dried and the leaf material in the case of *T. parva* was over 30 years old. In order to amplify rDNA from the leaf tissue, specific primers were designed from the ITS region. During the course of sequencing isolates from eastern and western Australia, it became apparent that there was intraspecific variation within the ITS region of both *T. parva* and *M. grandis* isolates, and that each of these species were con-specific. The reason for the intraspecific variation was out of the realm of this study, however, other studies of ascomycetes, including one of *M. punctiformis* also found that intraspecific variation does occur and at a much higher rate that first thought (Simon *et al*. 2008).

The other species that required clarification were *M. gregaria* and *M. aurantia*. The type specimen of *M. gregaria* was described pre-rDNA sequencing isolates in eastern Australia (Carnegie and Keane 1994) and hence during a review of the species isolated in WA, the *M. gregaria* described by Maxwell *et al*. (2003) could have been con-specific with that described by Carnegie and Keane (1994). This was confirmed by sequencing the rDNA ITS region of an isotype of *M. gregaria* from Victoria and several isolates of *M. aurantia* and the '*M. gregaria*' from WA. It was found that the isolates described as *M. gregaria* by Maxwell *et al*. (2003) were *M. ellipsoidea* and that the new species described by Maxwell *et al*. (2003) as '*M. aurantia*' was *M. gregaria*.

The taxonomy of the *Mycosphaerella* genus and its associated anamorphs has been undergoing a radical change in recent years. These changes have been largely based on molecular phylogenies of the ITS and LSU regions of the rDNA. Taxonomists face the dual challenges of reaching scientific consensus of a species, as well as dealing with the number of species that are yet to be discovered and described (Padial *et al*. 2010). Since molecular techniques are becoming more widely available, a more integrative approach of using traditional morphology and molecular phylogenies are being used for species descriptions. Padial *et al*. (2010) describe the advantages and disadvantages of these methods of identification and contrast tree-based and non-tree based methods for identifying relationships.

Hunter *et al*. (2006) was the first to use a multi-gene phylogeny to represent the different clades of *Mycosphaerella* species occurring on *Eucalyptus*. Three gene regions were used, the large subunit ribosomal DNA (LSU) partial sequence,

translation elongation factor 1-α (EF-1α) partial sequence and the internal transcribed spacers 1 and 2 flanking the 5.8S ribosomal DNA gene (ITS). A fourth gene (actin) was not used, as this did not support data from the other gene regions, indicating some variation within clades that were supported by both the other genes and also morphological characteristics. Based on the multi-gene phylogeny, Hunter *et al*. (2006) synonymised *M. ambiphylla*, *M. molleriana* and *M. vespa*. A study by Silva *et al*. (2009) singles out a particular isolate of *M. molleriana* as being different to those of *M. vespa* and questions the synonymy. However, this difference is also apparent in the ITS phylogeny by Hunter *et al*. (2006). Carnegie *et al*. (2007) described a new species that was morphologically similar to *M. vespa* with the exception of a slight constriction of the ascospore and germination pattern, however, upon sequencing was found to be quite different, falling outside the main *Mycosphaerella* clade represented in the paper. It also remains in the *Mycosphaerella* genus (Hunter *et al*. 2011). *Mycosphaerella ambiphylla* is also (mentioned as *M. ambiphylla* and *Teratosphaeria ambiphylla*) in Hunter *et al*. (2011) as a distinct species, however, according to Carnegie (pers comm.) this species remains synonymised with *M. molleriana*.

The focus of Chapter 7 was on the ecology *U. Dekkeri* in terms of determining whether it is hyperparasitic; or alternatively plant parasitic. There are important taxonomic implications around the ecology of this fungus and its generic identity as either *Dissoconium* or *Uwebraunia*. The genus *Dissoconium* was established based on *D. aciculare*, a suspected hyperparasite on *Erysiphe* (de Hoog *et al*. 1983). Whereas *Uwebraunia* was described as an anamorph genus for three species with *Mycosphaerella*-like teleomorphs associated with leaf spot diseases

of *Eucalyptus* spp. (Crous & Wingfield 1996, Crous 1998) and thought to be plant parasites.

The focus of Chapter 7 was on the ecology *U. dekkeri* in terms of determining whether it is hyperparasitic; or alternatively plant parasitic. There are important taxonomic implications around the ecology of this fungus and its generic identity as either *Dissoconium* or *Uwebraunia*. The genus *Dissoconium* was established based on *D. aciculare*, a suspected hyperparasite on *Erysiphe* (de Hoog et al. 1983). Whereas *Uwebraunia* was described as an anamorph genus for three species with *Mycosphaerella*-like teleomorphs associated with leaf spot diseases of Eucalyptus spp. (Crous & Wingfield 1996, Crous 1998) and thought to be plant parasites.

The results of Chapter 7 indicated that *U dekkeri* is not a hyperparasite of the two most common causes of MLD of eucalypts in Western Australia. The results of that chapter showed a possible plant parasitic ecology based on SEM evidence of leaf infection via leaf stomata. The SEM studies from that chapter also showed that conidiogenesis is both percurrent and sympodial. This finding contaradicted the former distinction between *Uwebraunia* and *Dissoconium*: *as* diagnostically percurrent in the former and sympodial in the latter. Following the publication of chapter 7 *Uwebraunia* was synonymised under *Dissoconium,* and then more recently separated again by Li et al (2012), who showed that species in that '*Dissoconium*' complex cluster in two well-supported clades. They resurrected the genus *Uwebraunia* to accommodate those species (*U. australiensis, U. commune, U. dekkeri and U. musae*) which have small, pyriform microconidia, do not form sclerotia or any yellow pigment in culture, and are associated with a

Mycosphaerella like teleomorph. The genus *Dissoconium* (*D. aciculare, D. eucalypti* and *D. protea*) has large, obclavate to ellipsoid microconidia, and produces sclerotia as well as a yellow pigment in culture." In doing this the authors cite the published work from Chapter 7 which found *U. dekkeri* to be a plant parasite rather than a hyperparasite; and that it has both sympodial and percurrent conidiogenesis. Therefore recognising the former characters used to delimit *Dissoconium* and *Uwebraunia* as being dubious; in part based on the findings of Chapter 7. The teleomorph of *U. dekkeri* was never isolated in the current study. The conidia of *U. dekkeri* look very similar to the ascospores of *M. lateralis* as drawn by Crous (1998). As many *Mycosphaerella* species produce asci on the same lesions, it is therefore plausible that asci of another species could have been mistakenly picked off the leaf and assumed to be those of *M. lateralis*.

The study by Hunter *et al*. (2006), using multiple gene regions, supports the finding made in the current study regarding *T. parva*, *M. grandis*, *M. aurantia* and *M. gregaria*. That is *M. grandis* should be formally synonymised with *T. parva* and *M. aurantia* with *M. gregaria*. From the ITS data generated in the current study, whereby multiple ITS sequences were sequenced from the same isolate, a small number of nucleotide changes (less than 1%) should not be used as evidence of a new species. The LSU may be a better gene region to use in such situations as it is considered by Hunter *et al*. (2006) to be a more conserved gene region, and shows less nucleotide differences than the ITS and EF-1α.

Taxonomy is important especially in Australia from a biosecurity perspective as quarantine or the potential to keep exotic pathogens out is easier than in other

countries sharing borders. A recent example was where taxonomy hindered the containment of Myrtle Rust caused by *Uredo rangelii* that causes disease on Myrtaceae (Carnegie and Cooper 2011). *Uredo rangelii* is considered part of the *Puccinia psidii sensu lato,* however, was not listed as a High Priority Pest in the Plantation Timber Industry Biosecurity Plan, unlike *P. psidii* which was later listed after the introduction in Schedule 13 (Level 1) of the Emergency plant Pest Response Deed. As a consequence, federal funding could not be immediately sourced after the initial diagnosis. After the introduction of the pathogen in 2010, even though Myrtle rust had not been recorded in Australia, it was not considered to be a major threat, which is very surprising. This demonstrates how different concepts in taxonomy can hinder biosecurity responses. This meant that there was confusion in enacting the containment policies (Carnegie *et al*. 2011). By the time molecular work was carried out and ITS sequences showed the two species were con-specific; the possibility of containment had decreased substantially. There remains some dissention around the taxonomic features that distinguished these two species, as the gene regions used to separate *Puccinia* species show a consensus (Carnegie *et al*. 2011, Carnegie *et al*. 2012). It is not uncommon for taxonomists to disagree; however, those disagreements can have disastrous effects to the biosecurity of Australia. Synonyms and anamorph/ teleomorph relationships should also be contained on any national database concerning quarantine.

9.4 Biology

In this section a number of issues concerning the epidemiology and pathogenicity of *Mycosphaerella* species are considered that arose in earlier chapters.

The biology of two key species, *M. nubilosa* and *M. cryp*tica have been previously investigated, however, this research was conducted 20–30 years ago, and on limited host genetic material (Park 1984). Since then there has been a lack of science regarding the biology and pathogenicity of the majority of the species that have been identified from eucalypts in Australia. There are several publications that incorrectly label certain species pathogenic or the causal agent of leaf damage when no formal testing has been conducted (Carnegie and Keane 1994; Carnegie *et al*. 2007). The trial in the current study followed on from a previous study (Jackson 2001). In both studies, *M. marksii* hyphae was observed to infect leaves of *E. globulus*, however, no lesions could be induced. It remains therefore uncertain if *M. marksii* is a pathogen, saprophyte or a hemi-biotroph.

In the present study, the observed slow onset of disease development of *M. cryptica* and *M. nubilosa* compared to those of Park (1984) could be because of host defence mechanisms. Lectins, cutinases and esterases, are involved in adhesion of several fungi (Tucker and Talbot 2001), although this has not been investigated on *Mycosphaerella* species on eucalypts. These materials often trigger recognition in the host. Recognition is important, both to a pathogen locating its hosts, as well as the host being able to detect a pathogen (Hahn,

1996). Recognition by the host is a crucial step of its defence system. The quicker the recognition signals occur the more likely an effective defence response can be triggered.

The side of the leaf infected is known to influence the mode of penetration. For example, the onset of disease in bananas is accelerated when leaves were inoculated on the abaxial surface compared to the adaxial surface (Washington *et al*., 1998). This could have important implications when using fungicides as a control measure. Washington *et al*. (1998) achieved 76–100% control of the disease when leaves were sprayed with a fungicide on the abaxial surface compared to 0–13% control on the adaxial surface. Although the control of MLD with fungicides in plantations is not commercially viable, it does require further study in eucalypts, as seedlings in nurseries are sprayed regularly to control leaf diseases such as MLD.

The epidemiology and aerobiology of *M. cryptica* on *E. delegatensis* and other *Eucalyptus* species was investigated in New Zealand in response to the devastating effects of MLD on plantations there (Cheah, 1977; Beresford, 1978). Beresford (1978) found that infection and disease development was seasonal. Leaves that had senesced had up to 25–50% of the leaf area infected by *M. cryptica*. The highest period of defoliation occurred in the summer months, however, the degree differed from year to year. The study by Cheah (1977) suggested that ascospores of *M. cryptica* were the primary inoculum source, but also that conidia were able to infect leaves. The relative humidity (RH) appeared to be the key factor for ascospore dispersal, with 98–100% RH needed (Cheah 1977). In Australia, Park (1988) also studied the epidemiology of *M. cryptica* and

M. nubilosa finding *M. nubilosa* to be mono or bicyclic whereas *M. cryptica* was polycyclic. Park (1984) also found that a RH of above 98% was needed for ascospore discharge for both species.

Park (1984) studied the infection process of *M. cryptica*, *M. nubilosa* and *T. parva* on several eucalypt species including *E. globulus*. He concluded that *M. cryptica* was able to form appressoria and could infect the upper surface of the leaf directly as well as through stomata on the lower surface. He found *M. nubilosa* to only infect leaves through stomata on the lower surface, and furthermore, concluded that *T. parva* was a saprophyte, as no infection of the leaves was observed.

In the present study, Koch's postulates were not fulfilled concerning disease development of *M. marksii*. Again this could be because of host-pathogen interactions, ascospore concentrations, ascospore viability or that more time is required for disease symptoms to develop. Although *M. marksii* ascospores were observed to enter stomata of *E. globulus* leaves, no lesions were evident on *E. globulus* plants; however, it is possible that lesions might develop with time.

9.5 Recommendations

From the research findings in this thesis, the following recommendations can be made:

- Plantations should be monitored regularly for MLD, particularly those eucalypt species/ hybrids new to WA. A risk assessment should be conducted for those species that may impact on yields and reduce returns on investment. As part of the risk assessment, a plan of action and

reporting procedure for new species incursions should be followed, and the possible effects on the native forest should also be taken into consideration.

- Spraying fungicide treatments for MLD once necrosis has been observed would not be as beneficial and would not be a commercially viable option of control. Climate modelling could be used to predict disease severity, in which case spraying pre-disease emergence may be more beneficial. This, however, needs further research.
- Use of species-specific primers could be optimised to assist in the rapid identification of particular species already present in plantations. Traditional methods of identification should still be conducted, however, as from the current study the number, abundance and distribution of *Mycosphaerella* species is not static in WA.

For the future success of eucalypt plantations in WA, forest companies need to be more proactive in their approach to disease management and also the protection of the surrounding native forest.

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APPENDIX

Table A4.1 Sequence alignment of *Teratosphaeria parva* and *M. grandis*

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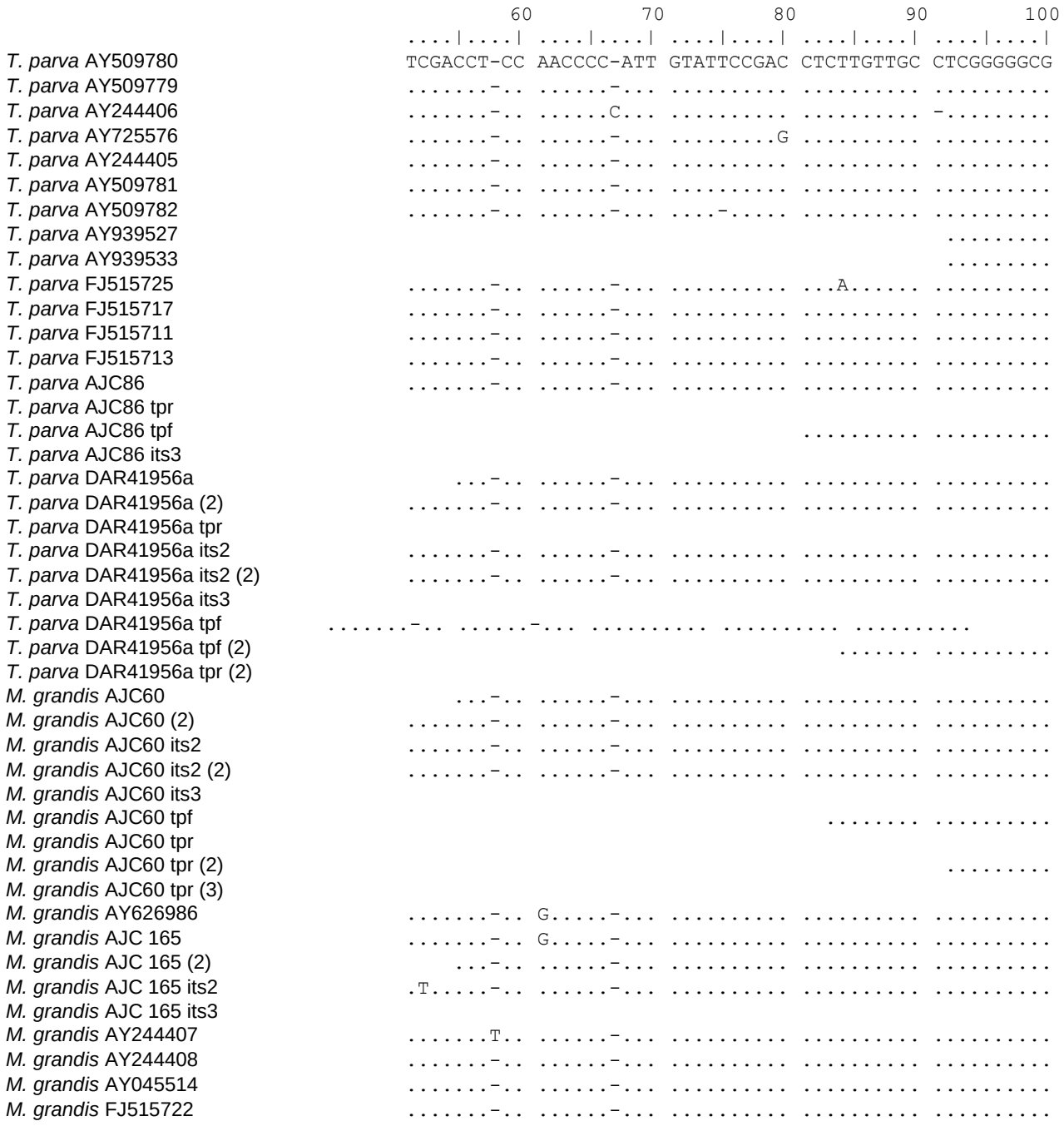
                                          10         20         30         40         50
                                   ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                  TCCGTAGGTG AACCTGCGGA GGGATCATTA CCGAGTGAGG GCCTCCGGGC
T. parva AY509779                  .......... .......... .......... .......... ..-.......
T. parva AY244406                  .......... .......... .........- .......... ..........
T. parva AY725576                  .......... .......... .......... .......... ..........
T. parva AY244405                  .......... .......... .......... .......... ..........
T. parva AY509781                  .......... .......... .......... .......... ..........
T. parva AY509782                  .......... .......... .......... .......... ..........
T. parva AY939527
T. parva AY939533
T. parva FJ515725                  .......... .......... .......... .......... ..........
T. parva FJ515717                             ......     .......... .......... ..........
T. parva FJ515711                             ......     .......... .......... ..........
T. parva FJ515713                             .........  .......... .......... ..........
T. parva AJC86                     .......... .......... .......... .......... ..........
T. parva AJC86 tpr
T. parva AJC86 tpf
T. parva AJC86 its3
T. parva DAR41956a
T. parva DAR41956a (2)             .......... .......... .......... .......... ..........
T. parva DAR41956a tpr
T. parva DAR41956a its2                                                          .........
T. parva DAR41956a its2 (2)                                                      .........
T. parva DAR41956a its3
T. parva DAR41956a tpf                                                            .........
T. parva DAR41956a tpf (2)
T. parva DAR41956a tpr (2)
M. grandis AJC60
M. grandis AJC60 (2)               .......... .......... .......... .......... ..........
M. grandis AJC60 its2                                                            .........
M. grandis AJC60 its2 (2)                                                        .........
M. grandis AJC60 its3
M. grandis AJC60 tpf
M. grandis AJC60 tpr
M. grandis AJC60 tpr (2)
M. grandis AJC60 tpr (3)
M. grandis AY626986                .......... .......... .......... .......... ..........
M. grandis AJC 165                 .......... .......... .......... .......... ..........
M. grandis AJC 165  (2)
M. grandis AJC 165 its2                                                          .........
M. grandis AJC 165 its3
M. grandis AY244407                .......... .......... .......... .......... ..........
M. grandis AY244408                .......... .......... .......... .......... ..........
M. grandis AY045514                .......... .......... .......... .......... ..........
M. grandis FJ515722                           .........  .......... .......... ..........
```


60 70 80 90 100
T. parva AY509780 TCGACCT-CC AACCCC-ATT GTATTCCGAC CTCTTGTTGC CTCGGGGGCG
T. parva AY509779
T. parva AY244406
T. parva AY725576
T. parva AY244405
T. parva AY509781
T. parva AY509782
T. parva AY939527
T. parva AY939533
T. parva FJ515725
T. parva FJ515717
T. parva FJ515711
T. parva FJ515713
T. parva AJC86
T. parva AJC86 tpr
T. parva AJC86 tpf
T. parva AJC86 its3
T. parva DAR41956a
T. parva DAR41956a (2)
T. parva DAR41956a tpr
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3
T. parva DAR41956a tpf
T. parva DAR41956a tpf (2)
T. parva DAR41956a tpr (2)
M. grandis AJC60
M. grandis AJC60 (2)
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3
M. grandis AJC60 tpf
M. grandis AJC60 tpr
M. grandis AJC60 tpr (2)
M. grandis AJC60 tpr (3)
M. grandis AY626986
M. grandis AJC 165
M. grandis AJC 165 (2)
M. grandis AJC 165 its2
M. grandis AJC 165 its3
M. grandis AY244407
M. grandis AY244408
M. grandis AY045514
M. grandis FJ515722

```
                                  110        120        130        140        150
                                  ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                 ACCCGGCCTT CGGGCGTCGG GGCCCCCGGT GGACCAT-CA AACTCTGCAT
T. parva AY509779                 .......... .......... .......... .......-.. ..........
T. parva AY244406                 .......... .......... .......... .......-.. ..........
T. parva AY725576                 .......... .......... .......... .......-.. ..........
T. parva AY244405                 .......... .......... .......... .......-.. ..........
T. parva AY509781                 .......... .......... .......... .......-.. ..........
T. parva AY509782                 .......... .......... .......... .......-.. ..........
T. parva AY939527                 .......... .......... .......... .......-.. ..........
T. parva AY939533                 .......... .......... .......... .......-.. ..........
T. parva FJ515725                 .......... .......... .......... .......-.. ..........
T. parva FJ515717                 .......... .......... .......... .......-.. ..........
T. parva FJ515711                 .......... .......... .......... .......-.. ..........
T. parva FJ515713                 .......... .......... .......... .......-.. ..........
T. parva AJC86                    .......... .......... .......... .......-.. ..........
T. parva AJC86 tpr
T. parva AJC86 tpf                .......... .......... .......... .......-.. ..........
T. parva AJC86 its3
T. parva DAR41956a                .......... .......... .......... .......-.. ..........
T. parva DAR41956a (2)            .......... .......... .......... .......-.. ..........
T. parva DAR41956a tpr                 ..... .......... .......... .......-.. ..........
T. parva DAR41956a its2           .......... .......... .......... .......-.. ..........
T. parva DAR41956a its2 (2)       .......... .......... .......... .....TAT.. ..........
T. parva DAR41956a its3
T. parva DAR41956a tpf            .......... .......... .......... .....TAT.. ..........
T. parva DAR41956a tpf(2)         .......... .......... .......... .......-.. ..........
T. parva DAR41956a tpr (2)
M. grandis AJC60                  .......... .......... .......... .......-.. ..........
M. grandis AJC60 (2)              .......... .......... .......... .......-.. ..........
M. grandis AJC60 its2             .......... .......... .......... .......-.. ..........
M. grandis AJC60 its2 (2)         .......... .......... .......... .....TAT.. ..........
M. grandis AJC60 its3
M. grandis AJC60 tpf              .......... .......... .......... .......-.. ..........
M. grandis AJC60 tpr
M. grandis AJC60 tpr (2)          .......... .......... .......... .......-.. ..........
M. grandis AJC60 tpr (3)
M. grandis AY626986               .......... .......... .......... .......-.. ..........
M. grandis AJC 165                .......... .......... .......... .......-.. ..........
M. grandis AJC 165 (2)            .......... .......... .......... .......-.. ..........
M. grandis AJC 165 its2           .......... .......... .......... .......-.. ..........
M. grandis AJC 165 its3
M. grandis AY244407               .......... .......... .......... .......-.. ..........
M. grandis AY244408               .......... .......... .......... .......-.. ..........
M. grandis AY045514               .......... .......... .......... .......-.. ..........
M. grandis FJ515722               .......... .......... .......... .......-.. ..........
```

```
                                  160        170        180        190        200
                                  ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                 CTTTGACGTC TGAGTAAATA TTGAATCAAT CAAAACTTTT AACAACGGAT
T. parva AY509779                 .......... .......... .......... .......... ..........
T. parva AY244406                 .......... .......... .......... .......... ..........
T. parva AY725576                 .......... .......... .......... .......... ..........
T. parva AY244405                 .......... .......... .......... .......... ..........
T. parva AY509781                 .......... .......... .......... .......... ..........
T. parva AY509782                 .......... .......... .......... .......... ..........
T. parva AY939527                 .......... .......... .......... .......... ..........
T. parva AY939533                 .......... .......... .......... .......... ..........
T. parva FJ515725                 .......... ........C. .......... .......... ..........
T. parva FJ515717                 .......... .......... .......... .......... ..........
T. parva FJ515711                 .......... .......... .......... .......... ..........
T. parva FJ515713                 .......... .......... .......... .......... ..........
T. parva AJC86                    .......... .......... .......... .......... ..........
T. parva AJC86 tpr
T. parva AJC86 tpf                .......... .......... .......... .......... ..........
T. parva AJC86 its3
T. parva DAR41956a                .......... .......... .......... .......... ..........
T. parva DAR41956a (2)            .......... .......... .......... .......... ..........
T. parva DAR41956a tpr            .......... .......... .......... .......... ..........
T. parva DAR41956a its2           .......... .......... .......... .......
T. parva DAR41956a its2 (2)       .......... .......... .....
T. parva DAR41956a its3
T. parva DAR41956a tpf            .......... .......... .......... .
T. parva DAR41956a tpf(2)         .......... .......... .......... .......... ..........
T. parva DAR41956atpr (2)
M. grandis AJC60                  .......... .......... .......... .......... ..........
M. grandis AJC60 (2)              .......... .......... .......... .......... ..........
M. grandis AJC60 its2             .......... .......... .-........ ..
M. grandis AJC60 its2 (2)         .......... .......... .-........ .......
M. grandis AJC60 its3
M. grandis AJC60 tpf              .......... .......... .......... .......... ..........
M. grandis AJC60 tpr
M. grandis AJC60 tpr (2)          .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (3)
M. grandis AY626986               .......... .......... .......... .......... ..........
M. grandis AJC 165                .......... .......... .......... .......... ..........
M. grandis AJC 165 (2)            .......... .......... .......... .......... ..........
M. grandis AJC 165 its2           .......... .......... .-........ ..
M. grandis AJC 165 its3
M. grandis AY244407               .......... .......... .......... .......... ..........
M. grandis AY244408               .......... .......... .......... .......... ..........
M. grandis AY045514               .......... .......... .......... .......... ..........
M. grandis FJ515722               .......... .......... .......... .......... ..........
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                                      210        220        230        240        250
                                 ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                CTCTTGGTTC TGGCATCGAT GAAGAACGCA GCGAAATGCG ATAAGTAATG
T. parva AY509779                .......... .......... .......... .......... ..........
T. parva AY244406                .......... .......... .......... .......... ..........
T. parva AY725576                .......... .......... .......... .......... ..........
T. parva AY244405                .......... .......... .......... .......... ..........
T. parva AY509781                .......... .......... .......... .......... ..........
T. parva AY509782                .......... .......... .......... .......... ..........
T. parva AY939527                .......... .......... .......... .......... ..........
T. parva AY939533                .......... .......... .......... .......... ..........
T. parva FJ515725                .......... .......... .......... .......... ..........
T. parva FJ515717                .......... .......... .......... .......... ..........
T. parva FJ515711                .......... .......... .......... .......... ..........
T. parva FJ515713                .......... .......... .......... .......... ..........
T. parva AJC86                   .......... .......... .......... .......... ..........
T. parva AJC86 tpr                         .......... .......... .......... ..........
T. parva AJC86 tpf               .......... .......... .......... .
T. parva AJC86 its3                                                                   ..
T. parva DAR41956a               .......... .......... .......... .......... ..........
T. parva DAR41956a (2)           .......... .......... .......... .......... ..........
T. parva DAR41956a tpr           .......... .......... .......... .......... ..........
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3
T. parva DAR41956a tpf
T. parva DAR41956a tpf(2)        .......... .......... .......... ..
T. parva DAR41956a tpr (2)                 .......... .......... .......... ..........
M. grandis AJC60                 .......... .......... .......... .......... ..........
M. grandis AJC60 (2)             .......... .......... .......... .......... ..........
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3                                                                 ..
M. grandis AJC60 tpf             .......... .......... .......... ..
M. grandis AJC60 tpr                       ......... .......... .......... ..........
M. grandis AJC60 tpr (2)         .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (3)                   .......... .......... .......... ..........
M. grandis AY626986              .......... .......... .......... .......... ..........
M. grandis AJC 165               .......... .......... .......... .......... ..........
M. grandis AJC 165 (2)           .......... .......... .......... .......... ..........
M. grandis AJC 165 its2
M. grandis AJC 165 its3
M. grandis AY244407              .......... .......... .......... .......... ..........
M. grandis AY244408              .......... .......... .......... .......... ..........
M. grandis AY045514              .......... .......... .......... .......... ..........
M. grandis FJ515722              .......... .......... .......... .......... ..........
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                                  260        270        280        290        300
                                  ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                 TGAATTGCAG AATTCAGTGA ATCATCGAAT CTTTGAACGC ACATTGCGCC
T. parva AY509779                 .......... .......... .......... .......... ..........
T. parva AY244406                 .......... .......... .......... .......... ..........
T. parva AY725576                 .......... .......... .......... .......... ..........
T. parva AY244405                 .......... .......... .......... .......... ..........
T. parva AY509781                 .......... .......... .......... .......... ..........
T. parva AY509782                 .......... .......... .......... .......... ..........
T. parva AY939527                 .......... .......... .......... .......... ..........
T. parva AY939533                 .......... .......... .......... .......... ..........
T. parva FJ515725                 .......... .......... .......... .......... ..........
T. parva FJ515717                 .......... .......... .......... .......... ..........
T. parva FJ515711                 .......... .......... .......... .......... ..........
T. parva FJ515713                 .......... .......... .......... .......... ..........
T. parva AJC86                    .......... .......... .......... .......... ..........
T. parva AJC86 tpr                .......... .......... .......... .......... ..........
T. parva AJC86 tpf
T. parva AJC86 its3               ...-...... .......... .......... .......... ..........
T. parva DAR41956a                .......... .......... .......... .......... ..........
T. parva DAR41956a (2)            .......... .......... .......... .......... ..........
T. parva DAR41956a tpr            .......... .......... .......... .......... ..........
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3             ........ .......... .......... .......... ..........
T. parva DAR41956a tpf
T. parva DAR41956a tpf(2)
T. parva DAR41956a tpr (2)        .......... .......... .......... .......... ..........
M. grandis AJC60                  .......... .......... .......... .......... ..........
M. grandis AJC60 (2)              .......... .......... .......... .......... ..........
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3             ...-...... .-........ .......... .......... ..........
M. grandis AJC60 tpf
M. grandis AJC60 tpr              .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (2)          .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (3)          .......... .......... .......... .......... ..........
M. grandis AY626986               .......... .......... .......... .......... ..........
M. grandis AJC 165                .......... .......... .......... .......... ..........
M. grandis AJC 165 (2)            .......... .......... .......... .......... ..........
M. grandis AJC 165 its2
M. grandis AJC 165 its3           .......... .......... .......... .......... ..........
M. grandis AY244407               .......... .......... .......... .......... ..........
M. grandis AY244408               .......... .......... .......... .......... ..........
M. grandis AY045514               .......... .......... .......... .......... ..........
M. grandis FJ515722               .......... .......... .......... .......... ..........
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                                  310        320        330        340        350
                                  ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                 CCTTGGTATT CCGAGGGGCA TGCCTGTTCG AGCGTCATTT CACCACTCAA
T. parva AY509779                 ...C...... .......... .......... .......... ..........
T. parva AY244406                 .......... .......... .......... .......... ..........
T. parva AY725576                 ...C...... .......... .......... .......... ..........
T. parva AY244405                 .......... .......... .......... .......... ..........
T. parva AY509781                 .......... .......... .......... .......... ..........
T. parva AY509782                 .......... .......... .......... .......... ..........
T. parva AY939527                 .......... .......... .......... .......... ..........
T. parva AY939533                 .......... .......... .......... .......... ..........
T. parva FJ515725                 .......... .......... .......... .......... ..........
T. parva FJ515717                 .......... .......... .......... .......... ..........
T. parva FJ515711                 .......... .......... .......... .......... ..........
T. parva FJ515713                 .......... .......... .......... .......... ..........
T. parva AJC86                    ...C...... .......... .......... .......... ..........
T. parva AJC86 tpr                .......... .......... .......... .......... ..........
T. parva AJC86 tpf
T. parva AJC86 its3               .......... .......... .......... .......... ..........
T. parva DAR41956a                .......... .......... .......... .......... ..........
T. parva DAR41956a (2)            ...C...... .......... .......... .......... ..........
T. parva DAR41956a tpr            .......... .......... .......... .......... ..........
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3           .......... .......... .......... .......... ..........
T. parva DAR41956a tpf
T. parva DAR41956a tpf(2)
T. parva DAR41956a tpr (2)        .......... .......... .......... .......... ..........
M. grandis AJC60                  .......... .......... .......... .......... ..........
M. grandis AJC60 (2)              .......... .......... .......... .......... ..........
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3             .......... .......... .......... .......... ..........
M. grandis AJC60 tpf
M. grandis AJC60 tpr              .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (2)          .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (3)          .......... .......... .......... .......... ..........
M. grandis AY626986               ...C...... .......... .......... .......... ..........
M. grandis AJC 165                ...C...... .......... .......... .......... ..........
M. grandis AJC 165 (2)            .......... .......... .......... .......... ..........
M. grandis AJC 165 its2
M. grandis AJC 165 its3           ...C...... .......... .......... .......... ..........
M. grandis AY244407               ...C...... .......... .......... .......... ..........
M. grandis AY244408               ...C...... .......... .......... .......... ..........
M. grandis AY045514               ...C...... .......... .......... .......... ..........
M. grandis FJ515722               ...C...... .......... .......... .......... ..........
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                                360        370        380        390        400
                           ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780          GCCTGGCTTG GTATTGGGCG CCGCGGTTTG CCGCGCGCCT CAAAGTCTCC
T. parva AY509779          .......... .......... .......... .......... ..........
T. parva AY244406          .......... .......... .......... .......... ..........
T. parva AY725576          .......... .......... .......... .......... ..........
T. parva AY244405          .......... .......... .......... .......... ..........
T. parva AY509781          .......... .......... .......... .......... ..........
T. parva AY509782          .......... .......... .......... .......... ..........
T. parva AY939527          .......... .......... .......... .......... ..........
T. parva AY939533          .......... .......... .......... .......... ..........
T. parva FJ515725          .......... .......... .......... .......... ..........
T. parva FJ515711          .......... .......... .......... .......... T.........
T. parva FJ515713          .......... .......... .......... .......... ..........
T. parva AJC86             .......... .......... .......... .......... ..........
T. parva AJC86 tpr         .......... .......... .......... .......... ..........
T. parva AJC86 tpf
T. parva AJC86 its3        .......... .......... .......... .......... ..........
T. parva DAR41956a         .......... .......... .......... .......... ..........
T. parva DAR41956a (2)     .......... .......... .......... .......... ..........
T. parva DAR41956a tpr     .......... .......... .......... .......... ..........
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3    .......... .......... .......... .......... ..........
T. parva DAR41956a tpf
T. parva DAR41956a tpf(2)
T. parva DAR41956a tpr (2) .......... .......... .......... .......... ..........
M. grandis AJC60           .......... .......... .......... .......... ..........
M. grandis AJC60 (2)       .......... .......... .......... .......... ..........
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3      .......... .......... .......... .......... ..........
M. grandis AJC60 tpf
M. grandis AJC60 tpr       .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (2)   .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (3)   .......... .......... .......... .......... ..........
M. grandis AY626986        .......... .......... .......... .......... ..........
M. grandis AJC 165         .......... .......... .......... .......... ..........
M. grandis AJC 165 (2)     .......... .......... .......... .......... ..........
M. grandis AJC 165 its2
M. grandis AJC 165 its3    .......... .......... .......... .......... ..........
M. grandis AY244407        .......... .......... .......... .......... ..........
M. grandis AY244408        .......... .......... .......... .......... ..........
M. grandis AY045514        .......... .......... .......... .......... ..........
M. grandis FJ515722        .......... .......... .......... .......... ..........
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                                  410        420        430        440        450
                                  ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                 GGCTGAGCCA ACTGTCTCTA AGCGTTGTGG TTTAATCATC CGCTTGCGAG
T. parva AY509779                 .......... .......... .......... .......... ......T...
T. parva AY244406                 .......... .......... .......... .......... ......T...
T. parva AY725576                 .......... .......... .......... .......... ......T...
T. parva AY244405                 .......... .......... .......... .......... ..........
T. parva AY509781                 .......... .......... .......... .......... ..........
T. parva AY509782                 .......... .......... .......... ..C....... ..........
T. parva AY939527                 .......... .......... .......... .......... ..........
T. parva AY939533                 .......... .......... .......... .......... ..........
T. parva FJ515725                 .......... .......... .......... ..C....... ..........
T. parva FJ515717                 .......... .......... .......... .......... ......T...
T. parva FJ515711                 .......... .......... .......... .......... ..........
T. parva FJ515713                 .......... .......... .......... .......... ..........
T. parva AJC86                    .......... .......... .......... .......... ..
T. parva AJC86 tpr                ....-..... ..-.......
T. parva AJC86 tpf
T. parva AJC86 its3               .......... .......... .......... .......... ..........
T. parva DAR41956a                .......... .......... .......... .......... ..........
T. parva DAR41956a (2)            .......... .......... .......... .......... ..........
T. parva DAR41956a tpr            .......... .......... .......... .......... ..........
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3           .......... .......... .......... .......... ..........
T. parva DAR41956a tpf
T. parva DAR41956a tpf(2)
T. parva DAR41956a tpr (2)        ....
M. grandis AJC60                  .......... .......... .......... .......... ..........
M. grandis AJC60 (2)              .......... .......... .......... .......... ..........
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3             .......... .......... .......... .......... ..........
M. grandis AJC60 tpf
M. grandis AJC60 tpr              ....
M. grandis AJC60 tpr (2)          .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (3)          ....
M. grandis AY626986               .......... .......... .......... .......... ......T...
M. grandis AJC 165                .......... .......... .......... .......... ......T...
M. grandis AJC 165 (2)            .......... .......... .......... .......... ..........
M. grandis AJC 165 its2
M. grandis AJC 165 its3           .......... .......... .......... .......... ......
M. grandis AY244407               .......... .......... .......... .......... ......T...
M. grandis AY244408               .......... .......... .......... .......... ......T...
M. grandis AY045514               .......... .......... .......... .......... ......T...
M. grandis FJ515722               .......... .......... .......... .......... ......T...
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                                460        470        480        490        500
                               ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780              ATCGAAGGCG ACGGCCGTTA AACTTATTCA AAGGTTGACC TCGGATCAGG
T. parva AY509779              .......... .......... .......... .......... ..........
T. parva AY244406              .......... .......... .......... .......... ..........
T. parva AY725576              .......... .......... .......... .......... ..........
T. parva AY244405              .......... .......... .......... .......... ..........
T. parva AY509781              .......... .......... .......... .......... ..........
T. parva AY509782              .......... .......... .......... .......... ..........
T. parva AY939527              ..
T. parva AY939533              .
T. parva FJ515725              .......... G......... .......... .......... ..........
T. parva FJ515717              .....T.... .......... .......... .......... ..........
T. parva FJ515711              .......... .......... .......... .......... ..........
T. parva FJ515713              .......... .......... .......... .......... ..........
T. parva AJC86
T. parva AJC86 tpr
T. parva AJC86 tpf
T. parva AJC86 its3
T. parva DAR41956a             .
T. parva DAR41956a (2)         .
T. parva DAR41956a tpr         .......... .......... .......... .......... ..........
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3        .
T. parva DAR41956a tpf
T. parva DAR41956a tpf(2)
T. parva DAR41956a tpr (2)
M. grandis AJC60
M. grandis AJC60 (2)           .
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3
M. grandis AJC60 tpf
M. grandis AJC60 tpr
M. grandis AJC60 tpr (2)       .......... .......... .......... .......... ..........
M. grandis AJC60 tpr (3)
M. grandis AY626986            .........- .......... .......... .......... ..........
M. grandis AJC 165             .........- .......... .......... .......... ..........
M. grandis AJC 165 (2)         .
M. grandis AJC 165 its2
M. grandis AJC 165 its3
M. grandis AY244407            .......... .......... .......... .......... ..........
M. grandis AY244408            .......... .......... .......... .......... ..........
M. grandis AY045514            .......... .......... .......... .......... ..........
M. grandis FJ515722            .......... .......... .......... .......... ..........
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                                      510        520        530        540        550
                                      ....|....| ....|....| ....|....| ....|....| ....|....|
T. parva AY509780                     TAGGGATACC CGCTGAACTT AAGCATATCA ATAAGCGGAG GA
T. parva AY509779                     ........
T. parva AY244406                     ........
T. parva AY725576                     ........
T. parva AY244405                     .......... .......... .......... .......... ..
T. parva AY509781                     .......... .......... .......... .......... ..
T. parva AY509782                     .......... .......... .......... .......... ..
T. parva AY939527
T. parva AY939533
T. parva FJ515725                     .......... .......... .......... ...
T. parva FJ515717                     .......... .......... .........A
T. parva FJ515711                     .......... .......... .......... TA
T. parva FJ515713                     .......... .......... .......... TA
T. parva AJC86
T. parva AJC86 tpr
T. parva AJC86 tpf
T. parva AJC86 its3
T. parva DAR41956a
T. parva DAR41956a (2)
T. parva DAR41956a tpr                .......... .......... ..........
T. parva DAR41956a its2
T. parva DAR41956a its2 (2)
T. parva DAR41956a its3
T. parva DAR41956a tpf
T. parva DAR41956a tpf(2)
T. parva DAR41956 a tpr (2)
M. grandis AJC60
M. grandis AJC60 (2)
M. grandis AJC60 its2
M. grandis AJC60 its2 (2)
M. grandis AJC60 its3
M. grandis AJC60 tpf
M. grandis AJC60 tpr
M. grandis AJC60 tpr (2)              .......... .......... .......... ...
M. grandis AJC60 tpr (3)
M. grandis AY626986                   .......... .......... .........
M. grandis AJC 165                    .......... .......... .........
M. grandis AJC 165 (2)
M. grandis AJC 165 its2
M. grandis AJC 165 its3
M. grandis AY244407                   ........
M. grandis AY244408                   ........
M. grandis AY045514                   .......... .......... .......... .........GA .
M. grandis FJ515722                   .......... .......... .......... ...G
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Table A4.2 Sequence alignment of *Mycosphaerella aurantia* and associated species

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                                10         20         30         40         50
                       ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742   TCCGTAGGTG AACCTGCGGA GGGATCATTA CTGAGTGAGG GCTCACGCCC
M. gregaria DAR72368                                                      ....
M. aurantia AY150331   .......... .......... .......... .......... ..........
M. aurantia AY509743   .......... .......... .......... .......... ..........
M. aurantia AY509744   .......... .......... .......... .......... ..........
M. aurantia EU042175   .......... .......... .......... .......... ..........
M. aurantia SJ31
M. aurantia SJ100                                                         .....
M. aurantia EU255896
M. aurantia DQ123604                                 ...... .......... ..GA.A....
M. buckinghamiae EU707856 .......... .......... .......... .......... ..........
M. africana AY626981   .......... .......... .......... .......... ..........

                                60         70         80         90        100
                       ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742   -GACCTCCAA CCCTTTGTGA ACCAACTCTG TTGCTTCGGG GGCGACCCCG
M. gregaria DAR72368   -.......... .......... .......... .......... ..........
M. aurantia AY150331   A.......... .......... .......... .......... ..........
M. aurantia AY509743   -.......... .......... .......... .......... ..........
M. aurantia AY509744   -.......... .......... .......... .......... ..........
M. aurantia EU042175   -.......... .......... .......... .......... ..........
M. aurantia SJ31
M. aurantia SJ100      -.......... .......... .......... .......... ..........
M. aurantia EU255896        ..... .......... .......... .........T ..........
M. aurantia DQ123604   -.......... .......... .......... .......... ..........
M. buckinghamiae EU707856 -.......... .......... .......... .......... ..........
M. africana AY626981   -.......... .......... .......... .......... ..........

                               110        120        130        140        150
                       ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742   CCGT-TTCGG CGACGGCGCC CCCGGAGGTC ATCAAACACT GCATCTTTGC
M. gregaria DAR72368   ....-..... .......... .......... .......... ..........
M. aurantia AY150331   ....-..... .......... .......... .......... ..........
M. aurantia AY509743   ....-..... .......... .......... .......... ..........
M. aurantia AY509744   ....-..... .......... .......... .......... ..........
M. aurantia EU042175   ....-..... .......... .......... .......... ..........
M. aurantia SJ31                  . .......... .......... .......... ..........
M. aurantia SJ100      ....-...AT .......... .......... .......... ..........
M. aurantia EU255896   ....G..... .......... .......... CATC...... ..........
M. aurantia DQ123604   ....-..... .......... .......... .......... ..........
M. buckinghamiae EU707856 ....-..... .......... .......... .......... ..........
M. africana AY626981   ....-..... .......... .......... .......... ..........
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                                160        170        180        190        200
                               ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742           GTCGGAGTCT TAAAGTAAAT TTAAACAAAA CTTTCAACAA CGGATCTCTT
M. gregaria DAR72368           .......... .......... .......... .......... ..........
M. aurantia AY150331           .......... .......... .......... .......... ..........
M. aurantia AY509743           .......... .......... .......... .......... ..........
M. aurantia AY509744           .......... .......... .......... .......... ..........
M. aurantia EU042175           .......... .......... .......... .......... ..........
M. aurantia SJ31               .......... .......... .......... .......... ..........
M. aurantia SJ100              .......... .......... .......... .......... ..........
M. aurantia EU255896           .......... .......... .......... .......... ..........
M. aurantia DQ123604           .......... .......... .......... .......... ..........
M. buckinghamiae EU707856      .......... .......... .......... .......... ..........
M. africana AY626981           .......... .......... .......... .......... ..........

                                210        220        230        240        250
                               ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742           GGTTCTGGCA TCGATGAAGA ACGCAGCGAA ATGCGATAAG TAATGTGAAT
M. gregaria DAR72368           .......... .......... .......... .......... ..........
M. aurantia AY150331           .......... .......... .......... .......... ..........
M. aurantia AY509743           .......... .......... .......... .......... ..........
M. aurantia AY509744           .......... .......... .......... .......... ..........
M. aurantia EU042175           .......... .......... .......... .......... ..........
M. aurantia SJ31               .......... .......... .......... .......... ..........
M. aurantia SJ100              .......... .......... .......... .......... ..........
M. aurantia EU255896           .......... .......... .......... .......... ..........
M. aurantia DQ123604           .......... .......... .......... .......... ..........
M. buckinghamiae EU707856      .......... .......... .......... .......... ..........
M. africana AY626981           .......... .......... .......... .......... ..........

                                260        270        280        290        300
                               ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742           TGCAGAATTC AGTGAATCAT CGAATCTTTG AACGCACATT GCGCCCCGTG
M. gregaria DAR72368           .......... .......... .......... .......... ..........
M. aurantia AY150331           .......... .......... .......... .......... ..........
M. aurantia AY509743           .......... .......... .......... .......... ..........
M. aurantia AY509744           .......... .......... .......... .......... ..........
M. aurantia EU042175           .......... .......... .......... .......... ..........
M. aurantia SJ31               .......... .......... .......... .......... ..........
M. aurantia SJ100              .......... .......... .......... .......... ..........
M. aurantia EU255896           .......... .......... .......... .......... ..........
M. aurantia DQ123604           .......... .......... .......... .......... ..........
M. buckinghamiae EU707856      .......... .......... .......... .......... ..........
M. africana AY626981           .......... .......... .......... .......... ..........

                                310        320        330        340        350
                               ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742           GTATTCCGCG GGGCATGCCT GTTCGAGCGT CATTTCACCA CTCAAGCCTA
M. gregaria DAR72368           .......... .......... .......... .......... ..........
M. aurantia AY150331           .......... .......... .......... .......... ..........
M. aurantia AY509743           .......... .......... .......... .......... ..........
M. aurantia AY509744           .......... .......... .......... .......... ..........
M. aurantia EU042175           .......... .......... .......... .......... ..........
M. aurantia SJ31               .......... .......... .......... .......... ..........
M. aurantia SJ100              .......... .......... .......... .......... ..........
M. aurantia EU255896           .......... .......... .......... .......... ..........
M. aurantia DQ123604           .......... .......... .......... .......... ..........
M. buckinghamiae EU707856      .......... .......... .......... .......... ..........
M. africana AY626981           .......... .......... .......... .......... ..........
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                                360        370        380        390        400
                               ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742           GCTTGGTATT GGGCGTCGCG GTTCCGCGCG CCTTAAAGTC TCCGGCTGAG
M. gregaria DAR72368           .......... .......... .......... .......... ..........
M. aurantia AY150331           .......... .......... .......... .......... ..........
M. aurantia AY509743           .......... .......... .......... .......... ..........
M. aurantia AY509744           .......... .......... .......... .......... ..........
M. aurantia EU042175           .......... .......... .......... .......... ..........
M. aurantia SJ31               .......... .......... .......... .......... ..........
M. aurantia SJ100              .......... .......... .......... .......... ..........
M. aurantia EU255896           .......... .......... .......... .......... ..........
M. aurantia DQ123604           .......... .......... .......... .......... ..........
M. buckinghamiae EU707856      .......... .......... .......... .......... ..........
M. africana AY626981           .......... .......... .......... .......... ..........

                                410        420        430        440        450
                               ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742           CAGTTCGTCT CTAAGCGTTG TGGCATATAT TTCGCTGAAG AGTTCGGACG
M. gregaria DAR72368           .......... .......... .......... .......... ..........
M. aurantia AY150331           .......... .......... .......... .......... ..........
M. aurantia AY509743           .......... .......... .......... .......... ..........
M. aurantia AY509744           .......... .......... .......... .......... ..........
M. aurantia EU042175           .......... .......... .......... .......... ..........
M. aurantia SJ31               .......... .......... .......... .......... ..........
M. aurantia SJ100              .......... .......... .......... .......... ..........
M. aurantia EU255896           .......... .......... .......... .......... ..........
M. aurantia DQ123604           .......... .......... .......... .......... ........T.
M. buckinghamiae EU707856      .......... .......... .......... .......... ..........
M. africana AY626981           .......... .......... .......... .......... ..........

                                460        470        480        490        500
                               ....|....| ....|....| ....|....| ....|....| ....|....|
M. aurantia AY509742           GCTTTTGGCC GTTAAATCTT TCTTAAGGTT GACCTCGGAT CAGGTAGGGA
M. gregaria DAR72368           .......... .......... .......... .......... ..........
M. aurantia AY150331           .......... .......... .........- .......... ..........
M. aurantia AY509743           .......... .......... .......... .......... ..........
M. aurantia AY509744           .......... .......... .......... .......... ..........
M. aurantia EU042175           .......... .......... .......... .......... ..........
M. aurantia SJ31               .......... .......... .......... .......... ...
M. aurantia SJ100              .......... .......... .........- ..........
M. aurantia EU255896           .......... .......... ...-.....- .......... ..........
M. aurantia DQ123604           .......... .......... .......... .......... ..........
M. buckinghamiae EU707856      .......... .......... .......... .......... ..........
M. africana AY626981           .......... .......... .......... .......... ..........

                                510        520        530
                               ....|....| ....|....| ....|....| ....|.
M. aurantia AY509742           TACCCGCTGA ACTTAAGCAT ATCAATAAGC GGAGGA
M. gregaria DAR72368           .......... .......... .......... ......
M. aurantia AY150331           .......... .....-.... .......... ......
M. aurantia AY509743           .......... .......... .......... ......
M. aurantia AY509744           .......... .......... .......... ......
M. aurantia EU042175           .......... .......... ...
M. aurantia SJ31
M. aurantia SJ100
M. aurantia EU255896           .......... .....
M. aurantia DQ123604           .......... ...
M. buckinghamiae EU707856      .......... .......... .......... ......
M. africana AY626981           .......... .......... .........
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Table A8.1 Summary of all effects of site(1), plot (2), treatment (3) and year (4) of repeated measures ANOVA for standardised tree volumes over 2002–2004 from Bills and Sixpenny.

Variable	DF Effect	MS Effect	DF Error	MS Error	F	p-level
1	1	57.3512	1949	0.146841	390.57	0.000000
2	8	1.0886	1949	0.146841	7.41	0.000000
3	3	12.4366	1949	0.146841	84.69	0.000000
4	1	298.1442	1949	0.013851	21525.45	0.000000
13	3	1.7773	1949	0.146841	12.10	0.000000
23	24	0.6164	1949	0.146841	4.20	0.000000
14	1	0.3703	1949	0.013851	26.74	0.000000
24	8	0.0624	1949	0.013851	4.51	0.000019
34	3	0.4976	1949	0.013851	35.93	0.000000
134	3	0.1974	1949	0.013851	14.25	0.000000
234	24	0.0413	1949	0.013851	2.98	0.000002

Table A8.2 Univariate tests comparing unweighted means of the Mycosphaerella damage index and Leaf-chew damage index in adult leaves from Bills and Sixpenny. Rao R (2,31) = 36.61; p<.0000

Site	Mycosphaerella index	Leaf-chew index
Bills	0.050000	2.685500
Sixpenny	0.423750	2.596500

Table A8.3 Univariate tests comparing logged means of the Mycosphaerella damage index and Leaf-chew damage index in adult leaves from Bills and Sixpenny. 1-SITE, 2-TREATMENT

	Mean sqr effect	Mean sqr Error	F(df1,2) 1,32	p-level
Log Mycosphaerella index	0.119357	0.001881	63.45905	0.000000
Log Leaf-chew index	0.007948	0.002879	2.76067	0.106375