The Development of Strategies for the Management and Research of Foliar Pathogens on Eucalypt Plantations: Using *Mycosphaerella* as a Case Study

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Declarations

This thesis does not contain any material, which has been accepted, for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge, this thesis contains no material published or written by any other person except where due acknowledgement has been made.

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Abstract

Foliar pathogens are capable of severely reducing the productivity and stem form of eucalypt trees in plantations. *Mycosphaerella* is one of the most severe defoliating pathogens to eucalypts worldwide. In Australia Mycosphaerella leaf disease (MLD) has produced episodes of severe defoliation in Tasmania, Gippsland and south-west Western Australia. *Mycosphaerella* is one of the most researched pathogens of eucalypts, however the majority of studies have concentrated on the taxonomy of pathogens and investigating their geographical and host ranges. In this thesis, MLD has been chosen as a model system to develop and apply new technologies to researching foliar pathogens. Research was conducted in the four main areas of disease assessment, growth impacts, molecular detection and resistance mechanisms.

Acquiring accurate and repeatable damage estimates at a tree level is essential for calculating damage at plantation and estate levels; especially where data will be used for computer generated modelling using programs such as Maestra or CABALA. Repeated assessments using the Crown Damage Index (CDI) tested the suitability of the method to provide reliable, objective and repeatable results. Nine assessors, with varying levels of experience, estimated damage on three plots of fifty trees each (3-4 years old), to obtain an understanding of the subjectivity of assessing damage caused by insects (e.g. *Chrysophtharta* spp.) and fungal pathogens (e.g. *Mycosphaerella* spp.) on *Eucalyptus globulus* Labill. Damage levels were measured by destructive sampling to enable direct comparisons between estimates and damage levels to be made. The most experienced assessors provided the most repeatable estimates and were generally the most accurate. The

incidence of foliar necrosis was the least subjective measure while defoliation was the most subjective and the least accurate of the indices measured. All assessors, regardless of experience, were able to predict the Crown Damage Index (a combined index of all damage classes) to within 12 % of measured damage levels. Further modification of the CDI in a separate study on younger trees (1-2 years old) further reduced the errors involved with damage estimates to within 4 % of actual damage levels.

Despite the importance of *Mycosphaerella* species as significant defoliating pathogens of temperate plantation eucalypts such as *E. globulus*, there have been no studies to investigate the effects of *Mycosphaerella* damage on growth of young trees measured through to rotation length. From the results of two growth trials, one short term and one longer term (tree growth was monitored until 3 and 6 years old, respectively), the damage threshold (level of damage before there were significant growth effects) was estimated to be approximately 20 %. In both trials, losses in volume were only observed until trees changed to adult foliage at which point the growth rate returned to that of the control trees. We predicted that with less than 80 % damage, growth rates follow a type 1 growth response, *i.e.* after an initial growth loss damaged trees recover and have a growth rate that is parallel with control trees. Above 80 % loss of effective leaf area, it is predicted that growth rates of control trees and damaged trees are permanently divergent. To give a long-term estimate of impact, the growth of trees in the longer term trial were modelled to rotation length. After 25 years growth (rotation length) it is estimated that a loss of one year's growth will occur as a result of the MLD damage observed in this trial. One year was also the length of time that juvenile foliage was exposed to greater than 20% damage.

A nested PCR detection method was applied to leaves and stems infected by MLD to detect the five most common *Mycosphaerella* species that occur in Tasmania. Leaf samples were taken from *E. globulus* and *Eucalyptus nitens* (Deane and Maid.) Maid. plantations in the northern regions of Tasmania and native re-growth in the north-east of Tasmania. For the first time it has been conclusively shown that in excess of five *Mycosphaerella* species can coexist in *E. globulus* leaves and four in *E. nitens* leaves, including a record of *Mycosphaerella nubilosa* (Cooke) Hansf. on *E. nitens* which has only been documented once before. Samples from native *Eucalyptus regnans* (Thüm) Lindaure provided evidence that the co-existence of several *Mycosphaerella* species on a single lesion may occur outside the plantation environment. The molecular detection test was a rapid, reliable and cost-effective method in comparison with classical mycological methods for the identification and differentiation of species associated with MLD on eucalypts. These studies have highlighted the potential for multiple pathogenic species of *Mycosphaerella* to simultaneously occupy the same niche.

The *Mycosphaerella* detection technique was also applied to determine the presence of species associated with MLD in leaf lesions of varying development, including asymptomatic tissue. Symptoms characteristic of putative *Mycosphaerella* lesions, collected from commercial *E. globulus* and *E. nitens* plantations, were categorised into five stages of development with asymptomatic tissue designated as the stage prior to any symptom expression. Lesions in all categories, including some asymptomatic leaf tissue, tested positive for the presence of up to four *Mycosphaerella* species. The number and composition of species within a lesion varied between early and late stage lesions, with trends occurring for the most pathogenic species to occupy necrotic and reproductive

lesions exclusively (*E. nitens*) or with only one other species (*E. globulus*). Early detection of *Mycosphaerella* species in asymptomatic leaves and at any stage of lesion maturity will facilitate more accurate, rapid and broad scale screening of plantations for ecological and epidemiological investigations at earlier stages of disease development. Effective and reproducible artificial inoculation techniques for MLD have not been developed; the confirmation of *Mycosphaerella* species in naturally infected early lesions using the nested PCR detection system enables the study of field infected leaves to determine the effects of infection on host physiology and resistance.

The timing and strength of necrophylactic periderm formation, deposition of defence chemicals and accumulation/retention of photosynthetic pigments were compared between MLD susceptible *E. globulus* and the more MLD resistant *E. nitens* after infection of the leaves with *Mycosphaerella* species. Resistance of *E. nitens*, as observed in southern Australia, was attributed to the speed of necrophylactic periderm formation, which was directly related to the amount and type of cell division occurring in the mesophyll cells. In *E. nitens* the necrophylactic periderm is formed early in lesion development by cellular division of mesophyll cells which were quickly reinforced with lignin, suberin and other polyphenolics. It is suggested that the rapid nature of necrophylactic periderm formation in leaves of *E. nitens* was due to the presence of isobilateral palisade layers and the need for less cellular division to fill intracellular spaces and form a continuous barrier of cells. In *E. globulus*, which has only one adaxial palisade layer, the necrophylactic periderm was formed more slowly and in a distorted fashion. It was primarily formed through hypertrophic changes to existing cells and limited cell divisions. Deposits of lignin and suberin in the cells of the necrophylactic periderm did not occur in *E. globulus* until later

stages of lesion development, and in many cases the necrophylactic periderm appeared to be ineffective in preventing further disease development. From this study of necrophylactic periderm formation, it was suggested that increased mesophyll density within a leaf may be linked with the speed and shape of necrophylactic periderms that are formed after infection by *Mycosphaerella* species; thus the more resistant species/families are able to restrict pathogen spread more effectively than susceptible species/families.

Under the same environmental conditions and inoculum load, northern NSW provenances of *E. nitens* have been observed to be more resistant to MLD than southern NSW provenances. Using histological methods, one provenance from each distribution were investigated with respect to constitutive anatomy. The cellular and histochemical changes after infection by *Mycosphaerella* species that led to barrier zone formation, including accumulation of defence compounds such as suberin, lignin and flavanoids were also compared. Leaves of resistant provenances were significantly thinner, had a higher proportion of palisade mesophyll and reduced intracellular airspace compared with those from the susceptible provenance. After infection, more cellular division was observed in sections from resistant leaves and the necrophylactic periderm formed was more organised, continuous, suberised and lignified than necrophylactic periderms formed in susceptible leaves. It is suggested that higher constitutive proportions of cell-dense palisade layers and thinner leaves can reduce the cellular division required to form of necrophylactic periderms after injury and compartmentalise pathogens more rapidly. More compact palisade layers may also play a role in the slowing or prevention of infection as some *Mycosphaerella* species may not be able to penetrate tightly packed mesophyll cells.

Resistance of *E. globulus* juvenile foliage to MLD has been shown to be under high genetic control. Differences between pairs of resistant and susceptible families, in constitutive traits of juvenile leaves such as stomatal density (counted with wax on and with wax removed), leaf density, total phenolics and total leaf wax was assessed on juvenile leaves. Four resistant and susceptible pairs of families were compared including one inter-provenance, one intra-provenance and two within family contrasts. Resistant families had significantly higher leaf density in three of the four contrasts and had a higher density of palisade mesophyll cells. Resistant genotypes also had a higher proportion of stomata covered by wax. The density of exposed stomata (both abaxial and adaxial) may influence resistance to initial *Mycosphaerella* infection with wax coverage or deposition identified as the main trait governing the exposure of stomatal openings. This study suggests that leaf density may be associated with a higher cellular density within the leaf which would increase the potential for necrophylactic periderm formation and compartmentalisation of the infected area once infection has occurred. Future studies are required to determine the relationship between leaf density and cellular density.

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Contents

Thesis structure

Each chapter/section of this thesis is a separate piece of work, assesses specific hypotheses and has been written in manuscript format. However to enhance the continuity of the thesis, abstracts have been omitted and references have been collated at the end of the thesis. Additional work, worthy of comment, has been included as appendices. The manuscripts embedded in this thesis have been reviewed or are under review in the CSIRO internal review process and are either published, accepted or submitted to their respective journals unless otherwise stated.

Chapter 1 investigates the sources of error associated with forest health assessment methods, in particular the Crown Damage Index (CDI). Using the recommendations for improved visual based assessment (outlined in Chapter 1), field staff used the CDI sampling strategy to calculate a mean plantation CDI. The errors involved with this method are discussed in Appendix 2. The CDI method was modified to reduce errors for research trials. The accuracy of the modified CDI method was investigated in Appendix 3. This method was used to collect data on tree growth and damage to directly assess growth effects of *Mycosphaerella* associated damage (Chapter 2.1) and to input into the Farm Forestry Toolbox to provide long term estimations of growth (Chapter 2.2). The use of molecular techniques to provide accurate and rapid detection of *Mycosphaerella* species was investigated in Chapter 3. The nested PCR detection method was applied a) in a field situation to provide information on the distribution of selected *Mycosphaerella* species within a plantation (Chapter 3.1) and b) to identify selected *Mycosphaerella* species within lesions of varying developmental stages (Chapter 3.2). The changes in anatomy and

histochemistry after infection in juvenile leaves of *E. globulus* and *E. nitens* were studied in Chapter 4.1. This suggested that constitutive mesophyll density may be influencing the timing and strength of a necrophylactic periderm for pathogen restriction. This hypothesis was tested in Chapter 4.2 where resistant and susceptible planting provenances of *E. nitens* in South Africa were investigated with respect to constitutive anatomy and necrophylactic periderm formation. Constitutive resistance mechanisms of resistant and susceptible *E. globulus* families were assessed in Chapter 4.3.

Manuscripts

The manuscripts embedded in this thesis are as follows:

1. Smith AH, Pinkard EA, Stone C, Battaglia M and Mohammed CL (2005) Precision and accuracy of pest and pathogen damage assessment in young eucalypt plantations. *Environmental Monitoring and Assessment.* 111**: 243-256.**

Acknowledgements: Thanks to Forestry Tasmania for access to plantations at Wielangta and Christmas Hills and the provision of trees for the destructive sampling work. Dr. Tim Wardlaw, Karl Wotherspoon, Stephen Scott, Jody Cartwright and David Fulford of Forestry Tasmania, Dr. Alieta Eyles of the Tasmanian institute of Agricultural Research, Anna Hopkins of the Co-operative Research Centre for Sustainable Production Forestry for their assistance with field studies. Dr. Chris Beadle of CSIRO Forestry and Forest Products for his assistance in the field and helpful comments. Malcolm Hall, Elizabeth Pietrzykowski and Julian Smith for their assistance in the field and laboratory.

2. Smith AH, Wardlaw TJ, Pinkard EA, Wotherspoon K and Mohammed CL (2006) Effects of Mycosphaerella leaf disease on the growth and wood quality of *Eucalyptus globulus.* To be submitted to Canadian Journal of Forest Research

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3. Wardlaw TJ, Smith AH, Pinkard EA, Wotherspoon K and Mohammed CL (2006) The impact of a single epidemic of *Mycosphaerella leaf disease* on the growth of *Eucalyptus globulus.* To be submitted to Canadian Journal of Forest Research

Acknowledgements: Many thanks to Forestry Tasmania for the use of their plantations in this study. Thanks to Lizz Pietrzykowski for her assistance in the field.

4. Glen M, Smith A, Langrell SRH and Mohammed CL (2005) Development of nested PCR detection of *Mycosphaerella* species and its application to the study of leaf disease in *Eucalyptus* plantations *Phytopathology* In press.

Acknowledgements: The authors are grateful for the assistance of Forestry Tasmania for allowing sampling and the establishment of field trials within their plantations. Thanks to Tim Wardlaw and Karl Wotherspoon for assistance with and co-ordination of chemical treatments. Thanks to Inez Tommerup for her invaluable input into the project.

The above manuscript includes all of the data obtained when the nested PCR was applied to *Mycosphaerella* infected plantations – Chapter 4 of this thesis. For the purpose of publication, these data have been combined with the information from the development of the nested PCR technique.

5. Smith AH, Glen M, Pinkard EA and Mohammed CL (2005) Early detection of *Mycosphaerella* species causing leaf spot on *Eucalyptus globulus* and *E. nitens* plantations. To be submitted to *Plant Pathology*.

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6. Smith AH, Gill WM, Pinkard EA and Mohammed CL (2005) Anatomical and histochemical defence responses in juvenile *Eucalyptus globulus* and *Eucalyptus nitens* leaves induced by *Mycosphaerella* infection. *Forest Pathology* In Press.

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7. Smith AH, Hunter G, Pinkard EA, Wingfield M, Mohammed CL (2005) Association of anatomical variation of juvenile *Eucalyptus nitens* leaves with resistance to Mycosphaerella leaf disease. *Australasian Plant Pathology* In press.

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8. Smith AH, Potts BM, Pinkard EA and Mohammed CL (2005) Associations of stomatal wax coverage and leaf density of juvenile *Eucalyptus globulus* leaves with resistance to Mycosphaerella leaf disease. Submitted to *Functional Plant Biology*.

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Additional Publications

Additional publications and presentations during candidature

Mohammed CL, Wardlaw TJ, Smith A, Pinkard EA, Battaglia M, Glen M, Tommerup I, Potts BM, Vaillancourt RE (2003) Mycosphaerella leaf diseases of temperate eucalypts around the southern Pacific Rim. *New Zealand Journal of Forest Research* **33**, 362-372.

Mohammed CL, Battaglia M, Pinkard EA, Glen M, Tommerup I, Smith A, Pietrzykowski E, Barry KM, Eyles A, Beadle CL (2004) New tools for cost effective health management in eucalypt plantations. In 'Proc. IUFRO Conf. Eucalyptus in a Changing World'. Aveiro, Portugal. (Eds NMG Borralho, JS Pereira, C Marques, J Coutinho, M Madeira and M Tomé) pp. 606-613. (RAIZ, Instituto Investigação da Floresta e Papel, Portugal).

Pietrzykowski E, Booth TH, Battaglia M, Stone C, Mohammed CL, Pinkard EA, Wardlaw TJ, Smith A (2003) Is the risk of an epidemic too high? Case study: Risk and remote sensing of Mycosphaerella leaf blight. Poster at CRC-SPF Annual Meeting, 21-23 October 2003. Cradle Mountain, Tasmania.

Smith A, Glen M, Tommerup I, Langrell S, Mohammed CL (2003) Rapid, in planta detection of pre-visual disease symptoms: a *Mycosphaerella* example. Poster at CRC-SPF Annual Meeting, 21-23 October 2003. Cradle Mountain, Tasmania.

Smith A, Wardlaw TJ, Pinkard EA, Battaglia M, Pietrzykowski E, Mohammed CL (2003) Is the Crown Damage Index damaged by scorer variation? Poster at CRC-SPF Annual Meeting 2003. 21-23 October 2003. Cradle Mountain, Tasmania.

Smith A (2003) Crown Damage Index. Presentation given at a 'Forest Health Workshop'. Manjimup, WA.

Smith A (2003) Development of an in planta PCR detection system for *Mycosphaerella* spp. of eucalypts. Presentation at a 'Forest Health Workshop'. Manjimup, WA.

Smith A, Wardlaw TJ, Pinkard EA, Pietrzykowski E, Mohammed CL, Battaglia M (2003) Is the Crown Damage Index damaged by scorer variation? Poster abstract In: Book of Abstracts of the 8th International Congress of Plant Pathology. 2-8 February, Christchurch, New Zealand.

Smith A (2003) Crown Damage Index: Some results. Seminar at 'LossFest' 26 February. Hobart, Tasmania.

Smith AH, Potts, BM, Pinkard EA and Mohammed CL (2005) Potential resistance mechanisms of juvenile eucalypt leaves to Mycosphaerella leaf disease. Poster abstract In: Book of Abstracts of Combio 2005. Adelaide Convention Centre, 25-29 September, 2005, pp115.

Smith AH, Gill WM, Pinkard EA, Hunter G, Wingfield MJ and Mohammed CL (2005) Defence responses in eucalypts to infection by *Mycosphaerella* species. Poster abstract In: Book of Abstracts of the 15th Biennial Australasian Plant Pathology Society Conference. 26th-29th September, 2005. Deakin University Geelong Australia, pp153.

Smith AH, Gill WM, Pinkard EA, Hunter G, Wingfield MJ and Mohammed CL (2005) Impact of MLD on the growth of *Eucalyptus globulus* plantations. Seminar at the *Mycosphaerella* Workshop of the 15th Biennial Australasian Plant Pathology Society Conference. 26th-29th September, 2005. Deakin University Geelong Australia.

Smith AH, Gill WM, Pinkard EA, Hunter G, Wingfield MJ and Mohammed CL (2005) Defence responses in eucalypts to infection by *Mycosphaerella* species. Seminar at the *Mycosphaerella* Workshop of the 15th Biennial Australasian Plant Pathology Society Conference. 26th-29th September, 2005. Deakin University Geelong Australia.

General Introduction

Eucalypt plantations of Australia

Eucalypt plantations are widespread in the southern hemisphere (Old, Wingfield *et al.* 2003) and are of principal importance worldwide as a source of hardwood for structural timber, veneer, fuel-wood, pulp and paper products. Hardwood plantations in Australia now comprise over 638, 000 ha (39% of the total plantation estate) (Commonwealth of Australia 2005) with *Eucalyptus globulus* Labill (> 50 %) and *Eucalyptus nitens* (Deane and Maid.) Maid. (> 5 %) as the most common species planted in southeastern and southwestern Australia. The high proportion of *E. globulus* reflects its high pulp yields, density (Farrington and Hickey 1989) and strength (McKinley, Shelbourne *et al.* 2002) compared with other plantation species. On cold or high-altitude sites where *E. globulus* cannot be grown successfully, *E. nitens* is a suitable alternative as it is frost tolerant (Volker, Owen *et al.* 1994) and has similar growth rates as *E. globulus*.

Commercial hardwood plantations are generally comprised of evenly spaced clones or races of a single fast growing species, on a 10-15 year rotation for pulpwood (20-30 years for solid wood products). Genetic selection for enhanced stem form, growth and structural properties coupled with the intensive management practices associated with plantations has considerably increased growth rates in plantations compared with those observed in native forest (Dargavel 1990). However, careful genetic selection and spatial deployment is required due to the increased risk of pest and pathogen attack associated with monoculture cropping (Park, Keane *et al.* 2000).

Foliar pathogens of *Eucalyptus* species

Fungal pathogens, browsing mammals, birds, insects, nutrient deficiencies, drought, frost and water logging are all damaging agents to plantation species. The subsequent damage can reduce growth and potentially lead to tree death. In native forests, the genetic variation within and between species reduces the likelihood of epidemics (Old, Wingfield *et al.* 2003). Factors that may increase the susceptibility of monoculture plantations to foliar epidemics include a) genetic homogeneity and uniformity (single species of the same age and foliage type) and b) spatial factors (such as closed canopies) reduce airflow and increased relative humidity creating conditions favourable for pathogen proliferation. Foliar pathogens of eucalypts of concern to forest managers worldwide include species of *Cylindrocladium* (e.g. Rodas, Lombard *et al.* 2005), *Cryptosporiopsis eucalypti* (e.g. Old, Dudzinski *et al.* 2002) and *Puccinia psidii* (e.g. Barber 2004). However one of the most damaging diseases, of concern to forest managers worldwide, is caused by species of *Mycosphaerella* and their anamorphs (Figures 1a, b & c) (e.g. Mohammed, Wardlaw *et al.* 2003).

Figure 1: Examples of damage caused by MLD in the juvenile leaf phase of *Eucalyptus globulus*. a) premature leaf senescence of juvenile foliage, branch death and stunting b) leaf and shoot blighting, c) leaf spotting.

MLD as a major disease of eucalypt plantations

MLD is a leaf spotting disease caused by *Mycosphaerella* species. MLD infection is spread through primary inoculums of ascospores and/or conidia. Windborne ascospores are ejected from lesions up to a height of 12-15 mm; however they require a relative humidity of 90 % and temperatures of 25 ºC for *Mycosphaerella nubilosa* (Cooke) Hansf. and 20 ºC for *Mycosphaerella cryptica* (Cooke) Hansf. before they are released (Crous 1998; Park 1988a). Conidia (from respective anamorphs) are splash dispersed over small distances and are mostly responsible for spread within the tree. Despite the requirement of moisture for release, spores are highly adapted to survive dehydration on leaf surfaces for up to 8 weeks for *M. nubilosa* and 4 weeks for *M. cryptica* with minimal loss of infectivity (Crous 1998).

Damage associated with MLD can include minor symptoms such as localized necrotic spots, but may also result in shoot dieback, defoliation, branch lesions/cankers, premature branch death and in some cases stunting and tree death. Severe but sporadic damage, caused by MLD, has occurred in *E. globulus* plantations in north-western Tasmania over the past 30 years (T. Wardlaw, pers. comm.). The incidence of MLD is also increasing in western Victoria and eastern South Australia and south Western Australia, where *E. globulus* plantations have rapidly expanded since the early 1990's (Mohammed, Wardlaw *et al.* 2003) Surveys in the coastal regions of Chile have identified a suite of species associated with MLD, which caused severe and sometimes complete defoliation of *E. globulus* (Ahumada, Hunter *et al.* 2003). In the central North Island of New Zealand damage caused by MLD has been so severe that *Eucalyptus* species are no longer planted (Hood, Gardner *et al.* 2002) and the importance of *Mycosphaerella* species in South Africa

has been recognized since the 1930's when severe defoliation stopped the planting of *E. globulus* (Lundquist and Purnell 1987).

Historically, research into the development of management strategies for MLD has received less attention than taxonomic studies and disease surveys describing host and geographical ranges (Mohammed, Battaglia *et al.* 2004). Only recently have the some management issues associated with MLD been directly addressed (eg. Carnegie and Ades 2002b; Carnegie, Keane *et al.* 1994; Mohammed, Battaglia *et al.* 2004; Mohammed, Wardlaw *et al.* 2003). Even so there is inadequate information available to develop operational management prescriptions and decision support systems (DSS) for MLD to prevent or reduce losses in growth and stem form caused by infection. Historically, investigation into the effects of MLD on growth and the development of management strategies has been delayed due to 1) The need for large-scale field and disease exclusion trials that are operationally restrictive, time consuming and costly. 2) The long rotations of forestry plantations (up to 30 years). 3) Damage by MLD generally occurs sporadically and is difficult to capture experimentally at a predetermined stage of tree growth in field trials. 4) Species of *Mycosphaerella* grow slowly and will not reproduce in culture. As a result there is a lack of effective inoculation techniques to obtain artificial infection in a controlled glasshouse environment.

MLD is a disease of worldwide concern and was chosen as a model system for this research. The primary objective of this study was to create a framework of research with MLD that can be applied to the biotic damage of eucalypts by pests, especially by foliar pathogens.

Research framework for MLD on eucalypts

A schematic diagram of the framework used to research MLD, the questions investigated in this thesis and how they are they are connected in a conceptual framework is presented in Figure 2. Four main research areas were investigated: 1. Assessment, 2. Growth Impact, 3. Detection and 4. Resistance.

ASSESSMENT:

What is the best field based method for assessing necrosis and defoliation?

The quantification of damage using visual estimation is often fraught with error. Data obtained from visual estimation are often used to calculate growth loss for plantations and input into computer modelling programs, such as CABALA (Battaglia, Sands *et al.* 2004) and Maestra (Medlyn 1997). These programs can inadvertently enhance even small errors; therefore it is important that techniques provide an accurate representation of actual damage levels to be able to provide sound estimates of growth- and economic impacts at a plantation level.

Previous studies of insect pests and pathogen damage to eucalypts have used scoring systems that have been developed for a particular purpose (e.g. scoring leaf scallops or the size of stem cankers). For foliar pathogens they are usually based on a score out of 10, where 1 is healthy and 10 is dead. Scoring strategies for *Mycosphaerella* damage have been devised by Lundquist (1987) and Carnegie, Keane *et al.* (1994) and were used to assess resistance trials of *E. nitens* and *E. globulus* respectively.

The use of a standardized and repeatable method is more favourable for use both operationally and in research so that damage levels can be compared between time-periods, plots, plantations, companies/research groups etc. In 2003 the Crown Damage Index (CDI) was suggested for use in Australia as a standardized and statistically sound method for the assessment of crown damage caused by pests and pathogens (Stone, Matsuki *et al.* 2003). Symptoms of tree damage and stress such as necrosis, defoliation and discoloration are quantified and collated into one CDI score representing the health of the tree. Categories can also be separated and reported individually without compromising the score. However, different scores are likely to be obtained for the same tree due to assessor variation and their perception of the damage, which will vary with the level of training or expertise. Scores may also vary due to non-uniform infection patterns (particularly on older trees) and variations in canopy shape and leaf type. The errors associated with CDI assessment have not been quantified to determine its suitability for use in research or forestry operations.

Hypotheses:

1. The CDI is an accurate and repeatable method for forest health assessment in forest operations (Chapter 1, Appendix 2).

2. Modification of the CDI increases the accuracy of quantifying MLD associated damage on young eucalypts for research (Appendix 3).

GROWTH IMPACT:

What impact does *Mycosphaerella* damage have on tree growth?

The extent of damage caused by *Mycosphaerella* species worldwide has been of major concern for over four decades. Research into the impact of forest pests and pathogens is often costly and time consuming. Forestry research is made difficult by long rotations that can be in excess of 30 years. In agriculture, where on average crops are harvested annually, there is more scope for the testing of control methods and the repetition of trials. In forestry even the spraying of fungicides is operationally challenging as plots are often difficult to access using ground-based machinery and trees can reach up to 4-5 metres in the first three years of growth. Exclusion of the target pathogen using fungicides is essential in growth trials and the timing of fungicide applications often coincides with periods of weather unsuitable for spraying. As a result, only one short-term study, with a fungicide sprayed control, was conducted on *E. globulus* (Carnegie and Ades 2002b). Another short-term study (on older trees) has been conducted on *E. nitens* in South Africa but did not include an uninfected control (Lundquist and Purnell 1987).

The impact of *Mycosphaerella* on eucalypt growth in a plantation is likely to be influenced by species, tree age and site variation (differences in climate and nutrition), therefore it is important not to generalize management prescriptions. The threshold levels of necrosis and defoliation to cause a) a growth loss and b) permanent deleterious effects on growth remain unknown in *E. globulus,* as do the effects of MLD on long term growth including losses at harvest. Advances in computer modelling, such as the development and use of the Farm

Forestry Toolbox (Private Forests Tasmania 2003), have enabled relatively accurate long-term predictions of the growth of forestry stands under varying conditions.

Hypotheses:

1. MLD reduces both short and long-term growth of *E. globulus*.

2. Infection by *Mycosphaerella* species can accelerate branch death which can potentially affect wood quality and pruning regimes.

DETECTION:

Can we detect multiple *Mycosphaerella* species within a leaf and determine their distribution at a plantation level?

Classification of ascospore shape, size and germination patterns is considered to be the most rapid means of *Mycosphaerella* identification. However intra-specific variation between *Mycosphaerella* species can be extensive and lead to false identification. *Mycosphaerella* species infecting lesions without reproductive structures cannot be identified. The positive identification of lesions with reproductive pseudothecia is often limited by poor ascospore production and release. *Mycosphaerella* species causing MLD on eucalypts are also slow growing and the co-existence of non-*Mycosphaerella* contaminants makes obtaining pure cultures from leaf material (to assess cultural and anamorphic characteristics) challenging and time consuming.

The use of classical taxonomy in epidemiological studies requires a high level of expertise due to the similarities between *Mycosphaerella* species. It has also partially limited these studies as non-reproductive pathogens are often overlooked, and due to the time-consuming nature of the studies sample sizes are reduced per plantation. To overcome some of these problems, a nested PCR technique was adapted to detect and discriminate the most commonly identified and potentially pathogenic species in the major plantation species of *E. globulus* and *E. nitens* (Yuan 1999). The nature of the technique allows for large samples to be collected and processed within 96 hours of collection and therefore there is great potential for large quantities of ecological and epidemiological data to be collected.

Hypotheses:

1. Species of *Mycosphaerella* in Tasmania can be detected in leaf and stem lesions using nested PCR.

2. The distribution of *Mycosphaerella* species in Tasmania can be reliably determined within a plantation/field trial using the nested PCR detection method.

How early in lesion development can *Mycosphaerella* species be detected?

The identification of *Mycosphaerella* species using classical techniques requires for lesions to be necrotic and have reproductive structures. Identification of species in lesions in early developmental stages is more difficult. The ability to detect *Mycosphaerella* species in early lesions will enhance research in two ways. Firstly the species occupying various developmental stages could be determined and used in epidemiological studies to provide

information on pathogen spread and pathogenicity. Secondly the confirmation of infection by *Mycosphaerella* species in these early leaf lesions allows for the research into host response and physiology using natural infection. The nested PCR detection technique can identify *Mycosphaerella* species in leaf tissue however the sensitivity of this technique has not been tested.

Hypotheses:

1. *Mycosphaerella* species can be detected in early lesions and asymptomatic tissue.

2. The composition of *Mycosphaerella* species will vary with lesion development.

RESISTANCE:

Can we identify constitutive and induced mechanisms associated with MLD resistance?

Excellent opportunities exist for selecting genotypes that provide resistance to MLD (Carnegie, Keane *et al.* 1994; Dungey, Potts *et al.* 1997) *Cryptosporiopsis eucalypti* (Old, Dudzinski *et al.* 2002) and *Aulographina eucalypti* (Carnegie and Keane 2003). However traits conveying genetic resistance need to be combined with commercially desirable traits to produce trees suitable for large-scale commercial use. There are two broad categories of resistance. Firstly, highly sophisticated recognition processes whereby plants can detect and respond to specific races of pathogens providing a high level of resistance to some pathogen races but none to others. This resistance is encoded by one or a few genes. The second category provides a broad level of resistance to many pathogen races (Lucas 1998).

This type of resistance is more favourable for the selection of durable resistance mechanisms, as resistance is observed against different genetic variants of the pathogen and cannot be overcome by a single mutation (Lucas 1998).

Plants can defend themselves using various polygenic strategies (Appendix 4), including pre-formed structural barriers (constitutive traits), which prevent the entry of the pathogen into the leaf fungi. Induced traits such as the production of anti microbial secondary metabolites (e.g.Angra-Sharma and Sharma 1994) and the formation of mechanical barriers in leaf tissue after infection (e.g. Schafer, Huckelhoven *et al.* 2004) limit the growth and activity of the pathogen in the host. The induced reinforcement of cell walls in barrier-zones prevents the transfer of a) phytotoxic metabolites to surrounding leaf cells (Ilarslan and Dolar 2002) and b) photosynthate, nutrients and water to the pathogen.

A primary component of the biochemical response is the release of various secondary compounds. These include exudations from the cuticle and leaves, and are broadly categorized into three main classes; phenolics, cyanogenic glycosides and phytoalexins (Blanchette and Biggs 1992). Although there are hundreds of chemicals classified under these headings, some of the main compounds include tannins, flavanoids, phenols, lignin, isoflavanoids and terpenoids. These compounds can act directly on the pathogen, catalyse the synthesis of antimicrobial compounds, induce structural multicellular changes (such as the formation of lignified, suberised or meristematic defence barriers) and repair, strengthen or alter cell walls (Aist 1983; Akai 1959; Beckman 1980; Heath 1980; Sherwood and Vance 1982).

Structural defence mechanisms play an important role in host response by delaying or even preventing entry into the plant. Access to leaves is generally gained through stomata or directly through the cuticle or through open wounds. There has been little work on the mechanisms of resistance to MLD. However it is known that *Mycosphaerella* resistance is under high genetic control (h^2=0.6) (Milgate, Potts *et al.* 2005) due to additive effects of many genes (Dungey, Potts *et al.* 1997). Other plant/disease interactions have indicated that epicuticular wax properties (Schwab, Noga *et al.* 1995), stomatal regulation (Ekanayake, Ortiz *et al.* 1998) and phosphorus (Carnegie and Ades 2002a) along with the production of secondary compounds may play a role in resistance.

Hypotheses:

1. Necrophylactic periderm formation will vary in cellular and chemical nature between resistant and susceptible varieties of eucalypts.
2. Variations in constitutive traits such as stomatal density, wax quantity and distribution, leaf anatomy and leaf phenolics are associated with resistance to MLD.

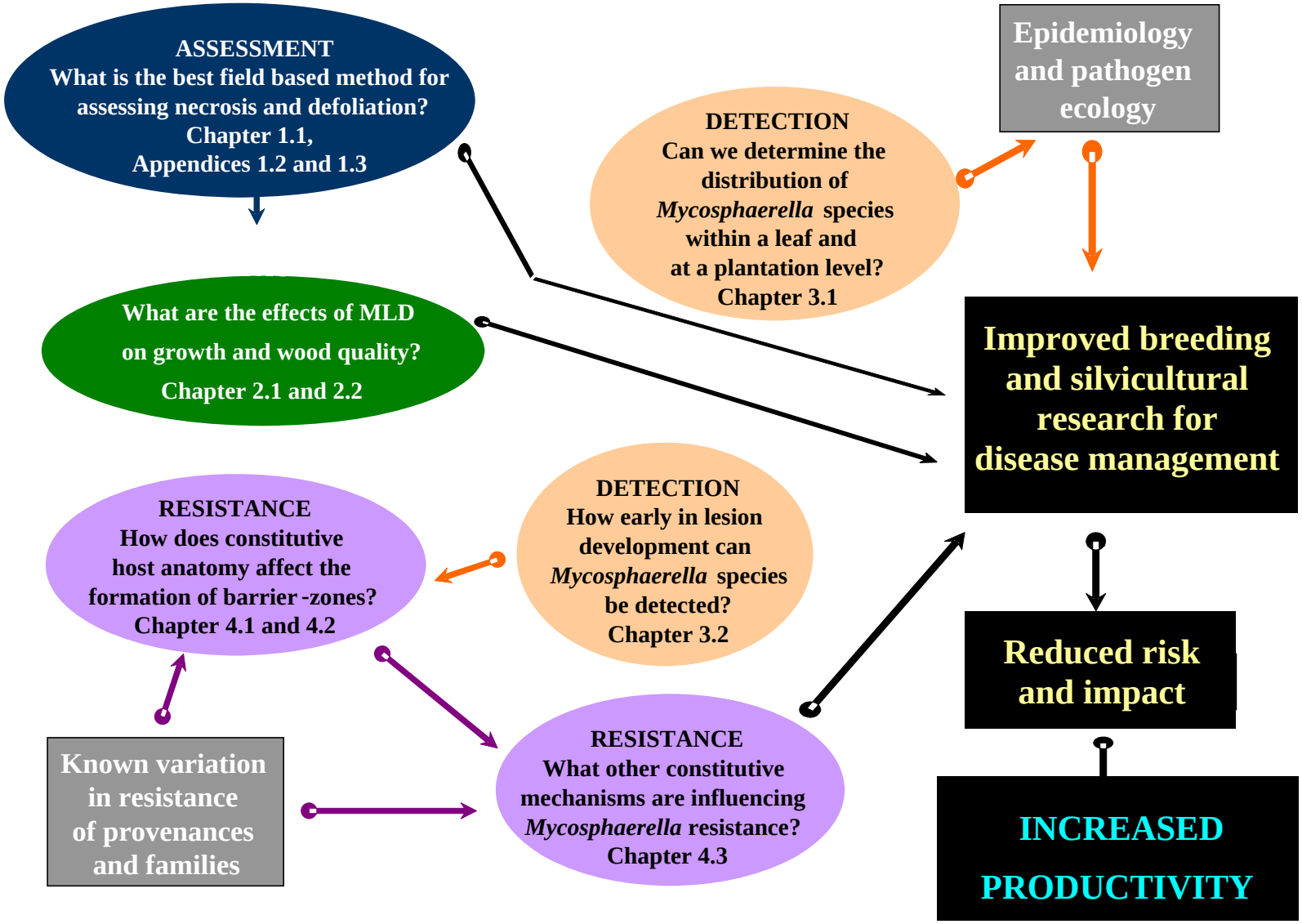


Figure 2: Conceptual framework for research (arrows indicate how each of the research questions are linked to management outcomes)

CHAPTER 1

1.1 Precision and accuracy of pest and pathogen damage assessment in young eucalypt plantations.

1.1.1 Introduction

Forest data are used by not only national governments for the development of forest policy but the forest industries, individual auditors, forestry professionals, conservation, environmental groups and the international scientific community (Matthews and Grainger 2003). The collection of repeatable, precise and accurate data worldwide has been limited, usually reactive to sporadic damaging events and with methodology that is not always compliant with reporting requirements within regions and countries (Michalak, Kelatwang *et al.* 2003). Visual assessment, involving incidence and severity estimations, has been adopted by assessors in forest and agricultural systems in most countries as they are currently the most time and cost-effective methods available to survey large areas (Lim and Gaunt 1981). In agricultural systems the harmonization and evaluation of assessment methodologies for accuracy and precision has been more progressive (Bussotti, Gerosa *et al.* 2003; Holmgren 2003; Innes 1993; Nutter, Gleason *et al.* 1993; O'Brien and van Bruggen 1992; Sherwood, Berg *et al.* 1983). Prior to 1993 the importance of evaluating methodology for forest heath assessment was underrated in many parts of the world (Bussotti, Gerosa *et al.* 2003; Holmgren 2003; Innes 1993). Whilst the problem has been less apparent in the United States where air pollution research has ensured forest monitoring programmes have incorporated quality assurance from the outset (US Department of Agriculture Forest Service 2002), Europe's main concern has been controlling the consistency of forest health observations within its

different countries (Innes 1993). Since 1993 the standardizing of methodology and the introduction of quality assurance programmes to regulate work methodologies and staff training has occurred in Europe (Bussotti, Gerosa *et al.* 2003; Innes 1988; Innes, Landmann *et al.* 1993; Kohl, Traub *et al.* 2000; Redfern and Boswell 2004).

Historically the responsibility for forest surveillance and pest management activities in Australian native and plantation forests has been that of the forest owners or managers (Stone 2003). The recent, rapid expansion of Australian's hardwood plantations to over 638, 000 hectares (National Forest Inventory 2005) has exposed the need for accurate, reliable and repeatable forest health assessment methods. The development of a repeatable and objective method for assessing damage in young eucalypts is essential to enable Australia to report on forest ecosystem health and vitality as recommended by the Montreal Process Implementation group. The Montreal Process includes important Criteria and Indicators for the Conservation and Sustainable Management of Temperate and Boreal Forests and is the framework adopted by Australia to monitor sustainable forest management. Also the requirement for countries to be more accountable for matters concerning forest management under the Montreal process has been driving the need for the development and testing of methodology that will enable comparisons to be made between programs and countries. It is also expected to contribute to the verification process for the Australian Forestry Standard – a certification of sustainable forest management (Stone, Matsuki *et al.* 2003).

Attempts to integrate forest health surveillance methodologies in Australia began with the development of a standardised index for assessing crown damage of young eucalypts. This standardised generic index, termed the Crown Damage Index (CDI)

(Stone 2003; Stone, Matsuki *et al.* 2003), can be statistically summarised for reporting obligations at regional, state and national levels (Commonwealth of Australia 1997). The CDI methodology was developed over a period of 24 months through consultation with the members of the Australian Government Forestry and Forest Products Committee Research Working Group 7 (Forest Health), with the major objectives of providing reliable data for reporting plantation health, evaluating the effectiveness of insect pest and disease control programs, assessing research trials and to make yield projections (Stone 2003). The CDI takes into account the incidence and severity of missing, necrotic or discoloured foliage at a whole tree level. Accompanying the CDI is a two-stage cluster sampling strategy designed to ensure that an unbiased estimate of the true plantation CDI mean is obtained efficiently. However, sources of error will occur at both the tree level (observer error) and at the plantation level (sampling error). This study focused on identifying the observer error associated with the assessment of damage present in the tree crowns. The results from the current study were also partially used to refine the CDI methodology.

Whilst much can be learned from assessment methods for deciduous and conifer species, the errors involved with the assessment of *Eucalyptus* species have not been evaluated. The purpose of the present study was to 1) quantify the bias among assessors (inter-rater repeatability) and between repeated assessments for a single assessor (intra-rater repeatability), 2) resolve the closeness of assessor estimates to measured damage levels and 3) ascertain the effects of assessor experience on these aspects.

1.1.2 Materials and Methods

1.1.2.1 Sites

Measurements were taken on plots of 50 *Eucalyptus globulus* Labill trees at two different sites. Two assessments were made at Wielangta (UTM 55G 569684 5273500) in southern Tasmania, Australia on the 15th of January 2003 and were used to determine the precision of the CDI by assessing the inter-rater and intra-rater repeatability of individuals. A third assessment was conducted at Christmas Hills (UTM 55G 331904 5469972) in north-western Tasmania, on the 25th of March 2003. Data from this assessment was used to determine the objectivity and accuracy of CDI components. Trees assessed at Wielangta exhibited a wide range of damage from insect herbivory and necrotic leaf lesions from fungal infection. Trees assessed at Christmas Hills had overall lower levels of leaf damage and particularly low levels of insect herbivory. An independent person that did not participate in assessments chose the plots prior to the exercise. The location of the plots was chosen to represent the best range of damage for all CDI components that the site could offer. Trees at Wielangta were 3-4 years of age and had a mean height of 7m; trees at Christmas hills were 2-3 years of age and had a mean height of 4 m. Both sites had just started phase change from juvenile to adult foliage but had not reached canopy closure.

1.1.2.2 Assessment of damage classes

Incidence (percentage of leaves affected in the crown) and severity (percentage of leaf area damaged on affected leaves) levels were assessed at a crown level for the following CDI components; foliar necrosis, partial loss of leaf tissue from insect feeding and whole leaf defoliation from premature leaf abscission or total leaf consumption by insects. For the purpose of the study the partial defoliation caused by insect chewing

was included as a separate index, whereas operationally the defoliation component of the CDI includes both the loss of entire leaves and parts of leaves missing (Stone 2003; Stone, Matsuki *et al.* 2003). A percentage estimate was given for all components of the CDI and then after the exercise was completed CDI values for each tree were calculated using the modified formula of (Stone 2003), where Incidence (I) = the estimated extent of damage type (*i.e.* defoliation, necrosis, discolouration) over the entire tree crown and Severity (S) = the estimated average level of severity at the leaf scale.

$$CDI = \frac{(IxS)_{defoliation}}{100} + \frac{(IxS)_{necrosis}}{100} + \frac{(IxS)_{discoloration}}{100}$$

Figure 1.1.1: Formula for the calculation of the CDI, modified from Stone, Wardlaw *et al*., (2003).

Visual standards were provided illustrating a range of damage levels for defoliation, partial leaf loss and necrotic leaf tissue per leaf (Stone 2003) to assist with estimates. A time limit was not set for each evaluation, but assessors were asked to walk around the tree and assess damage looking away from the sun. Prior to the assessments and regardless of experience, all assessors were given an explanation of the components of the method and how they are evaluated and then taken through three working examples.

1.1.2.3 Accuracy

Nine people assessed one plot of 50 *E. globulus* at Christmas Hills. The incidence and severity of necrosis, insect herbivory and whole leaf defoliation were estimated to the nearest 1%. A random sample of nine trees was harvested. The number of nodes and the number of defoliated nodes were counted on each harvested tree to determine the

incidence of the defoliation. The number of necrotic leaves was also determined to quantify the incidence of foliar necrosis. To calculate necrosis severity, each leaf was scanned in true colour using an Epson Expression 1680 colour scanner. Colour classes for depicting necrotic tissue and healthy tissue were determined and image analysis on each leaf was undertaken using Folia© (Regent Instruments Inc, Zurich, Switzerland). Ten leaves were randomly selected to check the accuracy of Folia© in measuring the percentage necrotic leaf area by manually selecting the necrotic area, changing it to black and analysing on a black and white threshold in Imagepro© (Media Cybernetrics, MD, USA). The standard error generated by Folia© when predicting necrotic and healthy tissue was ± 6.35%. The gross necrotic area and leaf area were determined for each leaf and hence the percentage of necrotic tissue for each tree could be calculated. Insect herbivory at the Christmas Hills site, if detected, was very low and would not have significantly contributed to the overall levels of damage of the tree crowns. The accuracy of individual scorers, experience groups and the whole group to predict the CDI and its individual components was established using linear regression in Microsoft Excel®. The coefficient of determination (R^2), slope and y-intercept were calculated. The relationship between the predicted R^2 and measured values was plotted to determine the ability of assessors to be more accurate when estimating lower and higher damage levels.

1.1.2.4 Inter-rater repeatability

To determine the variation between assessors for a single plot of trees, nine raters were asked to assess one plot of 50 *E. globulus* using a linear scale estimating damage to the nearest 1 %. The acceptable level of observer error was set at plus or minus 10 % between the grand mean of the observations for the group and the assessor's estimate

(Stone 2003).The percentage of estimates within an acceptable level of observer error was determined for each tree.

1.1.2.5 Experience

Assessors were placed into one of three groups based on their experience. Assessors in Group 1 had a coherent understanding of the factors influencing forest health and its assessment, had conducted regular forest health assessments using the CDI components and were considered capable of training others. Assessors in Group 2 had a good understanding of the assessment of CDI components and had conducted such assessments one to three times previously. Assessors in Group 3 were able to identify pests and diseases but were not familiar with assessing the components of the CDI. All assessors had a baseline knowledge level on identification of the common damaging agents and had some experience with assessing damage using other methodology. Nine assessors assessed a plot of 50 *E. globulus* at Wielangta and Christmas Hills. Damage estimates were placed into the "logarithmic" Horsfall-Barrat categories of 0-5, 6-11, 12-24, 25-49, 50-74, 75-86, 87-94 and 95-100 (Horsfall and Barrat, 1945). To determine the effects of experience on estimates for each CDI component, the frequency distribution of each assessor's estimates was analysed using a multinomial poisson analysis. This was conducted using a poisson link and the GLM function of S-Plus® (Venables and Ripley 1999).

1.1.2.6 Intra-rater repeatability

Nine assessors assessed 50 *E. globulus* at Wielangta in the morning and in the afternoon (one to two hours later). Estimations of whole leaf defoliation (total leaves missing), incidence of leaves with partial defoliation, average severity of the partial defoliation,

necrosis incidence and necrosis severity were placed into Horsfall-Barrat categories as above (Horsfall and Barrat 1945). Record sheets were collected after each assessment and raters were asked not to compare or talk about the assessment during this period. Chi^2 analyses were conducted on frequency distribution tables using Microsoft Excel® for each component of the CDI. Significant differences in the integrated index (CDI) of each assessor were determined using a mixed-effects analysis of variance in S-Plus® where the assessor was treated as a random effect and the significance of experience, "time of day" and their interaction were investigated. Differences between morning and afternoon assessments were determined for individual assessors, experience groups and the whole group of nine assessors.

1.1.3 Results

1.1.3.1 Accuracy

The measured levels for each component in the nine harvested trees are presented in Table 1.1.1. The relationships between damage estimates and measured values for each assessor are presented in Table 1.1.2 The most highly correlated index was the incidence of foliar necrosis with a mean R^2 (SE) for the group of 0.72 (0.07) and the least correlated index was whole leaf defoliation with a mean R^2 (SE) for the group of 0.07 (0.03). The parabolic relationship between measured levels and the predicted R^2 for the group is presented for CDI and whole leaf defoliation in Figures 1.1.4 and 1.1.5 respectively. Neither a parabolic nor a linear relationship was found between the predicted R^2 and the measured levels for the foliar necrosis components of the CDI.

1.1.3.2 Inter-rater repeatability

The closeness of estimates to measured levels is shown in Table 1.1.3. The consensus between assessors was good for all individual indices (>75%). However when these were combined into a single index (CDI), the percentage of estimates within the allowable field estimate error of ± 10% of the grand mean for the group was reduced to 68%. When compared to measured levels, the incidence of foliar necrosis was the most accurately estimated, with 75% of estimates within ± 10% of the grand mean for the group. Defoliation was the least accurate, where only 31% of estimates were within ± 10% of measured levels.

1.1.3.3 Experience

Effects of assessor experience were significant (P = <0.05) for all damage indices in all plots with the exception of the severity of foliar necrosis and the incidence of partial leaf defoliation (P = 0.46 and 0.09).

Table 1.1.1: Measured damage levels (%) for 9 trees at Christmas Hills in NW Tasmania that were harvested and destructively sampled after damage assessments.

Tree	Whole leaf defoliation	Incidence of foliar necrosis	Severity of foliar necrosis	Crown Damage Index
1	12	70	6	16
2	18	90	9	24
3	40	97	23	54
4	41	93	21	53
5	33	92	15	42
6	28	93	10	34
7	28	93	16	39
8	33	92	9	39
9	31	94	14	40

Table 1.1.2: Relationship between estimated damage levels for individual assessors and measured damage levels after destructive sampling (represented as R^2, slope and y-intercept).

	R^2								
					Assessor				
	1	2	3	4	5	6	7	8	9
Whole leaf defoliation	0.00	0.05	0.01	0.24	0.08	0.03	0.02	0.02	0.19
Incidence of foliar necrosis	0.75	0.83	0.75	0.47	0.33	0.59	0.90	0.92	0.96
Severity of foliar necrosis	0.35	0.80	0.88	0.62	0.34	0.83	0.72	0.86	0.75
Crown Damage Index	0.06	0.30	0.33	0.25	0.33	0.24	0.32	0.15	0.34
	Slope								
					Assessor				
	1	2	3	4	5	6	7	8	9
Whole leaf defoliation	0.00	0.26	0.17	0.13	0.53	0.24	0.11	0.10	0.23
Incidence of foliar necrosis	0.76	1.16	0.94	0.71	1.94	0.43	1.69	1.38	1.04
Severity of foliar necrosis	0.93	1.73	2.63	1.97	0.97	2.60	1.81	2.88	1.43
Crown Damage Index	0.22	0.41	0.95	0.49	0.42	0.87	0.66	0.34	1.08
	Y-intercept								
					Assessor				
	1	2	3	4	5	6	7	8	9
Whole leaf defoliation	29	6	11	4	4	8	7	15	0.4
Incidence of foliar necrosis	20	-22	4	15	-117	51	-68	-37	-3
Severity of foliar necrosis	12	-4	-5	-2	20	-5	4	-4	-1
Crown Damage Index	32	5	5	23	7	38	8	21	8

Table 1.1.3: Closeness of estimates by individual assessors to the grand mean of the group of assessors and measured levels obtained from destructive sampling.

Index	% Scores within +/- 10% of grand mean (n=450)	% Scores within +/- 10% of measured levels (n=81)
Whole leaf defoliation	75.80	30.87
Severity of foliar necrosis	86.87	46.91
Incidence of foliar necrosis	85.60	75.55
Incidence of partial leaf defoliation	98.88	*
Severity of partial leaf defoliation	89.78	*
CDI Plot 1	62.67	*
CDI Plot 2	56.00	*
CDI Plot 3	68.00	56.00

* Accuracy not measured on these plots

Table 1.1.4: Variation between morning and afternoon assessments of teams with different experience levels in pest and pathogen damage assessment when assessing damage on young eucalypts (< 4yo) at Wielangta in southern Tasmania.

Damage Index	Experience level (Teams of three)		
	1 (high)	1 (moderate)	3 (low)
Whole leaf defoliation	22.11	55.66*	92.84*
Incidence of foliar necrosis	31.29	17.25	42.67*
Severity of foliar necrosis	27.04	52.65*	68.88*
Incidence of partial defoliation	48.19*	164.32*	26.65
Severity of partial defoliation	20.41	66.49*	46.40*

*Significantly different AM and PM assessments.

Significance level = 31.40, confidence interval of 0.05%

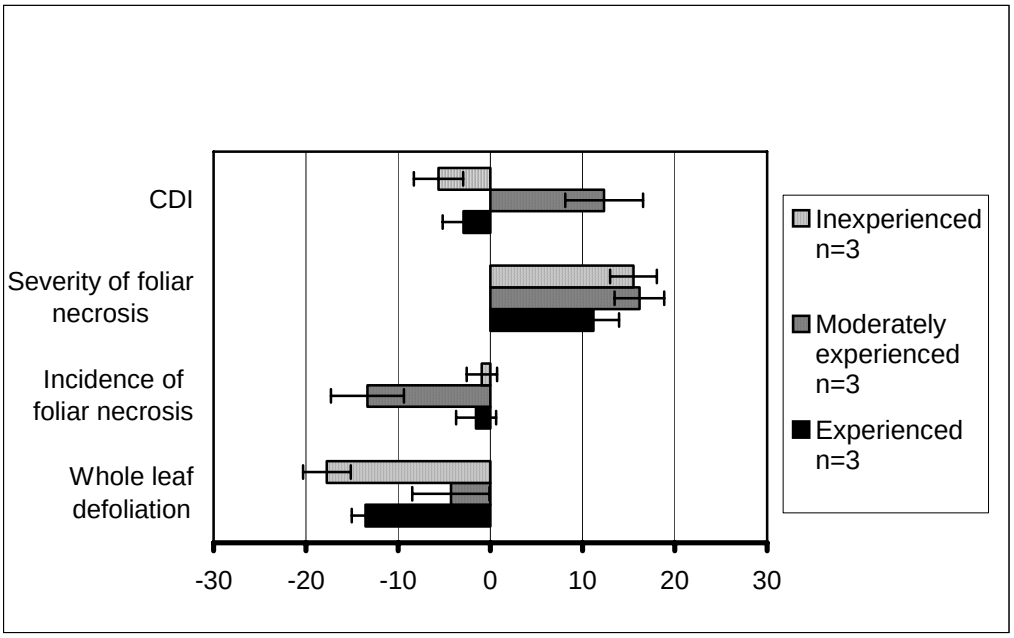


Figure 1.1.2: Mean deviation of visual estimates for assessors with varying levels of experience in pest and pathogen damage assessment from measured values (%) obtained from destructive sampling.

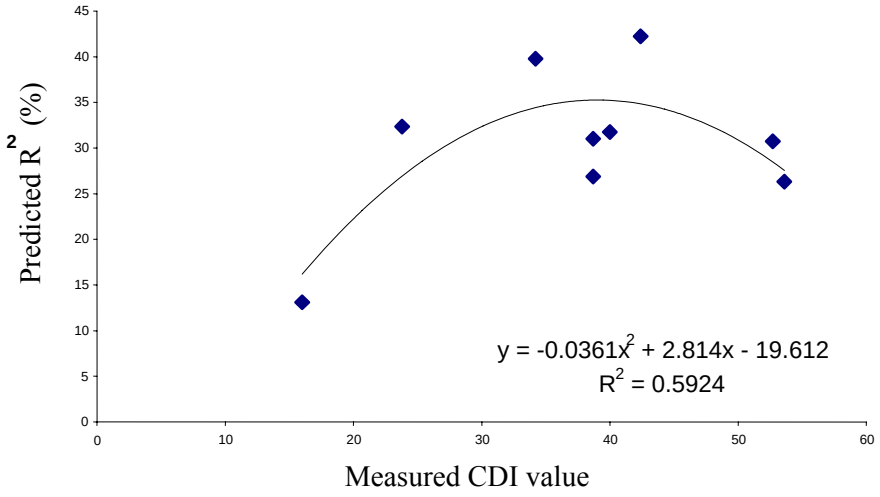


Figure 1.1.3: Parabolic relationship between measured CDI values and the predicted R^2 for the group of assessors.

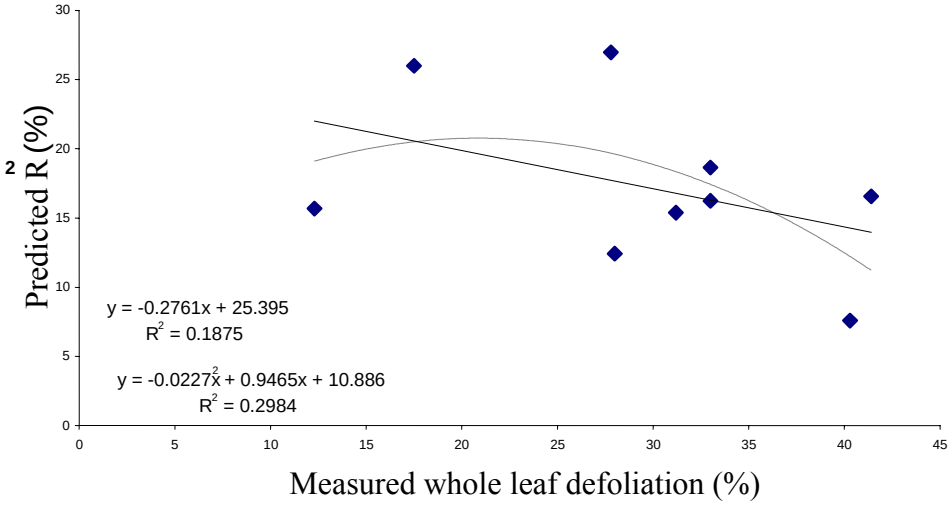


Figure 1.1.4: Parabolic relationship between measured whole leaf defoliation values and the predicted R^2 for the group of assessors.

1.1.3.4 Intra-rater repeatability

The variation in repeated assessments for CDI components is presented in Table 1.1.4. Two assessors were significantly different between morning and afternoon assessments for the incidence of foliar necrosis, five were significantly different for the severity of foliar necrosis, three were significantly different for the incidence of partial leaf defoliation and four were significantly different between repeated assessments for the severity of partial leaf defoliation and whole leaf defoliation. The least experienced group significantly changed their estimates between repeated assessments for every index except the incidence of partial leaf defoliation. The moderately experienced group significantly changed their estimates for all indices except the incidence of foliar necrosis and the most experienced group only changed their estimates for the incidence of partial leaf defoliation. Differences between morning and afternoon assessments for the group were significant for the severity of foliar necrosis (P = 0.01), the incidence of partial leaf defoliation (P = 0.00) and the severity of partial leaf defoliation (P = 0.00). There was no significant difference between the group's repeated assessments for whole leaf defoliation (P = 0.86), the incidence of foliar necrosis (P = 0.12) and the combined index (P = 0.19).

Results from this study are also summarised in poster format in Appendix 1

1.1.4 Discussion

A major advantage of this study is that the damage levels of assessed trees were quantified to allow the direct comparison between assessors and eliminate the potential bias of comparing with a reference or control observer. The parabolic relationship between the predicted R^2 and measured damage levels of both whole leaf defoliation

and the CDI indicated that assessors are able to more accurately predict damage at the high and low levels and not at the mid range, as the variation is at a maximum at the mid range and decreases at either end of the scale. Although the use of categorical assessment, as (suggested by Horsfall and Barrat, 1945), would be more suitable for these components, it would be of no additional benefit for assessing the incidence and severity of foliar necrosis. More importantly the change to using a linear scale (nearest 1 %) instead of categorical analysis has allowed for more robust and simplified statistical analysis. It has also eliminated the bias associated with the placement of trees into categories. For example if a tree did not have any defoliated leaves using a linear scale the assessor could record a 0 % for that tree - using categories the tree is recorded in the 0-10 % class. Therefore a bias of up to 10 % is recorded over all assessed trees even if the class mean is used.

The interpretation of whole leaf defoliation was the most problematic component of the CDI. Crown thinning, (transparency or defoliation) is not only one of the most widely assessed crown traits, but also one of the most subjective (Innes 1988; Innes 1993; Innes, Landmann *et al.* 1993; Kohl, Traub *et al.* 2000; Solberg and Strand 1999). In the current study, on most trees, defoliation appeared to be clearly visible and in a localized area on the internal mid to lower crown. However not all defoliation was actually visible to assessors. On nodal examination during destructive sampling it was found that the defoliation of either isolated leaves or small clusters of leaves had taken place throughout the entire crown. Hence the under-estimation observed in this study. The underestimation of defoliation has also occurred with the assessment of other tree species such as *Pinus ponderosa* (Muir and Armeanto 1998). One of the most difficult factors when estimating defoliation is the interpretation of the original leaf density when

the leaves are no longer present. One of the potential sources of error is the difference in individual opinion of a healthy phenotypic expression for a particular site. Therefore it is important to refer to the CDI as an indicator of crown damage and leaf loss due to the presence of damaging agents or processes and not as a surrogate for total leaf area, which is strongly influenced by many factors such as tree genetics and site quality. The provision of photographic standards (James 1971) becomes complicated with trees of the same species that vary in branch architecture and density. It is also particularly difficult to define defoliation in faster growing trees where the apical shoot determines the shape of the tree and natural senescence occurs in older leaves (Innes 1993). Studies by Redfern and Boswell (2004) have identified that the use of absolute standards - the perfect tree carrying the maximum amount of foliage for that species - in opposition to local standards, was the best way to detect changes in crown condition.

While there are underlying concerns about the repeatability and accuracy of assessing the incidence of whole leaf defoliation, other indices in the study had an acceptable level of agreement with group mean values and were usually within 10% of measured levels. Since the completion of the current study foliar discoloration, as a symptom of stress and early stages of infection in eucalypts, is assessed as a CDI component (Dell, Malajczuk *et al.* 2001). As it was not assessed in this study its effects on the precision and accuracy of the CDI are unknown and could be a potential source of error in the future. In addition, some of the errors involved with the sampling strategy outlined by Stone, Wardlaw *et al*., (2003), used to determine a mean CDI for the plantation, are outlined in Appendix 2.

Differences between morning and afternoon assessments decreased with increasing experience. (In a study by Innes 1988) observer experience was recognized as a major source of variation; subjective estimates were directly related to experience and training. Operator errors in precision and accuracy have been reduced in other pathosystems by calibration and method development, while repeatability has predominantly been improved with the amount of standardisation and training available (Gaunt 1987). However visual perception varies between individuals, which often influences how they score damage and affects their capacity to be trained. In this study the potential for both highly experienced groups and inexperienced groups to achieve estimates in most damage classes within the acceptable field estimate error of ± 10 % is not only attributed to training and experience but the adherence to instructions and efficient use of visual standards for each index. Moderately experienced assessors did not achieve accurate results in the majority of indices, possibly because the assessors eliminated their use of visual standards after a few trees due to overconfidence in their assessment abilities. The CDI field guide manual (Stone 2003) attempts to improve the accuracy of assessor estimates of leaf severity through the provision of photos illustrating a range of leaf areas affected by partial defoliation, necrosis and discoloration. Further attempts to improve the accuracy of the CDI for research purposes are outlined in Appendix 3.

Experimental literature covering self-assessment of skill and knowledge has frequently uncovered the general tendency for people to be inaccurate by being unaware of their own limitations (Ackerman 2002; Fischhoff, Slovic *et al.* 1977; Kreuger 1999; Kreuger and Dunning 1999). The use of computer-generated software for assessor self-assessment in order to generate personal knowledge of the limitations and inaccuracies of their own estimates is highly recommended (Nash, Saunders *et al.* 1992; Nutter

1989). The use of computer aided training mechanisms for disease assessment has greatly improved the accuracy and precision of visual estimates in agricultural pathosystems (Nutter 1989). Software that provides diagnostic assistance, training, quality assurance and standardization for forest health research projects and surveys in sugar maple (*Acer saccharum* Marsh.), red maple (*Acer rubrum* L.), black cherry (*Prunus serotinia* Ehrh.), white oak (*Quercus alba* L.) and northern red oak (*Quercus rubra* L.) has been developed (Nash, Saunders *et al.* 1992). We recommend that the development of similar assessment training software be developed for application to the health assessment of eucalypts. One of the main advantages of computer-aided training is that training can be conducted indoors on a wide array of disease symptoms, which may not be present in the environment at the time of training (Nutter and Schultz 1995). Meanwhile the use of highly trained and experienced assessors coupled with pre-assessment calibration among assessors is vital for attaining results with the highest level of repeatability and accuracy.

1.2 Assessment Summary

Assessors, regardless of experience, could estimate CDI values to within 12 % of measured damage levels. This error was reduced if assessors were highly trained. At a plantation level, groups with more field experience also had less error involved with scores. It is suggested that the use of visual standards, assessments in pairs and self calibration will help to make the method more accurate and repeatable for forest companies. The crown damage index was modified for research to improve its accuracy and repeatability. This method was also central in quantifying damage on MLD growth trials (Chapter 2). The use of the CDI in both operationally and in research will enable CDI estimates to be compared at a national level between companies, government agencies and research groups.

CHAPTER 2

2.1. Effects of Mycosphaerella leaf disease (MLD) on the growth and wood quality of *Eucalyptus globulus*.

2.1.1 Introduction

Eucalyptus globulus Labill is a primary plantation species, grown in temperate regions worldwide for structural and appearance grade timber, pulp and paper products. In Australia, a rapid expansion of monoculture plantations, particularly of *E. globulus*, has developed an area of approximately 715, 531 ha of hardwood plantation (National Forest Inventory 2005). Threatening these plantations is one of the most damaging fungal diseases of *Eucalyptus* species, Mycosphaerella leaf disease (MLD). Although several *Eucalyptus* species including *Eucalyptus nitens* (Deane and Maid.) Maid. are less susceptible to MLD in Australia, *E. globulus* is grown because of the high pulp yield, density (Farrington and Hickey 1989) and strength (McKinley, Shelbourne *et al.* 2002) exhibited by this species.

Pathogenic *Mycosphaerella* species are hemibiotrophic and are spread by wind-borne ascospores and splash-dispersed conidia (Park 1988a). Ascospore germination requires high humidity or free-water which can be provided by dewfall (Park and Keane 1982) or rainfall (Park 1988b). MLD is most damaging on juvenile foliage, in particular newly expanding leaves, with resistance increasing with leaf age and ontogenetic change to adult foliage. Although infection of adult foliage is rare, some growth effects have been documented at levels less than 15% (Carnegie and Ades 2002b). Severe defoliation has the potential to cause tree mortality, however no information has been collected on the amount of infection on the leaves or stems that will result in death or senescence of

branches or trees. Non-lethal damage from MLD has two components: a) necrosis (leaf or shoot spotting/blighting) and b) defoliation (Carnegie and Ades 2002b; Smith, Pinkard *et al.* 2005). However, the risk of stem defects (due to the pruning branches that have died as a result of infection) may potentially be a third component of MLD impact (Wardlaw 2001a). Therefore, in addition to causing losses in productivity (Carnegie, Keane *et al.* 1994) a severe MLD epidemic also has the potential to alter stem form and quality (Wardlaw 2001a) and increase the tree's susceptibility to other pests and pathogens as has been observed with severe insect defoliation (Kulman 1971).

In Tasmania, where *E. globulus* is native, severe MLD epidemics have occurred, leading to occasional tree death and the need for re-establishment of some plantations due to poor growth (Wardlaw 2001a). However, in Western Australia, large acreages of *E. globulus* have been successfully grown over the past 25 years with high growth rates and short rotations (~13-14 years). These plantations have been relatively disease free as *E. globulus* is not native and there is an absence of co-evolved pathogens in the native forests to infect plantation forests. However, surveys in Western Australia have indicated that *Mycosphaerella* incidence and severity is increasing in the major growing areas (Maxwell, Dell *et al.* 2003). In the future, growth losses caused by MLD may be evident in all *E. globulus* growing areas of Australia.

Tree growth responses to defoliation or treatment can be classified into two main groups. The first is a type 1 response where an initial decrease in growth is observed after damage but is not sustained in the long-term and growth rates return to parallel those of undamaged trees. The second is a type 2 response where reductions in growth

are infinitely reduced and trends are permanently divergent from those of undamaged trees (Snowdon 2002). These growth responses are often determined by the severity of damage, frequency of its occurrence, tree species, age of the tree when the damage occurs and nutritional status (Collett and Neumann 2002; Rapley 2005). Therefore it may be possible to have a type 1 growth response up to a threshold level of damage, and a type 2 response beyond that threshold. For example, a type 1 response was observed for *E. globulus* for up to 72% damage by *Mnesampela privata* but then a type 2 response beyond this damage level (Rapley 2005). Similarly, long-term growth effects were observed by pruning more than 80% of *E. nitens* foliage (Pinkard 2002). Some short-term growth impacts at a provenance level have been recorded (Carnegie, Keane *et al.* 1994). Prior to this only one study had been conducted on the effects of MLD on the growth of any *Eucalyptus* species (Lundquist 1987), and only recently have the effects of MLD on height and diameter of *E. globulus* been quantified with an uninfected control (Carnegie and Ades 2002b). Until growth effects are adequately quantified for MLD, over a range of sites and environmental conditions, it is difficult for forest managers to make decisions on planting *E. globulus* in high-risk areas.

We hypothesised that MLD is likely to reduce both stem growth and quality. The progression of damage caused by a *Mycosphaerella* epidemic was studied for 18 months. Growth effects were determined by comparing the volume of trees in plots treated with fungicide with those in untreated plots. The potential impact of MLD on stem quality was determined by comparing defoliation and the onset of branch senescence between treated and untreated plots.

2.1.2 Materials and methods

2.1.2.1 Field trial

Sixteen plots (8 treated with fungicide and 8 untreated) were established in an *E. globulus* plantation at Christmas Hills in north-western Tasmania (40˚55'09''S: 144˚58'58''E). Trees were subject to high levels of natural infection due to the presence of an epidemic on the adjacent plantation prior to planting. Plots were arranged in a randomised complete block design (Figure 2.1.1) with 24 measurement trees per plot. Measurement trees were surrounded by a 5 tree buffer zone before and a 4 tree buffer zone after the measurement plot. All blocks were separated by windrows except for block A and B, which were within the same windrow. These plots were established within the same windrow due to site limitations of aspect and soil type. Trees were planted in November 2001 with a spacing of 2.5m (along rows) x 3m (between rows). Primary fertilisation occurred in January 2002 using diammonium phosphate / urea at the rate of 100 kg ha^{-1}. All plots were aerially sprayed with the insecticide Fastac-duo® (250 ml product ha^{-1}) on 17th December 2002 to control an over-threshold population of *Chrysophtharta agricola* (Coleoptera: Chrysomelidae). A post-planting herbicide application was done between December 2002 and January 2003 using a tractor-mounted shrouded sprayer. Round-up® (3.5 litres/ha), Brush-off® (50 g/ha) and the surfactant Freeway® (200ml / 100 litres water) were sprayed between the planting mounds, and Lontrel (5 litres/ha) with the surfactant Freeway® (100ml / 100 litres water) was sprayed on the planting mounds. Slashing between the planting mounts was done in Jan 2004. Fungicide treatments commenced on 26th June 2002, and were regularly applied (~ every 3 weeks) until trees were too tall to be sprayed by hand (05/03). The objective of the fungicide applications was to limit *Mycosphaerella* infection and not to assess the viability of the fungicides; therefore several fungicides

were used throughout the trial. These included Nustar® (4g in 20L water with 20mL Agral surfactant), FS500 (125mL in 20L water). Fungicide was applied by hand to all foliage in treated plots until runoff.

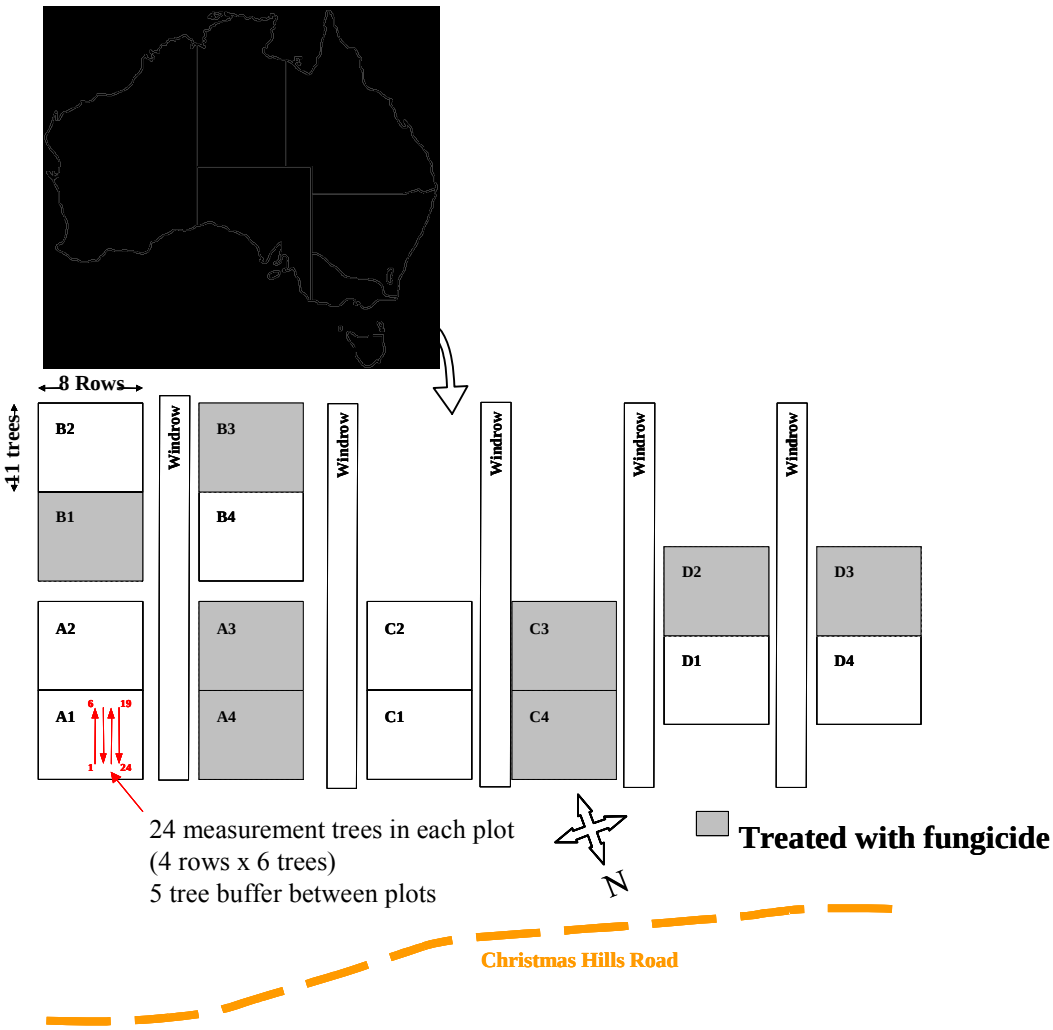


Figure 2.1.1: Layout of plots treated and untreated with fungicide to observe the short-term effects of *Mycosphaerella* associated damage.

2.1.2.2 Measurement of growth and damage assessment

Trees in measurement plots were measured and assessed for damage every 3 months (from June 2002 to September 2003) until leaf phase change from juvenile to adult leaves. Measurements included, height, diameter @ 15cm, crown diameter (along and across row), height and diameter of infection (along and across row), and where applicable the height and diameter of defoliation (along and across row).

The incidence of MLD damage in the trial was calculated using the crown dimensions mentioned above. The conical volume of the crown and its defoliated and necrotic components was calculated (Appendix 3). Defoliation was primarily "bottom up" in this trial and therefore we observed an inner lower crown defoliation, which progressed outwards as infection increased. Defoliation measurements were inside the measurements for necrosis, it was subtracted from the necrosis volume. Necrosis and defoliation were expressed as a percentage of crown volume.

The severity of infection and defoliation was visually assessed at each measurement interval as a percentage affected area of the measured crown incidence. Visual standards as described in Stone, Wardlaw *et al*., (2003) were used as a reference for severity measurements. An index of *Mycosphaerella* associated damage (necrosis and defoliation) was calculated using the formulas below:

Necrosis = ((necrosis incidence (%) - defoliation incidence (%))*severity (%))/100

Defoliation = (incidence (%)*severity (%))/100

Mycosphaerella index = Necrosis + Defoliation

Stem volume for treated and untreated trees was calculated at each measurement interval using an *E. globulus* taper function (Forestry Tasmania, unpublished).

2.1.2.3 Statistical analysis

Cumulative increments were calculated for height, diameter and stem volume for treated and untreated plots in the exclusion trial. Analyses of variance were conducted in Genstat® for each volume, height and diameter, using time as a factor and initial height, diameter or stem volume as co-variates. Block was also included as a factor to determine any site variations. Linear regressions of total volume and increments of height, diameter, and stem volume were conducted on data from fungicide treated and untreated plots over a) the period of the trial, b) the period of maximum infection (tree age: 17-20 months) and c) the period directly following leaf phase change (tree age: 20 - 26 months). Significant differences between slopes at each interval were determined by group regression analysis in Genstat® using tree age as the explanatory variable and treatment as a grouping to examine the hypothesis that regression lines have the same slope. This was also repeated to test the hypothesis that regression lines for each period of growth have the same intercept.

2.1.3 Results

2.1.3.1 Damage assessment

In the initial stages of infection lesions were first evident on young foliage (< 5 leaf pairs from the bud), but infection was not severe enough to cause blighting and shedding of these leaves. In the winter period from June 2002 to January 2003 there were only small amounts of defoliation. In treated plots a mean (SE) of 3.9 % (1.18) was recorded and untreated plots had only 6.8 % (0.98) defoliation. Over the first summer of the epidemic, as new leaves were formed on the outside of the crown, inner leaves were prematurely shed leaving a hollow inner crown. With the progression of the

summer period branches within the hollow crown began to senesce. Differences in the height of defoliation/branch senescence between fungicide treated and untreated plots became significant in May 2003 where the mean height of defoliation for treated plots was 0.76 m (0.05) and untreated plots was 1.26 m (0.04). In the final measurement at age 24 months, mean (SE) damage (*Mycosphaerella* index) in untreated plots was recorded as 22 % (0.94) while damage in treated plots was recorded at 13% (0.58). Therefore damage associated with MLD was reduced by 9 % in treated plots. Application of fungicide reduced mean defoliation (SE) in the peak infection period between January and August 2003 (tree age 14-21 months) by over half from 13.3 % (0.9) in untreated to 6.7 % (0.4) in treated plots.

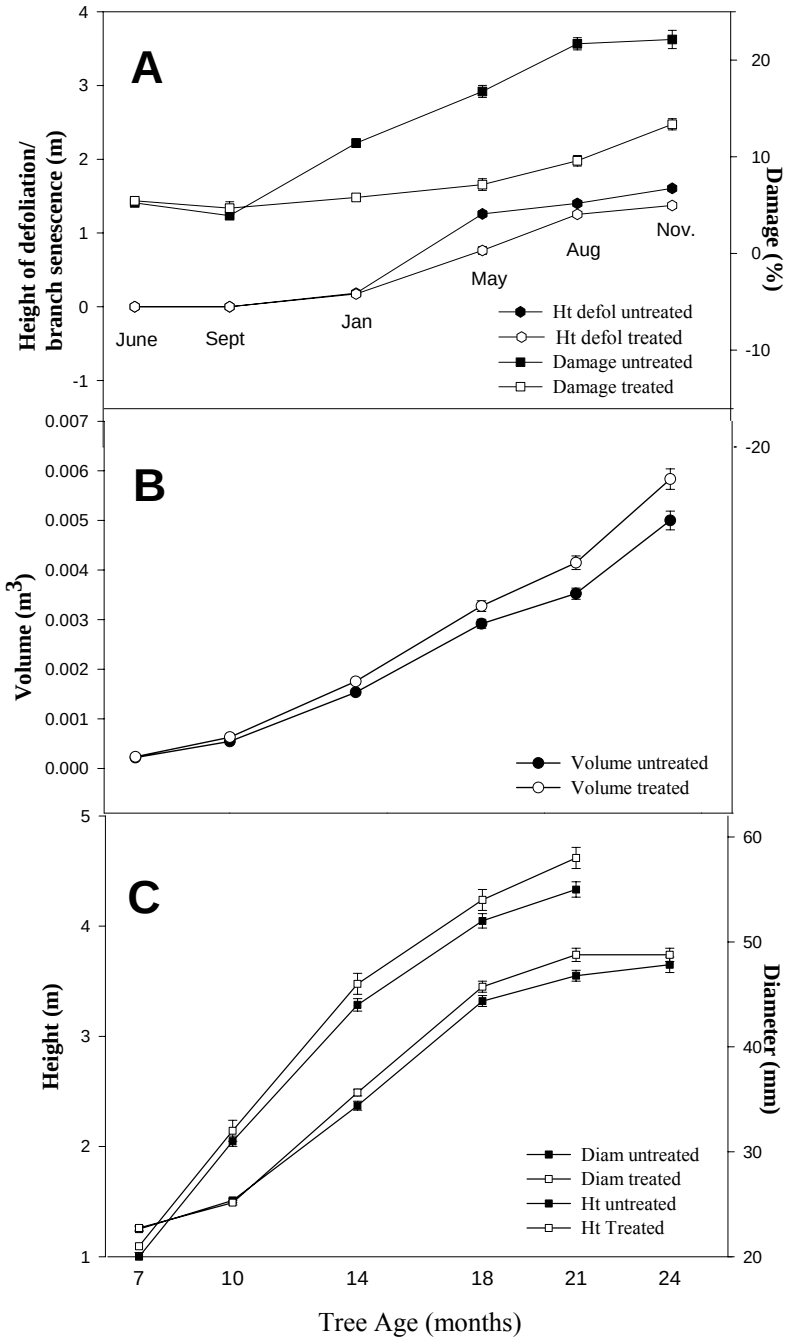


Figure 2.1.2: Measurement of treated and untreated trees over a period of two years. Measurement commenced when trees were seven months old with damage, height of defoliation/branch senescence, tree height and diameter measured every 3-4 months. Damage was measured as a *Mycosphaerella* index which is a cumulative score of necrosis and defoliation. This was related to the height of defoliation and branch senescence (Graph A). Volume (Graph B) was calculated from measured height and diameter values (Graph C) using a taper function.

2.1.3.2 Growth effects

While significant reductions in damage (P = < 0.001) were observed between treated and untreated plots early in the trial (tree age 14 months), significant reductions in height, diameter and stem volume were not observed until trees were 21 months old. Between January and August 2003 (tree age 14-21 months), an increase in mean (SE) crown damage from 17 % (1.7) to 22 % (1.2), resulted in differences between treated and untreated plots of 8 % for diameter and 7% for height (P < 0.001 for both). This resulted in a 17 % reduction in volume, which is the equivalent to 0.15 m^3 ha^{-1} at 21 months of age (Figure 2.1.2).

During this period trees had juvenile foliage. Differences in growth remained significant for the remainder of the trial (further 6 months). However when trees were 21-24 months old, ontogenetic change to adult foliage occurred. For the next six months, post phase change, further losses in stem diameter, height and volume did not occur, and the growth trajectory of both fungicide treated and untreated plots were parallel (Figure 2.1.2). Post phase change regressions between stem volume in fungicide treated and untreated plots and time did not vary in slope but were significantly different in the y-intercept (t pr = 0.063, t (1093) = 16.30).

2.1.4 Discussion

Potential losses due to *Mycosphaerella* infection of *E. globulus* were quantified at two levels, i) growth effects due to infection of juvenile foliage occurred between 20% damage and the onset of adult foliage and ii) defoliation/branch senescence was premature resulting in a height of dead branches above the recommended level for

pruning at this stage of development. The pruning of dead branches can result in stem defect if branch stubs are captured by the stem rather than ejected, which results in the formation of a kino trace behind the stub (Wardlaw 2001a). This study has highlighted the relationship between MLD and stem quality, with even low levels of damage accelerating branch death at an early stage of tree development. Management of plantations in *Mycosphaerella* prone sites may include carefully managed clear-wood regimes including the early pruning of lower branches before the senescence of branches due to *Mycosphaerella* infection can take place, or growing of plantations for pulpwood. The shoot and tip blighting commonly associated with MLD was not observed in this study. Blighting of the growing tips is likely to further enhance problems with stem form and quality by causing multiple leaders and stunting of growth.

Differences in tree volume between treated and untreated plots were significantly different at a threshold damage level of 20%. Whilst treatment with fungicide kept damage levels to below 13%, this threshold may have been reduced if the fungicidal control had been 100% effective. Significant growth losses were only observed between 20% damage and the onset of adult foliage. This suggests that the growth effects of up to 20% damage follow a type 1 growth response rather than a type 2 response (where growth rates of control trees and damaged trees are permanently divergent). The 20% threshold recorded in this study directly relates to a study of *Mycosphaerella* infection of *E. nitens* in South Africa, where a threshold damage level of 25% defoliation was recorded to significantly reduce stem growth (Lundquist and Purnell 1987). It is also directly comparable to results obtained by Pinkard (2002) where pruning of *E. nitens* to

remove 20% of foliage resulted in significantly lower stem volume than in unpruned trees.

At 20 percent defoliation we recorded a growth loss of approximately 17%, which is comparable to the growth losses (19.4%) caused by the same level of *Dothistroma pini* defoliation on *Pinus radiata* (van der Pas 1981). In contrast, a study by Milgate et al., (2005) suggests that an infection level of 20% would result in a 6.2% growth loss (based on a 3.1% growth reduction for every 10% increase in disease severity). Growth reductions in *E. globulus* have been recorded with as low as 10% *Mycosphaerella*-affected leaf area (Carnegie and Ades 2002b). The large variations in growth losses between the current study, Milgate et al., (2005) and Carnegie and Ades (2002b) highlight the importance of site factors such as nutrition and water availability on the growth of trees affected by MLD and the possibility of MLD effects being enhanced by sites with environmental stresses. Trials described by Milgate et al., (2005) were planted on ex-pasture of high nutritional value, the site used in the current study was ex-native forest of high nutritional value and the study by Carnegie and Ades (2002b) was conducted on a second rotation site with low levels of fertilizer applied only on planting. The effects of supplemented nutrition on *Mycosphaerella* severity has been recorded in a separate study by Carnegie and Ades (2002a) where trees that received a higher dose of phosphorus had significantly less infection than control trees. The effects of MLD may be reduced on nutrient rich sites as trees on high productivity sites have a greater capacity to produce leaf area after defoliation (Pinkard 2003). Further research on a wider range of sites and fertilizer treatments for *Mycosphaerella* management is necessary to determine the effects of nutrition on growth of infected trees.

Alternative management strategies for reducing the effects of *Mycosphaerella* damage include the planting of more tolerant *Eucalyptus* species in high-risk areas, weed control which can free up soil nutrients and increase airflow within the plantation, or fungicide application during the main infection periods. Aerial and/or ground based application of fungicides is not, however, a long-term solution for *Mycosphaerella* management, even though the potential control of infection with fungicides has been demonstrated in this study and previously (Carnegie and Ades 2002b). This is primarily due to i) the need to reduce chemical applications in commercial forestry to comply with forest certification, ii) the conditions required for spraying may not coincide with the infection periods and iii) chemicals need to be applied frequently for adequate control which is costly on a large scale.

The potential to control MLD on *E. globulus* may not be limited to treatment during high-risk periods. Significant variations in *Eucalyptus* species and *E. globulus* provenances have been identified for *Mycosphaerella* resistance (Carnegie, Keane *et al.* 1994; Dungey, Potts *et al.* 1997; Hood, Gardner *et al.* 2002; Milgate, Potts *et al.* 2005). Resistant *E. globulus* genotypes have demonstrated a high heritability ($H^2 = 0.6$) for traits contributing to *Mycosphaerella* resistance. However genotypes in these trials are not yet suitable for commercial plantings due to problems with stem form. The selection of genotypes with an early ontogenetic change to adult foliage may reduce growth effects in the long term as MLD is primarily a disease of juvenile foliage. This is providing MLD damage is below 80 %, after which tree growth is stunted and trees remain in their juvenile leaf stage.

2.2 Predicted impact of a single epidemic of Mycosphaerella leaf disease on the growth of *Eucalyptus globulus*

2.2.1 Introduction

There has been a rapid expansion of *Eucalyptus* plantations in Tasmania since the mid-1980's (Wood, Stephens *et al.* 2001). *Eucalyptus nitens* (Deane and Maid.) Maid. has been the dominant species planted (Wardlaw and de Little 2000). However, in 1997 Forestry Tasmania switched to planting *Eucalyptus globulus* Labill on frost-free sites because of advantages in pulp yield, density (Farrington and Hickey 1989) and strength (McKinley, Shelbourne *et al.* 2002).

By 2000, young *E. globulus* plantations in the Circular Head area, northwestern Tasmania, were beginning to suffer significant damage by the predominant leaf-infecting pathogen *Mycosphaerella nubilosa* (Cooke) Hansf. (Unpublished data, Forestry Tasmania). Infection by *M. nubilosa* produces large blighting lesions on the soft, juvenile foliage of young *E. globulus*. During epidemics, *M. nubilosa* can cause severe defoliation of *E. globulus* resulting from the premature shedding of heavily infected leaves. Mycosphaerella leaf disease (MLD) is the name given to the disease that results in this blighting and premature shedding of leaves. In 2002 there was a decision to cease planting *E. globulus* in the Circular Head area until a way of managing MLD was found.

A first step in this process was to evaluate the impact the disease was having on productivity. Carnegie, Keane *et al*. (1994) measured a significant negative correlation

in height and diameter growth with MLD severity in young *E. globulus*. However, that study only examined short-term growth impacts that were sustained during the disease epidemic. More recent research by Carnegie and Ades (2002b) did measure the growth impact of *Mycosphaerella* on *E. globulus* after the onset of adult foliage (trees ~ 4 years old). However, disease levels during that study were quite low and the plantation was older so most of the disease was due to *Mycosphaerella cryptica* (Cooke) Hansf. infection of adult foliage. Lundquist and Purnell (1987) did evaluate longer-term growth impacts for severe epidemic of a similar disease affecting *E. nitens* in South Africa but in that case the disease affected stands that were older than the *E. globulus* suffering MLD.

There was a severe epidemic of MLD in Circular Head (Murchison Forest District) area during the 2001-2 growing season. Health surveys recorded an average of 40 % leaf loss in 2000-age class *E. globulus* plantations across the district. *E. globulus* plantations of the 1999 and older age-classes escaped the MLD epidemic. This outbreak provided a unique opportunity to measure the growth impact of a severe disease outbreak.

2.2.2 Materials and Methods:

2.2.2.1 Study sites

Permanent plots were established in adjacent stands of *E. globulus* in Christmas Hills Block (latitude 40°55′09″ S, longitude 144°58′58″ E), north-western Tasmania (Figure 2.2.1). Compartment CH032E was planted in July 1999 and had reached adult foliage without suffering MLD. Compartment CH032F, which was planted in June 2000, suffered severe defoliation from the MLD epidemic.

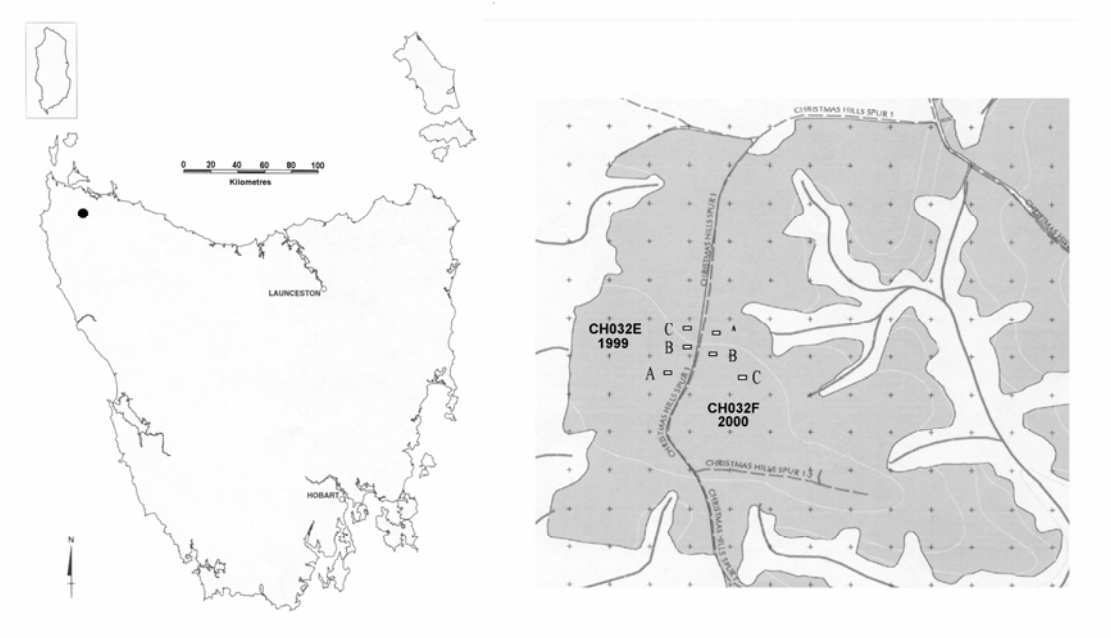


Figure 2.2.1: Location of *Mycosphaerella* leaf blight impact plots in Christmas Hills compartments 032E and 032F.

2.2.2.2 Disease measurement

Photographs of 10 trees in CH032F were taken at the beginning (August 2001) and end (February 2002) of the MLD epidemic. These were used to describe the pattern of damage.

Measurements of top-down defoliation were made in October 2001, midway through the epidemic. Eighteen plots each of 24 trees (four rows of six trees) were measured. The plots were arranged in six blocks of three plots. The blocks corresponded to bays between windrows that were aligned in an east-west direction and ran perpendicular to Christmas Hills Road Spur 1. In each block, plot 1 was closest and plot 3 the most distant to the spur road. The total height (Httot) and heights to the bottom (Ht1) and top

(Ht2) of the defoliation were measured on all trees in the 18 plots using a fibreglass height pole (Figure 2.2.2). Length of refoliation was calculated by subtracting height to the top of defoliation from total height: Httot-Ht2 (Figure 4.2). The severity of defoliation was calculated as the length of defoliation as a percentage of total tree height: ((Ht2-Ht1)/Httot)*100 (Figure 2.2.2). Analysis of variance was used to test whether there were significant differences in the severity of defoliation among blocks.

Figure 2.2.2: Height measurements of *E. globulus* that were taken to record the severity of top-down defoliation due to *Mycosphaerella* leaf blight.

After the MLD epidemic had subsided the total damage suffered was measured using the crown damage index (CDI) developed by Stone, Matsuki *et al.* (2003). CDI assessments were done on five randomly selected trees along the western section of CH032F. Components of CDI were assessed separately and then combined to provide a measure of the total percentage reduction in effective leaf area of the entire crown. Individual CDI scores were used to calculate an average CDI value for the compartment.

2.2.2.3 Growth measurement

Three plots were established within each of the two compartments. Each plot consisted of four rows of six trees (24 trees in total). Trees at the beginning and end of each row were marked using yellow flagging tape. After an initial measurement and another 6 months later, measurements of height and diameter were done annually. The first measurement was done in October 2002. Diameter measurements were taken at breast height over bark (DBHOB) using a diameter tape. Initial measurements of total tree height were done using a fibreglass height pole. As the trees became too tall to measure with a height pole (particularly in CH032E) remeasurements were done using a hypsometer (Vertex Forester®).

The data were checked for errors by plotting frequency histograms of diameter, height and their increments between remeasurements. Separate frequency histograms were plotted for each of the two age classes and each histogram was inspected for outliers that could indicate errors in data entry. Analysis of variance was used to test the significance of differences in height and DBHOB among the three plots in each of the

two compartments. Similarly, analysis of variance was used to test the significance of differences in height and diameter increments between the tow compartments.

Trajectories of height and diameter growth in CH032E and CH032F were plotted on a common stand-age scale. This enabled an estimate to be made of the duration of any transient growth effects from the MLD epidemic.

2.2.2.4 Calculation of site productivity

The area of the six plots in CH032E and CH032F were measured using a 50 metre tape. Each plot consisted of 24 trees in four rows of six trees. Because of gaps (missing trees) prior to establishing the plots some of the 6-tree rows were longer than the others. To account for this, the length of each 6-tree row was measured. The row-length measurement was taken midway between the first and last trees and their neighbouring trees outside the plots (Figure 2.2.3). Areas for each of the four rows within each plot were calculated and then summed to give the total plot area.

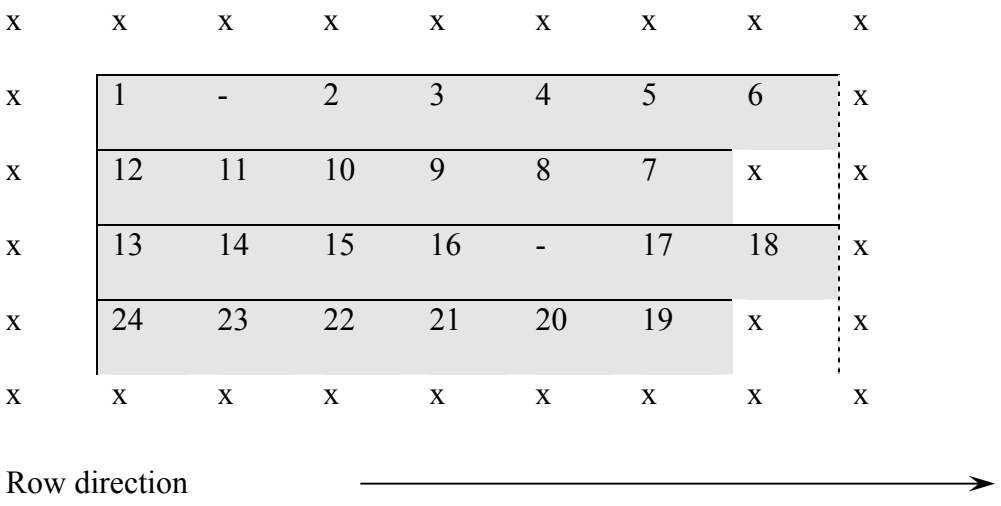


Figure 2.2.3. Layout of 24 tree plots consisting of four rows of 6 trees (shaded). The six lines indicate length and width measurements that were taken to calculate plot area. The dotted line indicates a width measurement that was done only in the 2000 age class plots.

Measurements of height and DBHOB taken at the latest remeasurement (May 2005) were entered into the inventory tool in Farm Forestry Toolbox Version 4.9© (Private Forests Tasmania 2003). Site index and total basal area of plot data created using the "Inventory" tool was then calculated. These calculated variables plus stand age were then used as inputs in the "Stand Manager" tool to calculate future harvest volumes, mean annual increment and economics (net present value and internal rate of return) of the plots in the two stands. The economics were based on an interest rate of 9 %. The log grade definitions and stand management regime used to calculate future yields and values are shown in Tables 2.2.1 and 2.2.2 respectively. The values used were "ball-park" figures approximating a solid wood regime for plantation eucalypts rather than any specific industry regime and log grade set.

Table 2.2.1 Log assortment table used by Farm Forestry Toolbox V 4.9 to calculate log yields and value.

Log category	Specifications	Value ($)
Pruned sawlog	≥40 cm diameter and ≥ 2.4 m long	80
Pruned veneer log	≥35 cm diameter and ≥ 2.6 m long	60
Pulpwood	≥10 cm diameter and ≥ 2.4 m long	20

Table 2.2.2. Stand management regime parameters used by Farm Forestry Toolbox V 4.9 to calculate log yields and value. NB. To model a growth lag of a year (due to MLD), ages for all operations were increased by 1 year.

Age	Activity	Cost ($/ha)
0	Clearing and site preparation	660
0	Weed control	75
0	Browsing mammal control	50
0	Seedlings (1100/ha)	400
0	Planting	110
0	Primary fertilisation	125
1	Browsing mammal control	150
1-11	Stand management (annually until age 11)	15
3	First pruning lift (300 sph to 2.7 metres)	400
4	Second pruning lift (300 sph to 4.5 metres)	450
6	Third pruning lift (300 sph to 6 metres)	600
11	Preparation of forest practices plan	50
11	Commercial thinning (5^{th} row outrow, 200 sph retained)	Revenue
25	Preparation of forest practices plan	50
25	Cleafall harvest	Revenue

2.2.3 Results

2.2.3.1 The 2001 MLD epidemic

The MLD epidemic commenced in CH32F during winter 2001. Soft leaves at the end of shoots suffered extensive blighting and were rapidly shed resulting in top-down defoliation. (Figure 2.2.4a). Older leaves in the lower crown were more resistant and instead of extensive blighting lesions, infection produced discrete circular lesions up to 1cm in diameter. These spots rarely covered more than 25 % of the leaf surface. By mid-spring (October 2001) almost 1/3 of the total crown length, on average, had been defoliated. There was, however, between 10 and 40 cm of new shoot growth. There was a strong east-west gradient of increasing disease severity apparent during the initial phase of the MLD epidemic in August 2001. The increase in disease severity along this gradient corresponded with proximity to CH032D. By mid-spring (October) 2001 this gradient in severity had disappeared and there was no significant difference in the amount of top-down defoliation among the three plots along that gradient in any of the six blocks that were measured.

By February 2002 all of the foliage present on the trees at the commencement of the epidemic had been shed. In addition, the spring and early-summer flush of foliage had been shed so that only the most recent foliage remained at the end of shoots (Figure 2.2.4b). The trees were effectively a hollow shell of soft, young foliage. Measurements taken in mid-winter (July) 2002 recorded an average crown damage index of 88 % (*i.e.* 88 % leaf area loss).

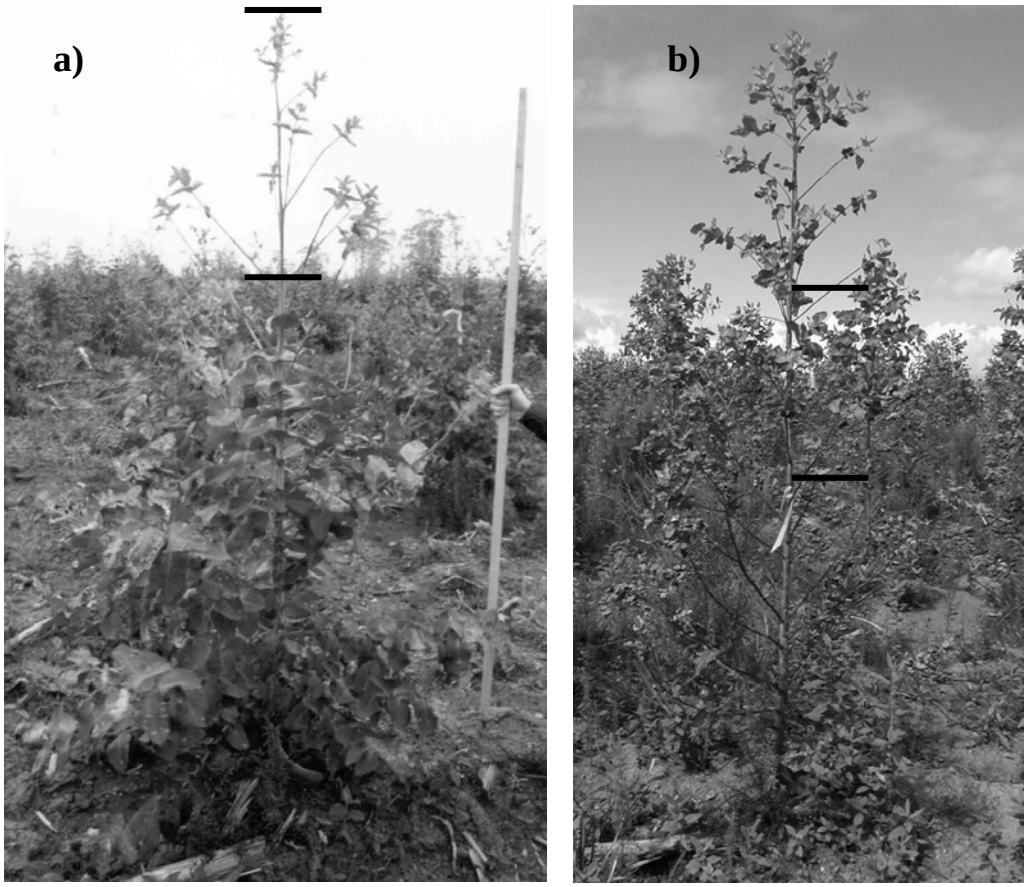


Figure 2.2.4. *Mycosphaerella* leaf blight epidemic in Christmas Hills compartment 032F. (a) Top-down defoliation in August 2001 following the shedding of heavily infected soft, young foliage at the end of shoots. (b) The same tree in February 2002 after the premature shedding of older, lower leaves resulting in a hollow crown: only new growth at the end of shoots remains. The bars on the two photographs indicate reference positions.

2.2.3.2 Tree height and diameter

Significant differences in height ($F_{2, 69}$ = 8.1; P = 0.0007, MSE = 0.899) and diameter ($F_{2, 69}$ =8.8; P = 0.0004, MSE = 2.286) among the plots in CH032E (1999 age class) were present at establishment and persisted. Trees in plot B were significantly larger (P < 0.01) than both plots A and C. These differences were increasing because the already

smaller trees in plot C had significantly lower diameter and height increments. There were no significant differences in either DBH or height among plots in the 2000 age class.

Trees in the 1999 age class were still significantly taller ($F_{1, 4}$= 45.1; P = 0.0026, MSE = 0.0518) and greater ($F_{1, 4}$ = 16.7; P = 0.015, MSE = 1.704) than trees in the 2000 age class at the last measurement in May 2005. However, there were no significant differences between the 1999 and 2000 age classes in diameter or height increment during increment periods between May 2003 and May 2005 (Figure 2.2.5).

Of greater interest was the comparison of tree sizes at comparable ages between the two age classes. This gives an indication of the growth impact of the 2001 MLD epidemic experienced by the 2000 age class. Figure 6 show plots of age versus average tree diameter and height of the 1999 and 2000 age classes. At age 4 years the 2000 age-class were 30 % smaller in diameter and 27 % shorter than the 1999 age class. The 2001 MLD epidemic was estimated to have retarded the growth of the 2000 age class by approximately 1.3 years and 1.2 years for diameter and height growth respectively.

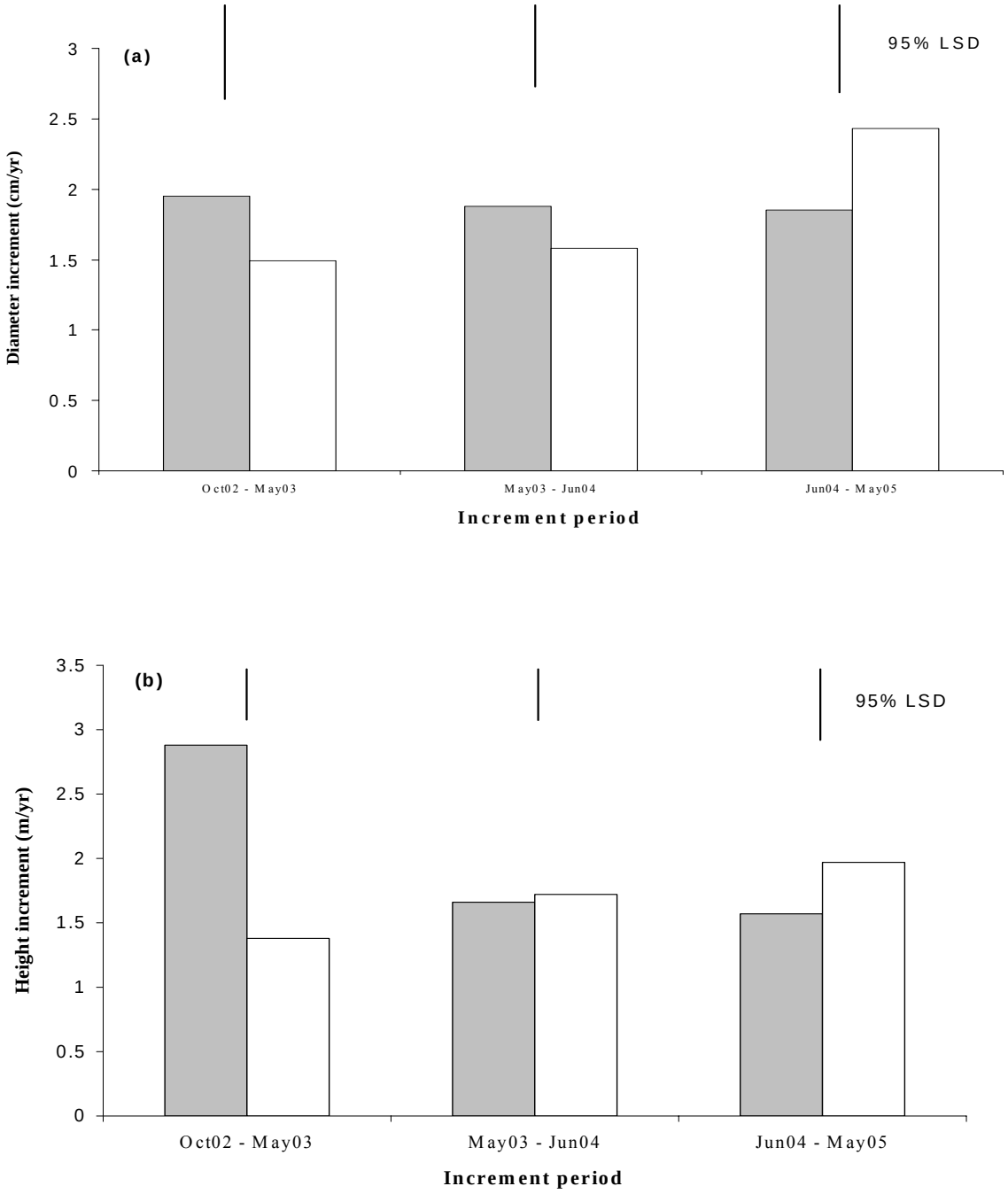


Figure 2.2.5. Average increments in (a) diameter and (b) height of trees from compartments CH032E (1999 age class) and the MLD-affected CH032F (2000 age class).

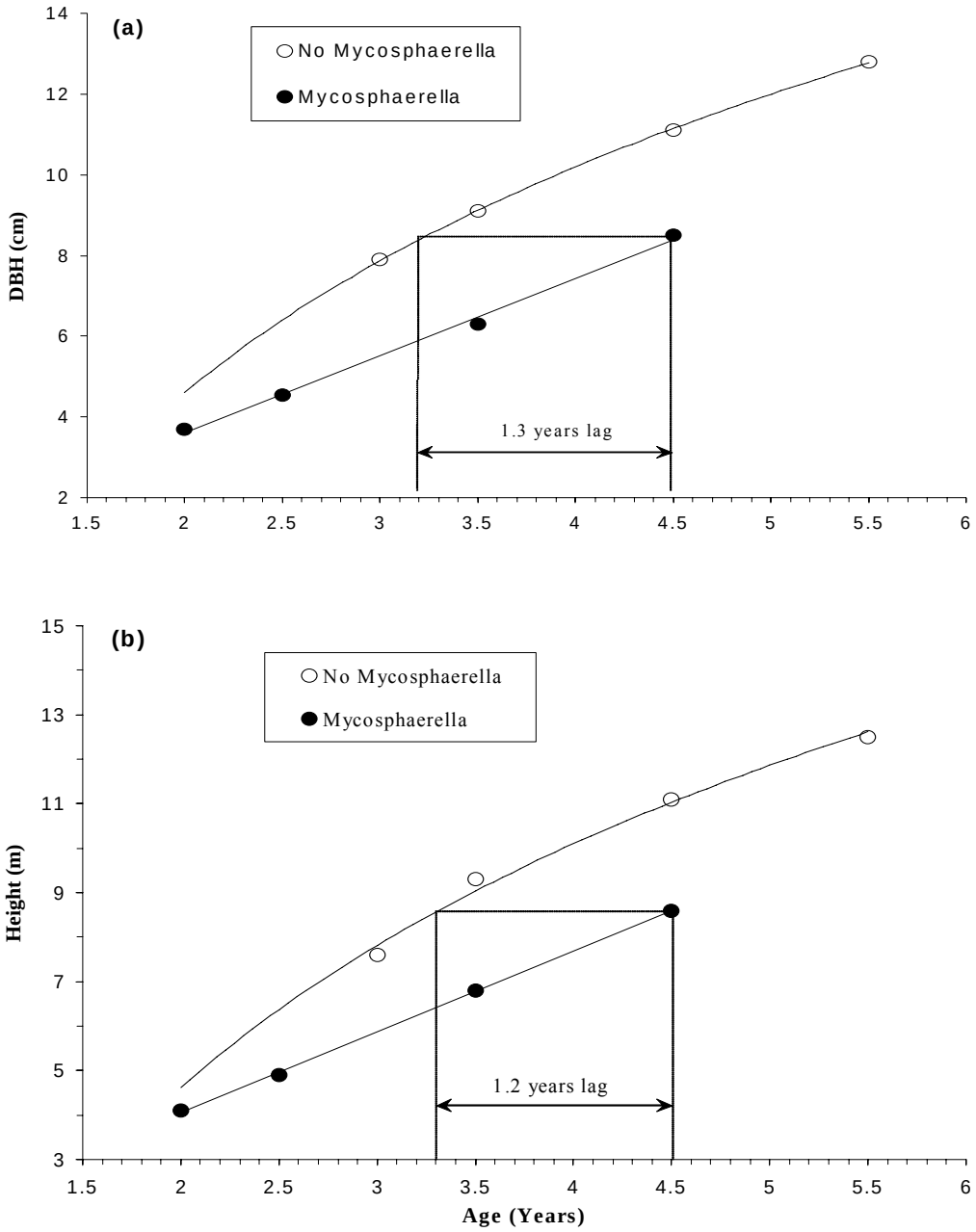


Figure 2.2.6. Plot of age versus average (a) diameter and (b) height of trees from CH032E (1999 age class) and the MLD-affected CH032F (2000 age class). Dotted lines show estimated retardation of height growth in the 2000 age class as a result of the 2001 MLB epidemic.

Table 2.2.3. Summary of stand statistics predicted using Farm Forestry Toolbox from May 2005 remeasurement of plots in 1999 and 2000-age classes of *E. globulus*

	1999 age class				**2000 age class (no age adjustment)**				**2000 age class (1 year lag in growth)**			
Plot	**1**	**2**	**3**	**All**	**1**	**2**	**3**	**All**	**1**	**2**	**3**	**All**
MDH (m)	14.4	15.1	14.6	14.7	10.8	10.3	8.9	10.8	10.8	10.3	8.9	10.8
Volume (m^3/ha)	53.4	78.7	57.2	64	25	22	19	22	25	22	19	22
Basal area (m^2/ha)	11.5	15.8	12.2	13.3	6.9	6.3	5.3	6.1	6.9	6.3	5.3	6.1
Site index (m)	28.6	29.6	28.8	29.0	25.5	24.6	26.5	25.5	29.7	28.7	30.7	29.7
MAI at harvest(m^3/ha/yr)	22	26	23	24	16	15	16	16	26	24	27	26
Harvest volume:												
Pruned sawlog (m^3)	47	108	73	72	5	2	7	5	112	72	133	100
Veneer (m^3)	83	56	74	76	39	22	40	33	50	77	33	59
Pulpwood (m^3)	291	330	305	304	266	254	274	264	346	310	371	340
Waste (m^3)	13	16	14	14	10	9	10	10	16	14	16	15
NPV ($ @ 9% interest)	-373	295	-27	-80	-1 267	-1 482	-1 283	-1 353	158	- 169	214	48
IRR (%)	8.2	9.6	8.9	8.8	5.3	4.3	5.3	5	9.3	8.6	9.4	9.1

2.2.3.3 Site productivity, yield and economics

At comparable ages (*i.e.* no age adjustment) the MLD epidemic in the 2000 (MLD affected) stand caused a predicted 12 % reduction in site index compared with the 1999 (MLD unaffected) stand (Table 2.2.3). A decline in site index of this magnitude would cause a 33 % reduction in mean annual increment and a 40 % reduction in the internal rate of return from the crop leading to a drop in the net present value of more than $1 000 / ha. However the growth impacts, at harvest, disappear if the growth impact of the MLD epidemic is modelled as a lag of 1-1.5 years in the stand growth (Table 2.2.3). This lag effect was modelled simply by delaying all stand management activities post-planting by a year and extending the rotation also by a year.

2.2.4 Discussion

The phenology and patterns of damage of the 2001-2 MLD epidemic at Christmas Hills parallels that described by Carnegie and Keane (1994) for an epidemic in a 2-year-old *E. globulus* plantation in East Gippsland. The Christmas Hills epidemic resulted in almost complete crown loss by late-summer (February 2002) in CH032F. Growth measurements did not commence until 12 months after the MLD epidemic by which stage growth increment had almost fully recovered as indicated by the parallel growth trajectories in Figures 4.6a and 4.6b. Assuming no significant difference in size between the two stands before the MLD epidemic (at age 1 in winter 2000 and 2001 for CH032E and CH032F respectively), the majority of the growth impact must have occurred between winter 2001 and mid-spring 2002. Lundquist and Purnell (1987) reported a virtual cessation of diameter and height increment in *E. nitens* that suffered >75 % defoliation: a level of defoliation comparable with that measured in CH032F. This accords well with the 1.2 and 1.3 years retardation of height and diameter growth respectively that is thought to have occurred in CH032F between winter 2001 and mid-spring 2002.

An important finding from this study was the speed of recovery of growth rates in CH032F after the MLD epidemic. This recovery from almost complete defoliation was considerably quicker that that measured by Candy (1999) and Rapley (2005) for similar intensities of defoliation in 2-year-old *E. nitens* and *E. globulus* plantation respectively. Candy (1999) measured growth reductions that were sustained for the 3-years of measurement after artificially defoliating 2-year-old *E. nitens* to simulate late-season feeding by chrysomelid beetle (adults). Similarly, Rapley (2005) recorded a sustained

(>24 months) reduction in growth rates in trees suffering severe (>70 % loss of leaf area) late summer defoliation by autumn gum moth, *Mnesampela privata* (Lepidoptera: Geometridae). In that study, trees suffering lesser level of damage (5-50 % loss of leaf are) recovered growth rates within 12 months. Candy (1999) showed that recovery of height and diameter growth was much more rapid when the defoliation occurred early in the growing season (spring-early summer) than if the defoliation occurred in late summer or autumn. The 2001-2 MLD epidemic reported here, while protracted, was most active during late-winter and spring 2001, when top – down defoliation due to blighting of young leaf was occurring. This period of defoliation matches the timing for the early season top – down defoliation treatment applied by Candy (1999).

Predictions of the rotation-length impact of the MLD epidemic depend strongly on how the short-term impacts on growth during and immediately following the epidemic are interpreted. Snowden (2002) introduces type 1 and type 2 growth responses to describe transient versus sustained growth responses. The growth trajectory following the MLD epidemic is consistent with the type 1 response of Snowden (2002). Therefore, it is more appropriate to predict future growth of MLD-affected stands by modelling a transient growth effect. This is equivalent to lagging the stand development for the time period it takes to recover growth rates to the same level as the unaffected stand. The consequences for assuming transient versus sustained growth reductions after an MLD epidemic have been shown to be significant, particularly if future projections of yield are being modelled from stand measurements taken relatively early in the rotation.

2.3 Summary of MLD growth impact

Over the course of a two year epidemic, damage mounting to ~20 % resulted in an 11 % loss of growth and damage of up to 80 % resulted in a 73 % growth loss. In the longer-term trial, these growth losses were maintained throughout the future growth of the tree (equivalent of a 1 year growth lag). After phase change to adult foliage growth rates of affected trees returned to a similar level as unaffected trees; suggesting that trees can withstand high levels of damage without long term impact on their growth rates. However the impact of damage is likely to be linked with site nutrition, with trees on poorer quality sites unable to recover as efficiently after damage. Fungicidal control in the exclusion trial was costly, laborious, and operationally difficult and not 100 % effective, therefore breeding for resistance was determined as the most suitable method for managing MLD in high-risk sites.

CHAPTER 3

3.1 Detection of species in *Mycosphaerella* complexes associated with crown damage of eucalypt plantations

3.1.1 Introduction

In 2002 Australia's hardwood plantation estate exceeded 715,531 ha and is predicted to be greater than 3.3 million ha by 2020 (National Forest Inventory 2005). Such rapid expansion has, and will, continue to generate large areas of young, single species plantations. It also creates a favourable environment for the transfer and spread of naturally occurring pathogens from forests to commercial plantations. *Mycosphaerella* species are some of the most significant pathogens causing defoliation in temperate eucalypt plantations in the Southern Hemisphere (Dick 1982; Milgate 2001; Park 1982; Park, Keane *et al.* 2000). *Mycosphaerella* species have the ability to infect and spread rapidly resulting in considerable loss of photosynthetic area by necrotic spotting and defoliation in plantations in medium to high rainfall zones (Dungey, Potts *et al.* 1997; Lundquist and Purnell 1987; Mohammed, Wardlaw *et al.* 2003).

The two most widespread and potentially hazardous pathogens associated with necrosis, shoot/leaf blighting, and defoliation in Australian temperate *Eucalyptus* plantations are considered to be *Mycosphaerella nubilosa* (Cooke) Hansf. and *Mycosphaerella cryptica* (Cooke) Hansf. These allegedly more dominant species have often been identified as the primary cause of outbreaks of Mycosphaerella leaf disease (MLD) in South Africa, Australia and New Zealand (Carnegie, Ades *et al.* 2001; Dungey, Potts *et al.* 1997; Hood, Gardner *et al.* 2002). In Australia, to overcome the losses in growth and the re-establishment of plantations, the substitution of *Eucalyptus nitens* (Deane and Maid.)

Maid. for *Eucalyptus globulus* Labill. plantings in *Mycosphaerella* prone areas has occurred due to *M. nubilosa* not being reported as a common pathogen of *E. nitens*. In South Africa however, the impact of *M. nubilosa* is very different. It has been a primary pathogen of *E. nitens* plantations for many years (Lundquist and Purnell 1987) and has recently been identified as the main cause of MLD in commercial plantations in the KwaZulu-Natal Province (Hunter 2002).

The taxonomy of the *Mycosphaerella* genus is complicated. On eucalypts there have been over 30 named species (Mohammed, Wardlaw *et al.* 2003). Classification of ascospore shape, size and germination patterns is considered to be the most rapid means of identification, however within species variation can be extensive and lead to false identification. Identification is also limited by poor ascospore release from pseudothecia. *Mycosphaerella* species causing MLD on eucalypts are slow growing and the co-existence of non-*Mycosphaerella* contaminants makes obtaining pure cultures from leaf material to assess cultural and anamorphic characteristics challenging and time consuming (Crous, Aptroot *et al.* 2000). Considering the limitations of isolating *Mycosphaerella* from *Eucalyptus* leaf, shoot and stem material it is possible that many species are overlooked during surveys. Macroscopic identifications made during large-scale routine surveys in plantations and forests cannot be readily validated by laboratory methods. Success in isolating *Mycosphaerella* from routine field surveys is highly variable and not always accurate.

Molecular-based technology using nested polymerase chain reaction (PCR) has recently enabled the development of a rapid and sensitive method for identification of several *Mycosphaerella* species occurring on Myrtaceae (Glen, Smith *et al.* 2006). Nested PCR

has been used with considerable success for the detection of multiple pathotypes in agricultural and plantation crop species including *Verticillium dahliae* (Verticillium wilt) (Mercado-Blanco, Rodriguez-Jurado *et al.* 2003), *Neonectria galligena* (Apple canker) (Langrell, Tommerup *et al.* 2003a) *Quambalaria eucalypti* (Eucalypt shoot blight) (Zauza, Alfenas *et al.* 2003), *Puccinia psidii* (Eucalypt rust) (Langrell, Tommerup *et al.* 2003a; Langrell, Tommerup *et al.* 2003c; Tommerup, Alfenas *et al.* 2004). *Mycosphaerella pini* (red-band needle cast) and *Fusarium circinatum* (Pine pitch canker) (Langrell, Tommerup *et al.* 2003b). Nested PCRs using taxa specific primers were developed for detection of the eucalypt pathogens *Mycosphaerella nubilosa, Mycosphaerella cryptica, Mycosphaerella vespa* Carnegie and Keane, *Mycosphaerella tasmaniensis* Crous & MJ Wingf. and *Mycosphaerella grandis* Park and Keane (Glen, Smith *et al.* 2006). ITS sequences for *Mycosphaerella. grandis* Carnegie and Keane will also amplify *M. parva* ITS sequences. However, recently these two species are considered to be synonymous (A. Carnegie pers. com.). Likewise, the *M. vespa* primers will also amplify ITS sequences from *Mycosphaerella molleriana* (Thüm) Lindau and *M. ambiphylla*. However, *M molleriana* and *M. ambiphylla* geographic range has not been recorded to include Tasmania. With this exception, the nested PCR method can detect and discriminate amongst these species in cultures, and leaf and stem tissue from eucalypts.

The main objectives of the present study were to (i) verify the concept that the nested PCR test for *Mycosphaerella* species on *Eucalyptus* is a practical and efficient tool for the routine discrimination of species in leaves from disease surveys or crown damage assessments and (ii) determine the effectiveness of using the nested PCR to identify

which species can co-exist and their frequency of occurrence in small segments of leaf or stem lesions from plantations or re-growth natural vegetation.

3.1.2 Materials and Methods

3.1.2.1 Sampling of leaf material from plantations and forest re-growth sites.

A survey of two-year-old *E. globulus* was conducted at Smithton in north-western Tasmania on 15/8/03 (S40º 55' 21": E144º 59' 39"). The plantation of approximately 21 ha was divided into 6 sections and 10 leaf samples taken from each section. Juvenile leaves were sampled from branches at breast height and selected at random, from trees up to 4 metres high. Leaves were taken from the 4th-5th leaf pair on each branch to minimise variation in leaf age, physiological development, height in the canopy and age of the lesions. Lesions from lateral branch stems were also sampled from seven randomly selected *E. globulus* trees. *E. nitens* samples were collected at three different sites on the north and north-east of Tasmania. A total of 16 samples were taken from plantations at Weldborough (S41º 13' 14": E 147º 51' 60"), St Helens (S41º 20' 41": E148º 3' 55") and Scottsdale (S41º 15' 34": E147º 25' 25"). Leaf material was sampled from branches at breast height, selected at random and sampled from the 4th to 5th pair on each branch. Trees were selected at random and were approximately 5-6 metres high.

A collection of 4 samples was made from natural forest regeneration of *E. regnans* F. Muell. at a roadside site near Bicheno (S41º 48' 19": E148º 14" 53") in the North East of Tasmania. Leaves were taken from the 4th pair on randomly selected branches. Trees were approximately 1.5 metres high and approximately 12 months old.

3.1.2.2 Sampling of lesions, DNA extraction and detection of **Mycosphaerella** *species.*

Each leaf was treated individually to avoid cross-contamination through the entire preparative process. Most lesions were > 5mm in diameter and contained necrotic tissue with or without a purple margin or reproductive structures. Some lesions were of earlier development (1 to 4 mm diameter, distinct, dispersed-purplish spot visible on either the abaxial or adaxial surface of the leaf). Dried leaves were removed from the paper bag and small sections of the lesions were cut using a new, sterile scalpel blade for each leaf. As leaves had different numbers of lesions, at different developmental stages, small sections were cut from every lesion up to a maximum of 15 lesions per leaf with approximately 1 cm^2 of lesion area analysed. If there were more than 15 lesions in a leaf, 15 were randomly selected. Each stem sample from *E. globulus* was obtained by scraping the surface of a stem lesion with a scalpel to produce shavings and approximately 80 mm^2 of stem surface area was taken. DNA was extracted and purified as described by Glen, Smith *et al*. (2006). *M. cryptica, M. nubilosa*, *M. vespa*, *M. tasmaniensis* and *M. grandis* were the chosen species for investigation. *Mycosphaerella* species in leaf samples were detected and discriminated using the accurate, sensitive and rapid taxa-specific, nested PCR method (Glen, Smith *et al.* 2006). The test incorporates a first-round amplification which is a positive control for DNA template quality. This ensures that false negatives can be detected. Negative controls were included in every round of PCR to check for false positives. Every set of PCR reactions included five samples containing DNA from cultures of each of the five species or taxa groups and one sample with no template DNA. The four non-target species in each of the species-specific tests were treated as additional negative controls. The standard isolates *M. cryptica, M. nubilosa, M. vespa, M. tasmaniensis* and *M. grandis* had been morphologically discriminated and have ITS sequences (GenBank

codes AY667576, AY667577, AY045500, AY667578, AY045514), which are consistent with their species designations. The test was repeated on about 30 % of samples to demonstrate the consistency of the analysis.

3.1.2.3 Statistical Analysis.

Linear regression tested the relationship between the number of lesions analysed and the number of species detected. Analyses of variance (ANOVA) were used to determine the differences in the detection of *Mycosphaerella* species in treated and untreated plots in the fungicide trial. The same analyses were used to determine the differences between tree height, tree diameter, or tree volume, and *Mycosphaerella* severity or defoliation in treated and untreated plots. All analyses were conducted using the statistical software package GenStat®.

Results from this study are also summarised in poster format in Appendix 4.

3.1.3 Results

Most leaves contained more than one lesion and therefore most 1 cm^2 samples consisted of sub-samples from more than one lesion. No relationship was found ($P>0.1$) between the number of lesions sampled per leaf and the number of species detected. Leaves from both *E. nitens* and *E. globulus* with only one lesion contained two to three *Mycosphaerella* species.

The *Mycosphaerella* species most frequently detected on *E. globulus* were *M. cryptica*, *M. nubilosa* and *M. grandis* and on *E. nitens*, *M. cryptica, M. tasmaniensis* and *M. grandis* (Table 3.1.1). All single leaf and stem lesions were analysed at an early

developmental stage. Single *E. globulus* leaf lesions contained *M. cryptica*, *M. grandis*, and either *M. vespa*, *M. tasmaniensis* or *M. nubilosa*. All stem lesions contained *M. grandis* with two samples testing positive for only that taxa. Five stem lesions recorded *M. tasmaniensis*. One sample contained *M. cryptica*. The number of species to simultaneously occur within a stem lesion is presented in Table 3.1.1.

Table 3.1.1: Frequency of detection for *Mycosphaerella* species

	Tree Species			
	Leaves			**Stems**
***Mycosphaerella* species**	***E. globulus* (n=60)**	***E. nitens* (n=13)**	***E. regnans*[a] (n=4)**	***E. globulus* (n=7)**
M. cryptica	92%	85%	Y	14%
M. nubilosa	83%	8%	Y	0
M. tasmaniensis	35%	77%	N	71%
M. vespa	62%	23%	Y	0
M. grandis	100%	100%	Y	100%

[a]-Due to the small sample size of the native re-growth site only a presence or absence of *Mycosphaerella* species have been presented

Of the *Mycosphaerella* species tested *M. grandis* occurred on every leaf from every sampling site irrespective of tree species. However, it always occurred with either *M. cryptica* or *M. nubilosa* on leaves and often with *M. tasmaniensis* and/or *M. vespa*. One *E. nitens* sample was positive for the presence of *M. nubilosa*. *M. tasmaniensis* was detected in 35 % of *E. globulus* and 77 % of *E. nitens* material but was the only *Mycosphaerella* species absent from re-growth *E. regnans*. *M. vespa* occurred in 62 % of *E. globulus* 23 % of *E. nitens* and in native re-growth *E. regnans*.

The absence of a species was confirmed as a true negative by a product from first-round amplification with general fungal ITS primers and/or one or more of the other four taxa-specific primer sets. Four samples that were initially negative for all species and the first-round amplification, gave positive results upon additional dilutions (*i.e.* from 10 to 100 fold). Why these samples were recalcitrant was not evident from the characteristics of the lesions. Every positive was confirmed by lack of amplification in all of the negative controls. Samples from the various treatments and controls were processed randomly, and there was no evidence of cross contamination.

All *E. nitens, E. globulus* and *E. regnans* leaves contained multiple *Mycosphaerella* species with generally greater than three species occurring per leaf (Table 3.1.2). The analysis for 30% of samples was independently repeated and produced consistent results.

Table 3.1.2: Co-existence of *Mycosphaerella* species in *E. nitens* and *E. globulus* leaves.

	Percentage of samples containing the corresponding number of *Mycosphaerella* species				
Leaf samples	1	2	3	4	5
***E. nitens* (n=13)**	0	22	67	11	0
***E. globulus* (n=60)**	0	10	37	33	20
***E. regnans** (n=3)**	N	Y	Y	N	N
Stem Samples					
***E. globulus* (n=7)**	29	57	14	0	0

*Due to the small sample size of the native re-growth site only a presence or absence of *Mycosphaerella* species has been presented.

3.1.4 Discussion

The nested PCR detection test for *Mycosphaerella* species from *Eucalyptus* produced rapid and reliable results for every leaf and stem sampled for the study. Five of the most predominant and/or pathogenic *Mycosphaerella* species were found to simultaneously co-exist in *E. globulus* leaves. In *E. nitens*, four out of the five *Mycosphaerella* species tested occurred simultaneously on leaves. It was also found that four species can co-occur in one leaf lesion alone and three in a stem lesion. All leaf and most stem samples had multiple *Mycosphaerella* species with the average of three species detected per cm^2.

Identification of *Mycosphaerella* lesions using classical identification techniques has been limited in the past by a lack of mature reproductive structures. Spore release from these structures is also highly variable with clusters of spores only released by one or two species per lesion (A. Carnegie, Pers. Comm., State Forests NSW, Park 1988a). Although these techniques have identified multiple *Mycosphaerella* species in lesions on Myrtaceous and Proteaceous hosts (Crous 2000), most MLD outbreaks in the past have usually been attributed to only one or two species (Carnegie and Keane 1994; Crous, Aptroot *et al.* 2000; Crous, Wingfield *et al.* 1998; Hood, Gardner *et al.* 2002; Maxwell, Hardy *et al.* 2001; Park 1982; Park and Keane 1982). There is substantial evidence from this study to suggest that the co-occurrence of multiple, pathogenic species in *Mycosphaerella* leaf lesions is typical for *Eucalyptus* species in Tasmania.

The co-occurrence of several *Mycosphaerella* species in a small leaf area raises the issue of species-species interaction and their roles as primary or secondary pathogens or saprophytes. In other systems, the co-occurrence of species is common. These highly

characterized disease systems include Fusarium head blight in cereals (Nicholson, Chandler *et al.* 2003; Nicholson, Simpson *et al.* 1998), *Melampsora* rust on willow and poplar (Royle and Ostry 1995) sooty blotch in apple trees (Johnson, Sutton *et al.* 1996) and the Dutch elm disease complex of *Ophiostoma ulmi*, *Ophiostoma novo-ulmi* and *Ophiostoma himal-ulmi* (Harrington, McNew *et al.* 2001). There is limited knowledge of the pathogenicity of most *Mycosphaerella* species on eucalypts including *M. tasmaniensis* and *M. vespa* which have been isolated frequently in past surveys in Tasmania (Milgate 2001; Yuan 1999). The detection of only sequences consistent with *M. grandis* in two stem lesions suggests one of two possibilities. 1) Another pathogen, not detected by the primers, was present in the stem lesion and *M. parva* was saprophytic or 2) *M. grandis* was a primary pathogen. *Mycosphaerella grandis* (now synonymous with *M. parva*) has been described as a leaf pathogen by Carnegie and Keane (1994), however there have not been any controlled inoculation experiments to verify this observation. Therefore some uncertainties remain about the pathogenicity of *M. grandis/M. parva*.

Our data also raises concerns about the usefulness of lesion appearance as a diagnostic tool for identifying the pathogens. *Phaeseolus vulgaris* co inoculated with both common bacterial blight (*Xanthomonas campestris* pv. Phaseoli) and rust (*Uromyces appendiculatus* var. appendiculatus) displays different necrosis symptoms than with single inoculations (Yuan 1999). Therefore the identification of *Mycosphaerella* species using lesion appearance, shape and position of pseudothecia may not be accurate due to the presence of several species in lesions.

The species detected in this study support the findings of a seasonally and geographically more comprehensive survey conducted by Milgate, Yuan *et al*. (2001) which demonstrated that *M. tasmaniensis* was widespread on *E. globulus*. However, the detection frequency for all species was much higher in this study presumably due to the sensitivity and specificity of the molecular detection methods and the ability of the test to detect species in tissue without reproductive structures. Using standard techniques based on lesion attributes, ascospore germination and cultural characteristics, rapid determination of the *Mycosphaerella* species associated with the large number of lesions sampled in this study would be impractical. Considering all the stems and most of the leaves analysed in the present study had lesions without reproductive structures, the use of classical techniques to rapidly identify species would also be impossible.

One of the more concerning results for the Australian *Eucalyptus* plantation industry from this study was the detection of *M. nubilosa* in an *E. nitens* sample. This raises questions about the potential for evolution or selection of aggressive strains from within the natural population of the pathogen. Recent results from a survey in South Africa have shown that *M. nubilosa* is the primary causative agent of MLD of *E. nitens* plantations (Hunter 2002). Species such as *M. cryptica* and *M. tasmaniensis* are frequently identified as the major species of MLD on *E. nitens* in Australia (Milgate 2001; Yuan 1999). Whilst these species were detected with a high frequency in *E. nitens* plantations at all sites, the presence of *M. nubilosa* on *E. nitens* has only been documented in Australia once previously (Carnegie and Ades, 2002c) and requires monitoring especially due to the increasing establishment of plantations of *E. nitens* in *Mycosphaerella* prone areas (Tibbits and Reid 1987a; Tibbits and Reid 1987b; Volker, Owen *et al.* 1994). In addition, the recent new record of *M. nubilosa* on *E. globulus* in

Western Australia and its isolation from *E. globulus* adult foliage (Mercado-Blanco, Rodriguez-Jurado *et al.* 2003), increases concerns about the spread of this highly destructive and "well characterized" species.

The discovery of up to and potentially more than 5 *Mycosphaerella* species in leaves and stems of *E. globulus* and *E. nitens* has challenged the concept of a primary pathogen in Mycosphaerella leaf disease. It is evident that in Tasmania where *E. globulus* is indigenous in native forests and *E. nitens* has been grown in plantations for over 30 years that species compositions are far more complex than original surveys using classical techniques have suggested. This study has illustrated that nested PCR is a useful technique to validate *Mycosphaerella* species in disease surveys and research trials. The detection technique enables rapid processing of large sample numbers, comprehensive- and reliable data. The nested PCR is a reliable and cost effective method useful for studies on the developmental ecology, epidemiology and biosecurity of MLD. It may also provide valuable information for the advance of management strategies for MLD on eucalypt plantations.

3.2 Early detection of *Mycosphaerella* species causing leaf spot on *Eucalyptus globulus* and *Eucalyptus nitens* plantations.

3.2.1 Introduction

The first indication of early *Mycosphaerella* lesions is the accumulation of anthocyanin in the epidermal cells above and around infected areas. This appears as a purple area usually within 10 days of infection. When these early lesions are transversely sectioned it can be seen that hyphal development is already advanced (Smith, Gill *et al.* 2005). Park and Keane (1982) also discovered a time differential of up to 8 weeks, depending on climatic variables (e.g. humidity, temperature and leaf wetness), between inoculation and the observation of necrotic lesions. Problems with obtaining large numbers of single species ascospores from lesion tissue or cultures have hampered inoculation trials. For this reason epidemiological trials have only been conducted for a limited number of *Mycosphaerella* species, namely *Mycosphaerella cryptica* (Cooke) Hansf. and *Mycosphaerella nubilosa* (Cooke) Hansf., *Mycosphaerella parva* Park and Keane and *Mycosphaerella lateralis* Crous and M.J. Wingf. Therefore the trophic roles (primary pathogen, secondary pathogen, saprophyte or endophyte) of most of the MLD species are ambiguous or unknown (Maxwell 2004). Until recently, identification of most species was only possible by comparing attributes of cultures, lesions and ascospores, all of which require actively fruiting lesions.

Molecular analyses have significantly improved the efficiency of identification for *Mycosphaerella* species in plantation eucalypts (Carnegie, Ades *et al.* 2001; Kularatne, Lawrie *et al.* 2004). Assays based on nested PCR, have been developed for several *Eucalyptus* diseases including species of *Mycosphaerella* (Glen, Smith *et al.* 2006),

Quambalaria eucalypti (Zauza, Alfenas *et al.* 2003) and *Puccinia psidii* (Langrell, Tommerup *et al.* 2003a; Langrell, Tommerup *et al.* 2003c; Tommerup, Alfenas *et al.* 2004). The *Mycosphaerella* diagnostic assay has been identified as an accurate, rapid and robust method for identifying small amounts of hyphae directly from eucalypt tissue and is able to detect multiple *Mycosphaerella* species in a single leaf or stem sample with or without reproductive pseudothecia (Glen, Smith *et al.* 2006). To advance current thinking about the ecological and epidemiological principles of the *Mycosphaerella* infection in cool temperate Australia, we applied the *Mycosphaerella* diagnostic assay to generate information about the sensitivity of the technique to detect *Mycosphaerella* species in early stage lesions and determine *Mycosphaerella* species compositions at varying stages of lesion development.

3.2.2 Materials and methods

3.2.2.1 Sampling Location

Naturally infected, commercial plantations, one each of young *Eucalyptus nitens* (Deane and Maid.) Maid. and *Eucalyptus globulus* Labill were sampled near Smithton (S40º 55'21" E144º 59'39") in Tasmania, Australia. A five-row buffer zone was allocated on both sides of the road separating the *E. globulus* and the *E. nitens* plantations. Samples were taken from within an area of 50 m^2 with approximately 50 m separating the sampling sites for both plantations. At the time of sampling the *E. globulus* plantation was approximately two years old (planted in November 2001) with a mean *Mycosphaerella* score of 35 % (± 16) and the *E. nitens* plantation was one year old (planted in November 2002) with a mean *Mycosphaerella* score of 2 % (± 4). The *Mycosphaerella* score was calculated by multiplying the incidence of infection

(*i.e.* percentage of leaves infected) by the severity (%) of the infection on those leaves. Juvenile foliage predominated and was sampled in both plantations.

3.2.2.2 Lesion development

Mycosphaerella lesions were allocated to 5 lesion categories based on their developmental status (Figure 3.2.1). Categories were adapted from those developed by Cheah and Hartill (1987) for *Eucalyptus delegatensis*. For the purpose of this study, asymptomatic tissue, three categories of early-stage lesions and two later developmental stages were sampled. *Eucalyptus globulus* and *E. nitens* lesions were categorised using the following criteria: 0 = No visual symptoms of infection. 1 = Vague area of discoloration/reddening (1-3 mm diameter), just discernable from surrounding leaf material at 10 × magnification. 2 = Indistinct, purple-tinged spot (1-3 mm diameter) visible on abaxial or adaxial leaf surface under 10 × magnification. 3 = Distinct, intense purple spot (1-3 mm diameter) visible on abaxial or adaxial leaf surface. 4 = Distinct necrotic spot (5-10 mm diameter) with a purple margin of >1 mm. 5 = Restricted necrotic lesion (usually >5mm diameter). Pseudothecia may or may not be present.

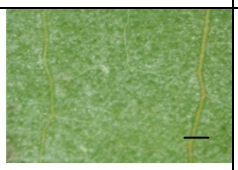
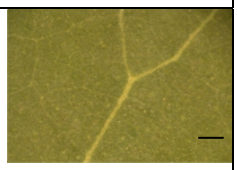
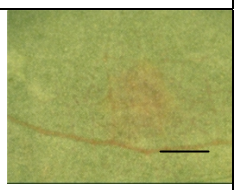
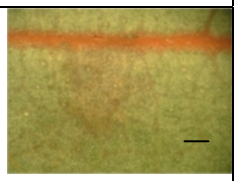
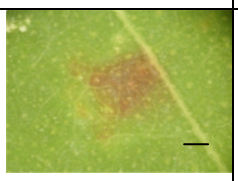
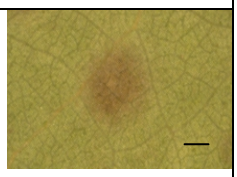
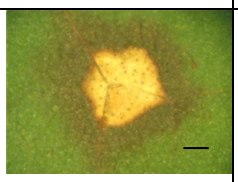
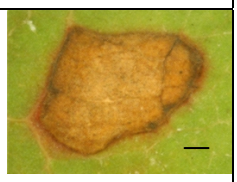
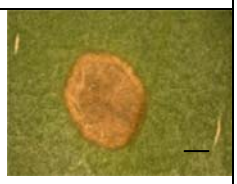
Category Bar = 1mm	***E. globulus***	***E. nitens***
Asymptomatic		
1		
2		
3		
4		
5		

Figure 3.2.1: Visual symptoms of *Mycosphaerella* lesion categories.

3.2.2.3 Sampling regime

3.2.2.3.1 Early detection of *Mycosphaerella* species – Set 1

Early lesion symptoms (Figure 3.2.1) were identified whilst leaves were still on the tree using the sun as a backlight. One replicate per tree of asymptomatic tissue and lesion categories 1 and 2 were sampled from within the first five leaf pairs of the upper crown of 30 *E. globulus* and 30 *E. nitens*. Leaves were separately sampled into paper bags by grasping the leaf through the bag and pulling it off the tree to avoid contamination. Leaves were dried at 28 ^{o}C and stored until analysis with the *Mycosphaerella* diagnostic assay.

3.2.2.3.2 *Mycosphaerella* species occupying lesions of varying age – Set 2

One sample of asymptomatic tissue and of each lesion category (1, 2, 3, 4 and 5) per tree was sampled. Samples were collected from five *E. globulus* and five *E. nitens* trees. Leaves from the upper crown and within the first five leaf pairs from the bud were sampled. Leaves were sampled using the same sampling procedure as for set 1, dried at 28 ^{o}C and stored until analysis with the *Mycosphaerella* diagnostic assay.

3.2.2.4 Mycosphaerella diagnostic assay

A sample area of approx. 1 cm^2 was excised from lesions of representative categories using a fresh scalpel blade for each sample to avoid DNA cross-contamination. A light box was used for categories 1 and 2 so that lesions could be seen. Asymptomatic tissue (1 cm^2) was excised from the middle of uninfected leaves to the right of the midrib. Each 1 cm^2 sample was ground in a 1.5 ml microfuge tube using a pellet pestle mixer in liquid nitrogen and DNA was extracted and nested PCR using *Mycosphaerella* taxon

specific primers was conducted as described by Glen Smith et al (2006). Three of the primer sets used were species specific for *Mycosphaerella cryptica*, *Mycosphaerella nubilosa* and *Mycosphaerella tasmaniensis* Crous and M.J. Wingf. The other two primer sets detect more than one species that may or may not be synonymous. A positive result for the primers M. vamF / M. vamR was scored as *Mycosphaerella vespa* Carnegie and Keane, even though these primers will also give a positive result for *Mycosphaerella ambiphylla* and *Mycosphaerella molleriana* (Thüm) Lindau. This is because *M. vespa* has been previously recorded from Tasmania and the other species have not. For similar reasons, a positive result from the primers M. gpF / M. gpR was scored as *Mycosphaerella parva*. The numbers of positive bands for each species in each lesion type were counted. Distinct and faint reactions on the gel were scored as a positive result. Repeated analysis was conducted for sample sets 1 and 2 with no differences found between analyses. Due to unknown reasons, out of the 230 samples in this study 10 samples did not amplify. These samples were evenly distributed within sample sets and tree species and therefore did not significantly impact results.

3.2.3 Results

3.2.3.1 Early detection of Mycosphaerella species

Mycosphaerella species could be easily detected at very early stages of lesion development (Table 3.2.1) and the mean number of species detected per lesion appeared to increase with lesion age (Figure 3.2.2). Category 1 lesions in *E. nitens* were the only samples not to produce a positive result for any of the five *Mycosphaerella* species tested in this study. A high proportion of asymptomatic *E. globulus* (59 %) and *E. nitens* (24 %) samples tested positive for one or more *Mycosphaerella* species

(Figure 3.2.2). Similar to the results of the first experiment, *M. parva* was the most frequently detected species in early stage lesions of *E. globulus* and *E. nitens*. *Mycosphaerella cryptica* and *M. tasmaniensis* were not detected in early stage lesions of *E. nitens* in this set of samples (Table 3.2.1).

Table 3.2.1: Detection of *Mycosphaerella* species (% of n) in asymptomatic tissue, early stage lesions (categories 1&2) and late stage lesions (categories 3-5).

		***E. globulus* (% of n)**					***E. nitens* (% of n)**				
	No. lesions	MC	MN	MV	MP	MT	MC	MN	MV	MP	MT
Asymptomatic (Set 1)	30	10	6	0	43	16	0	0	3	13	0
Asymptomatic (Set 2)	5	100	0	80	40	0	80	20	60	60	0
Categories 1 and 2 (Set 1)	60	32	15	0	75	10	0	2	0	48	0
Categories 1 and 2 (Set 2)	10	40	70	40	100	20	70	70	30	50	0
Categories 3-5 (Set 2)	15	40	73	13	100	0	100	20	13	20	0
Detection for all samples	120	31	24	8	71	11	22	10	8	37	0

MC = *M. cryptica*, MN = *M. nubilosa*, MV = *M. vespa*, MP = *M. parva* and MT = *M. tasmaniensis*

Set 1 = Sample set to detect *Mycosphaerella* species in early developmental stages only

Set 2 = Sample set to detect *Mycosphaerella* species occupying lesions of varying age

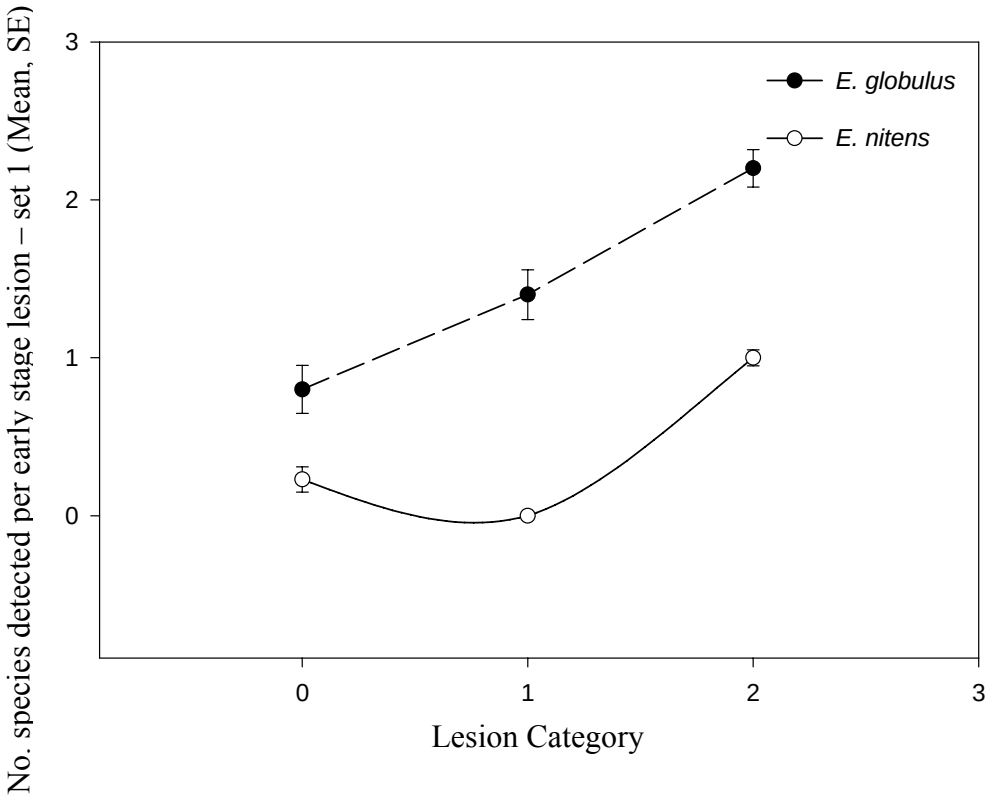
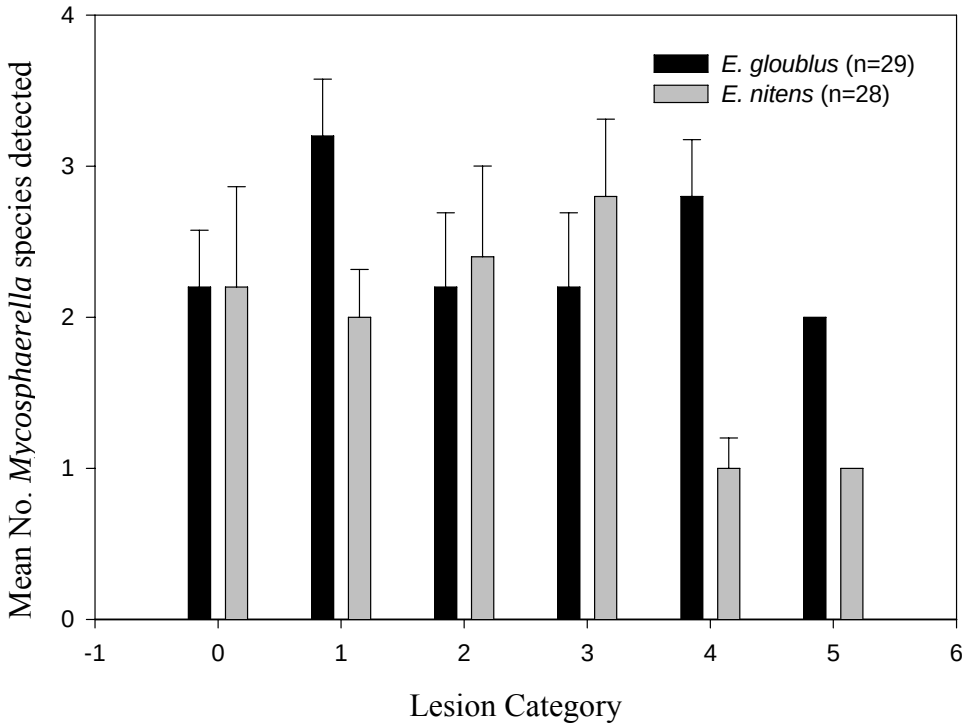


Figure 3.2.2: The number of *Mycosphaerella* species detected per lesion (Mean, SE) appears to increase with lesion age in early stage lesions on *E. globulus* and *E. nitens*.

3.2.3.2 Mycosphaerella species occupying lesions of varying age

Mycosphaerella species were detected in asymptomatic tissue and all lesion categories (Table 3.2.1). In asymptomatic tissue the number of species detected ranged from 0 to 4 in *E. nitens* and 1 to 3 in *E. globulus* with a mean of 2 species in lesions from both tree species (Figure 3.2.3). In *E. nitens*, the mean number of species per lesion appeared to increase with lesion age until categories 4 and 5 where one species was detected. In *E. globulus* there was no correlation between the mean number of species detected per lesion and lesion age (Figure 3.2.3). For all lesions in this sample set (asymptomatic tissue and lesion categories 1-5 combined), *M. parva* followed by *M. nubilosa* were the

most frequently detected in *E. globulus* and *M. cryptica* followed by *M. parva* were the most frequently detected species in *E. nitens* (Table 3.2.1).



* Standard error was 0 in category 5 lesions. There was no variation from the mean in *E. nitens* (Mean of 1 species) and *E. globulus* (Mean of 2 species).

Figure 3.2.3: The mean (SE) number of *Mycosphaerella* species detected per lesion appears to decrease in late stage lesions sampled from *E. globulus* and *E. nitens*.

The detection frequency of *M. cryptica* increased with increasing lesion age for both tree species with the exception of category 5 lesions in *E. nitens* (Figure 3.2.4a). *M. nubilosa* was only detected up until category 3 lesions in *E. nitens* but predominated in *E. globulus* with its occurrence in every lesion stage including 100 % and 80 % of

category 4 and 5 samples respectively (Figure 3.2.4b). *M. nubilosa* was not detected in asymptomatic *E. globulus* tissue. The occurrence of *M. vespa* decreased with increasing lesion age in both tree species (Figure 3.2.4c). A high detection frequency was initially observed for *M. vespa* on *E. globulus* (80 % detection in asymptomatic tissue) and *E. nitens* (60 % detection in asymptomatic tissue) (Figure 3.2.4c), but it was not detected in category 5 lesions of either tree species and was only detected in a limited number (20 %) of *E. globulus* category 4 lesions. *Mycosphaerella parva* was detected in every *E. globulus* sample of every lesion category and in 40 % of *E. globulus* asymptomatic samples (Figure 3.2.4d). Its occurrence was not as frequent in *E. nitens* compared with *E. globulus*, with detection of *M. parva* in only 20 % of category 4 samples and no detection in category 5 samples. It was, however, detected in 60 % of *E. nitens* asymptomatic tissue. The detection frequency of *M. tasmaniensis* was too low to discern any trends with time/lesion age.

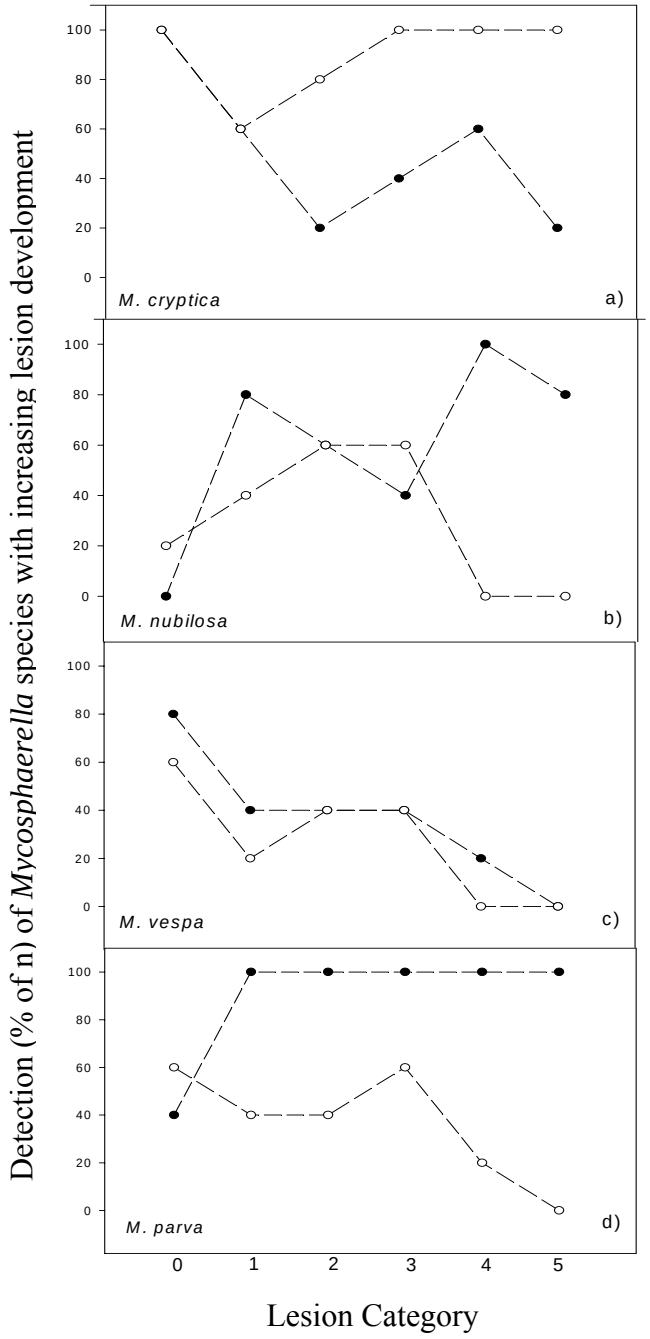


Figure 3.2.4: Detection of a) *M. cryptica*, b) *M. nubilosa*, c) *M. vespa* and d) *M. parva* in leaf lesion categories sampled from *E. globulus* (bold points) and *E. nitens* (hollow points).

3.2.4 Discussion

The presence of *Mycosphaerella* species was confirmed in asymptomatic tissue and visually diagnosed early stage lesions on both *E. globulus* and *E. nitens*. The co-occurrence of up to four pathogens at various stages of lesion development in the current study raises questions about the interaction of *Mycosphaerella* species within the lesion. Furthermore, some species were absent from advanced lesions including *M. vespa* and *M. tasmaniensis* in *E. globulus* and *M. nubilosa, M. parva* and *M. vespa* in *E. nitens*. *Mycosphaerella cryptica* was recorded as the only species in advanced lesions of *E. nitens*, even when other *Mycosphaerella* species were detected in earlier stage lesions from the same tree. Similarly in *E. globulus*, only *M. parva* and *M. nubilosa* occurred in advanced lesions. This suggests that either species in lesions are dependant on seasonal variations in inoculum or that competitive/antagonistic interactions may be occurring between *Mycosphaerella* species, with the eventual elimination of co-occurring species/strains.

Competition for space and resources (Paulitz 1990), excretion of lytic enzymes (Benyagoub, Benhamou *et al.* 1998; Chet 1990) and antibiosis (excretion of low molecular weight substances, toxic to other micro-organisms) (Cullen and Andrews 1984; Fravel 1988) are common mechanisms used by antagonistic fungi against other plant pathogens. The application of antagonistic pathogens is often a successful means of biocontrol (e.g. *Microsphaeropsis* sp. against *Venturia inaequalis* on apples (Benyagoub, Benhamou *et al.* 1998) and *Trichoderma harzianum* against *Botrytis cinerea* (Belanger, Dufour *et al.* 1995). However antagonistic interactions between species of the same genera, as proposed in this study, are rarely encountered (Mohammed and Guillamin 1988; Prospero, Holdenrieder *et al.* 2006). Further

investigation into phytopathogenic or antagonistic interactions between multiple pathogenic species in *E. globulus* and *E. nitens* is required to understand the principles involved with the coexistence of more than one pathogen in a leaf or lesion.

Valuable information about colonising *Mycosphaerella* species has been obtained before infection can be visually diagnosed or identified using classical techniques and less sensitive molecular technologies. This will allow for more complex ecological and epidemiological hypothesis testing in the future, including the study of inoculum threshold levels, host-pathogen interactions and the monitoring of infection/inoculation trials from the outset. It may also aid in the development and monitoring of other control measures such as systemic enhancers and fertiliser treatments. Visual diagnosis of MLD in the field was confirmed by molecular analyses for all samples except for category 1 lesions on *E. nitens* (sample set 1). Infection by other leaf spot fungi such as *Cercospora* spp. (Crous, Wingfield *et al.* 1989; Dick 1982) and *Phaeophleospora eucalypti* (Park, Keane *et al.* 2000) or damage from mechanical injury such as hail, can cause similar symptoms to those caused by MLD and therefore we recommend that visual diagnosis of early symptoms in the field should be supported by molecular analyses in future studies. The technique may also have a valuable application in obtaining biosecurity information on species occurrence in asymptomatic tissue and early lesions to help prevent the inadvertent transfer of pathogenic species on plant material or seed within Australia.

3.3 Detection Summary

Methodology to detect for *Mycosphaerella* species in naturally infected early stage lesions was applied. This research provided conclusive evidence that multiple species (up to 5) were occupying the same leaf or stem lesion simultaneously. In addition, *Mycosphaerella* species could be detected in all lesion stages including asymptomatic tissue. This provided a solid basis for studying the effects of early infection on host physiology and study the necrophylactic periderms formed in susceptible and resistant leaf material after infection by *Mycosphaerella* species.

CHAPTER 4

4.1 Anatomical and histochemical defence responses in juvenile *Eucalyptus globulus* and *Eucalyptus nitens* leaves induced by *Mycosphaerella* infection.

4.1.1 Introduction

Eucalyptus globulus (Labill) and *Eucalyptus nitens* (Deane and Maiden) Maiden are the two major *Eucalyptus* species chosen for plantations in cool temperate zones in Australia and exhibit varying levels of intra- and interspecific variation in susceptibility to Mycosphaerella leaf disease (MLD). When affected in plantations, *E. globulus* exhibit a range of symptoms that include spotting, blighting (Figure 1a), premature branch death (Figure 1b) poor growth rates (Carnegie and Ades 2002b; Lundquist and Purnell 1987; Milgate *et al.* 2005), altered tree form and defoliation (Figure 1b) that can lead to tree death (Wardlaw 2001). In contrast, *E. nitens* in the same planting areas appear to be tolerant of MLD when subjected to the same inoculum levels. Economically and environmentally viable control options for MLD on *E. globulus* have not been established and the development of resistant seedlings is still in its infancy even though significant genetic variation in *Mycosphaerella* resistance has been recorded for *E. globulus* (Milgate *et al.* 2005). Even though *E. globulus* is the preferred plantation species, the current management option in high-risk areas of Australia is plant *E. nitens* to avoid potential problems with *Mycosphaerella leaf disease* (MLD).

Mycosphaerella leaf disease is caused by a complex of *Mycosphaerella* species that can occur simultaneously on the same lesion (Glen *et al.* 2006). In general, infection by leaf

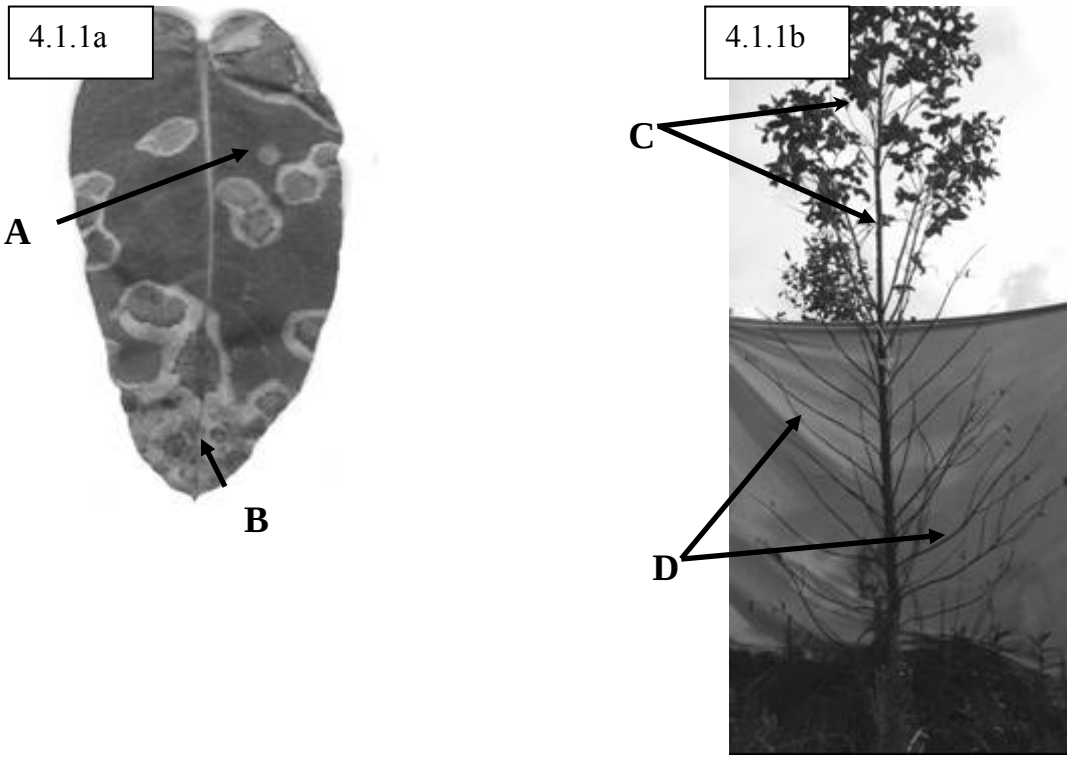
pathogens such as *Mycosphaerella* can result in a range of symptoms including spotting (effective compartmentalization of leaf tissue after infection) and blighting (rapid, extensive and unrestricted necrosis across all leaf cells and shoots) (Fink 1999). Initial infection in plant tissue induces many structural and physiological changes in the cells surrounding the infection to restrict the pathogen and reduce fluid loss (Moon *et al.* 1984). At a cellular level, mesophyll cells may swell (hypertrophy) and/or divide (hyperplasia) in response to infection. This creates a barrier termed a necrophylactic periderm in leaves (Fink 1999) which is similar to a barrier-zone formed in woody stem tissues (Jones and Fett 1987). In eucalypt stems, the first layers of intact cells of the barrier-zone, closest to the pathogen, are modified through the accumulation of antimicrobial and water impermeable substances such as suberin, lignin and other polyphenolics (Eyles and Mohammed 2002). Barrier zone formation cannot be cancelled by a single pathogen mutation and is therefore a more stable response to study than race-specific resistance as there are multiple genes involved in the response to infection (Prell and Day 2001). There is also substantial evidence that timing of this response is linked with disease resistance in other plant pathogen interactions such as *Cladosporium fulvum* (Lazarovits and Higgins 1976 a,b) and *Fusarium oxysporum* (Beckman *et al.* 1982) infection of tomato plants.

To obtain a more comprehensive understanding of induced resistance mechanisms between eucalypt species, we integrated epidemiological, molecular and histochemical techniques to characterize the timing of lesion progression and the cellular changes occurring in leaves of *E. globulus* and *E. nitens* that had been naturally infected with *Mycosphaerella* species. The objectives of this study were to compare the defence

responses induced by *Mycosphaerella* infection in commercial provenances of *E. globulus* and *E. nitens* grown in southern Australia with particular reference to:

1. Differences in constitutive host anatomy and induced anatomical changes with infection.

2. Histochemical localisation of induced suberin, lignin and other polyphenolics, and biochemical quantification of the levels of chlorophyll and anthocyanin in tissue representative of the varying stages of *Mycosphaerella* lesion development.



Figures 1a and 1b: Symptoms of Mycosphaerella leaf disease on eucalypts. At a leaf level (1a) these include spotting (A), blighting (B) and at a tree level defoliation (C) and premature branch death (D).

4.1.2 Materials and Methods

4.1.2.1 Leaf material

Juvenile *E. globulus* and *E. nitens* leaves were sampled from adjacent commercial plantations near Smithton (40˚55'09''S: 144˚58'58''E) in Tasmania, Australia. The two-year-old *E. globulus* and 1-year-old *E. nitens* plantations had been naturally infected with several different *Mycosphaerella* species. Naturally infected material was used to overcome problems with the low success rate of infection using artificial inoculation techniques. The approximate age of leaf lesion categories was estimated from spore release data collected by spore trapping. The *Mycosphaerella* species in leaf lesions were detected and identified using molecular techniques. Leaf lesions, collected from each plantation, were ascribed to one of four distinct morphological categories (Table 4.1.1). One lesion per category was randomly sampled for each tree for three *E. globulus* and three *E. nitens* trees (for fixing and embedding) and six *E. globulus* and six *E. nitens* trees (for fresh sections). Only young leaves (<5th pair from the bud) were sampled. Approximately 20 young leaves (<5th pair) were randomly sampled from the canopies of six different trees each of *E. globulus* and *E. nitens* to obtain the material required for chlorophyll and anthocyanin analyses.

Table 4.1.1: Morphology of lesion categories on *Eucalyptus globulus.*

Category	Approx. age	Diameter (mm)	Symptoms	Visibility
0	0	-	No apparent lesion symptoms	-
1	5-7 days	1-3	Purple/red spot on either abaxial or adaxial surface.	Indistinct, only visible only under ×10 magnification against strong light
2	16-18 days	1-3	Intense purple/red spot	Abaxial or adaxial surface of the leaf under ambient natural light
3	27-29 days	5-10	Distinct necrotic tissue with purple/red margin greater than 1mm in diameter.	Abaxial or adaxial surface of the leaf under ambient natural light
4	>37 days	>5	Defined necrotic tissue without a purple/red margin.	Abaxial or adaxial surface of the leaf under ambient natural light

NB: *Eucalyptus nitens* categories were as above except necrotic tissue was brown/grey and purple/red margins around category 3 lesions were generally restricted to < 3 mm.

4.1.2.2 Lesion age

The age of lesions sampled for this study was estimated by correlating the presence of ascospores (as indicated by spore trapping) with the probability of infection (appropriate temperature and humidity for spore germination). *Mycosphaerella* spores were collected onto a vaseline-coated plastic disc in a Quest volumetric spore trap, raised 2.3 m off the ground in the *E. globulus* plantation. A collective count of all *Mycosphaerella* spores, regardless of species, was taken on a daily basis (E.A. Pietrzykowski, unpublished data). A significant spore count was considered to be greater than 300 spores over three consecutive days. The key predictors of spore release and germination are considered to be humidity (> 95 %) (Cheah and Hartill 1987), temperature (15-20 ºC) and rainfall (leaf wetness, 5-7 days) (Park 1988a). A weather station, fitted with an automatic data logger, was located approximately 1.5 km from the *E. nitens* and *E. globulus* sampling sites. Information was collected hourly, daily and weekly for rainfall (mm), humidity (%), wind speed (m s^{-1}) and temperature (ºC). The timing of lesion development in this study was also compared with the timing of lesion development recorded in past studies (e.g. Cheah and Hartill 1987; Park 1988b)

4.1.2.3 Mycosphaerella *detection in leaf sections*

A molecular identification technique was used in this study rather than classical identification techniques due to the absence of reproductive structures in the majority of lesion categories. The tissue encompassing the samples that were fixed for sectioning (approximately 20 mm^2) was used to determine the *Mycosphaerella* species in the each lesion. Samples were ground in liquid nitrogen in 1.5 mL microfuge tubes with a pellet pestle mixer, mixed with extraction buffer (Raeder and Broda 1985) and incubated for

1 h at 65 °C. Tubes were centrifuged at 14 000 rpm for 15 min and the recovered supernatant was purified by binding to a silica matrix in the presence of sodium iodide (Glen, Tommerup *et al.* 2002). PCR was carried out in an Applied Biosystems GeneAmp PCR System 2700 thermocycler. First-round reactions contained 0.02 U/µl TTH+ Polymerase (Fisher Biotec) in 1 x Polymerisation buffer (Fisher Biotec), 2.0 mM MgCl2, 0.2 mg/ml BSA, 0.2 mM each dNTP and 0.25 µM each of primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White, Bruns *et al.* 1990). Second round PCR with species-specific primers (Glen, Smith *et al.* 2006) contained 0.01 U/µl TTH+ Polymerase and the same concentrations of all other reagents. The thermocycler program for first round PCR was: 94 °C for 3 minutes, 35 cycles of 94 ° C x 30 s, 55 °C x 30 s, 72 °C x 30 s, followed by an extension step of 72°C for 7 minutes, and for the second round: 94 °C for 3 min, 20 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 10 min.

4.1.2.4 Fixed sample preparation

Lesion sections of approximately 2 mm^2 were cut from the edge of each of the four categories from each of the three trees per species sampled for the study (*i.e.* three sections for each lesion category for each tree species). Each of these three sections was taken from a separate leaf from each of the three trees. Sections were fixed in 2.5 % glutaraldehyde in 0.1 M Sörensen's phosphate buffer (Sörensen 1909), pH 7.2, for 15 h at room temperature. Following two buffer washes (each for 20 min), the samples were dehydrated in an ascending acetone series in 20 % increments, finishing with two changes (each for 20 min) of 100 % propylene oxide before embedding in Spurr's resin (Spurr 1969).

4.1.2.5 Constitutive host anatomy and necrophylactic periderm formation

Transverse sections of control and category 1 and 2 lesions, 8-10 µm thick, were cut on a Reichart OmU2 ultramicrotome fitted with a glass knife. Sections were expanded and gently heat fixed onto clean glass microscope slides before staining for 1 minute in a toluidine blue/sodium borate solution (1 mL toluidine blue in 99 mL of 1 % sodium borate). Sections were rinsed in water and then de-stained by immersion in 70 % ethanol for 30 sec. Lignin deposits were examined by staining with a 1 % w/v phloroglucinol solution in 70 % ethanol for 5 min (Southerton and Deverall 1990). Excess phloroglucinol solution was poured off and sections were covered with two drops of concentrated HCl, covered with a glass cover slip and examined with a Zeiss compound light microscope. The sections were photographed immediately as the reaction faded with time. Bright pink/red areas appeared in the presence of lignin (Gahan 1984). A modified Maüles test for lignin was conducted to detect lignin high in syringyl groups (Ride 1975). Sections were washed with water and then placed in a 1 % aqueous $KMnO_4$ solution, washed with 12 M HCl and rinsed with 1 % w/v sodium sulphite solution. When counterstained with phloroglucinol to eliminate the autofluorescence of lignin (Biggs 1984) suberin was detected using a Zeiss Axiovert Fluorescence Microscope fitted with a mercury lamp between 340 and 380 nm. A colorimetric test was used to stain for catechol derivatives in fresh leaf sections as outlined by (Reeve 1951). One drop 10 % aqueous sodium nitrate (v/v), one drop of 10 % aqueous acetic acid and one drop of 20 % aqueous urea were added to each section. After 3 minutes, two drops of 2 N NaOH were added. Sections were mounted beneath a coverslip and examined immediately with a Zeiss compound light microscope. A deep cherry red colouration was observed in the presence of catechol derivatives. Dimethylaminocinnamaldehyde (DMACA) was used to specifically detect

flavan-3-ols. The DMACA solution was prepared by heating 3 M H_2SO_4 with 1-butanol to which 2 mL 0.1 % DMACA was added after cooling. Spurr's embedded sections were fixed to glass microscope slides and immersed in DMACA for 5 min. The slides were gently heated for approximately 10 sec on a hotplate. A blue/green product was observed in the presence of monomeric flavan-3-ols (Feucht 1992). Flavanoids were localized by immersing sections in 1 % 2-aminoethyl diphenylborinate in methanol for 10 min. Slides were rinsed with methanol and then observed between 535 and 550 nm using a Zeiss Axiovert Fluorescence microscope fitted with a mercury lamp.

4.1.2.6 Anthocyanin and chlorophyll quantification

Approximately 20 leaves were sampled per tree from which a representative sample of approximately 0.2 g of each lesion type was obtained. After sampling, leaves were transported in a refrigerator (4 ± 1ºC) before refrigeration at 4 ºC in the laboratory. Samples were processed within 48 h of sampling. Exact sample areas were determined using Folia® imaging software after scanning with an Epson Expression 1680 scanner at a resolution of 150 x 150 dpi. Chlorophyll was extracted using the method described in Close, Beadle *et al*. (2000). Absorbances at 663 nm and 645 nm were measured using a UV–visible spectrophotometer. Levels of chlorophyll a, b and total chlorophyll were determined using the following formulae:

Chlorophyll a = $(12.7 \times A_{663}) - (2.69 \times A_{645})$

Chlorophyll b = $(22.69 \times A_{645}) - (4.68 \times A_{663})$

Total chlorophyll = chlorophyll a + chlorophyll b (µg/mL) (Porra 1989).

Anthocyanins were extracted by homogenising samples in 10 mL of acidified methanol (40 mL H_2SO_4; 1760 mL methanol, pH 1) combined with one 10 mL washing of the homogeniser with acidified methanol. Samples were immersed in boiling water for 1.5 min and then left for 24 h at 4 ºC in darkness to extract. After centrifugation (2500 rpm; 10 min) absorbance was measured at 530 and 657 nm. Anthocyanin concentration (mg mL^{-1}) corrected for chlorophyll degradation products was determined using the formula A_{530}–0.25 × A_{657} (Mancinelli, Huang Yang *et al.* 1975). Chlorophyll and anthocyanin concentrations (mg mL^{-1}) were multiplied by corresponding dilution factors to determine pigment concentrations in the original sample. This was divided by the surface area of each sample to determine pigment concentrations in mg cm^{2-1} for each lesion type.

4.1.3 Results

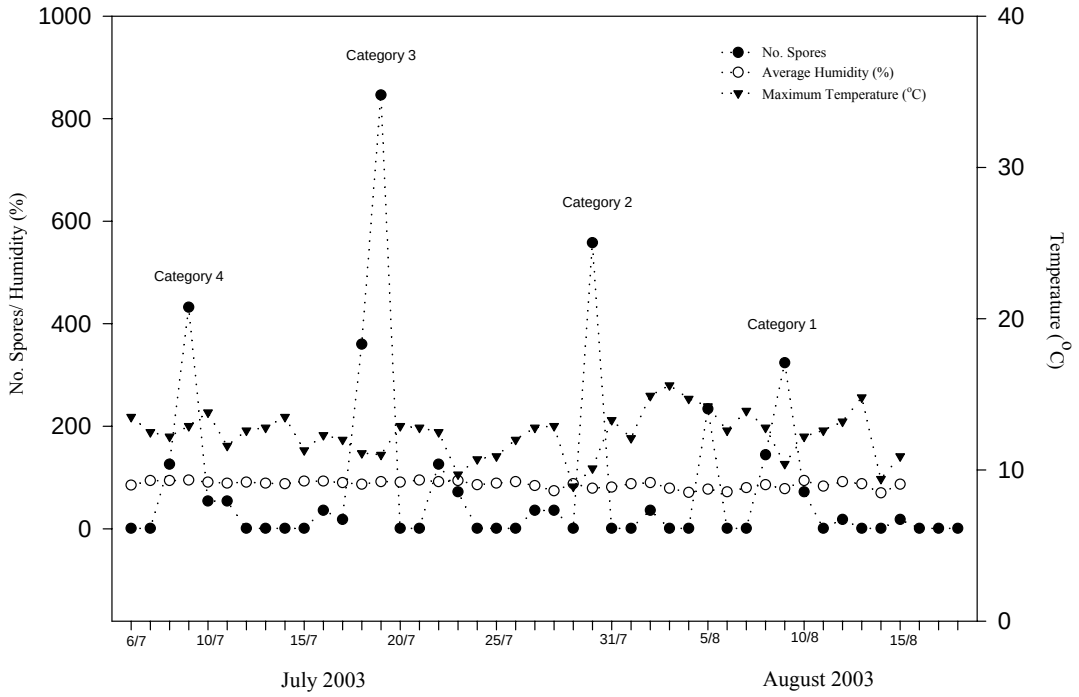
Results from this study are also summarised in poster format in Appendix 6.

4.1.3.1 Lesion age and Mycosphaerella *detection.*

There were four distinct spore release events where spore counts were in excess of 300 spores over 3 consecutive days. As spore counts in between these intervals were negligible it is assumed that these four intervals correspond with the four stages of lesion development observed in the field (Figure 4.1.2). Based on this assumption pre-visual lesions would be approximately 5-7 days and necrotic lesions would be greater than 37 days old which corresponds with lesion ages observed by Park (1988b) and Cheah and Hartill (1987). *Mycosphaerella* species were detected in all categories of lesion sampled for this study. Of the five *Mycosphaerella* species detected,

Mycosphaerella cryptica (Cooke) Hansf. and *Mycosphaerella parva* Park and Keane in *E. nitens* and *Mycosphaerella nubilosa* (Cooke) Hansf. and *M. parva* in *E. globulus* were the most frequently detected species. No correlation between specific *Mycosphaerella* species present and host response was observed (data not presented).

Figure 4.1.2: Four distinct intervals of *Mycosphaerella* spore release were assumed to be correlated with the four morphological lesion categories (see Table 1).



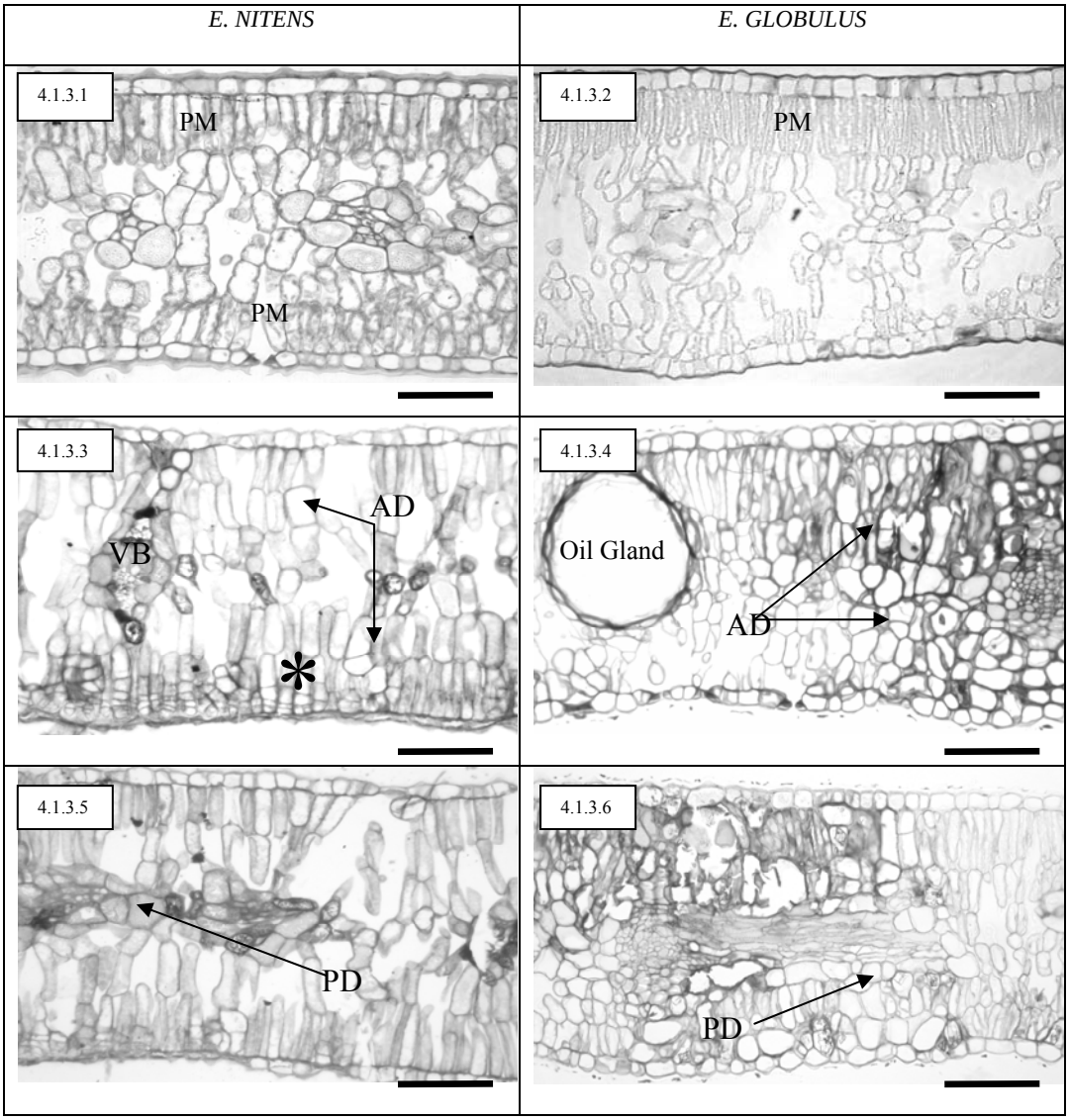


Figure 4.1.3: Transverse sections through *E. nitens* and *E. globulus* leaves showing constitutive anatomy (4.1.3.1 and 4.1.3.2) and the distribution of palisade mesophyll (PM) within each of these species (Bar = 0.50 mm in *E. globulus* and 0. 52 mm in *E. nitens*). Upon infection with MLD we can observe three types of induced cellular division. 1) Anticlinal division (AD) of mesophyll cells was initiated in category 1 lesions in both *E. nitens* and *E. globulus* (Figure 4.1.3.3 and 4.1.3.4). 2) Meristematic tissue divided periclinally (PD) around veins (Figure 4.1.3.5 and 4.1.3.6) and 3) Anaplasia (*) of palisade mesophyll was observed in *E. nitens* where multiple daughter cells were formed from a single larger cell.

4.1.3.2 Constitutive host anatomy and necrophylactic periderm formation

The control sections of *E. nitens* had single isobilateral layers of palisade mesophyll (Figure 4.1.4.1) whereas *E. globulus* sections had a single adaxial palisade layer only (Figure 4.1.4.2). Upon infection with *Mycosphaerella* species, necrophylactic periderm formation was initiated in both tree species by anticlinal and periclinal division of mesophyll cells (Figures 4.1.4.3 to 4.1.4.6). Considerable anaplastic division was observed in palisade mesophyll cells in *E. nitens* (Figure 4.1.4.3). Necrophylactic periderms in *E. globulus* were formed from multiple cells, some of which had been derived by cell division and others that had increased in size from hypertrophic swelling. In most cases necrophylactic periderms were distorted in category 3 and 4 lesions of *E. globulus* (Table 4.1.2). Necrophylactic periderms were formed at earlier stages of lesion development in *E. nitens* compared with *E. globulus* due to extensive cell division from both the adaxial and abaxial palisade parenchyma, which were infiltrated with defence chemicals such as suberin, lignin and other phenols within one week of infection (Table 4.1.2).

Table 4.1.2: Presence of defence chemicals with varying lesion symptom

	Lesion category (n=5 for each category)	**1**	**2**	**3**	**4**
	Lesion Age (days)	**5-7**	**16-18**	**27-29**	**>37**
Suberin	*E. nitens*	*	**	***	***
	E. globulus	-	*	**	***
Lignin	*E. nitens*	*	**	***	***
	E. globulus	-	*	**	***
Flavanols	*E. nitens*	-	-	***	***
	E. globulus			***	***
Catechol derivatives	*E. nitens*	*	*	**	**
	E. globulus	*	*	**	***
Flavanoids (epidermal cells only)	*E. nitens*	-	*	**	***
	E. globulus	-	-	**	***

Key: * Located within some cells that are scattered throughout the section.
** Located within cells that span adaxial and abaxial leaf surfaces – cells form a necrophylactic periderm of between 1-2 cells in thickness.

*** Located within cells that span adaxial and abaxial leaf surfaces – cells form a necrophylactic periderm of between 2-7 cells in thickness.

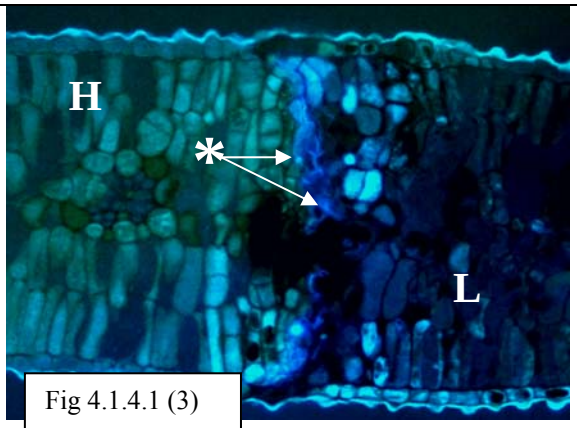
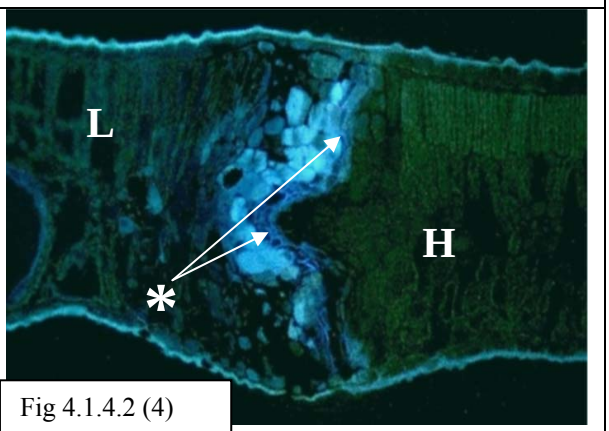
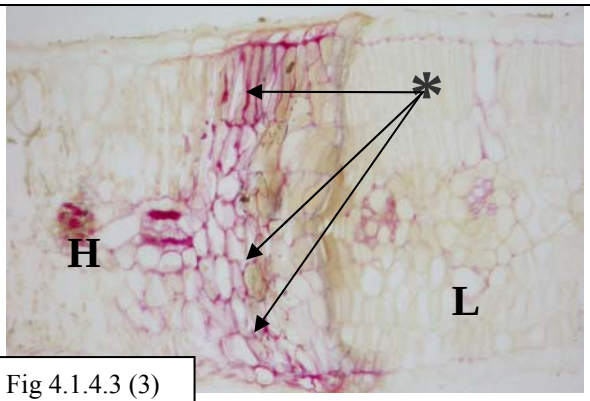
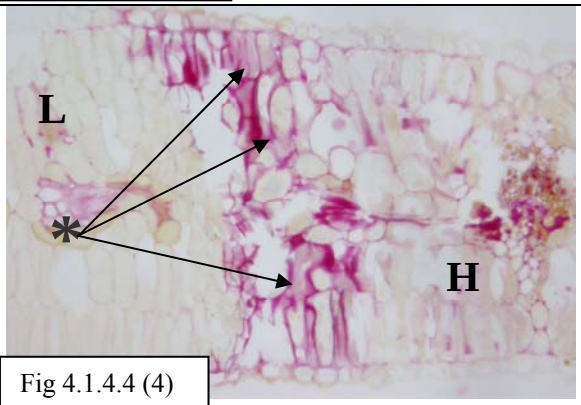
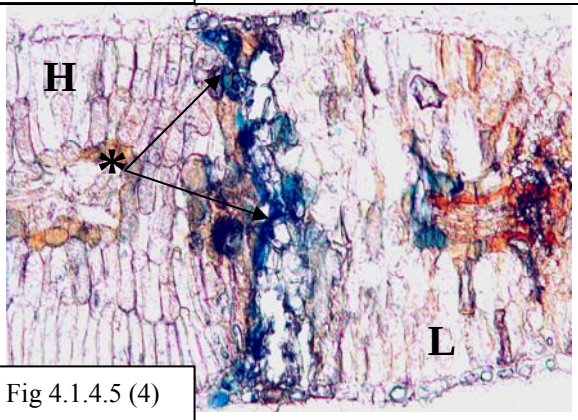
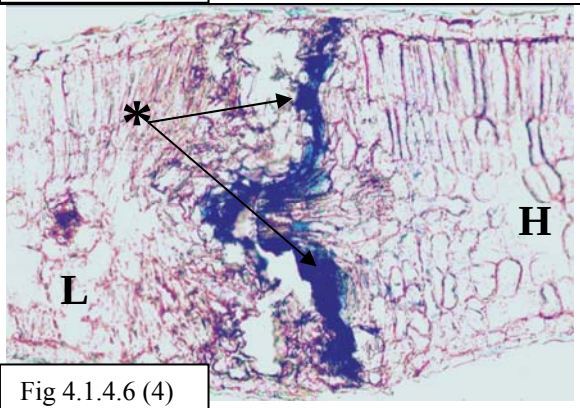
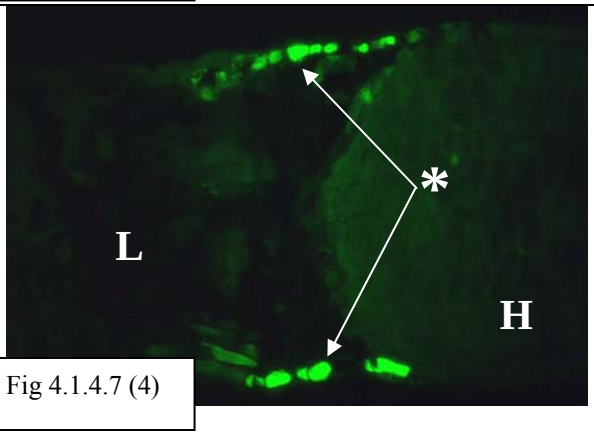
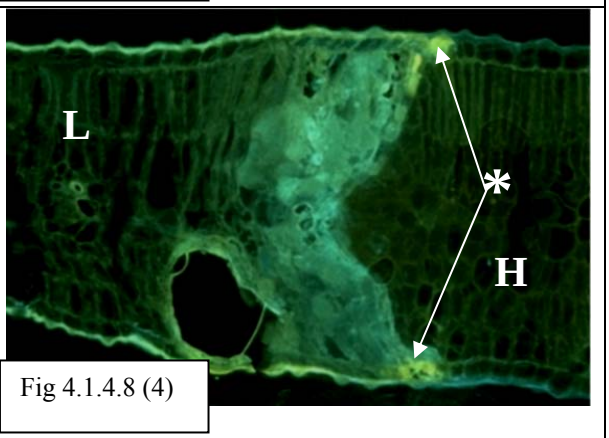
	E. NITENS	*E. GLOBULUS*
SUBERIN (purple fluorescence)	 Fig 4.1.4.1 (3)	 Fig 4.1.4.2 (4)
LIGNIN (pink stain)	 Fig 4.1.4.3 (3)	 Fig 4.1.4.4 (4)
MONOMERIC FLAVAN-3-OLS (blue stain)	 Fig 4.1.4.5 (4)	 Fig 4.1.4.6 (4)
FLAVANOIDS (Bright green/yellow fluorescence)	 Fig 4.1.4.7 (4)	 Fig 4.1.4.8 (4)

Figure 4.1.4: A comparison between tree species of the necrophylactic periderms formed to compartmentalise lesion or infected- (L) and healthy (H) tissue. Arrows leading from an asterisk (*) depict examples of the cells infiltrated with specific defence chemicals. The number in brackets on the figure labels depicts what category of lesion the section is from. A more direct and impermeable barrier can be observed in this *E. nitens* category 3 section (4.1.4.1) than the *E. globulus* category 4 section (4.1.4.2)**.** Suberin (4.1.4.1 and 4.1.4.2 – purple fluorescence) and lignin (4.1.4.3 and 4.1.4.4 – pink stain) are deposited within the cells of the barrier zone in both tree species. Monomeric flavan-3-ols (F1- blue stain) were not detected until category 4 lesions for both tree species (4.1.4.5 and 4.1.4.6). Flavanoids (bright green/yellow fluorescence) were distributed within epidermal cells of the necrophylactic periderm in *E. globulus* (4.1.4.7) and *E. nitens* (4.1.4.8).

4.1.3.3 Constitutive host anatomy and necrophylactic periderm formation (continued)

After necrophylactic periderm formation, in both tree species, inclusive and surrounding cells were re-enforced with, suberin (Figures 4.1.3.1 and 4.1.3.2), lignin (Figures 4.1.3.3 and 4.1.3.4), monomeric flavan-3-ols (Figures 4.1.3.5 and 4.1.3.6) and flavanoids (Figures 4.1.3.7 and 4.1.3.8). *Eucalyptus globulus* necrophylactic periderms were distorted in shape. The suberised zone of defence parenchyma ranged from one to four cells in thickness (Figure 4.1.3.2). Whereas *E. nitens* formed a necrophylactic periderm more directly between abaxial and adaxial leaf surfaces. The suberised zone was consistently two or three cells in thickness (Figure 4.1.3.1).

4.1.3.4 Anthocyanin and chlorophyll quantification

Anthocyanins were induced by infection in both *Eucalyptus* species (Figure 4.1.5) and were localised in high quantities on the healthy side of the necrophylactic periderm. Mean (SE) anthocyanin levels were significantly higher in category 1 tissue for both *E.*

globulus 3.38 (0.51) and *E. nitens* 3.56 (0.80) compared with mean levels (SE) of control tissue. Total chlorophyll concentrations decreased with increasing lesion development and were significantly reduced, from control values, in category 3 tissue for *E. globulus* (38.52 (4.85)) and category 4 (necrotic) tissue for *E. nitens* (49.14 (1.66)) (Figure 4.1.5). Significantly higher ($P < 0.05$) chlorophyll and anthocyanin was observed in necrotic tissue of *E. nitens* than in *E. globulus*.

Figure 4.1.5: Anthocyanin accumulation in tissue associated with *Mycosphaerella* lesion development

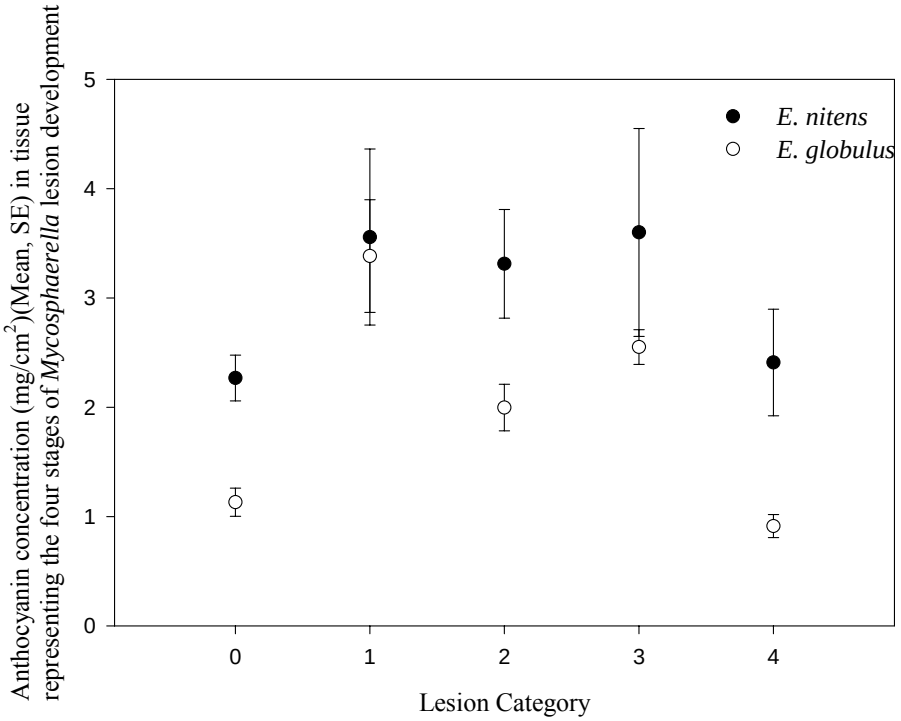
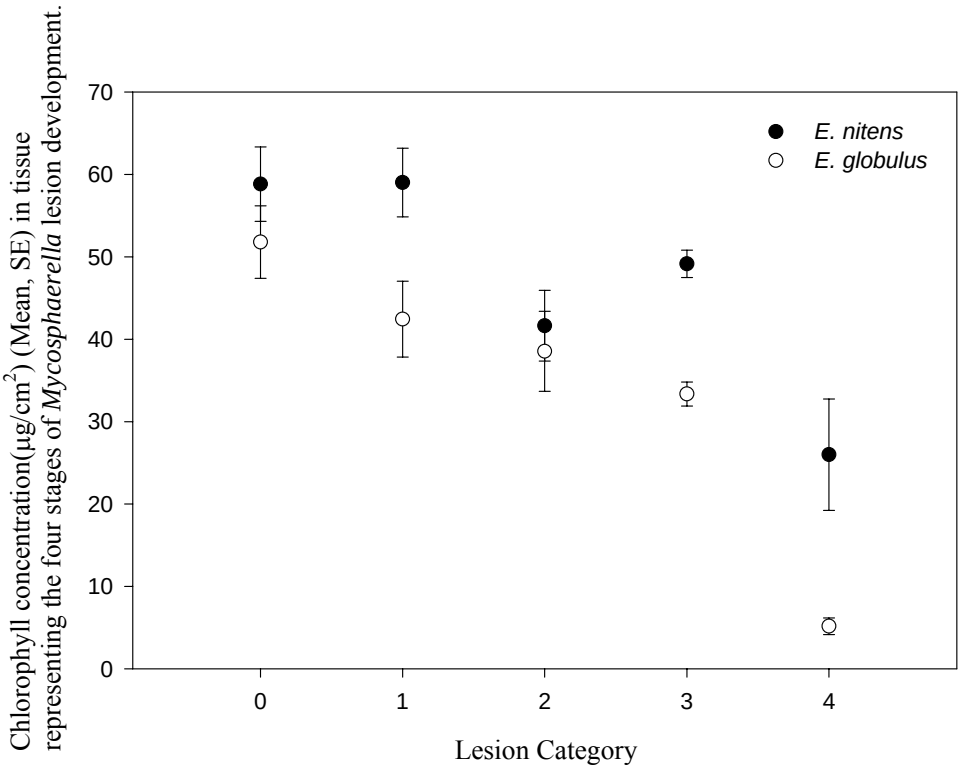


Figure 4.1.6: Chlorophyll retention in tissue associated with *Mycosphaerella* lesion development.



4.1.4 Discussion

The constitutive differences in the distribution of palisade mesophyll that occur between *E. nitens* and *E. globulus* may provide a primary basis of why plantations of *E. nitens* experience lower incidence and severity of Mycosphaerella leaf disease in southern Australia. Upon infection there are differences in timing and strength of necrophylactic periderm formation between *E. globulus* and *E. nitens.* There is strong evidence that mesophyll cells in *E. nitens* can undergo cell division including anaplasia in palisade mesophyll, whereby cells that are already differentiated can revert to embryonic, meristematic cells. This de-differentiation, not observed in *E. globulus,* allows for multiple regressive cell divisions and results in groups of smaller meristematic cells

forming from one or more larger cells (Fink 1999). A high capacity for cell division due to the presence of isobilateral palisade layers, and the occurrence of both anticlinal and periclinal division in *E. nitens* results in a more effective necrophylactic periderm than in juvenile *E. globulus* leaves. The absence of a palisade layer on the abaxial leaf surface of juvenile *E. globulus* leaves resulted in cell division from only the adaxial palisade layer and hypertrophy of existing palisade and spongy mesophyll cells to form a necrophylactic periderm. The periderm was slower to form and distorted compared with *E. nitens* which suggests that *E. globulus* produces cell wall strengthening compounds too slowly to provide resistance to *Mycosphaerella* pathogens. This is consistent with findings by Smith *et al.* (2006), who observed that a more resistant provenance of *E. nitens* was able to restrict pathogen spread at an earlier stage of *Mycosphaerella* lesion development than a more susceptible provenance grown in South Africa.

Rapid formation of necrophylactic periderms in *E. nitens* leaves allows for earlier deposition of defence chemicals, which react and cross link with each other to form complex polymers that reinforce cell walls and create an impermeable barrier to the pathogen (Agrios 2005). This confines the pathogen much earlier in its life cycle and prevents further disease development. Significant deposits of suberin and lignin were observed in the early developmental stages of *E. nitens* lesions, which did not occur in *E. globulus*. Early lignification of cells has enhanced resistance in many hosts under fungal attack (Dushnicky, Ballance *et al.* 1998; Hammerschmidt and Küc 1982; Southerton and Deverall 1990). This study indicates that early deposits of suberin and lignin increase the strength of cell walls in the necrophylactic periderm. This would enhance defence against compressive forces (such as that of a fungal infection peg), and

prevent a) the diffusion of toxins and enzymes from the fungus into host cells and b) translocation of water and nutrients from host cells to the fungus, as suggested in other host-pathogen interactions (Hawkins and Boudet 2003; Vance, Kirk *et al.* 1980).

Variation in *Mycosphaerella* resistance is not limited to inter-species differences within the *Eucalyptus* genus. Within *E. globulus*, tolerance to *Mycosphaerella* infection is increased both upon heteroblastic change to adult foliage and with leaf ageing. Correspondingly both adult foliage and older juvenile leaves have higher palisade densities than young juvenile *E. globulus* leaves (James and Bell 2001). There is some evidence that hyphae of some species of *Mycosphaerella* may not be able to penetrate between tightly packed cells such as those in palisade mesophyll layers (Park 1984). The presence of isobilateral palisade mesophyll layers in *E. nitens* may therefore provide an additional barrier to hyphal proliferation in *E. nitens* compared with only one palisade layer in juvenile *E. globulus* leaves.

Trends in the content of anthocyanin and chlorophyll were similar for both tree species (*i.e.* anthocyanin was accumulated in early stages of lesion development and chlorophyll concentrations decreased with increasing lesion development). However, concentrations of anthocyanin and chlorophyll at different stages of lesion development were different between tree species. Significantly higher levels of anthocyanin and chlorophyll were recorded in *E. nitens* for most lesion categories, compared with *E. globulus*. The accumulation of anthocyanin under these circumstances is likely to have a role in protecting cellular structures from photo-oxidative damage; as has been suggested by several authors in other plant species (e.g. Neil and Gould 2003; Neil,

Gould *et al.* 2002). Higher concentrations of chlorophyll in necrotic lesion tissue of *E. nitens* compared with *E. globulus* may potentially be due to the degree of hyphal proliferation within the leaf. Development of *Mycosphaerella* hyphae within *E. nitens* leaves may be hindered by cellular architecture or chemical components within the leaves of *E. nitens*. Future studies to investigate anatomical changes with *Mycosphaerella* infection in other eucalypt species are required. The development of techniques for artificial inoculation and quantification of hyphae within a lesion will aid these investigations.

4.2 Anatomical variation of juvenile *Eucalyptus nitens* leaves and resistance to Mycosphaerella leaf disease.

4.2.1 Introduction

Eucalyptus nitens is indigenous to south-eastern Australia and occurs naturally in populations ranging from northern New South Wales (NSW) to the Victorian Alps (Purnell and Lundquist 1986). Its success in plantation forestry has been due mainly to rapid growth, favourable wood properties and frost hardiness, making it suitable for planting in high altitude (600–1600 m) or cold/frosty areas where growth of *Eucalyptus globulus* (Labill) and *Eucalyptus grandis* (W. Hill) is unsatisfactory. *Eucalyptus nitens* was introduced into South Africa as a plantation species in 1926 (Poynton 1979) and is now a primary plantation species, grown for pulp and paper products (Hunter *et al.* 2004). However, the planting of *E. nitens* in the summer rainfall areas of South Africa has also increased the risk of disease epidemics such as Mycosphaerella leaf disease (MLD) (e.g. Mohammed *et al.* 2003), which is known to cause severe blighting and defoliation in warm, humid environments (18 º/24 ºC – night/day) where there is ample leaf wetness (Park 1988a) to assist spore maturation, ejection and dispersal.

In South Africa, *Mycosphaerella* species were first discovered on a *Eucalyptus globulus* plantation in the Eastern Cape Province in 1925 (Doidge 1950) and then again in 1933 near Capetown (Verwoerd and Du Plessis 1933). At this time *E. nitens* appeared to be relatively tolerant to MLD. Research was commenced in 1973 to decide the most suitable provenances to grow on high altitude sites, with selection for growth rate, stem form and wood quality (Purnell and Lindquist 1986). The emergence of *Mycosphaerella* species on *E. nitens* resulted in additional selection for MLD resistance in the Victorian, southern NSW and northern NSW provenances (Lundquist and Purnell 1987). The

results from this trial suggested that NSW provenances were considerably more resistant to MLD than Victorian provenances, with provenances from northern NSW demonstrating a higher tolerance to MLD than those from southern NSW.

Research into the response of *Eucalyptus* species to foliar pathogens at a cellular level is in its infancy. In *E. globulus* there is some evidence that leaf density (influenced by internal leaf structure) may contribute to resistance to *Mycosphaerella* species (Smith *et al.* 2005). Increased cell density may enhance plant resistance by preventing infection of certain *Mycosphaerella* species due to their inability to penetrate closely packed palisade cells (Park 1984). This theory is supported by studies associating the density of palisade parenchyma with resistance to several foliar diseases (Basra *et al.*, 1985; Mayee and Suryawanshi 1995; Yang 2000).

Parenchyma cells have various functions that include photosynthesis and the manufacture and transport of numerous chemicals used within the leaf and throughout the plant. They remain largely embryonic so that under the appropriate stimulation they may specialise further and develop into a large variety of other cell types (Weisz and Fuller 1962). Upon infection or injury, parenchyma cells undergo cellular division and abnormal cell swelling (hypertrophy) to fill airspaces in the leaf and form a continuous periderm to delimit the pathogen. Periderm formation not only restricts pathogen spread but retains the hydraulic integrity of the surrounding healthy tissue. Early lignification of cells after fungal attack has enhanced resistance in cucumber (Hammershmidt and Kuć 1982) and wheat (Southerton and Deverall 1990; Dushnicky *et al.* 1998).

The cellular response of *E. nitens* to infection by *Mycosphaerella* species has not been characterised. Plantings of both northern and southern NSW provenances of *E. nitens* in mixed plantations in the Mpumalanga province of South Africa have enabled a comparative study to test the working hypothesis that variations in leaf anatomy affect the formation of necrophylactic periderms and the effectiveness of pathogen restriction in *E. nitens.* The specific aims were to:

1. Quantify variations between provenances in leaf thickness, proportions of spongy and palisade mesophyll, cell size, density and relative proportion of airspace.
2. At two stages of infection (initial and necrotic) characterise the response to infection by studying the cellular divisions and modifications required to form necrophylactic periderms.

4.2.2 Materials and Methods

4.2.2.1 Leaf Material

Leaves with and without *Mycosphaerella* symptoms were sampled from both the Ebor provenance of northern NSW (herein referred to as resistant) and the Tallaganda provenance of southern NSW (herein referred to as susceptible) from a commercial plantation, at Rooihoogte (26°22'60S: 30°25'60E) in the Mpumalanga province of South Africa. The leaves of trees from the resistant and susceptible provenances were distinctly different and could be easily distinguished visually. At the time of sampling all trees in the plantation bore juvenile leaves. The resistant provenance had narrower, more vertically oriented leaves, whilst the susceptible provenance had wider, glaucous leaves. The plantation was naturally infected with three major species of *Mycosphaerella*, namely *Mycosphaerella nubilosa* (Cooke) Hansf., *Mycosphaerella*

marksii Carnegie and Keane and *Mycosphaerella lateralis* Crous and M.J. Wingf. Species were identified using ascospore size, shape and germination patterns as described by Hunter, Crous *et al*. (2004).

Five trees from each population were chosen by strategic sampling, which included starting at an initial row, moving up two trees and along one row where the next tree was sampled. If that tree was not of the correct provenance then the next tree of the correct provenance in that row was chosen. The leaves sampled were from the fourth leaf pair and assumed to be of the same age. Five leaves with initial and necrotic lesions were sampled from each tree. Five asymptomatic control leaves were also sampled that were from the same leaf stage and the same trees. Initial symptoms of lesion development were identified as 1–4 mm diameter distinct, dispersed purple spots visible on either the abaxial or adaxial surface. Initial symptoms were sampled to assess the type and quantity of induced cell division occurring after infection to form an initial necrophylactic periderm. Necrotic lesions were identified as >5 mm diameter necrotic lesion, light to dark brown/grey in coloration, without a purple margin. Necrotic lesions were sampled to assess the integrity and accumulation of defence chemicals (lignin, suberin and flavanoids) in advanced formation of necrophylactic periderms.

4.2.2.2 Fixed-sample preparation

Lesion sections of approximately 2 mm^2 were cut from the edge of each of the two lesion categories and from asymptomatic tissue. Asymptomatic tissue was taken from the middle section of uninfected leaves to the right of the midrib. Sections were fixed in 2.5 % glutaraldehyde in 0.1 M Sörensen's phosphate buffer, pH 7.2, for 15 h at room temperature. Following two buffer washes (each for 20 min), the samples were

dehydrated in an ascending ethanol series in 20 % increments, finishing with two changes (each for 20 min) of 100 % ethanol before embedding in Quetol resin. Transverse sections 2-6 µm thick were cut on a Reichart OmU2 ultramicrotome fitted with a glass knife. Sections were expanded and gently heat-fixed onto clean glass microscope slides before staining.

4.2.2.3 Constitutive cell density

Three Quetol-embedded sections 50 µm apart were stained for five minutes in Toluidene Blue O stain (1 % Toluidene Blue O in 1 % aqueous sodium borate which was diluted 1:10 with distilled water before staining). After staining, sections were rinsed thoroughly with distilled water; de-stained for 50 s in 70 % ethanol and then dehydrated in 100 % ethanol for 1 min. Sections were photographed at 100 ×. Three vertical transects through each leaf section (avoiding oil glands and vascular bundles) were used to assess the widths of cuticular, epidermal, palisade and spongy mesophyll cell layers and the proportion of palisade and spongy mesophyll of sample thickness measured per transect. Values for transects were averaged for each component before analysis. The same sections were then photographed at 200 ×, where cellular dimensions (length, width and area) and using a horizontal transect the number of palisade cells per 100µm and the proportion of airspace in the palisade mesophyll layer were determined. All data were analysed using general analyses of variance using the statistical package GenStat®, using resistance as treatment and leaf as a block.

4.2.2.4 Pathogen restriction

Quetol-embedded sections of initial and necrotic disease symptoms were assessed. Phloroglucinol-HCl was used to stain for lignin deposition. Sections were immersed in

1% w/v phloroglucinol solution in 70 % ethanol for 5 min. Excess phloroglucinol solution was poured off and sections were covered with two drops of concentrated HCl, covered with a glass cover slip and examined with a Zeiss compound light microscope. The sections were photographed immediately as the reaction faded with time. Bright red to orange areas appeared in the presence of lignin. Suberin was observed on the same sections at 365 nm using a Zeiss Axiovert fluorescence microscope fitted with a mercury lamp where the phloroglucinol was used as a counter-stain to eliminate the autofluorescence of lignin (Biggs 1984). Naturstoffreagenz A (1 % diphenylboric acid 2-aminoethyl ester in methanol) was used to detect flavanoids. Slides were flooded with the solution for 5 minutes. Flavanoids were detected at 364 nm (caffeic acid esters and styrylpyrones)(purple beam), 450–490 nm (styrylpyrones) (blue beam) and at >590 nm (red beam) (Hutzler, Fischbach *et al.* 1998) using a Zeiss Axiovert florescence microscope fitted with a mercury lamp.

4.2.3 Results

Results from this study are also summarised in poster format in Appendix 6.

4.2.3.1 Constitutive cell density

The resistant provenance was observed to have significantly thinner leaves ($p < 0.001$), thicker adaxial and abaxial cuticles ($p = 0.008$ and 0.002 respectively) and thinner adaxial and abaxial epidermal layers ($p = 0.002$ for both) than the susceptible provenance (Table 4.2.1). Leaves from the resistant provenance had a higher proportion of palisade cells ($p = 0.013$) and a lower proportion of spongy mesophyll ($p < 0.01$) (Table 1). Mean palisade cell dimensions (width and length) were not significantly different between provenances, but more palisade cells were recorded per mm of horizontal transects of leaves from the resistant provenance (Figure 4.2.1a), compared

with the susceptible provenance ($p < 0.001$) (Figure 4.2.1b) (Table 4.2.1). Palisade cells were more tightly packed in the resistant provenance as significantly less inter-cellular airspace was observed in the adaxial and abaxial palisade layers of the resistant provenance when compared with the susceptible provenance ($p = 0.002$ and 0.026 respectively).

Table 4.2.1: Quantitative anatomical features of *E. nitens* leaves that are resistant (NNSW) and susceptible (SNSW) to *Mycosphaerella leaf disease*. Mean values, standard errors of the differences of means (SE) and their statistical significance (P value) is given. Characteristics of susceptible and resistant leaves were statistically significant at $p < 0.05$.

	Mean		SE	p
Character	Susceptible	Resistant		
Total leaf thickness (µm)	329.7	280.9	3.6	< 0.001
Thickness of cuticle (µm)				
Adaxial	4.4	5.7	0.3	0.008
Abaxial	5.9	4.7	0.2	0.002
Thickness of epidermis (µm)				
Adaxial	12.7	11.4	0.3	0.002
Abaxial	13.3	11.3	0.5	0.002
Thickness of palisade mesophyll (µm)				
Adaxial	46.4	46.5	2.7	0.986
Abaxial	57.7	50.4	1.5	< 0.001
Thickness of spongy mesophyll layer (µm)	190.5	149.7	1.8	< 0.001
Total palisade as a proportion of leaf thickness (%)	31.6	34.5	5.3	0.013
Spongy mesophyll as a proportion of leaf thickness (%)	57.7	53.1	0.8	< 0.001
Airspace in spongy mesophyll layer as a proportion of leaf thickness (%)	43.2	35.6	0.7	0.152
Proportion of airspace in palisade layer (%)				
Adaxial	54.1	35.8	3.05	0.002
Abaxial	38.0	28.8	5.36	0.026
No. palisade cells per mm				
Adaxial	17	21	1.7	< 0.001
Abaxial	13	19	2.9	< 0.001
Width of palisade cells (µm)				
Adaxial	9.7	9.4	0.2	0.243
Abaxial	9.3	9.2	3.9	0.880
Length of palisade cells (µm)				
Adaxial	46.1	42.8	1.7	0.295
Abaxial	33.4	31.7	0.8	0.345

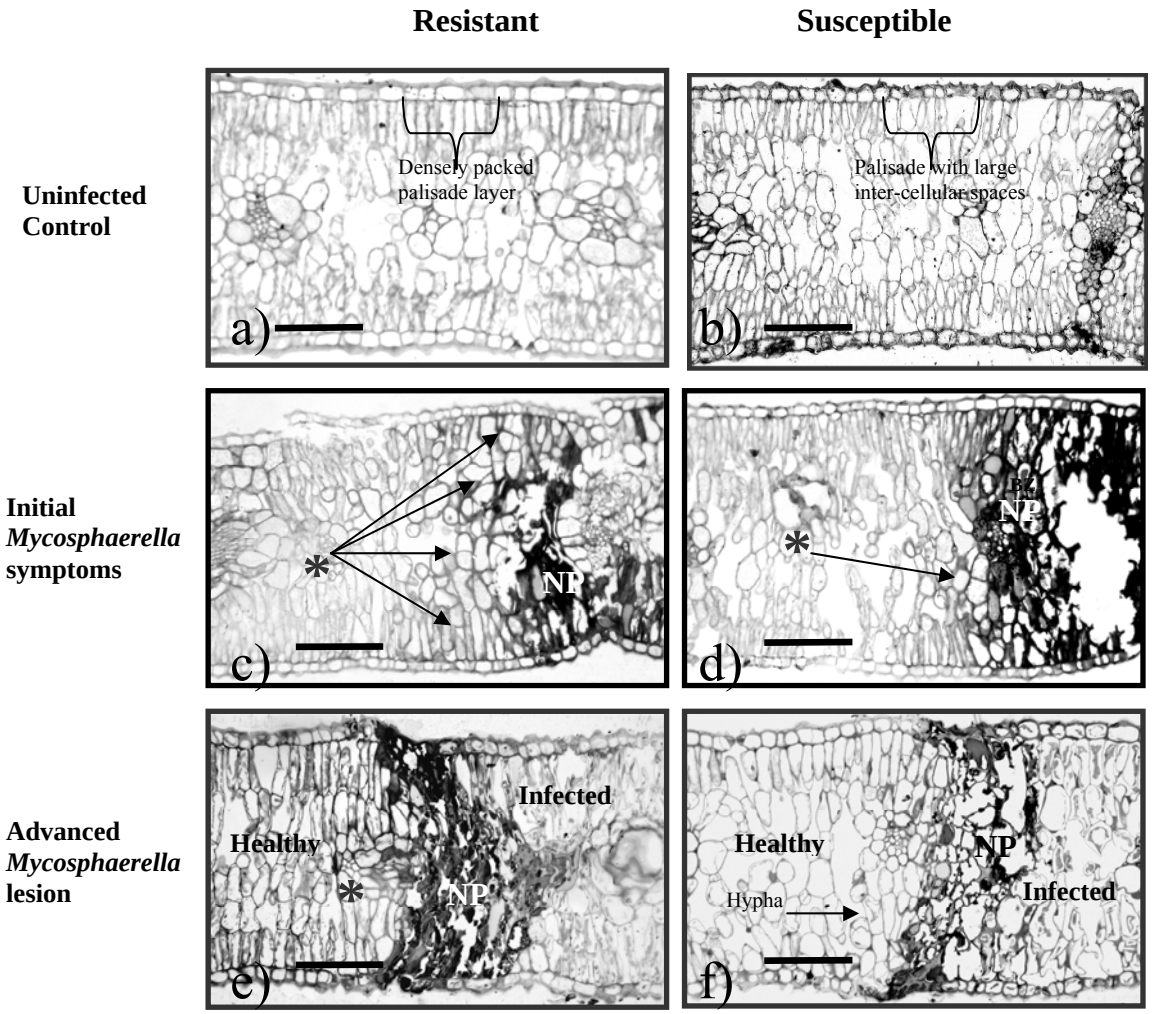
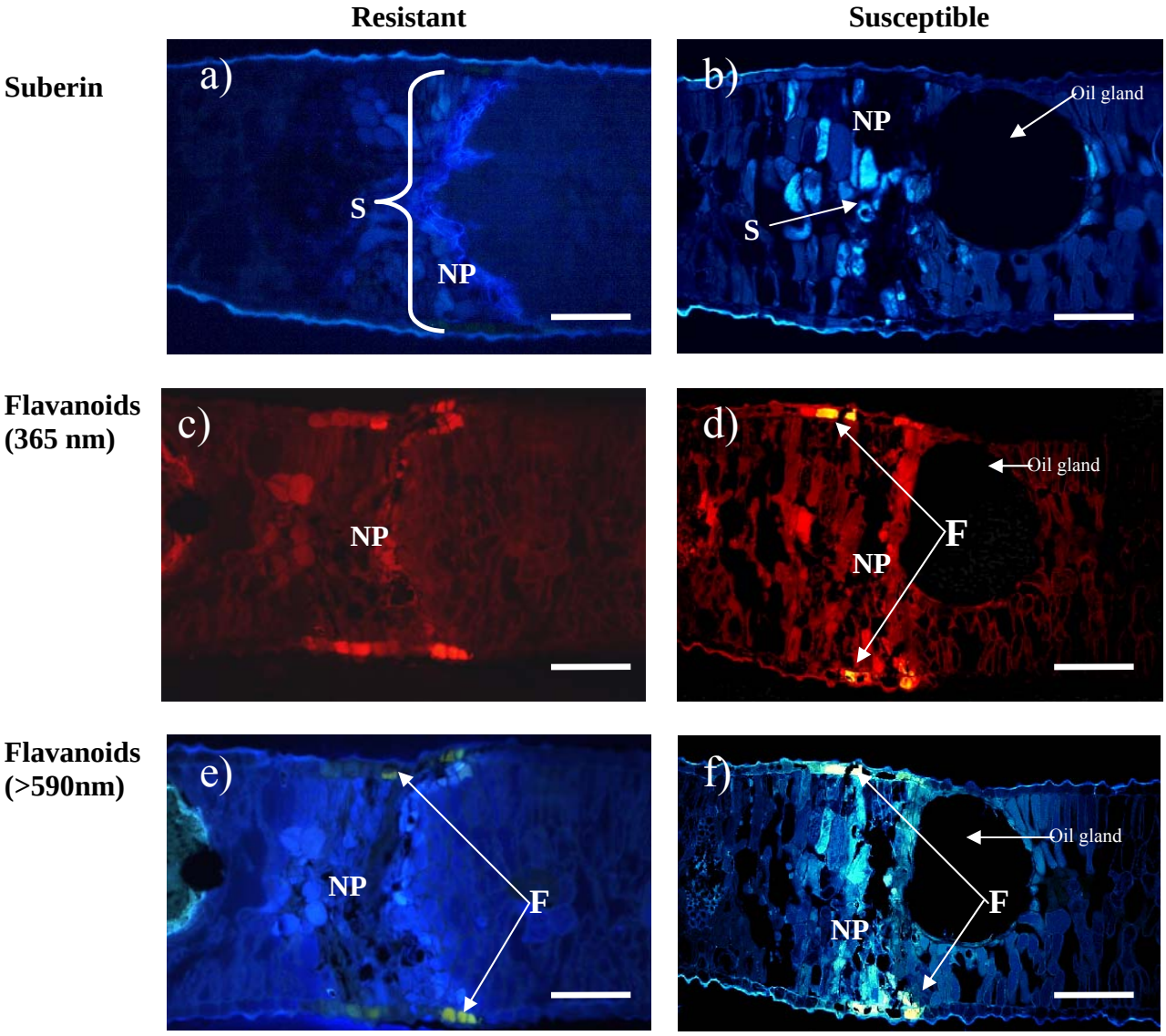


Figure 4.2.1: Typical transverse sections through resistant and susceptible leaves that were healthy (a&b), displaying initial *Mycosphaerella* lesion symptoms (c&d) and advanced *Mycosphaerella* lesion symptoms (e&f). Densely packed palisade mesophyll and reduced intracellular airspace between palisade mesophyll cells was observed for leaves of resistant (a) compared with susceptible (b). Upon infection this was associated with large amounts of cell division and the coalescing of cells to form a necrophylactic periderm in resistant leaves (c) whilst only a small amount of cell division was observed in susceptible leaves (d). In advanced lesions on resistant leaves (e) cells coalesced to form an organised necrophylactic periderm (> 5 cells in thickness) which was infiltrated with defence chemicals (darker regions) such as lignin, suberin and other phenolics and cells continued to divide (*) on the healthy side of the necrophylactic periderm (NP). In advanced lesions on susceptible leaves (f), a distinct and organised necrophylactic periderm was not observed. In some sections fungal hypahe had already begun to penetrate cells on the healthy side of the periderm (f).

4.2.3.2 Pathogen restriction

Transverse sections through initial lesions revealed a higher level of cell division adjacent to the infection site in the resistant (Figure 4.2.1c) compared with the susceptible provenance (Figure 4.2.1d) in all replicates. In resistant leaves, existing cells and divided cells coalesced to create a distinct zone that was often greater than five cell layers in necrotic samples. The zone was infiltrated heavily with suberin (Figure 4.2.2a), lignin (not presented) and flavanoids (Figures 4.2.2c and e). The necrophylactic periderm was continuous, organised and most probably impermeable to hyphae, water and toxins, as cells on the uninfected side of the barrier were healthy and had retained the capacity to continue cell division.

In the susceptible provenance, sections through initial lesions revealed that limited cell division occurred only in cells adjacent to the infection site (Figure 4.2.1d) in all replicates. In advanced lesions, the arrangement of cells into a necrophylactic periderm was unorganised (Figure 4.2.1f), which resulted in a dispersed accumulation of lignin, suberin (Figure 4.2.2b), flavanoids (365 nm and >590 nm; Figure 4.2.2c and e). In 40 % of sections through necrotic lesions of susceptible leaves, the necrophylactic periderm was not continuous, allowing hyphae to progress into healthy tissue. Flavanoids were observed in epidermal cells on the infected side of the necrophylactic periderm in both resistant and susceptible leaves in all replicates.



Bar = ~90 µm resistant, ~110 µm susceptible

Figure 4.2.2: Typical transverse sections through advanced *Mycosphaerella* lesions on resistant and susceptible leaves where the necrophylactic periderms (NP) have been histochemically stained for suberin (S) (blue fluorescence in a & b) and flavanoids (F) (yellow fluorescence in c-f).

4.2.4 Discussion

In eucalypts, variation in resistance to *Mycosphaerella* leaf spot pathogens has been observed between species (Carnegie *et al.* 1998), subspecies (*Eucalyptus globulus*, Carnegie and Ades, 2005; Carnegie *et al.* 1994) provenances (*Eucalyptus nitens*, Purnell and Lundquist 1986) and families (*E. globulus*, Milgate *et al.* 2005). In addition, resistance to *Mycosphaerella* infection increases with ontogenetic transition from juvenile to adult foliage (Carnegie and Ades 2002; Dungey *et al.* 1997) and within the juvenile leaf stage, whereby newly formed, soft and expanding leaves have been identified as the most susceptible whilst older leaves are more resistant (Park 1988b). Whilst resistance to *Mycosphaerella* infection is likely to be a combination of several traits, there appears to be a relationship between resistance and the proportion of palisade mesophyll. For example, *Eucalyptus nitens* (isobilateral palisade) has a higher tolerance to *Mycosphaerella* infection compared with *E. globulus* (unilateral palisade) (Smith *et al.* 2005) and palisade mesophyll layers increase in number from juvenile (one palisade layer) to adult foliage (~ 4–6 palisade layers) in *E. globulus* (James and Bell 2001).

Higher palisade densities (or volume fractions) have been associated with resistance to several leaf spot pathogens including *Cercospora* (Basra *et al.* 1985) and *Phaeoisariopsis* leaf spot in groundnut (Mayee and Suryawanshi 1995), Frog-eye leaf spot in soybean (Yang 2000) and *Septoria* leaf spot in celery leaves (Edwards *et al.* 1999). A tighter packing of palisade cells was also observed in resistant families of *E. globulus* when compared with susceptible families, however, this was a qualitative assessment (Smith *et al.* 2005). For the first time we have quantitative evidence that a tighter packing of palisade mesophyll cells may be associated with resistance to MLD.

Evidence from past and present studies suggests that compact palisade layers may slow or prevent hyphal development due to the inability of some *Mycosphaerella* species to penetrate and colonise tightly packed palisade cells (Smith *et al.* 2005; Park 1984). This, coupled with increases in cuticle thickness in resistant leaves compared with susceptible leaves, may act as a considerable barrier to hyphal penetration.

From histological examination, we can suggest that formation of necrophylactic periderms in *E. nitens* rely heavily upon cell division and the incorporation and coalescing of cells to link the adaxial and abaxial leaf surfaces. The host response of *E. nitens* to *Mycosphaerella* species in the current study was similar to that observed for the Victorian provenance of *E. nitens* (Smith *et al.* 2005) and *E. globulus* (Park and Keane 1982; Smith *et al.* 2005) and therefore the production of highly lignified and suberised periderm is assumed to be a generalised host response to wounding and fungal invasion. However, the differences between resistant and susceptible provenances are likely to be exhibited in the speed and strength of pathogen restriction. Our images of initial and advanced infection show that resistant leaves are more proficient in cellular division and the formation of effective necrophylactic periderms. It is hypothesised from the current study that resistant leaves are thinner and have a higher proportion of dense palisade cell layers and reduced proportions of spongy mesophyll. Therefore it is possible that resistant leaves would have a higher capacity to divide after infection and also require less cellular differentiation to form a necrophylactic periderm.

Whilst individual *Mycosphaerella* species can be detected within lesions using species-specific primers (Kularatne *et al.* 2004; Glen *et al.* 2006), the development of techniques such as real-time PCR will enable the hyphal mass within a lesion to be

compared between resistant and susceptible provenances after infection. Future studies might also concentrate on comparing host pathogen interactions with the pathogenicity of *Mycosphaerella* species and biotypes on resistant and susceptible trees. Further testing of *E. nitens* provenances and families will determine the suitability of using palisade density and internal leaf structure as an indicator of resistance in *E. nitens*.

4.3 Associations of stomatal wax coverage and leaf density of juvenile *Eucalyptus globulus* leaves with resistance to Mycosphaerella leaf disease.

4.3.1 Introduction

Mycosphaerella leaf disease (MLD) is caused by a complex of ascomycete fungi from the *Mycosphaerella* genus (Glen, Smith *et al.* 2006) and is one of the most important foliar diseases of *Eucalyptus globulus* Labill plantations worldwide (Mohammed, Wardlaw *et al.* 2003). Productivity of eucalypt plantations is affected when MLD results in greater than 25% defoliation (Lundquist and Purnell 1987). Decreases in volume (Carnegie, Keane *et al.* 1994; Milgate, Potts *et al.* 2005) and alterations to stem form and quality (Wardlaw 2001b) have been reported as a result of MLD and will potentially cause delays in harvesting due to slower growth rates. The use of fungicides is not suitable for controlling MLD due to the impracticalities and cost of regular application during peak infection periods. Weather conditions are usually not favourable for spraying, large equipment is required for trees over 3 m and sites are often inaccessible. Genetic variation of *E. globulus* at a provenance and family level to *Mycosphaerella* species has been identified (Carnegie, Keane *et al.* 1994; Dungey, Potts *et al.* 1997; Hood, Gardner *et al.* 2002; Milgate, Potts *et al.* 2005) and the development of resistant genotypes may provide an effective and economical way to minimise the effects of MLD on productivity (Milgate, Potts *et al.* 2005). The expression of resistance in field trials depends on the type and severity of disease, with reported narrow-sense heritabilities of disease severity range from 0.12 and 0.34 following a mild infestation (Dungey, Potts *et al.* 1997) up to 0.6 following a heavy infestation (Milgate, Potts *et al.* 2005). The latter study suggests that foliar resistance to MLD is

under strong genetic control in *E. globulus*, however little is known of the underlying mechanisms.

MLD infects predominantly juvenile foliage, with newly formed leaves (first three pairs from the bud) being the most susceptible to infection and lesion development (Park 1988a). *Mycosphaerella* species infect via wind-dispersed ascospores and/or splash dispersed conidia (Park 1988a). The two species regularly associated with MLD epidemics are *Mycosphaerella cryptica* (Cooke) Hansf. and *Mycosphaerella nubilosa* (Cooke) Hansf. (e.g. Carnegie and Keane 1994; Milgate, Vaillancourt *et al.* 2005). Whilst there is some evidence for *M. cryptica* directly penetrating the leaf cuticle, both species require stomatal openings as entry points (Park and Keane 1982). Associations between stomatal frequency and resistance have been recorded for several *Mycosphaerella* and *Cercospora* pathogens including *Mycosphaerella fijiensis* on banana (Craenen, Coosemans *et al.* 1997), frog-eye leaf spot (*Cercospora sojina*) on soybean (Yang 2000) and *Mycosphaerella berkelayi* on groundnut (Jyosthna, Reddy *et al.* 2004). Resistance due to lower stomatal frequency can also be enhanced by thick wax coverage of the leaf cuticle (Craenen, Coosemans *et al.* 1997). Epicuticular wax morphology is distinctive for eucalypt species and is often used in taxonomy; however, considerable variation has been observed for quantity and coverage within species (Li, Madden *et al.* 1997). Epicuticular waxes, in particular aliphatic benzyl and phenylethyl alkonate components, also enhance resistance to insect attack in *Eucalyptus globulus* (Jones, Potts *et al.* 2002) by deterring *Mnesampela privata* oviposition (Rapley, Allen *et al.* 2004). For fungal resistance, wax properties reduce wettability and hinder water adhesion to the leaf surface, which is necessary for the germination of pathogens

(Neinhuis and Barthlott 1997). This also lengthens the incubation time of the fungus (Craenen, Coosemans *et al.* 1997).

Alternatively, post infectional mechanisms such as the formation of necrophylactic periderms and the accumulation of toxic secondary metabolites to delimit pathogens may provide a more durable form of resistance. Recent studies have suggested that a high constitutive cellular density may be advantageous for necrophylactic periderm formation (Smith, Gill *et al.* 2005) and may be linked with increased leaf density measured on a dry weight per unit leaf area basis (Castro-Diez, Puyravaud *et al.* 2000). Higher palisade densities have also been linked with resistance to leaf spot pathogens in soybean (Yang 2000) and groundnut (Mayee and Suryawanshi 1995).

The present study compared leaf traits between pairs of resistant and susceptible *E. globulus* plants of varying levels of genetic relatedness. Leaf traits such as stomatal density (wax-on and wax-off), amounts and distribution of epicuticular wax, palisade density, mass to area ratio (LMA) and leaf phenolics were studied to identify traits that may confer resistance of *E. globulus* to MLD.

4.3.2 Materials and Methods

4.3.2.1 Plant material

Leaf traits were examined on resistant and susceptible genotypes of *E. globulus* that were derived from the same seed used to establish two adjacent *Mycosphaerella* field trials as described by Milgate, Potts *et al*. (2005) (Table 4.3.1). The first field trial consisted of 864 trees originating from 53 open pollinated families and was assessed in its juvenile leaf phase for *Mycosphaerella* damage. A ten point scoring system for

% severity of the whole tree was used (1=0-3 %, 2=4-6 %, 3=7-12 %, 4=13-17 %, 5=18-25 %, 6=26-38 %, 7=39-50 %, 8=51-63 %, 9=64-75 %, 10=76-100 %) (Milgate, Potts *et al*., 2005a). Milgate, Potts *et al*. (2005a) ranked families as resistant and susceptible after they had experienced a severe epidemic (mean damage = 34 %). Damage in the trial was extremely variable at the tree level with the damage to foliage ranging from complete to virtually unaffected. The narrow sense heritability for disease severity was recorded as 0.6 suggesting that resistance to *Mycosphaerella* in *E. globulus* was under high genetic control (Milgate, Potts *et al.* 2005a). The adjacent field trial comprised seedlings of 20 open pollinated families from the Jeeralang provenance of *E. globulus*. Resistant and susceptible genotypes were studied at the following levels of genetic relatedness, 1. inter-provenance (Taranna – resistant and Sth Flinders - susceptible), 2. intra-provenance (resistant and susceptible families from the Jeeralang provenance) and 3. within family (Leprenna and Seymour).

Table 4.3.1: The most divergent resistant and susceptible families were selected on the basis of their mean severity score recorded for the two adjacent trials in NW Tasmania. Four levels of genetic relationship were investigated.

Pair	Relationship	Provenance/ Family	Family code	Least square mean damage (%) (SE)	n	Ranking
1	Inter-provenance	Taranna	T7_08	15.4 (4.94)	10	R
		Sth Flinders	SF_13	62.8 (3.41)	21	S
2	Intra- provenance	Jeeralang	JN2	5.9 (2.99)	20	R
		Jeeralang	JN21	18.2 (2.38)	20	S
3	Within family	Leprenna	L6_05	20.8 (4.71)	11	R
		Leprenna	L6_10	33.9 (4.71)	11	S
4	Within family	Seymour	S2_09	24.2 (3.19)	24	R
		Seymour	S2_10	39.0 (4.50)	10	S

Further information on the material selected from the Woolnorth seed orchard for the first trial is described in detail in Volker (1990) and Dutkowski and Potts (1999)

Seed was germinated in October 2003 in seed raising trays. Seedlings were re-potted three times during the course of the experiment (4 cm seeding tubes in October 2003, 15 cm pots in January 2004 and 20 cm pots in June 2004). Every three weeks, until June 2004, plants from each family were randomised in their location and were fertilised until run-off with Nitrosol liquid fertiliser (4:1:3, N: P: K) (Envirogreen Pty.Ltd. Stapylton QLD). Upon re-potting, plants were fertilised with Osmocote slow-release pellet fertiliser (Scotts Australia Pty. Ltd., Baulham Hills, NSW) (10:1:5, N: P: K –

2 teaspoons per plant). Plants were kept outdoors for the duration of the study and all plants bore juvenile foliage. To eliminate the effects of induced responses due to sampling, plants were divided into three separate sets with each set being sampled only once. The first set was sampled at an age of 6 months to assess mass to area ratio and leaf morphology. The second set was sampled at 12 months to assess total leaf wax and stomata density and the third was sampled for constitutive total phenolics when the trees were 19 months old.

4.3.2.2 Leaf Mass to Area Ratio (LMA)

Six juvenile leaves (first three pairs from the leaf bud – pairs 1-3) were sampled per plant from ten plants of each family. The first three leaf pairs were chosen for all experiments in this study, as newly formed leaves are most susceptible to *Mycosphaerella* infection (Park 1984). Leaf area was measured using Winfolia® analysis of scanned images (Regent Instruments, www.regent.qc.ca). Leaves were placed into paper bags and dried at 70 ºC for 72 h before weighing. LMA was calculated by dividing dry weight (g) by fresh area (m^2).

4.3.2.3 Leaf morphology

Leaf sections (~3 mm^2) of third pair juvenile leaves were sampled from three plants per family and fixed in FAA (formaldehyde: acetic acid: 70 % ethanol, 5: 5: 9) for a minimum of 24 h at 4 ºC, dehydrated with an ethanol series and infiltrated in a LR White acrylic resin (Proscitech, Brisbane, Queensland, Australia) series. Samples were then polymerised in 100 % LR White resin for 8-10 h at 60 ºC. Transverse sections 8-10 µm thick were cut on a Reichart OmU2 ultramicrotome fitted with a glass knife.

Sections were expanded and gently heat fixed onto clean glass microscope slides before staining with Toluidine Blue (1 % w/v TBO in 1 % w/w sodium borate buffer).

4.3.2.4 Constitutive total leaf phenolics

One pair of leaves from the first three nodes was sampled per tree from 3 plants per family. Leaf material was prepared and extracted following the method outlined by Hagerman (1995). Freeze-dried, ground leaf material (0.5 g) was placed into a glass test tube with 5 mL of 70 % acetone. Samples were sonicated for 30 min at 4º C, centrifuged at 2800 g min^{-1} for 10 min. The supernatant was decanted and stored at 4 ºC. The process was repeated a further three times with supernatants combined for each sample. Leaf phenolics were determined using the modified Prussian blue assay for total phenolics (Graham 1992). The concentration of phenols (per unit of dry weight) was determined in relation to a gallic acid standard (SIGMA G-7384).

4.3.2.5 Stomatal Counts

Wax-on and wax-off stomatal counts were conducted on the upper and lower surfaces of three juvenile leaves per tree (pair 1-3), with three replicates per family. Within each leaf, three fields of view (FOV) were counted along a diagonal transect of each sample. Tissue samples (1 cm^2) were excised from the right hand side of the leaf margin in the middle of the leaf. For the first leaf pair, where the size of the leaf was too small to excise two samples, a sample was taken, spanning the leaf margin, from each leaf in the pair. One sample was de-waxed by rubbing the surface with dichloromethane soaked cotton buds and the number of stomata on both sides of the leaf was counted at 200 × magnification. The epicuticular wax was left on the second sample, which was gold

coated (40 nm) using a Bal-tec SCD050 sputter coater and observed under high vacuum (accelerated voltage of 15 kv) with an ElectroScan 2020 Environmental Scanning Electron Microscope (ESEM). Counts were made at 150 × as described above. For wax-on counts, stomata were included in the count if any part was visible through the wax. To enable a comparison between the two counts, wax-off counts were scaled up by 27 % to standardise variation in FOV between the light microscope (0.09 mm^2) and the ESEM (0.33 mm^2). The number of stomata per mm^2 was calculated.

4.3.2.6 Total Leaf Wax

A bulk sample of thirty leaves per tree was sampled from the first three pairs of five plants per genotype. Each leaf was removed at the base using tweezers and then dipped several times into a beaker filled with 40 mL dichloromethane to remove wax. The initial weight of the 50 mL beakers was obtained and then each beaker was re-weighed, to four decimal places, after the dichloromethane had evaporated. Leaf area was determined with Winfolia® as specified in the LMA method. An estimate of the relative amount of wax on a per unit leaf area (g/m^2) was determined.

4.3.2.7 Statistical analysis

For traits measured from the seedlings, a two-way fixed effects model including the effects of pair, resistance class (resistant, susceptible) and their interaction was fitted to the seedling data using the Proc Mixed of SAS (SAS Institute, version 9.1). The difference between resistant and susceptible genotypes was also tested using analyses of variance for each of the contrasts. Results from this study were regressed against the damage recorded from the Jeeralang and Woolnorth trials (Milgate, Potts *et al.* 2005) which had genetically identical material to this study.

4.3.3 Results

Results form this study are also summarised in poster format in Appendix 7.

4.3.3.1 Mass to area ratio and leaf morphology

The major difference between resistant and susceptible genotypes was found for LMA (Table 4.3.2) where leaves from resistant plants had a significantly higher LMA than their susceptible counterparts ($P < 0.001$) (Figure 4.3.1a). This difference was significant in the inter-provenance ($P = 0.011$), intra-provenance ($P = 0.034$) and one within family (Leprenna $P = 0.0005$) pair. Increased leaf mass per unit area did not explain the variation in susceptibility within the Seymour family pair ($P = 0.441$). LMA explained 0.49 % of the variation in MLD resistance observed in the two field trials assessed by Milgate, Potts *et al*. (2005) (Figure 4.3.2). Transverse sections through the leaves revealed more closely packed palisade mesophyll cells in uninfected resistant genotypes than in uninfected susceptible genotypes (Figures 4.3.3a and b).

Table 4.3.2: Least squares means and significance of pair, resistance class and their interaction for selected leaf traits.

Trait	Least square mean		Significance of effects		
	Resistant	**Susceptible**	**Pair (df=3)**	**Resistance (df=1)**	**Pair x resistance class (df=3)**
Stomata counts on the lower leaf surface (mm^{-1})					
Visible stomata (wax on)	16.71	21.91	0.682	0.061†	0.906
Total (wax off)	37.98	33.31	0.000 ***	0.238	0.127
Hidden (wax off – wax on)	21.4	11.40	0.0027 **	0.048 *	0.173
Stomata counts on the upper leaf surface (mm^{-1})					
Visible stomata (wax on)	0.28	1.93	0.029 *	0.029 *	0.096
Total (wax off)	0.39	0.82	0.665	0.337	0.468
M:A (g dry wt./m^2)	1.08	0.96	0.005 **	0.0003 ***	0.018 *
Total leaf wax (g/m^2)	0.14	0.14	0.062	0.824	0.000 ***
Constitutive leaf phenolics (ul/ug)	0.93	0.88	0.001 ***	0.146	0.000 ***

Significance: * $p < 0.05$ ** $p < 0.1$ and *** $p < 0.001$.
† Significant when non significant terms are excluded from the model ($F_{1, 38} = 4.2$; $p = 0.048$)

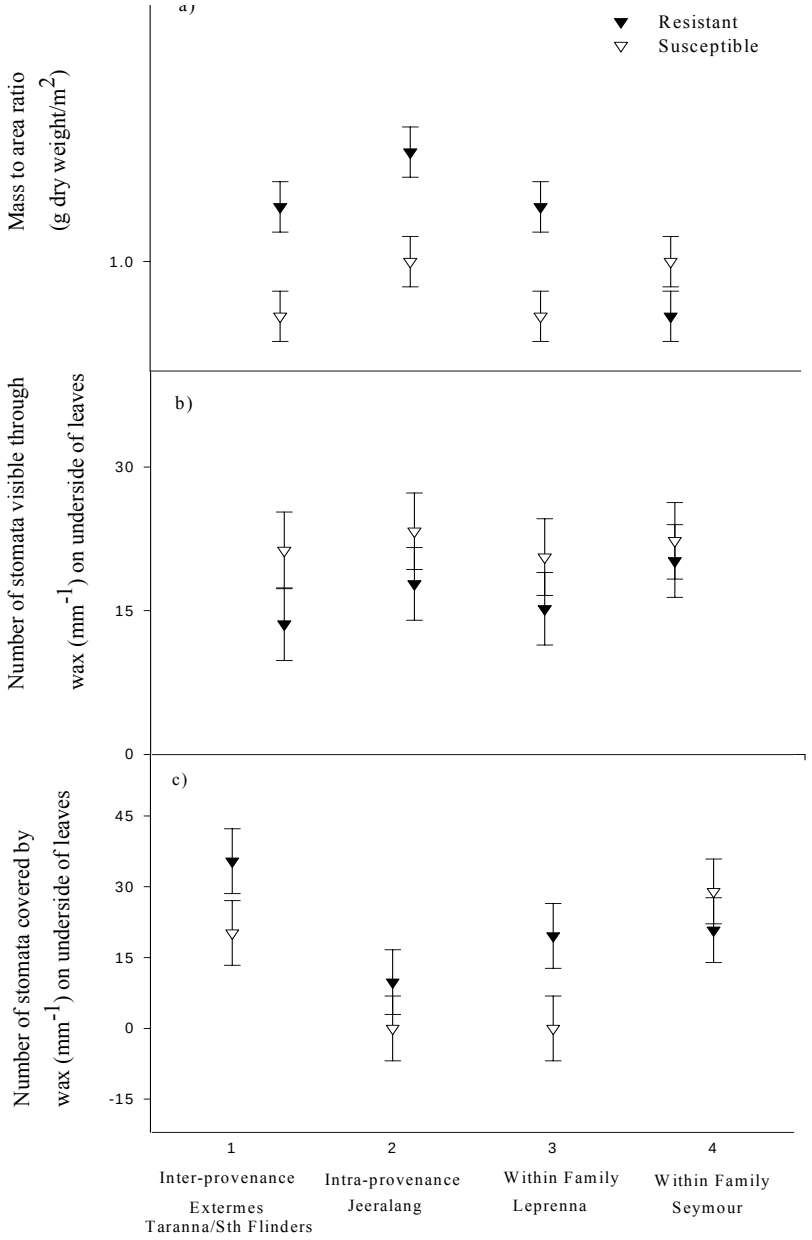


Figure 4.3.1: Mean (SE) mass to area ratio and stomata coverage by wax of resistant and susceptible genotypes for each level of genetic relatedness (inter, intra provenance and within family). Resistant leaves (a) were heavier per unit area, (b) had less visible stomata through the epicuticular wax layer and (c) significantly more stomata covered by wax than susceptible leaves.

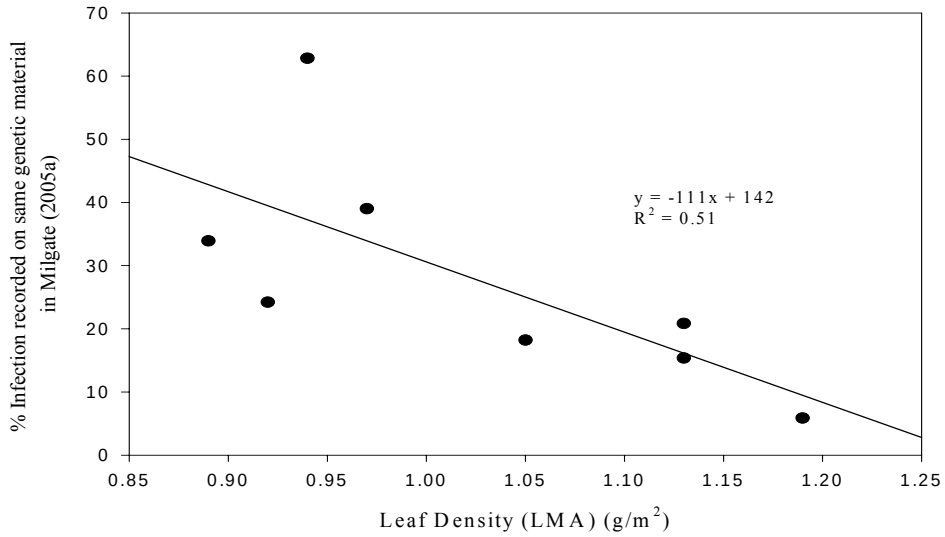
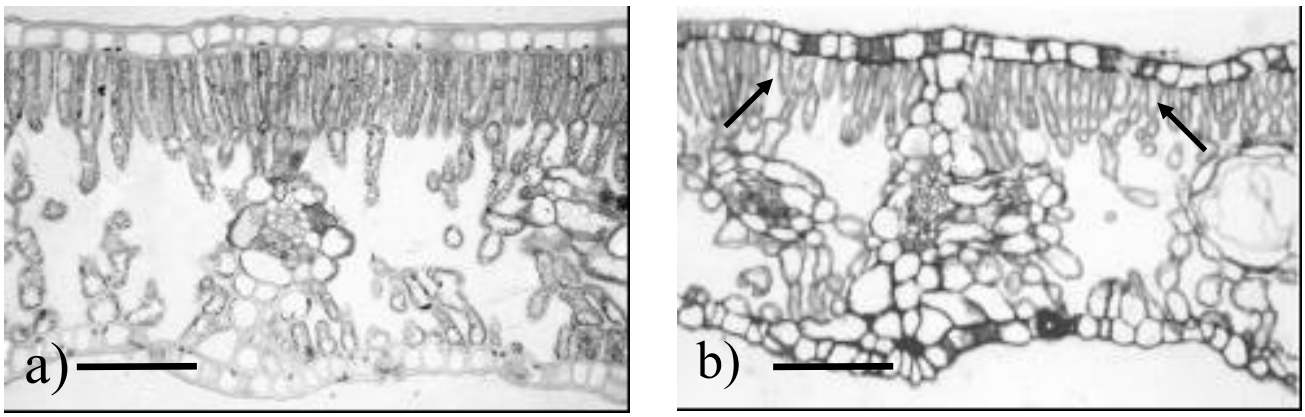


Figure 4.3.2: Correlation between severities of *Mycosphaerella* infection (% of effective leaf area lost) recorded in the initial adjacent field trials (Table 4.3.1) and LMA of replicate families recorded in this study.



Bar = 60 µm

Figure 4.3.3: Typical transverse sections through Leprenna resistant (a) and susceptible (b) leaves. Palisade mesophyll variation can be observed at an intra- family level with more intercellular airspace observed between cells of the susceptible (indicated by the arrows) than the resistant family.

4.3.3.2 Constitutive phenolics

Significant differences in constitutive leaf phenolics were observed between pairs and the interaction between pairs and resistance (P = <0.0001) (Table 4.3.2). The significant difference between pairs was mainly due to the low total phenols in the Seymour families compared to those from Leprenna and Jeeralang. The highly significant interaction term was due to the resistant family in the Taranna/Sth Flinders pair having greater phenols than the susceptible family. This trend was not evident in other pairs where significant differences between families were found.

4.3.3.3 Stomatal density and wax coverage

With wax on there was a trend for resistant families to have less stomata visible on the underside of the leaves (P = 0.06). This effect became significant when non-significant terms were excluded from the statistical model ($F_{1, 38}$ = 4.20; pz = 0.048) (Table 4.3.2) and held up over intra-provenance, inter-provenance and within family pairs. The upper surface of resistant leaves had significantly less visible stomata with wax on (P = 0.029) but not after the removal of epicuticular wax (Table 4.3.2). The number of stomata covered by wax (*i.e.* the difference between stomatal counts with wax on and with wax removed) was therefore significantly higher for resistant genotypes overall (P = 0.04). At a pair wise level the inter-provenance and the Leprenna contrasts had significantly more hidden stomata in resistant genotypes. Resistant genotypes from the Seymour contrast had less, but not significantly less, hidden stomata than their susceptible counterpart (Figure 4.3.1). The total amount of leaf wax did not vary significantly between resistant and susceptible families overall (Table 4.3.2) however higher wax coverage of stomata and stomatal guard cells was observed in resistant genotypes under SEM.

4.3.4 Discussion

The results from this study suggest that resistance to MLD may be correlated with leaf density (LMA) and the density of stomata exposed by epicuticular wax. The most significant variation between resistant and susceptible plants was observed for LMA and this occurred at an intra-provenance, inter-provenance and within family level.

LMA will increase if leaves have more cells (*i.e.* increased proportions of palisade and spongy parenchyma, epidermis, air space and sclerified tissues) and/or by increased density of individual cells (Castro-Diez, Puyravaud *et al.* 2000). Levels of leaf phenolics were not significantly different between resistant and susceptible pairs. This is with the exception of the South Flinders/Taranna comparison, which is most probably due to natural variation between provenances (O'Reilly-Wapstra, McArthur *et al.* 2004). Leaf oils were not assessed in this study, however a study by Li (1993) observed no significant differences between or within provenances of *E. globulus* (Geeveston, Tasmania and Otways, Victoria). Therefore it is unlikely that the variation in LMA observed in this study is due to high molecular weight compounds such as leaf phenolics and terpenes. The link between palisade density and LMA in eucalypts was initially made by Sefton (2002). Transverse sections through resistant and susceptible leaves in the current study suggest that resistant plants have a higher cellular density of mesophyll layers. More palisade mesophyll layers are also observed in *E. globulus* adult leaves (James and Bell 2001), which may explain the low incidence of MLD recorded on adult foliage (e.g. Barber, Kularatne *et al.* 2005; Carnegie, Keane *et al.* 1994; Dungey, Potts *et al.* 1997).

Palisade density has been associated with MLD resistance of juvenile *Eucalyptus nitens* leaves (Smith, Gill *et al.* 2005) and many other studies provide evidence that a higher palisade mesophyll density in leaves can provide resistance to leaf spot fungi in crops such as groundnut (Basra et al., 1985; Mayee and Suryawanshi, 1995) soybean (Yang, 2000) and celery (Edwards et al., 1999). The mechanism that associates closely arranged palisade cells with resistance to MLD remains largely unknown. It has been suggested that:

i) *Mycosphaerella* hypahe may not be able to penetrate between closely packed cells (Park 1984).

ii) An increased number of cells in leaves may allow for rapid compartmentalisation of the pathogen after infection. This is because fewer cell divisions are required to form a necrophylactic periderm from the abaxial to the adaxial leaf surfaces.

iii) There are more cell walls for the fungus to overcome to obtain resources (as a result of an increased number of cells per unit area) ; thereby slowing pathogen progression.

A reduced number of exposed stomata on resistant leaves (with wax on) may be a second genetic based mechanism for MLD resistance on *E. globulus*. In general, increased pathogen resistance is correlated with a decrease in overall stomatal density (Jyosthna, Reddy *et al.* 2004; Mahajan and Dhillon 2003; Ramos, Narayanan *et al.* 1992b), as this reduces the number of entry points into the leaf. Counts obtained after removing the epicuticular wax revealed that the stomatal density underneath of resistant leaves was not significantly different from, and was often higher than susceptible families, with the number of exposed/visible stomata reduced in resistant leaves by thick deposits of wax. It is possible that wax covered stomata are less efficient in gas exchange and therefore a higher stomatal density may have evolved to compensate for

this loss in resistant plants (Rubiales and Niks 1996). With extensive wax covering, pathogens are more likely to overgrow stomata rather than penetrating and infecting the leaf (Rubiales and Niks 1992). Hydrophobic wax coatings can also assist in the removal of water, spores and other particulate depositions from the leaf whilst also reducing the contact area of particles (spores etc.) with the leaf surface (Neinhuis and Barthlott 1997). Free water is required for *Mycosphaerella* ascospore germination (Park 1984) and leaves that have patchy depositions of epicuticular wax can become water congested and are particularly susceptible to infection by pathogens.

In commercial plantations, a trait such as wax deposition is unlikely to provide a reliable MLD resistance as wax may be dissolved by surfactants used in insecticide sprays. In addition, the deposition of wax on leaves varies with environmental conditions and site nutrition, even if trees are genetically pre-disposed to producing more wax or distributing wax more evenly. However LMA may provide tree breeders with an easily assessable trait for determining MLD resistant *E. globulus*. Therefore, it is recommended that LMA be quantified in a larger scale field trial with a greater variation of genotypes to test the robustness of the association between LMA and MLD resistance.

4.4 Resistance Summary

Variations in constitutive anatomy, such as increased mesophyll density and reduced leaf thickness, are playing a major role in determining the timing, shape and effectiveness of necrophylactic periderm formation that restricts pathogen spread after infection. Other constitutive variations that may convey resistance MLD in *E. globulus,* and potentially other plantation species, is the absence of visible stomata due to a thick layer of epicuticular wax distributed in and around stomata openings. It is assumed that this layer will play several roles including the restriction of initial infection by encouraging hyphae to grow over the stomatal openings that provide an entry point into the leaf. It is proposed that the resistance mechanisms identified throughout this research may assist in the selection of genotypes providing both increased resistance to MLD and commercially favourable traits such as stem form and wood properties.

General discussion and future prospects

The Crown Damage Index is recommended for operational use at a tree and a plantation level. This research concluded that most assessors could estimate damage to within the allowed ± 10 % error margin that is described in the methodology. However damage levels of up to only 54 % were recorded in this study and it is not known whether greater levels of damage (e.g. 50-100 %) would increase or decrease the accuracy of assessors. To overcome problems with accuracy and repeatability, it is recommended that pairs of pre-calibrated and trained assessors conduct assessments. The development of software, which provides diagnostic assistance, training, quality assurance and standardization for forest health research, has improved assessments in other forest species such as sugar maple, red maple black cherry, white oak and northern red oak. It is also a suitable means of providing training indoors on a wide array of disease symptoms which may not be present in the environment at the time of training. A version of this software, at a leaf- and tree level, is recommended to improve the accuracy and precision of assessors.

From a management perspective, the CDI provides information on current tree health but does not provide information concerning future damage (such as egg batches or larval numbers) nor indicate whether pre-emptive measures such as insecticide sprays are necessary. To overcome this problem it is suggested that the CDI assessments be carried out in conjunction with current surveys. Using the CDI, forest companies should be able to compare damage levels, including *Mycosphaerella* spread and severity, over plantations, estates, companies, districts and states. However Australia covers approximately 4000 km from north to south, which encompasses a large range of

environmental conditions. There are often large variations in canopy architecture and leaf density between planting provenances of the same species (primarily due to genetics, site nutrition and climatic variables). CDI assessments are made using a healthy phenotype as a reference and there may be large variations in what is considered to be a healthy phenotype in different states.

To reduce errors involved with CDI estimates, the method was modified for research and tailored to the patterns of MLD damage observed in the exclusion trial detailed in Chapter 2. After a single infection event, uniform infection patterns involving the loss of a large amount of older leaves in the centre of the tree (infection cone) were observed. Although the modified method may not be suitable for measuring more arbitrary damage caused by other biotic and abiotic agents, it is the most suitable and accurate method for measuring *Mycosphaerella* damage and recording the location of damage throughout the crown.

The results of this research suggest that *E. globulus* is highly resilient to *Mycosphaerella* damage. After initial growth loss during the epidemic (< 80 % defoliation), affected trees reached adult foliage and subsequently maintained growth rates similar to those of unaffected trees for the duration of the study. This provides some evidence that *E. globulus* has a large capacity to replace leaf area after a single defoliation event. However, the effects of multiple attacks by pathogens- and/or pathogen-insect combinations are likely to be more detrimental. The ability to recover after damage will also rely largely on site and climatic variables. Poor nutrition and drought stress are likely to impact on both the extent of the damage caused during an event and the rate of recovery of leaf area afterwards. There is also the possibility of i)

tree death, ii) weed infestations or iii) permanent stunting of trees in plantations. Data collected from this thesis will feed into a computer model such as Maestra to provide estimates on the effects of i) leaf loss in different parts of the canopy and ii) different types of damage caused by a wide array of pests and pathogens at a tree- and stand level. These estimates can then be incorporated into a process based model such as Cabala that will enable site-specific predictions on the effects of abiotic and biotic damage to be made.

Silvicultural practices such as early weed control and nutritional application have the potential to reduce negative growth effects caused by foliar damage. Weed control reduces numbers of weed species that compete for nutrients and water, producing trees that are fitter and more able to defend themselves against pathogens. The reduction of foliage surrounding juvenile *E. globulus* leaves also increases airflow and reduces humidity within tree crowns, providing conditions that are less favourable for *Mycosphaerella* ascospore germination. The positive effects of increased nutrition on *Mycosphaerella* severity has previously recorded by Carnegie (1994), where trees which received a higher dose of phosphorus had significantly less infection than control trees. It has also been observed in plantations where windrow trees are planted in highly nutritional ash beds have significantly more growth and less infection than trees planted between windrows (E. Pietrzykowski, pers. com.). There is potential for nutritional application in the management of MLD however further research into nutrient combinations and dose is required. The potential of micronutrients (e.g. copper, zinc) to increase palisade mesophyll density also requires investigation.

The selection of genotypes that are proficient in avoiding or restricting *Mycosphaerella* infection are the most likely prospect for managing MLD on a large scale. Although the slowing of *Mycosphaerella* disease progression using fungicides has been demonstrated in this study and in studies in Victoria (Carnegie and Ades, 2002) and Western Australia (G. Hardy, Pers. Comm.), the economics of fungicide application are not always favourable. To control *Mycosphaerella* infection, regular applications every 2-3 weeks were required during high-risk periods (Carnegie and Ades, 2002). Often plantations are unsuitable for spraying due to adverse climatic conditions and site variations (Wardlaw, 2001). Even if fungicide sprays and their application were more cost effective, forestry companies are avoiding the use of chemicals to control insects and fungal pathogens as they are detrimental to the environment. Increased *Mycosphaerella* resistance of *E. globulus* at a provenance- and family level (Dungey et al., 1997) has both increased growth and reduced the rate of disease progression (Milgate et al., 2005). This thesis has discussed the potential for hosts to evolve over time to combat increased disease pressure. For example the northern NSW provenance of *E. nitens* which grows in conditions more suitable for the proliferation of fungal pathogens has a distinctly different leaf anatomy than the southern NSW provenance. Plantings of *E. globulus,* outside its natural range, have resulted in relatively disease-free and highly-productive plantations in Australia and worldwide. However, due to disease incursions and adaptations of local pathogens, the incidence and severity of disease tends to increase with time (Maxwell, Dell *et al.* 2003). It is now evident that in conjunction with host evolution we are also observing the evolution of the pathogens such as *M. nubilosa* (G. Hunter Pers. Com.) and *M. cryptica* (Milgate et al., 2005) suggesting that this system is in a constant state of flux.

Infection by *Mycosphaerella* species can not only reduce growth but decrease stem quality. Current management of plantations for clear-wood involves the pruning of the lower branches before natural senescence occurs (green pruning). This reduces the chance of branch trace defects in the wood. The infection of trees with *Mycosphaerella* species often results in branch lesions and premature branch death. The pruning of dead branches increases the risk loose knots and kino trace in solid wood products (Wardlaw, 2001). To reduce the quantitative and qualitative impacts of MLD, future research may focus on methods to reduce inoculum, pruning regimes suited to plantations in high-risk areas or treatments to improve plantation health, therefore reducing the effects of energy trade-offs between resistance and growth.

The nested PCR detection method is suggested as a fast and effective means of identifying selected *Mycosphaerella* species in the leaves and branches of research trials. By applying this method to both *E. globulus* and *E. nitens* leaves we detected multiple species at all stages of lesion development. There has been little work conducted on the pathogenicity of *Mycosphaerella* species infectious to eucalypts and the possibility of interaction between species has not been previously discussed. The ability to detect pathogens at an early stage of development, and without the reproductive pseudothecia required for classical taxonomy, has dramatically improved prospects for researching. Aspects such as *Mycosphaerella* lesion development, the co-existence of species in lesions over time, and the seasonal variation in species present in plantations and airborne inoculum can be researched using the nested PCR technique. This study was able to identify the presence of *Mycosphaerella* species in lesions of varying age, however there are still some knowledge gaps with regard to a) the

localization of highly pathogenic species in a lesion, and b) the relative quantities of each species in a lesion.

There was no observed link between which species was present in a particular lesion and the host response, although each lesion contained at least one of the know *Mycosphaerella* pathogens (*i.e. M. cryptica* or *M. nubilosa*). Classical taxonomy was used to identify the species infecting the leaves studied in Chapter 4.2. An extension of this research, not within the scope of the thesis, could use cloning techniques to provide additional information on the fungal species present in each lesion and link this information to host response. Although the development of species-specific inoculation techniques is central to studying the effects of a single species on host response, future studies can utilize inoculation methods (developed in Appendix 3), using mixed-species lesion suspension, to study species-species interactions within a lesion.

The nested PCR has potential application in a) taxonomic studies, b) investigation of ecological and epidemiological principles and c) monitoring of hyphal development after nutritional or other control applications (e.g. systemic enhancers and antifungal treatments). It is likely that species-specific primers can be developed a large range of *Mycosphaerella* species as well as other foliar pathogens. The careful monitoring of species movement using molecular detection will play a key role in the restricting the transfer of highly pathogenic species between states and countries.

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Appendix 1

Is the Crown Damage Index damaged by scorer variation?

A.H. Smith [1,2,4], T.J. Wardlaw[3], E.A Pinkard [1,2], M. Battaglia [2,4], E.A Pietrzykowski [1,2] & C.L. Mohammed [1,2,4]

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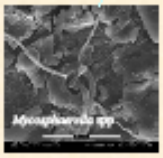
The Crown Damage Index (CDI) was developed as a standardised method to provide core data on eucalypt crown health, comparable Australia-wide*.

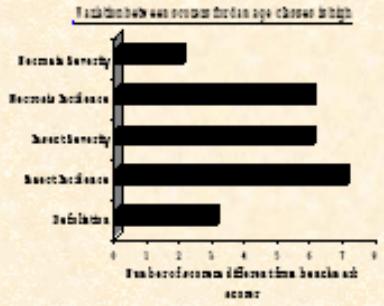
Measures include defoliation & discoloration* and the incidence and severity of insect/browsing and necrotic spots.

CDI is calculated by adding (% incidence x % average level of severity) for all damage classes.

Nine scorers, with varying levels of experience, made AM and PM assessments of 50, 2-3 yo *E.globulus*.

Typical damage assessed by the CDI





Within scorer variation high between morning and afternoon assessments

	Scorer No.									No. scorers different from one to pair
	1	2	3	4	5	6	7	8	9	
Defoliation	O	O	Π	Π	Π	O	O	Π	O	4/9
Insect Incidence	O	Π	Π	Π	O	Π	Π	O	O	5/9
Insect Severity	O	O	Π	Π	Π	O	Π	Π	O	5/9
Necrosis Incidence	O	Π	O	O	O	O	O	Π	O	2/9
Necrosis Severity	O	Π	Π	Π	O	O	Π	O	Π	5/9

O = not significantly different from any scores
Π = significantly different from any scores

Experienced
Moderately Experienced
No experience

Scoring damage classes

- Scorer variation was high in three out of five damage classes.
- Defoliation and necrosis severity were the damage classes scored most consistently.
- Individual scorers tended to change their scores between morning and afternoon assessments.

Implications for the CDI

- Variation in scoring the damage classes within an experience level was not significant and did not influence CDI values.
- CDI values calculated from the experienced group were significantly different from the other two experience levels. This may indicate that training and accurate visual standards are required for reliable results.
- Whilst the CDI is a useful tool for scoring at a plantation level for management purposes, it is not suitable for scoring single trees for research.

Appendix 2: CDI repeatability at a plantation level

A2.1 Background

The CDI (Stone, Matsuki *et al.* 2003) is a single value, which represents the total amount of damage present on a tree. To eliminate the impact of site variation on the overall CDI score for a plantation, a strategic sampling process has been developed. To determine the CDI at a plantation level, there are seven steps in CDI data collection as outlined in Stone, Matsuki *et al*. (2003) which can be conducted in plantations of varying size and shape. In brief these include:

1. defining the plantation
2. dividing the plantation into 8 sections
3. dividing the sections into grid cells
4. selecting one grid cell per section
5. locating the grid cell in the field
6. selecting 6 trees per grid cell and
7. assessing the trees

The objective of this study was to determine the variation between pairs of assessors in estimating the CDI of a plantation.

A2.2 Methods

The feasibility of using the CDI to assess damage at a plantation level was investigated at two sites in Western Australia (WA). Operational and management staff from WA timber companies completed a CDI workshop. The workshop included a 2.5 hour seminar on a) the assessment of CDI categories and plantation and b) sampling strategies to obtain an accurate representation of the plantation CDI. The workshop also included a field component whereby training of assessors on the recognition and

assessment of necrosis, discoloration and defoliation was undertaken. The errors associated with scorer variation were partially eliminated by applying the findings of Chapter 1.1, which included the following;

1. Calibration with other assessors.
2. Assessments in pairs.
3. Recognising the tendency to miss smaller areas of defoliated leaves from the score.
4. The use of visual standards (Figure A2.1) to calibrate assessments despite the experience level of the assessor.

After each workshop, five scoring pairs assessed two plots of 6 trees. Therefore a total of ten plots were assessed per plantation. Two different two-year-old *E. globulus* plantations were assessed. Each plantation was assessed on a different day by different groups of people (the scorers that were most experienced assessed both plantations). The plantations were deemed to be uniform in damage distribution and therefore scoring pairs should be able to estimate the plantation CDI to within ±10 % by assessing different trees. The mean CDI and the 95% confidence intervals were calculated for each pair of assessors for each plantation using the formula described by Stone, Matsuki et *al*. (2003). Plantation means were calculated as the average CDI of the five pairs.

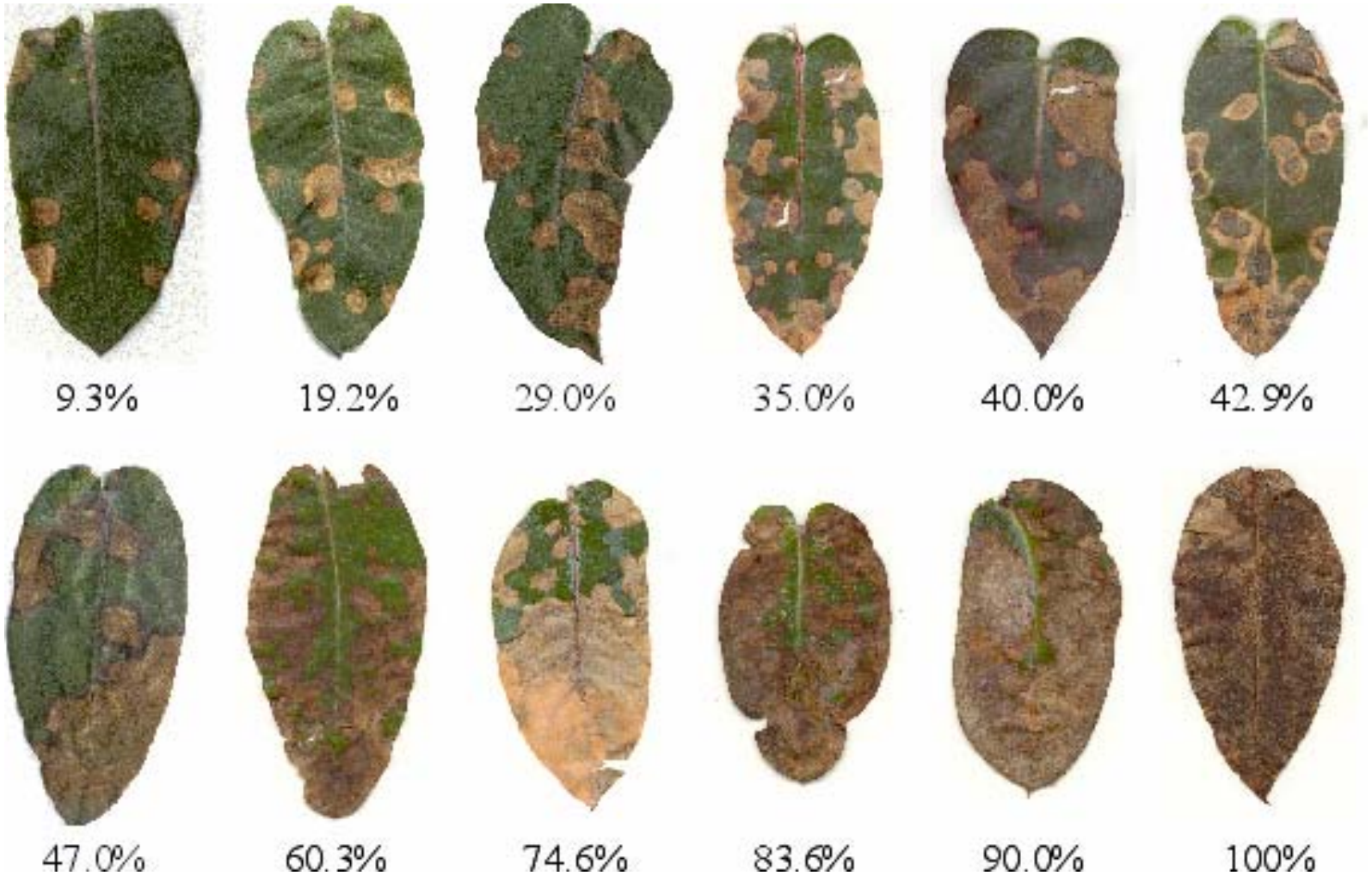


Figure A2.1: Visual standard used to assess leaf necrosis (partly published in Stone et al., 2003)

Tables A2.1 and A2.2: CDI estimates (Mean, 95% Confidence Interval) of the five pairs of assessors in Plantations 1 and 2.

	Scoring Pair					
Table A1.1(Plantation 1)	**1***	**2**	**3**	**4**	**5**	**Mean**
Mean	27	70	56	58	36	49
95%CI	11	15	23	17	10	15
number of trees assessed	12	11	12	12	12	12

*Group furthest from the mean score for the plantation

	Scoring Pair					
Table A1.2(Plantation 2)	**1***	**2**	**3**	**4**	**5**	**Mean**
Mean	19	25	38	35	27	29
95%CI	11	2	12	15	2	8
number of trees assessed	12	12	12	12	12	12

*Group furthest from the mean score for the plantation

A2.3 Results and Discussion

Summaries of the mean CDI scores and their 95% confidence intervals for each pair of assessors in each plantation are presented in Tables A2.1 and A2.2. The ability of the scorers to estimate plantation CDI differed between the two sites. A plantation mean CDI (SD) of 49 (15) was recorded in plantation 1 and a plantation mean CDI (SD) of 29 (8) was recorded in plantation 2. There was a large variability around the mean CDI scores for the first plantation. This was potentially was due to (1) the reduced time for training (2) greater tree-to-tree variation within the plantation and (3) there were fewer

field operatives, accustomed to seeing and assessing pest and pathogen damage, in the group.

At each exercise the two people with the most experience in CDI assessment consistently produced lower plantation CDI values than untrained participants in both field exercises. It is proposed that the assessors in this study had different perceptions of damage depending on which state they worked in. Assessors with the most CDI experience were from Tasmania where there are frequently high levels of damage in the field whilst the presence of high damage levels is less frequent in of WA and assessors are used to observing relatively undamaged crowns.

The presence of damage by different biotic and abiotic agents and the impact these have on tree growth can vary considerably between the states of Australia. Results from this study and Chapter 1.1 have outlined several errors involved in the CDI applied at a tree and plantation level. However the benefits of using a standardised system throughout Australia outweigh the associated errors. Therefore the CDI is recommended for monitoring crown health to fulfil company- and forest certification reporting requirements.

In research, small errors can lead to large over/underestimations of results, (especially when data is used to input into computer modelling systems such as CABALA). For application in research trials the method may require some modification to reduce these errors.

Appendix 3: Modifications to the Crown Damage Index (CDI) to improve its accuracy for research trials.

A3.1 Background

Different levels of accuracy are required for forestry operations and research. Whilst operationally the CDI can be used as a monitoring tool; in research, data may be required for the calculation of growth loss on at a tree stand level and can be fed into computer models to estimate long-term effects of pest and pathogen damage. It is therefore crucial that scoring techniques provide an accurate representation of "actual" disease intensity.

The CDI is a standardised and statistically sound method, but is not always accurate across a range of assessors. Experienced assessors were able to predict the CDI of a tree to within 3%, but not all research personnel are experienced at foliar damage assessment. This study was undertaken to determine whether the accuracy and repeatability of the CDI method can be enhanced by modification so that it is reliable and accurate for research projects.

A3.2 Methods

A small experiment was designed to compare damage estimates using a modified CDI methodology (whereby incidence estimates were measured and calculated as a proportion of the tree area). Actual damage estimates were obtained for comparison using destructive sampling. Five trees less than 1.5m in height were selected in a plantation at Christmas Hills (UTM 55G 331904 5469972) in northwestern Tasmania.

For five trees:

1. Incidence of leaf spotting/necrosis was calculated as a proportion of the whole tree. Measurements required for this calculation included: total crown width (along and across row), width of infection (within the crown), total crown height and height of infection (Figure A3.1). The "severity" of spotting on the leaves within the measured area was estimated by an experienced assessor using visual standards (Carnegie, Keane *et al.* 1994). The CDI was calculated by multiplying the incidence by the severity score.

2. An actual level of leaf spotting was obtained by destructively sampling the same five trees. All leaves were removed from the trees after harvesting.

The number of leaves with infection was determined to be the "actual" incidence of leaf spotting.

To calculate severity of leaf spotting/necrosis, leaves were separated into four severity categories.

No infection	Healthy
1-10%	1-5 spots, 5 mm diameter (midpoint severity = 5 %)
11-15%	5-15 spots, 5-10mm diameter (midpoint severity = 13 %)
16-35%	Irregular necrotic areas and coalescing spots (midpoint severity = 26 %)

The number of leaves in each category was totalled and the "actual" severity of *Mycosphaerella* spotting was calculated by multiplying the midpoint severity by the number of leaves in each category.

The scoring error between actual damage levels and levels estimated using the modified CDI methodology was compared.

A3.3 Results

Average scorer error between estimated and actual damage levels was 3.3 % (SE = 0.9 %). One tree was overestimated by 7.4 %; however this was the smallest tree with the least number of branches (Table A3.1).

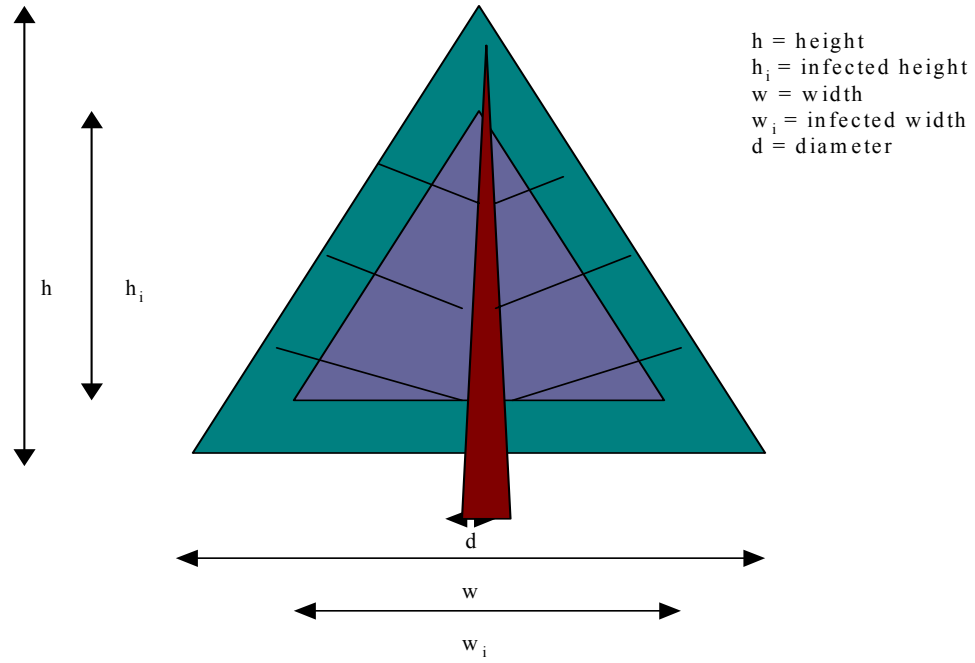


Figure A3.1: Strategy for measuring necrosis in conical shaped canopies (violet).

Table A3.1: Comparison between estimated damage levels (obtained using the modified CDI method) and actual damage determined by destructive sampling.

	Estimated CDI	Actual CDI	Error	No branches	Tree height (m)
1	16.5	14.14	2.4	28	1.5
2	13.2	12.30	0.9	24	1.2
3	19.6	12.20	7.4	11	1.0
4	12.7	14.10	1.4	21	1.2
5	12.1	17.49	5.4	18	1.2

A3.4 Discussion

By measuring (rather than estimating) crown damage incidence we were able to reduce the errors involved with the CDI, observed in Chapter 1.1, for an experienced assessor by up to 10%. Field trials will often have many assessors, all of which will vary in experience and visual perception. The modified CDI appears to be a more reliable method for research as it is reproducible on crowns of varying density and reduces the number of visual estimates required to calculate the CDI. A higher error was associated with the smallest tree with the least dense crown. The CDI for this tree was influenced by overestimation of necrotic severity. Often less dense crowns make damage more visible; hence a greater severity score will be given. However due to the small sample size, in this study, it is difficult to discern whether crown density was influencing results.

The modified CDI provided a more accurate estimation of damage than visual assessment alone and was therefore used to estimate damage in the growth trials in Chapter 2.1. The modified method from this thesis is therefore recommended for field trials where an accurate representation of damage is required for trees in their first 1-3 years of growth, that have a conical crown shape and relatively uniform pattern of damage. Infection/defoliation patterns can become complex as trees get older and the shape of the crown changes, making them difficult to measure. The method is not suitable operationally as measurements are extensive and time consuming. The method is also only suitable for trees in the juvenile leaf phase as there is a distinct change in crown shape upon phase change to adult foliage.

Appendix 4

Rapid, *in planta* detection of pre-visual disease symptoms: a *Mycosphaerella* example

Anna Smith[1,2,3], Morag Glen[3], Inez Tommerup[3], Stephen Langrell[3] and Caroline Mohammed[1,2,3].
[1] University of Tasmania, [2] CRC for Sustainable Production Forestry, [3] CSIRO Forestry and Forest Products.

The detection and identification of disease causing organisms is of priority for Australian biosecurity. Using this system we are able to detect minute amounts of the fungus directly from leaf material within 2 days. This allows the fungus to be identified before disease symptoms are obvious and before it has caused damage. This technology is also useful for the identification and control of *Mycosphaerella* species 8-20 weeks sooner than conventional methods.

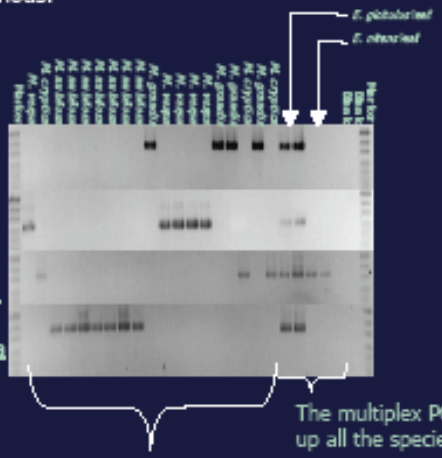
In comparison with other diagnostic techniques the nested PCR is:

More reliable

Highly specific

Over 40× more sensitive

Able to detect very small amounts of fungus in the leaf



Mycosphaerella is a destructive pathogen of concern worldwide

The signs of infection are:

Necrotic spots and blighting.
Leaf senescence and defoliation.
Branch death before pruning – loss of clearwood for sawn timber.
Growth losses, tree death and re-establishment.

Applications for *Mycosphaerella* management:

1

Confirmation of classical taxonomy

Eliminate confusion between species with similar
1. germination.
2. spore attributes and
3. cultures

2

Early warning system

Identification at this stage can buy forest managers 8-20 weeks.
This may be enough time to control infection before damage occurs.

3

Australian biosecurity

Mycosphaerella is a worldwide problem. This technology will help to restrict the spread of highly pathogenic species.

Appendix 5: Mechanisms of resistance of plants against pests and pathogens

Type 1 – prevention of infection			
Resistance mechanism	**Pest/Pathogen**	**Host**	**Example**
↑ Trichome density	Russian wheat aphid (*Diuraphis noxia*)	Wheat	(Bahlmann, Govender *et al.* 2003)
↑ Size and frequency of trichomes thicker cuticle ↓ Stomata	Late blight (*Phytophthora infestans*)	Potato	(Mahajan and Dhillon 2003)
↑ Aliphatic phenylethyl and benzyl wax esters	Autumn gum moth (*Mnesampela privata* Guenee)	*Eucalyptus globulus*	(Jones, Potts *et al.* 2002)
↑ Wax coverage of stomata	Leaf rust (*Puccinia hordei*)	*Hordeum chilense*	(Vaz Patto, Rubiales *et al.* 2003)
↓ Size and frequency of stomata ↑ Trichome density	*Alternaria mali*	Apple trees	(Yong and Chool 1992)
↓ Size and frequency of stomata Persistent, hydrophobic wax coverage	*Xanthomonas campestris*	*Lycopersicon* spp.	(Ramos, Narayanan *et al.* 1992a)
↑ Trichome density	*Glomerella cingulata*	Bean	(Jerba, Rodella *et al.* 2005)
Presence of anamorphous abaxial sheet waxes	Powdery Mildew (*Erysiphe graminis*)	*Lolium* spp.	(Carver and Thomas 1990; Carver, Thomas *et al.* 1990)
↑ Deposition of leaf surface wax and cuticle thickness.	*Phoma sorghina*	Winged bean	(Kumar, Jain *et al.* 1985)
↑ Deposition of leaf surface wax	*Mycosphaerella fijiensis*	Banana and plantain	(Craenen, Coosemans *et al.* 1997)
Thicker cuticle	*Colletotrichum coffaenum* *Sphaerotheca macularis* *Botrytis cinerea*	coffee, strawberry, sorghum, rose	(Hammer and Eversen 1994; Jenks, Joly *et al.* 1994; Nutman and Roberts 1960; Peries 1962)

↑ = increased, ↓ = reduced

Type 2 – restriction of pathogen spread			
Resistance mechanism	**Pest/Pathogen**	**Host**	**Example**
Hydrogen peroxide formation Papilla production	*Blumeria graminis*	Barley	(Hűckelhoven, Fodor *et al.* 1999; Kumar, Schafer *et al.* 2002)
↑ Foliage retention and colour	Swiss needle cast (*Phaeocryptopus gaeumannii*)	Douglas Fir	(Temel, Johnson *et al.* 2005)
Stomatal callose deposits	*Plasmopara viticola*	*Vitis vinifera*	(Gindro, Pezet *et al.* 2003)
↑ Lignification	*Ascochyta rabiei* *Pyrenophora tritici-repentis* *Cercospora zea-maydis*	Chickpea Wheat Corn	(Dushnicky, Ballance *et al.* 1998; HongLian, GenWu *et al.* 2003; Ilarslan and Dolar 2002)
↑ Lignification and Rapid necrosis	Stem rust (*Triticum aesivum*)	Wheat	(Beardmore, Ride *et al.* 1983; Reisenser, Tiburzy *et al.* 1986)
↑ Hydrogen peroxide formation	Stem rust (*Triticum aesivum*)	Wheat	(Flott, Moerschbacher *et al.* 1989)
↑ Anthocyanin production	*Xanthomonas campestris*	Cotton	(Kangatharalingham, Pierce *et al.* 2002)
↑ Early transcription of PR proteins	*Fusarium graminearum*	Wheat	(Pritsch, Muehlbauer *et al.* 2000)
Effective barrier zone formation. Peroxidase formation	*Bipolaris sorokiniana*	White Barley	(Schafer, Huckelhoven *et al.* 2004)
↑ Levels of proteins, lignin, phenolics and callose	*Helminthosporium maydis*	Maize	(Angra-Sharma and Sharma 1994)
↓Penetration hyphae and infection vesicles Rapid barrier zone formation leading to smaller lesions	*Mycosphaerella pinodes*	Pea	(Nasir, Hoppe *et al.* 1992)
↑Lignin in cell walls ↓ Accumulation of Fusrium toxins	*Fusarium culmorum*	Wheat	(Kang and Buchenauer 2000)
Tightly packed palisade and collenchyma cells	*Septoria apiicola*	Celery	(Edwards, Isaac *et al.* 1999)

↑ = increased, ↓ = reduced

Appendix 6

Anatomical variation in juvenile eucalypt leaves associated with resistance to Mycosphaerella Leaf Disease (MLD)

A Smith[1,2,3], E Pinkard[1,2], W Gill[4], G. Hunter[5], M Wingfield[5] & C Mohammed[1,2,3]
[1] University of Tasmania, [2] CRC for Forestry, [3]Forest Biosecurity and Protection, Ensis, [4]TIAR, [5]FABI.

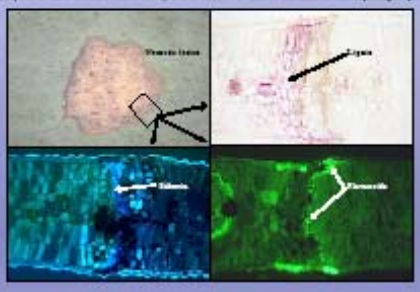
Introduction

Infection by species of *Mycosphaerella*, the cause of MLD, result in quantitative & qualitative losses due to necrosis, premature leaf senescence, shoot, branch & tree death.

Upon infection, defensive responses occur in adjacent undamaged cells & tissues to delineate pathogens.

Existing cells, daughter cells & swollen cells become impregnated with defence chemicals such as lignin, suberin & flavanoids to form a continuous barrier to restrict pathogen spread & transfer of nutrients to the fungus and prevents toxin transfer to healthy tissue.

Methods



Resistance to MLD occurs at inter & intra-specific levels. Three comparisons were studied:

1. *E. nitens* (resistant) & *E. globulus* (susceptible) in southern Australia,
2. northern NSW provenances (resistant) & southern NSW provenances (susceptible) of *E. nitens* in South Africa &
3. resistant & susceptible families of *E. globulus* from a family trial*.

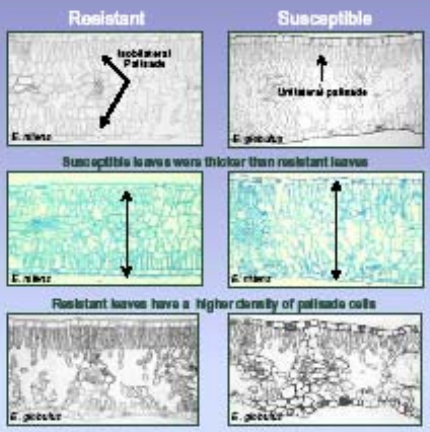
Constitutive leaf anatomy (uninfected tissue) such as leaf thickness, proportions of spongy & palisade mesophyll, cell size, density & relative proportion of airspace were quantified as they play a major role in the speed & integrity of barrier-zone formation.

Results and Discussion

1. *E. globulus* v *E. nitens*
   Compared with *E. globulus*, *E. nitens* leaves:
   had a higher density of mesophyll due to isobilateral palisade layers & barrier-zone formation was more rapid.

2. *E. nitens* v *E. nitens*
   Compared with susceptible leaves, resistant leaves were thinner & higher in palisade and spongy mesophyll density.
   There was also less intracellular spacing of mesophyll cells.
   Barrier-zone formation was thicker and more efficient at restricting pathogen spread.

3. *E. globulus* v *E. globulus*
   Compared with susceptible families, resistant families of *E. globulus* had a higher palisade cell density & thinner sections. Higher mesophyll density was linked with an increase in specific leaf weight.



Conclusions

- Leaves with high mesophyll densities (caused by reduced leaf thickness, reduced intracellular spacing and greater proportions of spongy and palisade mesophyll), have greater potential for large amounts of rapid cell division to form a thick and effective barrier-zone than susceptible leaves.
- Variations in sample thickness & cell density may explain significant increases in specific leaf weight of resistant families
- Specific leaf weight is suggested as an easy-to-assess screening trait for Mycosphaerella resistance in *E. globulus* and *E. nitens*.

Acknowledgements: Thanks to the microscopy department of the University of Pretoria for their valuable assistance into this project. The first author is funded by an Australian Postgraduate Award & a CSIRO scholarship.
Reference: * Milgate, A.W., Potts, B.M., Joyce, K, Mohammed, C. & Vaillancourt, R.E. 2005. Genetic variation in *Eucalyptus globulus* for susceptibility to *Mycosphaerella nubilosa* & its association with tree growth. Aust. Plant. Path. 34:11-16.

Appendix 7

Potential resistance mechanisms of juvenile eucalypt leaves to Mycosphaerella leaf disease

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MLD is a major cause of leaf and shoot blight, premature leaf and branch senescence and tree death in temperate eucalypt plantations worldwide.

Financial losses may be due to complete re-establishment, reduced growth, rotational lags and decreased stem quality due to branch defects.

- Resistant and susceptible pairs* were contrasted at inter-provenance, intra-provenance and within family levels.
- Assessed traits (pre-infection) included specific leaf weight (SLW), waxed and de-waxed stomatal density on both upper and lower leaf surfaces, total leaf wax and phenolics.

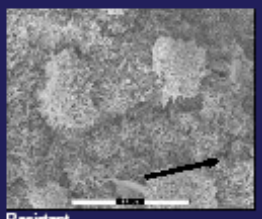
Two potential resistance mechanisms were identified

Type 1 Resistance to initial infection

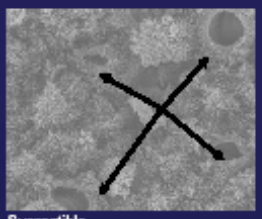
Resistant families had increased wax coverage of stomata. Pathogens are more likely to grow over stomata rather than penetrating and infecting the leaf.

The hydrophobic nature of the wax reduces free water required for ascospore germination.

Stomata are covered with wax in resistant leaves



Resistant



Susceptible

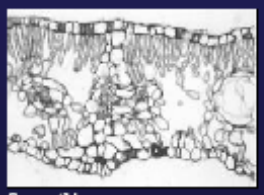
Type 2 Restriction of pathogen after infection

Resistant families had a higher SLW than susceptible genotypes. Anatomical observations reveal a higher cell density of mesophyll in resistant genotypes.

A greater constitutive cell density increases the prospective amount and type of cell division required to form barrier-zones.

Palisade density is higher in resistant leaves

Resistant



Susceptible

Therefore barrier-zone formation is more rapid and pathogen spread is restricted more efficiently compared with susceptible genotypes.

Increases in mesophyll density may also explain increased MLD resistance in

1. Nth NSW provenances of *E. nitens* compared with Sth NSW provenances in South Africa.
2. *E. nitens* (isobilateral palisade) compared with *E. globulus* (unilateral palisade) in southern Australia.
3. Older juvenile leaves & adult leaves.

Acknowledgements: Thanks to Gunns Ltd for the provision of seed for this study. The first author is funded by an Australian Postgraduate Award and a CSIRO scholarship.
* Mitgale, A.W., Potts, B.M., Joyce, K., Mohammed, C. and Vaillancourt, R.E. 2005. Genetic variation in *Eucalyptus globulus* for susceptibility to *Mycosphaerella nubilosa* and its association with tree growth. Aust. Plant. Path. 34:11-18.

Appendix 8: Development of a technique for *Mycosphaerella* inoculation.

A8.1 Background

Artificial inoculation of plants is an important part of researching infection biology, host specificity and pathogenicity of fungal species. However this process is hindered for most species of *Mycosphaerella* due to an inability to produce ascospores and conidia in culture. Whilst some infection has been achieved by clipping lesions with reproductive structures to healthy leaves (Park 1984), inoculum is generally produced by harvesting ascospores from field-inoculated leaves (Crous 1998). This technique has provided the majority of information about modes of infection, lesion development and host specificity (Cheah 1977; Park 1984). However the technique is reliant on the development and maturation of pseudothecia in the field, which may be influenced by seasonal variation in different regions of Australia. As the numbers of ascospores to be obtained from each harvest can vary from ten to thousands, the application of the technique to obtain large-numbers of ascospores for a simultaneous inoculation of a large-scale trial may not be feasible or reliable. This study investigated the spray inoculation of homogenised lesions onto resistant and susceptible *E. globulus* families and monitored the resultant infection occurring from inoculation.

A8.2 Methods

The susceptibility of selected resistant and susceptible families (Table A8.1), as discussed in Chapter 4.3, was tested by spray inoculating plants on 23/3/2005 with a lesion suspension. Plants were germinated, potted, fertilised as previously mentioned in Chapter 4.3 until the last re-potting. At this stage of growth, plants remained in smaller pots and were not fertilised to induce stress. At the time of the study trees were

approximately 1.5 m in height. Plants were kept in shade-house conditions for the first month of the trial and were then moved into a glass-house after some frosts and low temperatures were recorded outdoors. Approximately 60 lesions were pulse blended (× 3) with 200 mL of water. The lesion suspension was centrifuged for 5 minutes at 2800 g/min. The supernatant was sprayed onto the underside of leaves of three trees per family. The remaining unsprayable solution was painted onto the underside of leaves. A sample of inoculated leaves were cleared (submersion in 95 % ethanol for 3 hours at 70 ºC) and stained with lactophenol cotton blue to record the presence and germination of ascospores on the leaf surface. The incidence and severity of lesions were assessed during April and May 2005 until an outbreak of foliar oedema (5/6/2005) changed the anatomy of the leaves. Significant differences in the incidence and severity of *Mycosphaerella* lesions on resistant and susceptible plants were tested using t-tests assuming unequal variance in Splus®. Incidence and severity measurements and the diameter of selected/tagged lesions were monitored on 14/4/2005 and 5/6/2005 for resistant and susceptible trees. The relationship between foliar oedema and its expression on trees resistant and susceptible to *Mycosphaerella* infection was also investigated.

Table A8.1: Families from the Woolnorth and Jeeralang trials, assessed for *Mycosphaerella* resistance by Milgate et al., (2005), were reproduced from seed to produce plants for the investigations in Chapter 4.3 and the development of methods for artificial inoculation.

Pair	Provenance/ Family	ID	% of crown damaged in Woolnorth trial*	Ranking
1	Taranna	T7_08	15.38 (4.94)	R
	Sth Flinders	SF_13	62.82 (3.41)	S
2	Jeeralang	JN2	5.86 (± 2.99)	R
	Jeeralang	JN21	18.19 (± 2.38)	S
3	Leprenna	L6_05	20.84 (4.70)	R
	Leprenna	L6_10	33.91 (4.70)	S
4	Seymour	S2_09	24.20 (3.18)	R
	Seymour	S2_10	39.00 (4.50)	S

* *Mycosphaerella* resistance in the Jeeralang pair was assessed in the Jeeralang family trial not the Woolnorth trial.

Table A8.2: Incidence and severity of *Mycosphaerella* lesions 74 days after spray inoculation with lesion suspension.

	Mycosphaerella **Incidence**	***Mycosphaerella*** **Severity**	***Mycosphaerella*** **score (Incidence x severity**	**Oedema score (Incidence x severity)**
1R	33.3 (13)	4.3 (1)	1.7 (1)	9.2 (3)
1S	50.0 (10)	8.0 (1)	3.9 (1)	35.3 (7)
2R	9.3 (1)	5.7 (0)	0.5 (0)	17.2 (5)
2S	18.3 (4)	4.7 (0)	0.9 (0)	10.3 (3)
3R	21.7 (4)	5.0 (0)	1.1 (0)	5.7 (2)
3S	48.3 (20)	6.7 (1)	3.1 (1)	14.3 (7)
4R	40.0 (17)	12.7 (6)	5.2 (3)	4.8 (1)
4S	21.7 (9)	7.0 (1)	1.3 (0)	26.3 (9)

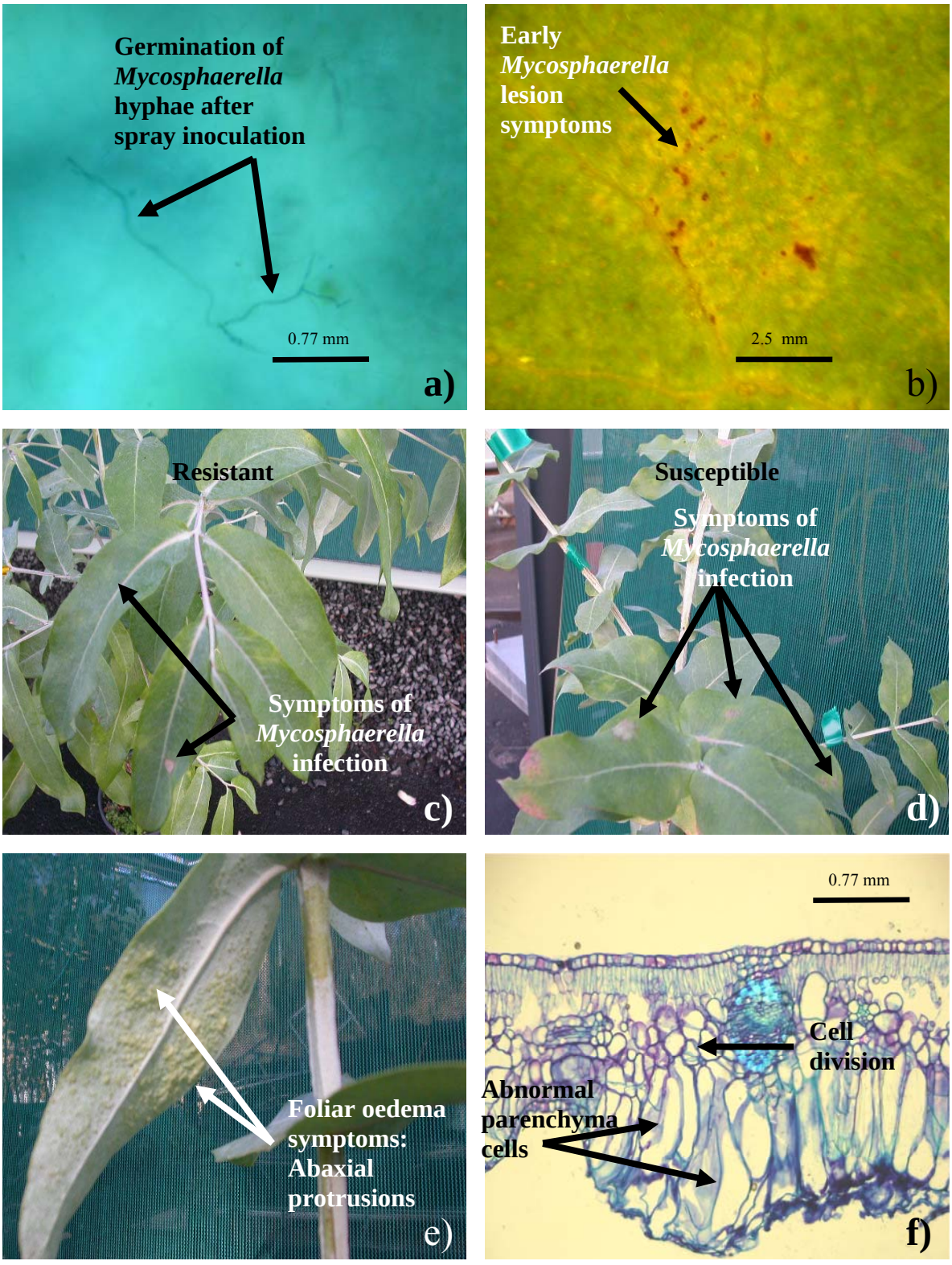


Figure A8.1: (a) Ascospores germinated within four days of inoculation; (b) Initial lesion symptoms were chlorotic with some reddening. Symptoms of infection on resistant (c) and susceptible (d) leaves. Symptoms (e) and induced differentiation of abnormal parenchyma cells in (f) caused by foliar oedema.

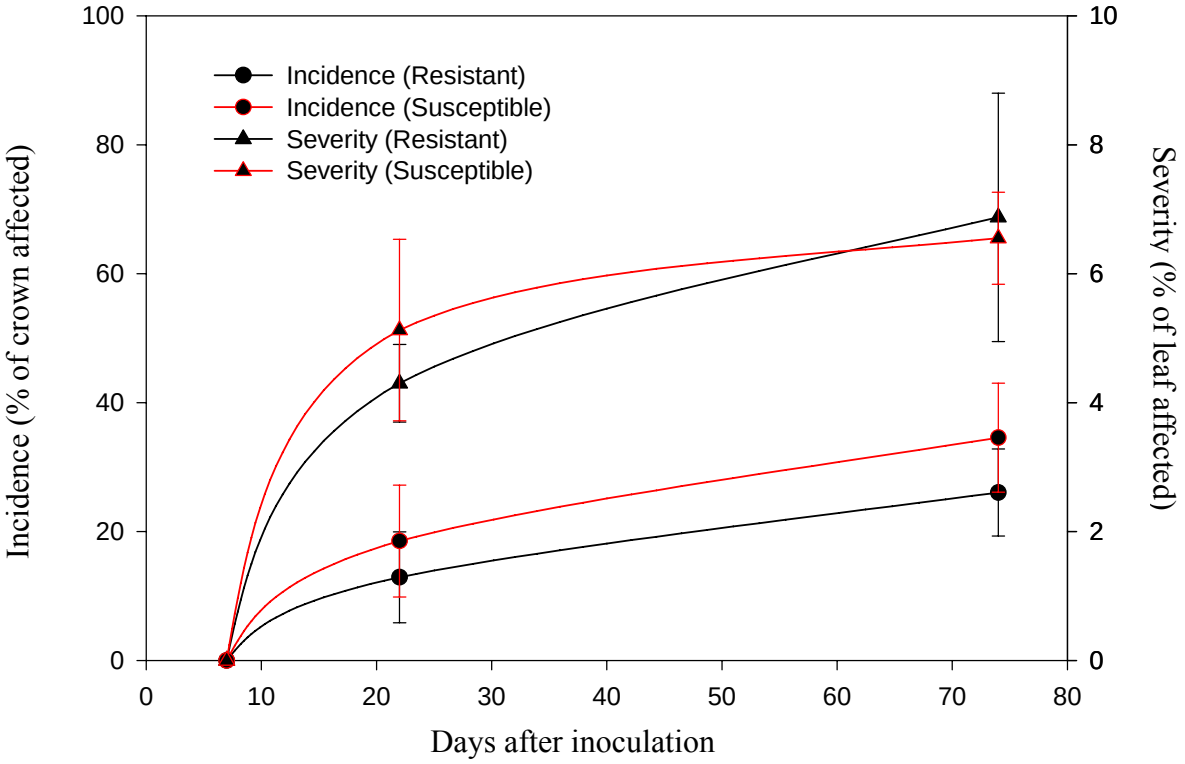


Figure A8.2: Incidence and severity of *Mycosphaerella* lesions on resistant and susceptible *E. globulus* families.

A8.3 Results and Discussion

Ascospores germinated within four days of inoculation (Figure A8.1a) with the first sign of lesions 8-9 days after inoculation as faint chlorotic spots with reddening occurring in some (Figure A8.1b) but not all families. Most lesions were at least partially necrotic 74 days after inoculation. Park (1984) observed necrotic lesions after 40 days between 15 and 20 ºC and Cheah (1977) observed lesions developed from stage 1 to stage 5 within 2 months between November 1975 and January 1976. The slower development of symptoms observed in the current study was possibly due to the unfavourable conditions for *Mycosphaerella* growth such as low temperatures (< 5 ºC) outdoors. Trees were moved into a controlled temperature glasshouse environment to

encourage *Mycosphaerella* symptom progression. However, symptoms of infection progressed slowly after moving trees into the glasshouse. This trial was successful in demonstrating inoculation of *E. globulus* with crushed lesion suspension, which has not been previously demonstrated. Combined with molecular detection methods such as those described in Glen, Smith *et al*., (2006) the species present in the suspension can be identified and the inoculum may be more comparable to natural conditions than attempting to obtain ascocpores of a single *Mycosphaerella* species as airborne inoculum rarely consists of only one species (E. Pietrzykowski, pers. com.).

At the completion of the trial, there was a higher incidence of *Mycosphaerella* lesions on susceptible families compared with resistant families (Figures A8.1c and d; Table A8.1) however the severity of leaf lesions was not necessarily higher in susceptible families (Figure A8.2; Table A8.2). The damage levels (% per tree) were not significant between resistant and susceptible pairs due to low infection levels (<10%), a low number of replicates and high variation within families (Table A8.2). The incidence of *Mycosphaerella* lesions was higher in susceptible families for all pairs but Seymour. In addition the Seymour pair did not produce results consistent with the other pairs when resistance mechanisms were evaluated in Chapter 4.3. It is possible that the ranking of this pair was incorrect in the original Woolnorth trial (see Chapter 4.3).

One of the major problems affecting inoculation studies in the glasshouse environment is the development of an anatomical abnormality termed foliar oedema. It is thought that the high humidity can bring about formation of lenticel like structures in the leaves causing rapid and uncontrolled cell division and the appearance of callus like structures

on the surface of leaves (Pinkard, Gill *et al.* 2006). The development of foliar oedema changed the course of this study however the incidence and severity of the callus (Figures A8.1 e and f) was consistent with resistance rankings for all pairs but Jeeralang (Table A8.2). The difficulties with inoculating *Mycosphaerella* in the glasshouse have also outlined the need for a surrogate pathogen or syndrome such as oedema that can be more easily inoculated in a controlled environment but also provide information indicative of resistance and susceptibility to *Mycosphaerella* and other foliar pathogens.

This study was relatively small and conducted on stressed trees (there is possibly a greater chance of *Mycosphaerella* infection if the trees are stressed). Further studies need to be conducted on a wider range of resistant and susceptible pairs of different ages and nutritional status to determine if the correlation between *Mycosphaerella* ranking and the development of foliar oedema was an artefact of the results. Further testing on more resistant and susceptible pairs will also determine whether the *Mycosphaerella* rankings and resistance mechanisms for this pair are consistent with the Woolnorth trial.