The Role of mycorrhizal fungi in *Eucalyptus gomphocephala* (tuart) health

By

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August 2014
Declaration

I hereby declare that the work in this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution. To the best of my knowledge, all work performed by others, published or unpublished, has been acknowledged.

Lily F. Ishaq

August 2014
Abstract

_Eucalyptus gomphocephala_ DC (tuart) is a woodland tree endemic to the Swan Coastal Plain of Western Australia. The species is of importance for ecological, economic and cultural values. However, _E. gomphocephala_ is in severe decline and the cause of the decline is poorly understood. Due to their important role in ecosystem function, mycorrhizal fungi are hypothesized to play a role in the health status of _E. gomphocephala_. Recently a fungal pathogen, _Phytophthora multivora_ has been implicated in tree decline.

_In vitro_ and glasshouse trials were conducted to examine the impact of _P. multivora_ on ectomycorrhizal (ECM) fungi and _E. gomphocephala_ seedlings. _Phytophthora multivora_ was negatively affected by _Pisolithus albus_ (Cooke and Massee) Priest and a _Pisolithus_ sp., and the ECM fungi were not/or only slightly impacted by _P. multivora_. The ECM fungal inoculation did not increase shoot growth of _E. gomphocephala_ seedlings in the presence of _P. multivora_, however, root growth increased and shoots appeared healthy throughout the trial.

Intact soil cores for bioassay were collected from 12 sites with _E. gomphocephala_ canopy condition ranging from healthy to declining. Sites were classified into three classes; healthy, moderately healthy and declining based on total crown health index. In each site, four trees were randomly selected and eight intact soil cores (0 - 20 cm depth) were collected. Soil samples for arbuscular mycorrhizal (AM) fungi spore assessment and chemical analysis were collected adjacent to the intact soil core. Sites differed for seventeen soil chemistry parameters, and nine variables measured were significantly related to the canopy condition of _E. gomphocephala_.

iii
in the field. Although spore density of AM fungi differed between the sites there was no relationship between spores and canopy condition of *E. gomphocephala*.

A bioassay pot experiment with *E. gomphocephala* as the trap plant was set up using intact soil cores to evaluate inoculum potential of mycorrhizal fungi. Soil cores for AM fungi spore assessment were taken one month prior to harvest and plants were harvested at seven months. Roots were assessed for mycorrhizal colonization and the relationship between mycorrhizal colonization and crown health was evaluated. ECM fungi colonization dominated seedling roots produced in soils taken from under healthy canopies whereas AM fungi dominated seedling roots in soils taken from under trees with declining canopies. From the seventeen soil chemical properties measured, eight and ten variables were related to ECM and AM colonization, respectively.

Molecular analysis was undertaken to evaluate mycorrhizal communities associated with seedling roots from the bioassay trial. This confirmed observations on root colonization, namely there were more ECM fungi associated with seedling roots grown in soil collected from under healthy canopies, and more AM fungi were found in seedling roots grown in soil collected from under declining canopies. Also, there were more saprophytic fungi for seedling roots grown in soil collected from healthy canopies than for seedlings in declining canopy soil, but the reverse was the case for pathogenic and root rot fungi.

A second glasshouse trial was undertaken to assess mycorrhizal colonization over time using soils taken from contrasting sites. A similar trend in dominance of ECM in seedlings grown in soil collected from under a healthy canopy was observed even when the seedlings were still quite young. For example, at 5 weeks root tips of
seedlings in a healthy site soil were 24% colonized by ECM fungi compared with 3% in the declining site soil.

This is the first study to investigate mycorrhizal formation in relation to the health status of *E. gomphocephala*. The low ECM fungal colonization of *E. gomphocephala* seedlings grown in declining sites soil might indicate reduced ECM inocula in the sites where the soil were collected. Reasons for the changes in populations of mycorrhizal fungi under declining trees are discussed. This research now needs to be extended to the field by investigating colonization patterns in seedlings and also the molecular analysis of mycorrhizal fungi populations in sites with a range of canopy conditions. An approach for managing health of *E. gomphocephala*, thus could be gained in the future.
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Publication

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Conference presentations


### List of abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>Arbuscular mycorrhiza</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANOSIM</td>
<td>Analysis of similarities</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>CD</td>
<td>Crown density</td>
</tr>
<tr>
<td>CDR</td>
<td>Crown dieback ratio</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylene-triamine-penta-acetic acid</td>
</tr>
<tr>
<td>E. Eucalyptus</td>
<td>Eucalyptus</td>
</tr>
<tr>
<td>ECM</td>
<td>Ectomycorrhiza</td>
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<tr>
<td>EI</td>
<td>Epicormic index</td>
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<tr>
<td>et al.</td>
<td>et alia</td>
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<tr>
<td>Exc.</td>
<td>exchangeable</td>
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<tr>
<td>FT</td>
<td>Foliage transparency</td>
</tr>
<tr>
<td>IPCC</td>
<td>Intergovernmental Panel Climate Change</td>
</tr>
<tr>
<td>INVAM</td>
<td>International collection of arbuscular and vesicular-arbuscular mycorrhizal fungi</td>
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<tr>
<td>ITS</td>
<td>Internal transcribed spacer</td>
</tr>
<tr>
<td>MANOVA</td>
<td>Multivariate analysis of variance</td>
</tr>
<tr>
<td>MURU</td>
<td>Murdoch University, herbarium index</td>
</tr>
<tr>
<td>MMN medium</td>
<td>Modified Melin Norkrans medium</td>
</tr>
<tr>
<td>NARPH</td>
<td>Nilstat Ampicilin Rifadin Piramicin Hymexazol</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>PAST</td>
<td>Paleontological Statistics Software Package for Education and Data Analysis</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
</tr>
<tr>
<td>P. Phytophthora</td>
<td>Phytophthora</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDA</td>
<td>Potato dextrose agar</td>
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<td>Pi. Pisolithus</td>
<td>Pisolithus</td>
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<tr>
<td>PVLG</td>
<td>Polyvinyl alcohol-lacto-glycerol</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>R</td>
<td>Statistical value of ANOSIM</td>
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<tr>
<td>RA</td>
<td>Relative abundance</td>
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<tr>
<td>SD</td>
<td>Spore density</td>
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<tr>
<td>SE</td>
<td>Standard error of the mean</td>
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<tr>
<td>SIMPER analysis</td>
<td>Similarity percentage analysis</td>
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<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>SR</td>
<td>Species richness</td>
</tr>
<tr>
<td>SWWA</td>
<td>Southwest Western Australia</td>
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<tr>
<td>TCHI</td>
<td>Total crown health index</td>
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<tr>
<td>TRG</td>
<td>Tuart Response Group</td>
</tr>
<tr>
<td>THRG</td>
<td>Tuart Health Research Group</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>YNP</td>
<td>Yalgorup National Park</td>
</tr>
</tbody>
</table>
# Table of contents

Declaration .............................................................................................................. i  
Abstract ................................................................................................................. iii  
Acknowledgements ................................................................................................... vi  
Publication and Conference presentations .............................................................. viii  
Abbreviations .......................................................................................................... ix  

## Chapter 1. General introduction and thesis aims .................................................. 1  
1.1 Introduction ....................................................................................................... 1  
1.2 Thesis aims ....................................................................................................... 3  
1.3 Organization of the thesis ............................................................................... 4  

## Chapter 2. Literature review .............................................................................. 7  
2.1 Tree decline ..................................................................................................... 7  
2.2 Causes of tree decline .................................................................................... 9  
2.3 Key factors associated with tree decline ......................................................... 11  
  2.3.1 Climate change ...................................................................................... 11  
  2.3.2 Diseases and pests ............................................................................... 14  
  2.3.3 Air pollution ....................................................................................... 14  
  2.3.4 Herbicides ......................................................................................... 15  
  2.3.5 Decreased soil health ......................................................................... 16  
  2.3.6 Mycorrhizal fungi and forest health ................................................... 18  
2.4 Environment for eucalypts in southwestern Australia ..................................... 20  
  2.4.1 Climate .............................................................................................. 20  
  2.4.2 Soil ..................................................................................................... 23  
2.5 *Eucalyptus gomphocephala* decline ............................................................... 24  
2.6 Possible causes of *E. gomphocephala* decline ............................................ 26  
2.7 Concluding remarks ...................................................................................... 29  

## Chapter 3. Can ECM fungi protect *E. gomphocephala* from infection by *Phytophthora multivora*? .............................................................. 31  
3.1 Introduction ..................................................................................................... 31  
3.2 Materials and methods .................................................................................. 34  
  3.2.1 Experiment 1 ..................................................................................... 34  
    *ECM fungi isolate* ..................................................................................... 34  
    *Phytophthora multivora isolate* ............................................................... 36  
    *Secondary metabolite antagonism* ......................................................... 36  
    *Direct interaction* ................................................................................... 37
4.3.3. Predicting crown health from soil chemical properties .............. 83
4.3.4. Spore density of AM fungi between sites ............................... 85
4.3.5. Abundance and species richness of AM fungi spore .................. 86
4.3.6. Correlation between spore density and species richness of AM fungi ................................................................. 88
4.3.7. Predicting spore density and species richness of AM fungi from soil chemical properties .............................................. 88
4.3.8. Correlation between AM fungi (spore density and species richness) and crown health ................................................. 89
4.4 Discussion .............................................................................. 89

4.4.1 Soil chemical properties of study sites ..................................... 89
4.4.2 Soil chemical properties and crown health ............................... 91
4.4.3 AM fungi abundance and diversity between the sites ................. 92
4.4.4 AM fungi (abundance and diversity) and crown health ............. 93

Chapter 5. Soil inoculum potential of mycorrhizal fungi related to the health status of *Eucalyptus gomphocephala* ........................................ 97

5.1 Introduction ............................................................................ 97
5.2 Materials and methods ............................................................ 99
5.2.1 General approach ............................................................... 99
5.2.2 Crown health assessment ..................................................... 99
5.2.3 Soil collection ..................................................................... 100
5.2.4 Bioassay trial ..................................................................... 100
5.2.5 Mycorrhizal assessment ....................................................... 104
5.2.6 Plating for *Phytophthora* ..................................................... 105
5.2.6 Statistical analysis ............................................................... 105
5.3 Results .................................................................................. 107
5.3.1 Seedling growth ................................................................. 107
5.3.2 AM fungi spore density ....................................................... 108
5.3.3 Abundance and diversity of AM fungi spore ......................... 110
5.3.4 Correlation between spore density and species richness of AM fungi ............................................................................. 111
5.3.5 Predicting spore density and species richness of AM fungi from soil chemical properties ......................................................... 114
5.3.6 Correlation between spore density of AM fungi and crown health... 115
5.3.7 Correlation between species richness of AM fungi and crown health ............................................................................... 116
5.3.8 Arbuscular mycorrhizal colonization ...................................... 116
Chapter 1

General introduction and thesis aims

1.1 Introduction

_Eucalyptus gomphocephala_ (tuart) is a woodland tree endemic to the Swan Coastal Plain of Western Australia (Elridge et al. 1994). The species is of importance for ecological, economic and cultural values. In its natural range, it provides habitat for vulnerable fauna and threatened flora, and the trees have historical and aesthetic values (Powell and Keighery 2002). Moreover, it is considered as a resilient species as it can survive in a wide variety of soil types, including calcareous and saline soils, and can tolerate stressful conditions such as floods and drought (Elridge et al. 1994).

More than 112,000 hectares of _E. gomphocephala_ forest existed at the time of European settlement. However, due to clearing for agriculture and urban development only about 30,000 hectares of _E. gomphocephala_ remain (TRG 2002; Government of Western Australia 2003). Over the past decade _E. gomphocephala_ has experienced an alarming reduction in health and vitality (Archibald et al. 2010; Cai et al. 2010). The decline in tree health, known locally as tuart decline, has been very noticeable in the Yalgorup Region between Mandurah and Bunbury, approximately 100 km south of Perth, with the most severe decline near Preston beach in Yalgorup National Park (YNP) and on nearby freehold land. In some areas, the morbidity and mortality rates of _E. gomphocephala_ trees across all age classes has reached 90% (TRG 2002). The rate of spread and the intensity of decline have raised concern about the impact this may have on ecosystem health and function (THRG 2006; Close et al. 2009; Archibald et al. 2010).
Currently, the cause of *E. gomphocephala* decline is poorly understood. However, a number of physical and biological factors have been considered to be the primary cause of this decline (Longman and Keighery 2002; THRG 2006). It is likely that more than one factor is involved and interactions between two or more factors may be responsible for triggering the decline. The main factors undergoing investigation include hydrology and water quality, fire and competition, pests and pathogens, soil microbes and nutrition (THRG 2006). The role of soil bacteria, oomycetes and fungi in *E. gomphocephala* decline is poorly understood but they may be important (Scott et al. 2009; Cai et al. 2010; Scott et al. 2011) as they are critical factors in ecosystem function. In particular, mycorrhizal fungi, one of the most abundant and important beneficial below-ground functional groups, are suspected of being important for *E. gomphocephala* health. It is widely known that some mycorrhizal fungi can enhance plant growth and productivity by making nutrients available to plants and providing resistance to drought, disease and other stressors (Smith and Read 2008).

Eucalypts are well known to form mycorrhizal associations, predominantly with ectomycorrhizal (ECM) fungi, but can also have associations with arbuscular mycorrhizal fungi (AM) and both ECM/AM (Lapeyrie and Chilvers 1985; Brundrett and Abbott 1995; Chen et al. 2000a; Adams et al. 2006; Jasper 2007). Eucalypts may benefit from this association, especially those species that have been introduced to inhospitable environments or in their natural environments where nutrient availability becomes limiting for growth due to disturbance and other factors. Thus, it is likely that mycorrhizal fungi are involved in the healthy functioning of *E. gomphocephala* forests and woodlands as, in general, the soils in the region are of low fertility (Moore 1998), and the environment can be severe due to climate change (increased temperature and reduced precipitation) (Bates et al. 2008b).
A number of studies have investigated the correlation between mycorrhizal fungi and plant health (Ellis and Pennington 1992; Causin et al. 1996; Montecchio et al. 2004; Mosca et al. 2007; Horton et al. 2013). In a preliminary study on *E. gomphocephala* decline, some baseline data were collected on the above-ground abundance and diversity of ECM fungal sporocarps and unpublished observations suggested that sporocarps may be less abundant in declining stands (Legault 2005). A study by Scott et al. (2011) suggested that loss of fine roots was caused by disease associated with *Phytophthora multivora*, and this pathogen appeared to be widespread (Barber et al. 2013). Despite this, in studies conducted so far on *E. gomphocephala* decline, little attention has been paid to the role of mycorrhizal fungi in facilitating colonization by *E. gomphocephala* seedlings in the inhospitable soils of the coastal plain. Similarly, little information is available about the diversity of mycorrhizal fungi in *E. gomphocephala* woodlands and their sensitivity to environmental or biotic perturbations. Recently, it has been reported that there was a decrease in ECM extramatrical networks (a network of ECM hyphae in the soil) in declining sites (Scott et al. 2013a), which was related to the presence of *P. multivora*. However, there is no detailed information on the impact of *P. multivora* on ECM fungi. Whether the decreased ECM extramatrical network in declining sites is related to *P. multivora*, as previously hypothesized, or is caused by other factors needs to be elucidated.

**1.2 Thesis aims**

The aims of this study were to determine if:

1. Ectomycorrhizal fungi abundance on roots is affected by fine feeder root disease caused by *Phytophthora multivora*,
2. Arbuscular mycorrhizal diversity and abundance varies with changes in the health status of *E. gomphocephala*,

3. The soil inoculum potential of ecto and endo-mycorrhiza change with the health status of *E. gomphocephala*,

4. Mycorrhizal fungi communities colonizing the roots of *E. gomphocephala* change with the health status of the tree, and

5. The sequence of development of mycorrhizas over time differs between healthy and declining sites.

### 1.3 Organization of the thesis

Chapter 1 includes a general introduction as a background to the study followed by the objectives of the study. The literature review (Chapter 2) examines tree decline phenomena, the causes of tree decline, *E. gomphocephala* decline and possible causes of decline. The interaction between ECM fungi and *P. multivora* is examined *in vitro* and in a glasshouse trial (Chapter 3). The mycorrhizal status of healthy and declining sites is examined including density and diversity of AM spores (Chapter 4) and inoculum potential of mycorrhizal fungi (Chapter 5). Molecular analysis to identify mycorrhizal fungi colonizing *E. gomphocephala* seedlings is presented in Chapter 6. The sequence of development of mycorrhizal fungi in healthy and declining sites is explored in Chapter 7. The General Discussion presents an integration and interpretation of the key results from the experiments conducted and recommends areas for further research. A schematic representation of and relationship between the chapters is presented in Fig. 1.1.
Fig. 1.1. Scheme of the relationships between chapters of the thesis.
Chapter 2

Literature review

2.1 Tree decline

Decline is a gradual and general loss of vigor often leading to death of individual trees or stands of trees. In contrast, dieback is a general response to environmental stress that also refers to relatively sudden death or immediate impacts of acute stress. Dieback can lead to further dieback or decline (Manion 1991; Ciesla and Donaubauer 1994; Jurkis 2005).

Tree decline is a major environmental issue affecting a wide range of tree species throughout the world (Allen et al. 2010). Examples of major tree decline are given below.

a. Europe. Mortality of many species of woody flora and forests has occurred across the Mediterranean regions including Spain (Penuelas et al. 2001) and of various tree species such as Quercus (oak), Abies (fir), Picea (spruce), Fagus (beech) and Pinus (pine) in France (Breda et al. 2006). In Sweden, tree mortality occurred in Quercus robur (pedunculate oak) (Drobysheva et al. 2007), while in Greece, tree mortality has been reported to occur in Abies cephalonica (greek fir) and Pinus halapensis sub. brutia (allepo pine) (Tsopelas et al. 2004; Korner et al. 2005).

b. America. Decline and dieback of forests such as Betula (birch) dieback, Fraxinus (ash) dieback, and Acer (maple) decline have been documented in North America since early last century (Heatwole and Lowman 1986; Ciesla and Donaubauer 1994). Recently, numerous reports indicated episodes of forest decline have increased considerably in the last two decades. Perhaps the most severe forest mortality occurred from Alaska to Mexico beginning in 1997 and affecting around
20 million ha of many tree species (Raffa et al. 2008). In British Columbia, the mortality of *Pinus concorta* (lodgepole pine) reached more than 10 million ha (Kurz et al. 2008), while mortality of around 1 million ha of *Populus tremuloides* (quaking aspen) was reported in Saskatchewan and Alberta (Hogg et al. 2008; Michaelian et al. 2011). In the eastern part of North America, one of the most well known decline and mortality events in trees was the decline syndrome affecting the oak family from Missouri to South Carolina (Clinton et al. 1993; Voelker et al. 2008). Extensive increases in tree mortality have also been reported to occur in South and Central America including increased mortality of tropical forests in northwest and southeast Brazil (Williamson et al. 2000; Rolim et al. 2005), and mortality of *Nothofagus dombeyi* (evergreen beech) in Patagonia (Suarez et al. 2004).

c. Africa and Asia. Some noticeable increases in tree mortality in Africa include those of tropical moist forests in Uganda (Lwanga 2003), *Brachystegia glaucescens* (mountain acacia) in Zimbabwe (Tafangenyasha 2001), and die-off of mesic tree species in Senegal (Gonzalez 2001). In Asia, increased tree mortality included examples from tropical forests in Malaysian and Indonesian Borneo (Nakagawa et al. 2000; van Nieustadt and Sheil 2005), and tropical dry forests in India (Khan et al. 1994).

d. Australia. Tree declines, particularly of eucalypts, have occurred since the late 19th century following agricultural and urban development, and have increased considerably in recent decades (TRG 2003; Jurkis 2005; Stone and Simpson 2006). Some major eucalypt decline events in eastern Australia included high altitude dieback of *E. delegatensis* (alpine ash) in Tasmania (Ellis et al. 1980; Ellis and Pennington 1992), bell miner associated dieback (BMAD) of *E. saligna* (Sydney blue gum) in New South Wales (Heatwole and Lowman 1986; Jurkis and Turner
2002), and ‘New England dieback’ or rural dieback. Rural dieback was probably the most well known canopy decline initially affecting paddock trees in the rural areas of New England, but then became widespread throughout Australia. It affected numerous species of eucalypts and other native trees in a wide range of climate regimes including humid, sub-humid and semi-arid (Heatwole and Lowman 1986; Ciesla and Donaubauer 1994; Close and Davidson 2004). This dieback has become extensive over three decades, with an estimated cost of over AUD $20 billion for revegetation of the affected areas (Close and Davidson 2004). In eucalypts, rural dieback was characterized by a thinning of the crown that began at the branch ends and progressed towards the main trunk, leaving typical dead branches protruding beyond the remaining foliage. The new leaves developed subsequently from epicormic buds which may re-occur for years before eventual death (Close and Davidson 2004).

In Western Australia, one of the most serious dieback was associated with *E. marginata* (jarrah). This dieback has been known since the 1920’s and has increased considerably since that time (Shea and Dell 1981; Shearer and Tippett 1988), although the causal agent *Phytophthora cinnamomi* was not isolated until 1964 (Podger et al. 1965). Other eucalypts that have declined rapidly over recent decades included *Corymbia calophylla* (marri) (Paap et al. 2008) and *E. rudis* (flooded gum) (Wallace et al. 2006; Edwards 2011). Recently, prominent declines have also occurred in *E. gomphocephala*, *E. wandoo* (wandoo), *E. loxophleba* (York gum) and *E. salmonophloia* (salmon gum) (THRG 2006; Robinson 2008).

### 2.2 Causes of tree decline

Based on the disease triangle, the three components, namely susceptible host, virulent pathogen and favourable environment, must be present and interact appropriately for the
development of a disease (Schuman 1991). Tree decline however, is a more complex situation. It can be caused by a number of abiotic and biotic factors, which may interact synergistically to promote tree decline (Heatwole and Lowman 1986; Manion 1991; Ciesla and Donaubauer 1994). Abiotic factors include non-anthropogenic agents such as climatic factors (e.g. drought, excessive rainfall), salt spray from the ocean or mechanical injury, and anthropogenic agents such as air pollution and forest management practice. The biotic factors are living or infectious disease-causing agents such as pathogens, insects, viruses, and other parasitic organisms (Ciesla and Donaubauer 1994).

Several theories have been proposed to globally explain tree decline phenomena. For example, the host-stress-saprogen model was proposed by Houston (1992), climatic perturbation by Auclair et al. (1992), and cohort senescence by Mueller-Dombois (1992). One of the concepts that has been widely used to analyze a number of decline situations is the decline spiral model developed by Manion (1991) (Figure 2.1). According to Manion (1991), forest declines cannot be identified by a single biotic or physiological cause. Instead they are considered to be more complex diseases combining abiotic environmental stresses and biotic agents. A conjunction of three factors, predisposing, inciting and contributing factors, must occur for the onset of decline.

Predisposing factors are long term factors that change the ability of trees to withstand or respond to injury inducing agents. Inciting factors are short-term stresses that trigger the decline, and contributing factors are long term factors, mostly opportunistic organisms, that subsequently further weaken and ultimately kill trees. Accordingly, decline is induced by a sequential occurrence of one stressing factor
from each ring (Fig. 2.1), and factors within each ring are interchangeable (Manion 1991).

![Diagram of tree decline factors]

Fig. 2.1. Manion’s concept of decline (Manion, 1991).

2.3 Key factors associated with tree decline

As introduced above, a large number of factors might be involved in tree decline phenomena. For the purpose of this thesis, this section focuses on those factors that are considered to be the most important in *E. gomphocephala* decline, the topic of this thesis. These factors are described below.

2.3.1 Climate change

The world climate has changed, which is widely accepted as a result primarily from the effects of increasing emissions of carbon dioxide (CO₂) and other greenhouse
gases originating mainly from human activities (IPCC 2007; Moore and Allard 2008). In particular, an increase in global mean temperature (about 0.5°C since 1970) and changes in the world’s hydrological cycle associated with climate change are considered to have already impacted upon some of the world’s ecosystems. In addition, climate models predict a greater shift in climatic patterns over the coming decades in many regions including much warmer temperatures and altered precipitation patterns (IPCC 2007), which might result in increased forest stress and mortality risk (Moore and Allard 2008; Allen et al. 2010).

Forests and woodlands growing in marginal climate zones where net primary vegetation productivity is strongly water limited such as in semi-arid regions, and tree species at the drier edges of their range distribution might display substantial growth decline and mortality (Allen 2009). Mediterranean climate regions are particularly vulnerable (Klausmeyer and Shaw 2009; Matusick et al. 2012). However, drought or warming temperature might also impact on tropical moist forests, as well as boreal and temperate forests (Allen 2009). More detail of recent drought-induced mortalities of trees around the world is discussed by Allen et al. (2010).

Drought and rising temperature can have direct impacts on trees or interactive impacts on trees and biotic agents (Desperez-Loustau et al. 2006; Moore and Allard 2008; Allen et al. 2010). Climate-induced water stress may directly cause tree mortality through short-term acute effects such as irreversible disruption of xylem water columns within the stem and leaves (cavitation). Over long periods, chronic water stress may weaken or ultimately kill trees either directly through carbon deficit or indirectly through pest attack, which can overwhelm stressed trees (McDowell et al. 2008). Trees in a stressed or weakened condition are prone to attack by
pathogens (Tainter and Baker 1996), which is possibly due to changes in the nutritional status of plants and a decrease in plant defense mechanisms (Boyer 1995; Desperez-Loustau et al. 2006; Moore and Allard 2008).

Warming temperatures can also directly have a big influence on biotic agents, especially insect population dynamics. Insect development and reproduction can be accelerated resulting in increasing infestation pressure (Moore and Allard 2008; Netherer and Schopf 2010). In particular, outbreaks of some aggressive bark beetle species have been correlated with rising temperature (Rouault et al. 2006; Netherer and Schopf 2010).

Beside impacting on tree physiology and biotic agents, warmer temperatures may also affect the distribution of a tree species, particularly those at latitudinal and altitudinal extremes (Parmesan 2006). It is expected that a significant upward movement of the tree line may occur. Species with shorter life spans and faster reproduction cycles such as herbs, ferns and mosses are likely to be unaffected by climate warming, whereas larger long-lived trees and shrubs might be under great threat (Lenoir et al. 2008).

Climate change might also impact on the below ground/soil-rhizosphere, particularly mycorrhiza. The impact of climate change on a plant’s rhizosphere, physiology and/or ecology may either directly impact on mycorrhiza through changes in resource availability and distribution of mycorrhizas, or indirectly through changes in below ground allocation of carbon (C) to roots and changes in plant species distribution (Bellgard and Williams 2011).
2.3.2 Diseases and pests

There are large numbers of diseases and pests that have contributed to tree decline worldwide, acting either as primary or secondary agents (Schuman 1991; Tainter and Baker 1996; Old 2000; Jung et al. 2005; Jung and Nechwatal 2008). As primary pathogens, they can attack and kill healthy trees, while as secondary agents they infect the host after the establishment of a primary pathogen, or after hosts have experienced predisposing or inciting stress agents. There are well known examples of tree decline worldwide that have been implicated with the involvement of diseases and pests. For instance, *Phytophthora* spp. have been considered as the cause of devastating diseases in some Australian native plants (Cahill et al. 2008; Scott et al. 2009), as well as in *Quercus* and *Fagus* in Europe and USA (Jung et al. 2005; Grunwald et al. 2012). Further examples are the mortality of deciduous and coniferous trees in North America caused by *Armillaria* spp., Chestnut blight (canker disease) in USA by the fungus *Cryphonectria parasitica*, Dutch elm disease in North America and Europe by the fungus *Ophiostoma ulmi*, and pine wilt disease in North America by nematodes (Schuman 1991; Tainter and Baker 1996). In addition, an outbreak of *Acer* decline in New England, New York and Canada, and *Quercus* decline in the United States were triggered by complex insect defoliation (Houston 1992), and mortality of *Pinus* forests in Canada and North America was related to an outbreak of bark beetles (Williams and Liebhold 2002; Kurz et al. 2008).

2.3.3 Air pollution

Anthropogenic air pollution resulting from industrialization such as acid deposition, sulphur (S) deposition and elevated ozone has been suggested as a possible cause of forest decline in Europe and America (Ciesla and Donaubauer 1994). For example, decline of *Picea rubens* (red spruce) in the northeastern U.S. and *Acer* in Europe has
been related to acid deposition (DeHayes et al. 1999; Horsley et al. 2000), while
decline of *Pinus* forests in Eurasia has been related to S deposition (Savva and
Berninger 2010).

Acid deposition can cause loss of basic cations from soil affecting many ecosystem
processes such as the mobilization and leaching of essential nutrients as well as toxic
substances (Kreutzer 1992). In some cases the acidification can be ameliorated by
the addition of calcium (Ca) to the soil as shown by Juice et al. (2006) for stands of
*A. saccharum* in northern USA.

Air pollution may also occur synergistically with climatic stress to promote tree
decline. For instance, increased ground ozone levels and UV radiation combined
with frost shock and extreme temperature fluctuation have been associated with the
decline of *Picea abies* (Norway spruce) stands in the region of Moravian Silesian-
Boskids of the Czech Republic (Haddas 2010). Bytnerowicz et al. (2007) and Woo
(2009) suggested mechanisms by which air pollution and climate change impact on
tree decline. Accordingly, these two factors may interactively impact on forests in a
number of ways, including changes in soil processes, tree growth, species
composition and distribution, and increased plant susceptibility to stress. However,
the processes occur over long periods of time, and the magnitude of the interaction
depends on the sensitivity of individual trees and species to the pollutants, and
complex interactions between trees, the prevailing environment and other organisms.

2.3.4 Herbicide

The application of herbicides might be related to tree decline especially those
adjacent to crops or areas where herbicides are frequently used. However, trees near
right-of-ways, roadsides, railroads, and trees in urban areas where lawn spray,
fertilizers containing herbicides and soil herbicides are applied, might also have a high risk of damage (Boutz and Stack 1986). The effect of herbicides on trees could be through spray drift, movement of volatiles, application to the soil and movement in soil and water. Trees might be injured either through the absorption of a toxic chemical by roots or from direct application onto the above-ground tissues (Boutz and Stack 1986).

There are several examples of the negative impact of herbicide on non-target trees/forest including the mortality of neighboring trees at a distance within 3 m where injected herbicide (imazapyr) was applied on Ailanthus altissima (tree-of-heaven) (Lewis and McCarthy 2008), and widespread mangrove dieback in north-eastern Australian due to agricultural herbicides (Duke et al. 2005). Furthermore, it has been reported that herbicides may also negatively impact on the rhizosphere and soil microorganisms (Kremer et al. 2005).

2.3.5 Decreased soil health

Deleterious changes in the physical, chemical and biological properties of soil might be considered as important factors associated with tree decline. For instance, decline of Austrocedrus chilensis (mal del ciprés) in southern Argentina had been reported as due to poor soil drainage (Manna and Rajchenberg 2004), while decline of sugar maple in Europe and North America was related to disruption of nutrient cycles, increased soil acidification, and increased availability of toxic metals (Horsley et al. 2000; St. Clair et al. 2005; Kogelmann and Sharpe 2006). In particular, deficiencies and imbalances of various nutrients such as nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), manganese (Mn) or Ca have been implicated with sugar maple decline (Horsley et al. 2000). Moreover, Schaberg et al. (2006) suggested that although the specific element associated with sugar maple decline might vary
between sites, Ca deficiencies could be the more likely potential contributor of decline.

In Australia, a study to investigate the cause of Mundulla Yellows decline in a range of tree species found that adverse changes in soil chemical properties including increased alkalinity and salinity, accumulation of carbonate and bicarbonates, induced deficiency of Fe and Mn (Czerniakowski et al. 2006), and lime chlorosis (Parsons and Uren 2007) was the likely cause of decline. Grigg et al. (2009) studied *E. marginata* and *C. calophylla* decline in an urban parkland in south Western Australia. These authors found that plant nutritional problems, particularly Mn deficiency due to alkaline irrigation, contributed to tree decline. Recently, Fife and Michael (2009) suggested that severe growth and tree mortality within *E. globulus* (blue gum) plantations in southern Australia resulted from previous irrigation of agricultural crops with ground water containing high levels of bicarbonates.

Decreased soil health below ground might also impact on rhizosphere-associated microorganisms, in particular soil bacteria and fungi as the most numerous soil microbes (Coleman et al. 2004). Beneficial soil microorganisms are known to support plant growth (Marschner 1995; van der Heijden et al. 1998b; van Bruggen and Semenov 2000). They can have crucial roles in the functioning of terrestrial ecosystems (van der Heijden et al. 1998b; van der Heijden and van Straalen 2008), and can respond quickly to changes in soil ecological systems (Williamson and Wardle 2007). Moreover, their abundance and diversity may strongly interact with ecosystem functioning (Coleman et al. 2004). Consequently, any adverse change in below ground soil conditions might be related to a decline in plant growth.
2.3.6 Mycorrhizal fungi and forest health

Importance of mycorrhizal fungi

Mycorrhizal fungi constitute one of the most abundant and important beneficial below-ground functional groups. These fungi form associations with the roots of around 80% of all terrestrial plant species (Smith and Read 2008), can influence plant diversity and productivity (van der Heijden et al. 1998b; van der Heijden and van Straalen 2008), and fulfill important roles in ecosystem processes including carbon (C) transport and cycling (Simard et al. 1997; Wu et al. 2001; Hogber and Hogber 2002; Hobbie and Hobbie 2006), mineral nutrient acquisition and cycling (Bolan 1991; Marschner and Dell 1994; Clark and Zeto 2000; Plassard and Dell 2010), and improving soil structure (Tisdall 1991; Bethlenfalvay et al. 1999; Muchane et al. 2013). Furthermore, mycorrhiza may increase plant resistance to some pathogens (Ramachela and Theron 2010; Ismail et al. 2013) and may improve plant growth on degraded land (Sahraoui et al. 2013).

The presence of ECM fungi is of pivotal importance in most forests. In native eucalypt ecosystems, nutrient availability is usually low and litter is important as a nutrient source (Read and Perez-Moreno 2003). In general, ECM fungi can mobilise P, N and other nutrients from litter to tree roots (Attiwill and Adams 1993; Perez-Moreno and Read 2000). The diversity of mycorrhiza may indicate a wide functional capacity that in turn may provide resilience to cope with variable climates and perturbations. Thus, mycorrhiza might help to perpetuate woodlands by aiding their recovery in the face of environmental disturbances (Amaranthus 1998; Tommerup and Bougher 2000).
**Factors affecting mycorrhizal fungi**

There are numerous factors that might govern the abundance and distribution of mycorrhizal fungi in space and time. First, the structure of the above ground plant community may affect mycorrhizal fungi through specificity of preference. In AM symbioses, the level of specificity among host-plant and fungi is generally low (Smith and Read, 2008). In contrast, ECM fungi present different levels of specificity, ranging from broad to restricted associations (Mollina et al. 1992).

Second, soil conditions may directly or indirectly influence mycorrhizal fungi. Abiotic soil factors such as pH, soil fertility, soil moisture, characteristics of soil organic matter, and soil temperature have been suggested to affect mycorrhizal fungi (Brundrett 1991; Conn and Dighton 2000; Erland and Taylor 2002). Lastly, other factors that might affect mycorrhizal fungi include interaction with other soil microorganisms (Klett et al. 2007; Rigamonte et al. 2010), anthropogenic disturbance including agricultural practice such as tillage (Kabir 2005) and fertilization (Pampolina et al. 2002; Schmidt et al. 2010), fire (Cairney and Bastias 2007), pollution (Cairney and Meharg 1999; Kraigher et al. 2007), and climate change (Bellgard and Williams 2011). The relationship between environmental factors and mycorrhizal fungi might occur in two ways. Environmental factors could affect photosynthesis and thereby alter carbon allocation to mycorrhizal fungi. Alternatively, changes in mycorrhizal fungi composition may alter plant nutrient uptake and photosynthetic performance (Kernaghan 2005).

**Mycorrhizal fungi and tree decline**

A number of studies exploring the relationship between mycorrhizal fungi and tree decline have revealed that fine roots of declining trees are less mycorrhizal than
healthy trees (Ellis and Pennington 1992; Causin et al. 1996; Coughland et al. 2000; Montecchio et al. 2004; Scott et al. 2013a). A pot study by Coughland et al. (2000), evaluating the effect of changes in soil pH on AM fungi colonization related to Acer decline, found that a declining site with more acidic soil had a larger spore population but was lower in taxonomic diversity than the healthy site, and liming could stimulate sporulation and quantity of colonization. Mothecechio et al. (2004) studied ectomycorrhizal diversity in declining Quercus in a coastal forest of northern Italy. The decline symptoms occurred after heavy land reclamation in the adjoining area which caused a lowering of the ground water table and increased intrusion of sea water. They found that ectomycorrhizal morphotypes differed markedly among the healthy, weakly and severely declining trees, suggesting the occurrence and distribution of only a limited number of morphotypes as an indicator of the severity of decline. Moreover, it has been suggested that the decreased ectomycorrhizal diversity in declining stands might be related to the level of stress such as drought (Swaty et al. 2004) and defoliation status of host plants (Barto and Rillig 2010), or might be related to soil properties (Peter et al. 2008). Loss of some mycorrhizal taxa from declining sites may reduce host tolerance to certain exogenous stresses, as well as affect nutrient uptake because of alterations in colonization intensity and development of extra radical hyphae (Coughland et al. 2000).

2.4 Environment for eucalypts in southwest Western Australia

2.4.1 Climate

Southwest Western Australia (SWWA) has a Mediterranean type climate and is characterized by warm to hot, dry summers and mild to cool, wet winters (Gentilli 1989). Most (>80%) rain falls between April and October (Bates et al. 2008a) and a prolonged dry period occurs between October and April lasting between 4 to 7
months (Gentilli 1989). Mediterranean ecosystems have been recognized as highly sensitive to climate driven ecosystem shifts (Klausmeyer and Shaw 2009), and SWWA in particular is likely to be affected (Laurance et al. 2011). It is projected that the climate in Mediterranean regions will undergo a process of warming, considerable drying and experience more frequent extreme weather events over the next century (Klausmeyer and Shaw 2009).

Since the 1970s, there has been a dramatic change in the SWWA climate. The annual mean temperature has increased +0.15°C per decade, whilst early winter rainfall has decreased. The mean total rainfall over the period 1975-2004 decreased by around 14% compared to the periods from the mid-1890 to 1974, with a major feature of the rainfall decline being the absence of the very high rainfall years, which commonly occurred throughout the last century (Bates et al. 2008a). Furthermore, it is projected that the drying and warming trend in this region will continue with estimates of up to 40% rainfall reduction and mean annual temperature increases of up to 5°C by 2070 (CSIRO and BoM 2007). Some factors that have been proposed as contributors to the decline in rainfall in SWWA include natural climate variability, enhanced greenhouse effect (Bates et al. 2008a), and land cover change (Pitman et al. 2004).

Recent reports of increased temperature in Australia suggested that summer 2012 was the hottest on record, exceeding the last record in 1972 (www.climatecommission.gov.au). It also reported that more than 70% of Australia experienced extreme temperatures during the heat wave on December-January 2012-2013. Western Australia, in particular, experienced unusual hot and dry conditions from September to December 2012 with an extreme heat build-up in December followed by a one week heat wave at the end of December 2012 (www.climatecommission.gov.au). A more detailed overview of the extreme
temperature event recorded in Western Australia and trend of changes in climate are illustrated in Figs. 2.2a and 2.2b. It has been considered that in addition to increased temperature, more frequent occurrences of frost events in SWWA (unpublished data) might also severely impact on tree health leading to tree decline (G. Hardy, Personal communication). The bell curve (Fig. 2.2b), thus could be stretched two ways.

Fig. 2.2. (a) extreme events recorded in Western Australia in late 2012 to early 2013, (b) predicted changes in climate (www.climatecommission.gov.au, accessed on 4 March 2013).
It has been suggested that the projected future change in climate will either directly or indirectly affect SWWA forest species and ecosystems. The direct effect could be through changes in temperature, rainfall and the frequency of extreme climate events including heat waves and droughts, whilst indirect effects could be through altering species interactions, fire regimes, stream flow and hydrology, and the nature and intensity of existing biodiversity threats (Maher et al. 2010).

In addition, the climate may become increasingly unsuitable for fauna and flora species that the ecosystems currently support (Laurance et al. 2011). Several dominant tree species endemic to SWWA have experienced a decline in health, which has been related to changes in climate (Brouwers et al. 2013a; Brouwers et al. 2013b; Matusick et al. 2013). Furthermore, it has been suggested that species may differ in their resilience to change in climate, resulting in shifts in species composition, range and ecosystem functioning (Hansen et al. 2001).

2.4.2 Soil

Soils in SWWA are mostly highly weathered. In general, these soils are low in fertility and organic matter, and the surface horizon is dominated by sand to sandy loam with kaolinite as the dominant sub-soil clay mineral (Moore 1998). In the Swan Coastal Plain of SWWA, three dune systems run parallel to the coast. Closest to the coast, and the youngest, is the Quindalup dune system (calcareous deep sand), and behind this is the Spearwood dune system (siliceous deep sand overlying limestone). The third and furthest from the coast, and the oldest, is the Bassendean dune system which is a complex of low dunes, sand plains, and poorly drained plains and swampy flats (Moore and Griffin 1998). Thus, the soils in the Swan Coastal Plain range from uniform calcareous sands showing variable depth and surface darkening, from very
shallow to deep siliceous yellow-brown and grey brown sands, or pale, sometimes bleached sands with yellow-brown subsoil (McArthur and Bartle 1980).

2.5 *Eucalyptus gomphocephala* decline

Since the early 1990’s the remnant of *E. gomphocephala* tree populations in the Yalgorup region between Mandurah and Bunbury, approximately 100 km south of Perth, have suffered an alarming reduction in health and vitality (Longman and Keighery 2002; TRG 2003; THRG 2006). In the most affected area within YNP, 80% of the mature trees have died (Longman and Keighery, 2002), whereas, near Preston beach, 30 km south of Mandurah, the mortality rates of *E. gomphocephala* trees across all age classes have reached 90% (TRG 2002; THRG 2006) (Fig. 2.3). The *E. gomphocephala* decline is initially characterized by discolouration, desiccation and death of foliage on individual small outer canopy branches. Defoliation follows, leading to a thinning crown and stag headed appearance, the dead branches protrude above the top of the crown like the antlers of a stag. The death of the branches usually occurs from August to May, and in the following spring and summer, the trees resprout from epicormic buds, typical of eucalypts recovering from stress. However, this new epicormic growth dies in the next cycle. The sprouting becomes weaker and weaker until the tree eventually dies (Longman and Keighery 2002). The typical decline of *E. gomphocephala* can be seen in Fig. 2.4.
Fig. 2.3. Severe *E. gomphocephala* decline at Lake Clifton within Yalgorup National Park, 2007. Photo courtesy of Paul Barber.

Fig. 2.4. Characteristic of *E. gomphocephala* decline: (a) with epicormic growth, (b) death of epicormic shoots and entire tree collapse. Photo courtesy of Giles Hardy.

There is considerable concern about the extent of *E. gomphocephala* decline and the subsequent impact it may have on the ecosystem (Edwards 2004; THRG 2006; Close et al. 2009; Archibald et al. 2010). The decline not only could have a serious impact on ecosystem function and health but also could lead to loss of biodiversity. For
example, some understorey species in *E. gomphocephala* woodlands such as *Acacia benthamii* (bentham’s wattle), *Jacksonia sericea* (waldumi), *Lasiopetalum membranaceum* and *Dodonaea hackettiana* (hackett’s hop-bush) are classified as threatened (THRG 2006). Moreover, the loss of *E. gomphocephala* may result in a severe impact on fauna, abundance and biodiversity. For instance, the vulnerable western ringtail possum lives in the northern part of the range of *E. gomphocephala*. Loss of invertebrate biodiversity as well as loss of water birds on the Yalgoorup Lake is also predicted due to the loss of *E. gomphocephala* woodlands (Dell et al. 2002b; THRG 2006). Recently, Wentzel (2010) evaluated the effect of *E. gomphocephala* decline on fauna and found that the decline resulted in significant changes to fauna habitat, which was related to canopy characteristics, litter and understorey density. The degree of impact on fauna, however, was considered to be related to successional patterns of plant species in *E. gomphocephala* woodland.

### 2.5.2 Possible causes of *E. gomphocephala* decline

Although a number of biotic and abiotic factors have been linked to *E. gomphocephala* decline, the syndrome still remains complex with regards to the predisposing, inciting and contributing factors. These have been discussed by various authors (Table 2.1). Edwards (2004) examined a range of environmental correlates and associations of *E. gomphocephala* canopy condition throughout its range including within YNP, in an attempt to identify environmental factors that might be related to *E. gomphocephala* decline. This author proposed that severe canopy decline in Yalgormup may be associated with unique hydrology, higher rainfall, and fine and shallow soils coupled with groundwater depth fluctuations, and groundwater chemistry changes, especially higher salinity and alkalinity. More widely across the range of *E. gomphocephala*
where slight to moderate decline occurred, increasing fragmentation, nutrient enrichment and ground water change were suggested as possible causes (Edwards 2004). However, since there was no strong correlation with a single factor found in this study, it was suggested that further research on environmental factors was required.

Table 2.1. Factors that may play a role in *E. gomphocephala* decline

<table>
<thead>
<tr>
<th>Suggested factors</th>
<th>References</th>
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<tbody>
<tr>
<td>Environmental changes including</td>
<td>Longman and Keighery (2002)</td>
</tr>
<tr>
<td>a) physical environmental changes: climate change (low rainfall, frost) and hydrological changes (increased ground water and salinity)</td>
<td></td>
</tr>
<tr>
<td>b) change in soil condition (nutrient deficiency and toxicity)</td>
<td></td>
</tr>
<tr>
<td>c) biological factors (herbivore, pathogen and understorey competition)</td>
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<tr>
<td>d) anthropogenic disturbance (clearing, fragmentation, pollution)</td>
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<tr>
<td>e) changes in fire regimes</td>
<td></td>
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<tr>
<td>Disease of complex etiology with a number of predisposing and contributing factors:</td>
<td>(Archibald et al. 2010)</td>
</tr>
<tr>
<td>a) environmental factors including declining water table, salinity, decreasing annual rainfall, nutrient enrichment of soil and water bodies, fire exclusion, drought stress caused by understorey species as predisposing factors</td>
<td>THRG (2006)</td>
</tr>
<tr>
<td>b) biotic agents (insect and pathogen) as contributing factors</td>
<td></td>
</tr>
<tr>
<td>Land management practices (fire exclusion) resulting in water stress and nutrient deficiency</td>
<td>(Close et al. 2009; Close et al. 2011)</td>
</tr>
<tr>
<td>Soil-borne plant pathogen <em>Phytophthora multivora</em> causing loss of fine roots</td>
<td>(Scott et al. 2009; Scott et al. 2011)</td>
</tr>
<tr>
<td>Decreased soil microbial functional diversity</td>
<td>(Cai et al. 2010)</td>
</tr>
<tr>
<td>Drought and heat</td>
<td>(Matusick et al. 2012)</td>
</tr>
<tr>
<td>Decreased ECM extramatrical networks</td>
<td>(Scott et al. 2013a)</td>
</tr>
</tbody>
</table>

Land management practices, particularly the exclusion of fire, has been suggested as a factor contributing to *E. gomphocephala* decline (Longman and Keighery 2002; THRG 2006; Close et al. 2009; Archibald et al. 2010; Close et al. 2011). Close et al. (2009) suggested exclusion of fire could result in two conditions; 1) water stress due
to competition with understorey and drought, and 2) nutrient deficiency resulting from changes in soil conditions and adverse impacts on mycorrhizal associations. In contrast to these authors, a previous study by Malajczuk and Hingston (1981) in *E. marginata* forest in SWWA found that the number of ectomycorrhizal root tips were higher in an unburnt site (45 years) than sites unburnt for 6 years or 1 year. These authors proposed that fire not only could eliminate the substrate of certain ectomycorrhiza but also could have a sterilizing effect, which could reduce the inoculum potential of the fungal symbionts.

As mentioned earlier, climate change (decreased rainfall, increased temperature) is a factor considered to be related to forest decline worldwide (Moore and Allard 2008; Allen 2009; Allen et al. 2010) including SWWA (Brouwers et al. 2013a; Brouwers et al. 2013b; Matusick et al. 2013). Matusick et al. (2012) found that a severe and sudden dieback event in *E. gomphocephala* corresponded with extreme drought and heat conditions in early 2011. It was hypothesized that site conditions, in particular the precipitation drainage pattern, might play important roles in woodland survival during drought. Trees located on water shedding sites with low soil water holding capacity were more susceptible, whilst those located on water gaining sites were more resistant to stressful periods of aridity.

Currently, there is an integrated research project to determine the cause(s) of *E. gomphocephala* decline, with the main emphasis on hydrology and water quality, fire and competition, pests and pathogens, soil microbes and nutrition (THRG 2006). These series of studies suggest that factors including soil health (soil beneficial microorganisms and nutrition), and biotic factors might have important roles in tree decline. For example, it was found that ectomycorrhizal sporocarps were less abundant under declining stands (Legault 2005), and fewer mycorrhizal pads were
associated with declining trees than healthy trees (Dell et al. 2006b; Scott et al. 2013a). A study by Cai et al. (2010) also revealed that soil microbial functional groups decreased with a decline in health of *E. gomphocephala*. Deficiency symptoms of nutrients such as manganese (Mn), iron (Fe), zinc (Zn), boron (B), and toxicities of P have been observed in *E. gomphocephala* foliage (Eslick 2005; Dell et al. 2006a). The presence of opportunistic pathogens such as the aggressive foliar pathogen *Mycosphaerella cryptica*, stem-infecting fungi (*Botryosphaeria* spp.) and soil-borne pathogens (Pythiaceous species) (Taylor et al. 2009; Scott et al. 2011) have been observed and might have a role as contributing factors to *E. gomphocephala* decline.

### 2.6 Concluding remarks

In its natural ecosystem *E. gomphocephala* is experiencing severe decline, and a number of factors might be involved (Longman and Keighery 2002; Edwards 2004; THRG 2006; Close et al. 2009; Archibald et al. 2010; Cai et al. 2010; Matusick et al. 2012; Scott et al. 2013a). Considering their role in ecosystem function, it is likely that mycorrhiza may play an important role in *E. gomphocephala* health.

A few studies have reported the functional and anatomical changes in the ectomycorrhizal status of declining trees compared with healthy trees (Causin et al. 1996; Montecchio et al. 2004; Peter et al. 2008). However, it remains to be determined whether these findings could be extended to *E. gomphocephala* decline, especially where the habitat and environmental conditions are different. In addition, disturbance and stress factors affecting *E. gomphocephala* woodlands in YNP such as fertilizer history, fire regime change, soil condition change, groundwater changes, and drought and heat associated with climate change may directly or indirectly affect mycorrhiza. However, very little is known about how important mycorrhizal fungi
are for healthy functioning of *E. gomphocephala*, in particular, their resilience and sensitivity to environmental or biotic perturbations already evident in the region.

*Eucalyptus gomphocephala* woodlands at YNP are dominated by calcareous soils, and the dominant tree might be classified as a calcicole since it occurs naturally on this soil type. Taking into account the importance of ECM fungi for the growth of eucalypts in their native habitat, it not known whether *E. gomphocephala* is a pure calcicole plant species or it is a symbio-calcicole plant, which depends on fungal symbionts for growth. Thus, further research is required to evaluate how dependent *E. gomphocephala* is on ECM fungi for healthy functioning, particularly where environmental factors such as soil conditions and presence of soil-borne pathogens may not be favourable for tree growth.
Chapter 3

Can ectomycorrhizal fungi protect *Eucalyptus gomphocephala* from infection by *Phytophthora multivora*?

3.1 Introduction

The genus *Phytophthora* is one of the most important groups of soil-borne pathogens causing disease and mortality of forests worldwide. Species within this genus have been associated with worldwide forest and tree decline (Balci and Halmschlager 2003; Jung et al. 2005; Scott et al. 2009; Barber et al. 2013). Ectomycorrhizal fungi are considered to provide protection to plant roots from soil-borne root pathogens by a number of mechanisms which include the provision of a physical barrier to infection, utilizing surplus carbohydrates that might reduce attractiveness of the roots to pathogens, secreting antibiotics inhibitory to pathogens, and favouring protective rhizosphere organisms (Zak 1964). It has also been suggested that improved nutrient uptake by mycorrhizal fungi may also help the host plant to develop defence mechanisms against root diseases (Ramachela and Theron 2010).

A number of studies have highlighted the protective potential of ECM fungi against soil-borne pathogens in general either *in vitro* or on seedlings (Perrin 1990; Zengpu et al. 1995; Pinto et al. 2006). Using filtrates from four ECM fungi, namely *Boletus edulis*, *Rhizopogon roseolus*, *Laccaria laccata* and *Lactarius deliciosus* to investigate their effect on spore germination of *Fusarium* damping off *in vitro*, Pinto et al. (2006) found that *L. laccata* strongly reduced *Fusarium* spore germination, suggesting the role of volatile anti-fungal compounds released by the ECM fungi. The protective potential of ECM fungi against *Phytophthora* species on seedlings has been reported such as *P. cinnamomi* on *E. marginata* (Malajczuk 1988). *P.
cambivora and P. cinnamomi on Castanea sativa (Chesnut ink disease) (Branzanti et al. 1999), and P. parasitica on Uapaca kirkiana (Ramachela and Theron 2010).

In contrast, at the stand or natural forest level some studies have reported a deleterious impact of Phytophthora on ECM fungi. For example, Blom et al. (2009) found that ECM were less abundant and root tip diameters were smaller on declining Castanea sativa sites where Phytophthora was present, compared to healthy Castanea sites where ECM were more abundant. In addition, Scatollin et al. (2012) compared the ECM community structure at healthy Castanea sites with declining sites which had been infested by Phytophthora cambivora and P. cinnamomi. They found that the decrease in mycorrhizal community composition was highly related to an increased disease incidence.

Phytophthora multivora has been isolated from declining E. gomphocephala sites at YNP, and demonstrated to cause fine root loss in E. gomphocephala seedlings (Scott et al. 2011). By using a soil infestation technique utilizing isolates of P. multivora recovered from the rhizosphere of declining trees, Scott et al. (2011) showed that the isolates caused significant fine root loss, suggesting P. multivora is a pathogen to E. gomphocephala. More recently, it has been reported that ECM extramatrical networks were less abundant in declining E. gomphocephala sites compared to healthy sites (Scott et al. 2013a).

It was hypothesized that the presence of P. multivora might adversely impact on ECM (Scott et al. 2013a). However, this remains to be further clarified as there is no information available on the interaction between ECM fungi and this oomycete. Whether the ECM fungi would be impacted by P. multivora or whether ECM fungi could protect E. gomphocephala from P. multivora is not yet known. Thus, the interaction between P. multivora and ECM fungi, and the impact of P. multivora on
ECM fungi colonization as well as its impact on the growth of *E. gomphocephala* in the presence/absence of ECM fungi need to be elucidated. Therefore, the current study aimed to:

- Investigate the effect of *P. multivora* on the *in vitro* growth of ECM fungi,
- Evaluate the protective effect of ECM fungi on *E. gomphocephala* seedlings from *P. multivora*,
- Examine the impact of *P. multivora* on ECM colonization of *E. gomphocephala* roots, and
- Enhance our knowledge on the interaction between ECM fungi and *P. multivora*.

The following hypotheses were examined:

H1. *Phytophthora multivora* is antagonistic to ECM fungi *in vitro* and on seedlings,

H2. ECM inoculation provides protection to *E. gomphocephala* seedlings from *P. multivora* and

H3. ECM fungi differ in their ability to promote growth of *E. gomphocephala* seedlings.

In order to achieve these aims, two experiments were undertaken. The first experiment examined the interaction between *P. multivora* and ECM fungi *in vitro*. The second experiment explored the effect of *P. multivora* on ECM colonization and growth of *E. gomphocephala* seedlings in a glasshouse trial.
3.2 Materials and methods

3.2.1 Experiment 1

To investigate whether *P. multivora* was antagonistic to ECM fungi, and *vice versa* for ECM fungi to *P. multivora*, three methods were explored, namely the production of secondary metabolites, and shared container systems including direct interactions (shared dishes) and volatile compounds (taping two cultures together).

*ECM fungi isolates*

Three ECM fungi isolates, obtained from the Murdoch University collection, *Pisolithus albus* (isolate MURU 7353), *Scleroderma areolatum* Ehrenb (isolate MURU 7271) and *Pisolithus* sp. (isolate MURU 7354, a new species discovered in Western Australia, the taxonomy and identification is in process) were chosen for this trial. *Pisolithus* and *Scleroderma* were chosen as they are widely distributed in Australia and commonly found in *E. gomphocephala* woodland. These isolates were cultured from sporocarps collected from YNP. The procedures to culture ECM fungi from sporocarps were based on the methods described by Brundrett et al. (1996). Briefly, the sporocarps were cleaned with a fine bush to remove all adhering soil followed by surface sterilisation with a paper towel soaked with 70% ethanol for 5 seconds. In a laminar flow, sporocarps were fractured carefully to avoid any internal surface contamination. Small pieces of mycelium from the centre of sporocarps were taken with a sterile razor blade and placed onto Potato Dextrose Agar (PDA) medium. The pure mycelia were then sub-cultured onto PDA and/or MMN (Modified Melin Nokrans) media (Fig. 3.1).
Fig. 3.1. Sporocarp of; (a, b) *Pi. albus* (isolate MURU 7353), (c, d) *(S. areolatum* (isolate MURU 7271), and (e) *Pisolithus* sp. (isolate MURU 7353), and their mycelia (f, g and h for *Pi. albus*, *S. areolatum* and *Pisolithus* sp., respectively).
Phytophthora multivora isolate

The *P. multivora* isolate (isolate 14926 VHS) was provided by the Centre of Phytophthora Science and Management (CPSM), Murdoch University. The isolate was sub-cultured onto half-strength PDA (½ PDA; Becton, Dickinson, Sparks, USA) and placed in a 20°C room in the dark.

Secondary metabolite antagonism

Cellophane was utilized to evaluate whether secondary metabolites of ECM fungi were antagonistic to *P. multivora*, and *vice-versa* for *P. multivora* to ECM fungi. Two media, MMN and PDA, were used for this trial. Petri dishes (90 mm diameter) each were partly filled with MMN or PDA media. The media were then overlain with a cellophane disc that had been washed, boiled, dried and autoclaved for 2 consecutive days (autoclaved for the first day and repeated on the next day) before use. These cellophane discs were cut from porous transparent cellophane sheets, purchased from a supplier to the florist trade and without a trade name, with the size being slightly smaller than the Petri dish diameter.

To evaluate the effect of secondary metabolites of ECM fungi on the growth of *P. multivora*, ECM fungi were grown on PDA and MMN media overlain with cellophane. A piece of mycelium of *Pi. albus*, *S. areolatum* or *Pisolithus* sp., taken from 4-week-old cultures using a cork borer (5 mm diameter), was placed centrally in each Petri dish, and incubated at 25°C in the dark. Three replicates were set up for each of the ECM fungi. After two weeks of incubation, the cellophane was discarded and a 5 mm diameter mycelial plug of *P. multivora*, taken from a 7-day-old culture of *P. multivora* using a cork borer, was placed on the plate and incubated at 20°C in the dark. For the control, *P. multivora* was grown on PDA and MMN media after discarding clean cellophane discs from the plates. Growth of *P. multivora* was
measured on day 7. The diameter of the mycelial colony was measured at two
directions at right angles, and the initial agar plug size subtracted.

To investigate the effect of secondary metabolites of \textit{P. multivora} on the growth of
ECM fungi, \textit{P. multivora} was grown on PDA and MMN media overlain with
cellophane. \textit{Phytophthora multivora} mycelium, taken from 7-day-old cultures of \textit{P.}
multivora using a 5 mm cork borer, was placed centrally in each Petri dish, and
incubated at 20\textdegree C in the dark. Three replicates were set up for \textit{P. multivora}. After 3
days of incubation, the cellophane was discarded and a 5 mm diameter mycelial plug,
taken from 3-weeks-old cultures of the ECM fungi using a cork borer, was placed on
the plate and incubated at 25\textdegree C in the dark. For the control, the ECM fungi were
grown on PDA and MMN media after discarding clean cellophane from the plates.
The colony diameter growth of the ECM fungi was measured on day 14.

\textit{Direct interaction (shared dishes)}

Petri dishes each were partly filled with PDA or MMN media. A line, that equally
divided the Petri dish area into two sides, was made on the base of each Petri dish
using a marker pen. One side was used to grow ECM fungi, whilst the second side
was for \textit{P. multivora}. Ectomycorrhizal fungi were plated prior to \textit{P. multivora}. Since
\textit{Pi. albus} grew quite fast, it was plated 2 weeks prior to \textit{P. multivora}, whereas
\textit{S. areolatum} and \textit{Pisolithus sp.} were grown 3 weeks prior to plating \textit{P. multivora}. For
the controls, the ECM fungi were grown on PDA and MMN media on one side of the
Petri dish without \textit{P. multivora} on the other side, and \textit{vice versa} for \textit{P. multivora}.
The diameter growth of ECM fungi and \textit{P. multivora} were measured 6 days after
treatment.
Volatile compound antagonism (taping two cultures together)

A piece of mycelium (5 mm diameter) of the ECM fungi *Pi. albus*, *S. areolatum* and *Pisolithus* sp., taken from 2-week-old cultures of *P. albus*, and 3-week-old cultures of *S. areolatum* and *Pisolithus* sp., was placed centrally in each Petri dish containing MMN medium, and incubated at 25°C in the dark for 10 days. Meanwhile, 5 mm diameter mycelia plugs, taken from a 7-day-old culture of *P. multivora*, was placed centrally in Petri dishes containing PDA medium, and incubated at 20°C in the dark.

After 3 days of incubation, *P. multivora* was challenged against the ECM fungi. The Petri dish lids of the *P. multivora* and ECM fungi cultures were discarded and the base of the Petri dishes, each with *P. multivora* and the respective ECM fungi, were combined together and sealed with Parafilm™. The cultures were then incubated at 25°C in the dark. The diameters of the *P. multivora* and the ECM fungi were measured before the challenge, and 5 days after treatment. There were three replicate plates for each treatment combination and control treatments.

3.2.2 Experiment 2

A glasshouse trial was undertaken to investigate whether ECM can protect *E. gomphocephala* from *P. multivora*. The trial used ECM fungal mycelium (ECM synthesis) as the inoculum source of ECM fungi, and two species of ECM fungi, the fast-growing *Pi. albus* (isolate number MURU 7353) and the slow-growing *S. areolatum* (isolate number MURU 7271) were chosen. These mycelial cultures were obtained from sporocarps of each species collected in association with *E. gomphocephala* at YNP, as previously described. There were six *E. gomphocephala* treatments in the trial: 1) *Pi. albus* alone; 2) *S. areolatum* alone; 3) no ECM (control 1); 4) *Pi. albus* + *P. multivora*; 5) *S. areolatum* + *P. multivora*, and 6) *P. multivora*
alone (control 2), with 6 replicate containers per treatment with two seedlings in each container.

*ECM culture and mycorrhizal synthesis*

Petri dishes (90 mm diameter) were filled with MMN medium. The medium was then overlain with a cellophane disc that had been prepared as described earlier. Aseptically, three mycelial plugs approximately 5 mm in diameter were taken from 4 week-old colonies of the ECM fungal isolates grown on MMN, and placed approximately 1 cm apart in a row in the centre of each Petri dish. The plates were then sealed with Parafilm™ and incubated in the dark at 25°C until the colonies were 1 - 2 cm in diameter after approximately 2 and 4 weeks for *P. albus* and *S. areolatum*, respectively.

Seeds

Seeds of *E. gomphocephala* were collected from YNP, provided by the State Centre of Excellence for Climate Change, Woodland and Forest Health (Murdoch University). Seeds were surface sterilized by dipping them briefly (5 seconds) in 70% ethanol, soaked in a solution of 3% sodium hypochlorite (5 minutes), and then washed three times in sterile distilled water. The surface sterilized seeds were placed on 0.75% (w/v) water agar for 6 days to germinate the seeds and to check for contamination.

The synthesis of ECM associations

Axenically, four germinating seeds with short radicles (6 days old) were gently removed from the water agar, and placed in a row 0.5 cm above the outermost hyphal growth in the plate with ECM fungi. The plates were then sealed with Parafilm™ and resealed with electrical tape to avoid contamination. Plates with the fungi and
seedlings were placed on a tray and incubated at 25°C with 16 hours light (150 µE m⁻¹ s⁻¹ light intensity) and 8 hours dark cycle. On the first day of incubation, the plates were placed horizontally on the trays, and thereafter on a slant (approximately 20° from the vertical). This allowed the seedling roots to grow towards the fungi and adhere to the cellophane surface, whilst allowing excess water to drain away from roots. The plates were incubated for 4 weeks until the roots were sufficiently colonized by ECM hyphae (Fig. 3.2).

For the control treatment, germinating seeds (4 - 5) were grown in a plate filled with MMN medium overlain with cellophane.

![Fig. 3.2. Mycorrhizal synthesis on *E. gomphocephala* seedlings using MMN medium overlain with cellophane; (a) *Pisolithus albus* (isolate MURU 7353), and (b) *Scleroderma areolatum* (isolate 7271).](image)

*Growing medium preparation*

A mixture of washed white river sand and yellow sand (2:1) was used as the growing medium for the trial. The mix was pasteurized with steam for 3 hours at 85°C before use. Five hundred grams of the pasteurized medium was placed into a clear polyethylene plastic bag (15 x 10 cm), which in turn was placed into a rigid polyethylene plastic container (16.5 x 10.5 cm) inserted into a black plastic bag (13 x 11 cm), which functioned to keep the roots in the dark. The plastic bags and the rigid
polyethylene plastic container were UV sterilized (12 hours) before use. The rigid polyethylene plastic container was composed of two transparent plastic food container lids (17.5 x 12 cm) which were cut around the edges and attached to each other using a stapler. A small window (8 cm length and 2 cm width) was made approximately 7 cm from the top and 1 cm from the left and right sides of the container to facilitate the placement of *P. multivora* inoculum onto the seedling roots (Figs. 3.3a and 3.3b).

**Planting**

Planting of *E. gomphocephala* seedlings was undertaken on a clean bench in the laboratory with sterilized equipment. Briefly, two holes around 1 - 2 cm deep were made using a sterile glass rod (5 mm diameter). For *S. areolatum* synthesis, the cellophane around the roots of each seedling was cut using a sterile blade and then the seedlings gently removed from Petri dishes. The attached cellophane was then removed from the roots, and a single seedling was placed into each hole.

The *P. albus* densely colonized the seedling roots (Fig. 3.2), and instead of cutting the cellophane around the roots, the colonized seedlings together with attached mycelia were gently removed from the plates, and the seedlings directly planted into the medium. To supplement the ECM fungi colonised roots, a 0.5 cm$^2$ of colonized cellophane of each fungus was placed into each hole prior to planting the seedlings.

For the control treatment, 0.5 cm$^2$ sterile non-colonized cellophane was also included when planting the seedlings.

The containers with the seedlings were placed onto a tray and grown in a controlled temperature room (25 ± 0.5°C) with 16 hours light (150 µE m$^{-1}$ light intensity) and 8 hours dark cycle. Seedlings with the same treatment were placed in the same tray to avoid any cross contamination. To avoid any seedling stress immediately after
transferral from humid Petri dishes into containers, the seedlings were covered with food plastic wrap (Glad Wrap, Glad® Product) immediately after planting. One day later, small holes were made in the plastic wrap to allow air circulation. The holes were enlarged daily and the plastic cover was removed 7 days after planting. Holes were made into the base of each plastic bag to allow drainage.

The seedlings were watered overhead every two days to container capacity (20 ml) for the first 2 weeks and then daily thereafter. The trays were randomly moved around the bench weekly. The seedlings were removed to an evaporatively cooled glasshouse with controlled temperature (25 ± 1°C) 24 days after planting. A complete liquid fertilizer of low nutrient content was applied (a quarter strength of recommended dose) weekly once the seedlings were 27 days old. The composition of the fertilizer used was: 500 µM N, 10.9 µM P, 214.8 µM K, 55 µM Ca, 19.5 µM Mg, 81.8 µM S, 0.63 µM Fe, 0.3 µM Mn, 0.6 µM B, 0.1 µM Zn, 0.05 µM Cu and 0.02 µM Mo (Chapter 4). The fertilizer regime was based on that developed for mycorrhizal eucalypts in forest nurseries (Dell and Malajczuk 1995).

**Phytophthora multivora treatment**

**Phytophthora multivora** isolate

The *P. multivora* isolate, as stated in Exp. 1, was sub-cultured onto ½ PDA and grown at 20°C in the dark. The culture was grown for 8 days before being inoculated onto millet (*Panicum miliaceum*) seed.

**Phytophthora multivora** inoculum

Millet seeds were used as an inoculum source to infest the sand medium (1% per gram sand medium). It was prepared as follows: 50 mL of millet seed and 40 mL of water were placed into 250 mL Erlenmeyer flasks, sealed with non-absorbent cotton
and covered with aluminium foil. The flasks were autoclaved at 121°C for 30 minutes over three consecutive days, agitated after each treatment and were inoculated on the third day once the millet seed had cooled. The flasks were aseptically inoculated in a laminar flow using eight agar plugs (10 mm²) colonized with *P. multivora* for 10 days per 250 mL Erlenmeyer flask and then placed inside clear zip lock plastic bags in order to avoid contamination, and incubated at 20°C in the dark. The flasks were shaken by hand every 3 days to evenly distribute the inoculum. Millet seeds were incubated for 8 weeks prior to inoculating the seedlings.

*Phytophthora multivora* inoculation

Seedlings were inoculated with *P. multivora* 6 weeks after planting into the containers. The plastic bag behind the small window of the rigid plastic container was horizontally cut around 5 cm length and 2 cm width with a sterile blade, and some of the sand from this area was removed taking care not to damage roots, and placed on the soil surface. Then, *P. multivora* inoculum (5 g per container) was placed uniformly into the sand medium, covered with the sand, and both the plastic bag and the window sealed with electrical tape (Fig. 3.3c). After inoculation, the seedlings were flooded overnight (12 hours) to encourage sporangia production by placing each container into an individual plastic bag filled with 200 mL deionised water (Fig. 3.3d). In order to confirm the viability of the inoculum, sub-samples were also placed onto half-strength PDA and incubated at 20°C in the dark to confirm that *P. multivora* grew from the colonized millet seed (Fig. 3.4).

Around 6 weeks after inoculation, two seedlings (control seedlings with/without *P. multivora* treatment) were destructively sampled to observe the root systems. As no necrosis was observed in the roots with *P. multivora* inoculum, all the seedlings were then flooded again for 24 hours to encourage sporangia production and zoospore
release. One week after the second flooding, some excess water from *P. multivora*, *P. multivora* + ECM, and control treatments (without *P. multivora* and ECM) was collected from the black plastic bags outside the containers, and approximately 0.5 ml water was plated onto NARPH agar, a selective medium specific for the isolation of *Phytophthora* species (Hüberli et al. 2000). Hyphae of *P. multivora* observed on the plates were compared to hyphal structure of *P. multivora* described by Scott et al. (2009) and thus confirming that *P. multivora* was still viable.

Fig. 3.3. Inoculation steps; (a) ‘furrow’ approximately 5 cm long, 2 cm wide and 1 cm deep was made for inoculation, (b) *P. multivora* inoculum was inserted into the ‘furrow’ and covered with the sand, (c) the window was closed and sealed with electrical tape, and (d) seedlings were flooded for 12 hours.
Seedlings were harvested when they were 4-months old, which was 10 weeks after inoculation. One replicate across each treatment was harvested daily. shoots were cut at the collar (soil line), and fresh weights determined. The plastic bag was cut over a 1.5 mm sieve and the sand medium was washed slowly from the root systems with tap water. The clean roots were then placed in a Zip-Lock (19.5 x 13 cm) plastic bag. The roots were cleaned again under a dissecting microscope to remove sand grains and millet seeds from the root systems. The roots were then examined for evidence of necrosis under a stereomicroscope Zeiss Stemi SV 11 at 12x magnification, and then scored based on the following disease rating scale: 0 = no damage, 1 = ≤ 10%, 2 = 11 - 25%, 3 = 26 - 50%, 4 = 51 - 75%, and 5 = > 75% necrosis. After that, roots were blotted dry and fresh weights taken. Ten 0.5 cm sections of root fragments including white and necrotic roots (brown and black roots if present) were removed and placed onto NARPH agar. The root segments were plated with and without surface sterilization. For surface sterilization, the root segments were dipped for 10 seconds in 70% ethanol followed by rinsing 3 times in sterile distilled water and then dried with paper towel before plating. The roots were observed daily under a dissecting microscope (Olympus CX 31) at 200x magnification for morphological
structures of \textit{P. multivora} for three days, and then the percentage of root fragments infected by \textit{P. multivora} was calculated for each seedling and treatment.

Ectomycorrhizal tip assessment was based on the protocols described by Brundrett et al. (1996). Briefly, root samples, approximately 25% of the root system were cleared by heating in 10% (w/v) KOH in an autoclave for 15 minutes at 121°C, strained through a fine nylon mesh (160 µm), rinsed with water and placed into a vial for staining. A staining solution of 0.05% (w/v) trypan blue (CI 23850) in lactoglycerol (1:1:1 lactic acid, glycerol and water) was added into the vials and left overnight. The stained roots were then strained again through the fine nylon mesh, rinsed with water, placed back into the vial, and then destained with lactoglycerol for around one hour to remove excess dye.

Ectomycorrhizal assessment was based on the proportion of ECM root tips over the total number of root tips. The ECM were characterized by swollen tips stained blue. Roots were poured and spread into a 9 cm Petri dish, and the non ECM and ECM tips were counted under a stereomicroscope (Zeiss Stemi SV 11) with a graticule at 6 times magnification. Assessment was repeated two to three times to obtain the total number of root tips, generally around 50 - 100 in each sample.

3.2.3 Statistical analysis

\textit{Experiment 1}

The interaction between \textit{P. multivora} and ECM fungi through secondary metabolites, direct antagonism and volatile compounds was investigated using independent-sample T-tests. The growth of \textit{P. multivora} and ECM fungi in the challenge treatment was compared with the control. For secondary metabolites and direct antagonism, T-tests were run for each medium (PDA and MMN), separately. These
analyses were conducted using SPSS software package version 17.0 developed by SPSS Inc., Chicago, USA.

Experiment 2

The effect of ECM and *P. multivora* inoculation on plant growth was investigated using a two-way MANOVA with factors of ECM fungi (none, *Pisolithus* MURU 7353 or *Scleroderma* MURU 7271) and *P. multivora* (with and without), and dependent variables of root fresh weight and shoot fresh weight. This was followed by Univariate analysis of each dependent variable. The dependent variables were log transformed to correct for heteroscedascity.

The possible ability of ECM to mitigate the effects of *P. multivora* on root infection was investigated in a one-way MANOVA with a factor of ECM (none, *Pisolithus* MURU 7353 or *Scleroderma* MURU 7271) and dependent variables of proportion of ECM tips and percentage root segment infected by *P. multivora*. This was followed by Univariate analysis for each dependent variable. Data conformed to all assumptions of the analysis, so no transformations were applied.

Root necrosis was recorded as a rank variable, with values of 0 (0%), 1 (≤ 10%); 2 (11 - 25%); 3 (26 - 50%); 4 (51 - 75%), and 5 (> 75%). For those samples exposed to *P. multivora*, the rank scores for necrotic tips without ECM, *Pisolithus* MURU 7353 and *Scleroderma* MURU 7271, were compared using a non-parametric Kruskal-Wallis analysis of variance. Data conformed to all assumptions of the analysis, so no transformations were applied. These analyses were conducted using STATISTICA 8 (Stat Soft. Inc. USA).
3.3 Results

3.3.1 Experiment 1

*Secondary metabolite antagonism*

Independent-sample T-tests indicated that growth of *P. multivora* both on PDA and MMN media containing secondary metabolites of *Pi. albus* was significantly (P = 0.0001 and P = 0.003, respectively) less than the control (Fig. 3.5). However, the ECM fungi did not completely inhibit growth. Out of the two media used for this trial, growth of *P. multivora* on PDA was superior to growth on MMN.

![Fig. 3.5. The effect of secondary metabolites of *Pi. albus* on the growth of *P. multivora* on PDA and MMN media. Values are means (n = 3) ± SE.](image)

Growth of *P. multivora* on PDA medium containing secondary metabolites of *Pisolithus* sp. was significantly (P = 0.03) less than the control. However, growth of *P. multivora* on the MMN medium containing secondary metabolites of *Pisolithus* sp. was not significantly (P = 0.06) different from the control (Fig. 3.6). In contrast to *Pi. albus* and *Pisolithus* sp., *S. areolatum* appeared not to have an inhibitory effect on the growth of *P. multivora* on either PDA or MMN as *P. multivora* grew well on both the media containing secondary metabolites of *Scleroderma* and was not significantly (P = 0.499 and P = 0.1, respectively) different from the control.
Fig. 3.6. The effect of secondary metabolites of *Pisolithus* sp. on the growth (cm) of *P. multivora* on PDA and MMN media. Values are means (n = 3) ± SE.

For secondary metabolite antagonism between *P. multivora* and ECM fungi, data were not analyzed since the ECM fungi did not grow well on the media containing secondary metabolites of *P. multivora*. It is likely that the secondary metabolites released by *P. multivora* negatively impacted on the growth of ECM fungi. Of the three ECM fungi observed, none of the ECM fungi grew well on PDA. On the other hand, the ECM fungi grew well in the absence of *P. multivora* in the controls with mean growth of *Pi. abus*, *S. areolatum* and *Pisolithus* sp. on day 14 being 2.3, 0.8 and 0.9 cm, respectively. On MMN medium containing secondary metabolites of *P. multivora*, *S. areolatum* could not grow, but *Pi. abus* and *Pisolithus* sp. could grow, although growth was poor. The mean growth of *Pi. abus* on day 14 was 2.1 cm compared with 2.5 cm in the control, whilst for *Pisolithus* sp. the mean growth was 0.9 cm compared with 1.5 cm in the control.

**Direct interaction**

Growth of *Pi. abus* in culture paired with *P. multivora* on PDA medium was significantly (P = 0.035) less compared with the controls, where they were grown individually. The control culture of *Pi. abus* had a mean growth of 5.2 cm compared
to 4.7 cm when paired with *P. multivora*. In contrast, growth of *Pi. albus* when paired with *P. multivora* on MMN medium was not different (*P* = 1.0) to the control (Fig. 3.7), which could indicate that *Pi. albus* was not impacted by *P. multivora*. Growth of *P. multivora* on PDA in paired culture was significantly (*P* = 0.0005) less than the control treatment. However, on MMN medium, growth of *P. multivora* was not different from the control (Fig. 3.7).

![Fig. 3.7. Direct antagonisms between *Pi. albus* and *P. multivora* on PDA and MMN media. Values are means (n = 3) ± SE.](image)

There was no significant difference between growth of *S. areolatum* in culture paired with *P. multivora* to growth without *P. multivora* (control treatment) on PDA. However, on MMN medium, growth of *Scleroderma* in paired culture was slightly less (1.8 cm) than the control (2.1 cm) (Fig. 3.8). Growth of *P. multivora* in culture paired with *Scleroderma* on PDA medium was significantly (*P* = 0.001) less than the control treatment. However, on MMN medium, growth of *P. multivora* in paired culture was not significantly (*P* = 0.07) different to the control.

Growth of *Pisolithus* sp. in culture paired with *P. multivora* both on PDA and MMN media was not significantly (*P* = 0.6 and *P* = 0.6, respectively) different from the control (Fig. 3.9). Growth of *P. multivora* in paired culture with *Pisolithus* sp. on
PDA was significantly ($P = 0.001$) less than the control treatment. However, on MMN, growth of *P. multivora* in paired culture was not significantly different from the control.

![Graph](image1)

**Fig. 3.8.** Direct antagonism between *S. areolatum* and *P. multivora* on PDA and MMN media. Values are means ($n = 3$) ± SE.

![Graph](image2)

**Fig. 3.9.** Direct antagonism between *Pisolithus* sp. and *P. multivora* on PDA and MMN media. Values are means ($n = 3$) ± SE.

The effect of direct antagonism between *P. multivora* and the three ECM fungi was only measured once because the *P. multivora* and ECM mycelium started to overlap each other. Growth of *Pi. albus* and *Pisolithus* sp. was slow at the beginning of confrontation (5 days after challenge) compared with the control, however, when the paired cultures were left longer, the ECM fungi continued to grow and seemed to
have an inhibitory effect on the growth of *P. multivora*. The same trend was observed for the paired culture between *S. areolatum* and *P. multivora*. These conditions are illustrated in Fig. 3.10.

![Fig. 3.10. Trend in growth of ECM fungi subjected to direct antagonism with *P. multivora*; (a) *P. albus*, 10 d after treatment, (b) *S. areolatum*, 14 days after treatment and (c) *Pisolithus* sp. 10 days after treatment. The upper part was *P. multivora*, and the lower was ECM fungi.](image)

**Volatile compound antagonism**

There was a significant (*P = 0.006*) effect of *P. multivora* on the growth of *Pi. albus* when the two cultures were taped together. Growth of *Pi. albus* was less (4.3 cm) in culture paired with *P. multivora* than in the control (4.9 cm). On the other hand, growth of *P. multivora* in paired culture was not significantly different from the control (Fig 3.11), indicating that volatile compounds of *Pi. albus* had no negative impact on the growth of *P. multivora*.

Growth of *S. areolatum* in culture paired with *P. multivora* was not significantly different from the control, which could indicate no negative impact of volatile compounds released by *S. areolatum* on the growth of *P. multivora*, and *vice versa* for *P. multivora* on *S. areolatum*. 
Growth of *Pisolithus* sp. in culture paired with *P. multivora* was slightly (P = 0.048) less than the control. Similarly, growth of *P. multivora* in paired culture was also significantly (P = 0.02) less than the control (Fig. 3.12). This may indicate that the volatile compounds released by *Pisolithus* sp. had a negative impact on the growth of *P. multivora*, and *vice versa* for *P. multivora* on *Pisolithus* sp. The impact of volatile compounds on the ECM fungi and *P. multivora* interaction are shown in Fig. 3.13.

Compared with control treatment, it seemed that the mycelial growth of *P. multivora* in paired culture with ECM fungi was slightly thinner and less rosette (Fig. 3.13).
Fig. 3.13. Volatile compound antagonism between ECM fungi and *P. multivora* in dual culture; (a1) growth of *P. albus* when exposed to *P. multivora* and control, (a2) growth *P. multivora* in the same culture and control, (b1) growth of *S. areolatum* paired with *P. multivora* and control, (b2) growth *P. multivora* in the same culture and control, (c1) growth of *Pisolithus* sp. paired with *P. multivora* and control, and (c2) growth *P. multivora* in the same culture and control.

### 3.3.2 Experiment 2

*Pisolithus albus* (MURU 7353) is a fast growing ECM fungus which only took 2 weeks to form a 1 - 2 cm diameter colony compared with *S. areolatum* (MURU 7271) which took 4 weeks to achieve the same colony diameter. Furthermore, *Pi. albus* was able to colonize *E. gomphocephala* seedlings faster than the *S. areolatum*,...
and formed quite dense mycelium during the synthesis. After inoculation with *P. multivora*, the seedlings above ground remained healthy throughout the trial; however, the control treatment, particularly those seedlings that were not inoculated with ECM fungi had relatively poor growth.

**Effect of ECM fungus and *P. multivora* inoculation on growth of seedlings**

Multivariate analysis revealed significant effects of ECM fungi (*F*(4, 58) = 2.608, *P* = 0.044) and *P. multivora* (*F*(2, 29) = 19.674, *P* = 0.00004) inoculation on shoot and root fresh weight, but the interaction was not significant (*F*(4, 58) = 1.415, *P* = 0.24). Univariate results showed that ECM fungi inoculation significantly (*F*(2, 30) = 5.076, *P* = 0.012) increased root fresh weight (Fig. 3.14), but was not significant for shoot fresh weight (*F*(2, 30) = 2.835, *P* = 0.074).

Furthermore, the root growth of *E. gomphocephala* seedlings responded differently to the two types of ECM fungi used in this trial. Seedlings inoculated with *Pi. albus* had better root growth than those inoculated with *S. areolatum*. Surprisingly, *P. multivora* inoculation significantly elevated both shoot (*F*(1, 30) = 37.27, *P* = 0.000001) and root fresh weight (*F*(1, 30) = 39.45, *P* = 0.000001) (Figs. 3.15a and b, 13.6).

![Graph](image)

Fig. 3.14. Root fresh weight (g) of seedlings without ECM fungal inoculation and inoculated with *Pisolithus albus* (isolate MURU 7353) or *Scleroderma areolatum* (isolate MURU 7271). Data were logarithm transformed (log₁₀ (√4+1)). Values are means (n = 6) ± SE.
Fig. 3.15. Shoot (a) and root (b) fresh weight of seedlings without inoculation and inoculated with *P. multivora*. Data were logarithm transformed: \( \log_{10}(\sqrt{3}+1) \) for shoot fresh weigh, and \( \log_{10}(\sqrt{4}+1) \) for root fresh weight. Values are means (n = 18) ± SE.

Fig. 3.16. Growth of *E. gomphocephala* seedlings inoculated with *P. multivora* and ECM fungi; (a) *Pi. albus* isolate MURU 7353 and (b) *S. areolatum* isolate MURU 7271. Seedlings inoculated with *P. multivora* (left), with ECM fungus (middle), and with both ECM fungus and *P. multivora* (right). Seedlings were 3-months-old.

**Effect of ECM fungi inoculation on the effect of P. multivora**

Multivariate analysis indicated a significant effect of ECM fungi inoculation (\( F_{(3, 26)} = 6.61, P = 0.00025 \)) on dependent variables including the proportion of root tips and percentage of root segments infected by *P. multivora*. Univariate results revealed that inoculation with ECM fungi significantly (\( F_{(2, 15)} = 2.185, P = 0.000008 \)) increased root tips colonized by ECM fungi. Inoculation with *Pi. albus* MURU 7353 resulted
in 21.9% colonized root tips, whilst the treatment inoculated with *S. areolatum* MURU 7271 had 13.17% infected root tips. Surprisingly, *P. multivora* inoculation increased the proportion of root tips colonized by ECM fungi, where 30% and 29% of root tips were colonized by *P. albus* and *S. areolatum*, respectively (Fig. 3.17). The effect of millet seed inoculum used to introduce *P. multivora* on ECM fungi is illustrated in Fig. 3.18.

Fig. 3.17. Proportion of ECM tips of seedlings inoculated with *Pisolithus albus* MURU 7353 or *Scleroderma areolatum* MURU 7271, without and with *P. multivora* inoculation. Values are means (n = 6) ± SE.

Fig. 3.18. Root tips colonized by *Pi. albus* MURU 7353 (black arrow) growing inside a millet seed (white arrow).
Root necrosis

The seedlings inoculated with *P. multivora* had necrotic lesions in some roots. The percentage of roots with necrotic lesions varied from < 10% (score ‘1’) to 11 - 25% (score ‘2’), with more than 50% of roots with *P. multivora* inoculation treatment having a score of ≤ 10% (score ‘1’). In contrast, the roots both in the control (without ECM) and ECM fungal inoculation treatments were mostly white, healthy and no lesions were observed (Fig. 3.19).

Fig. 3.19. Appearance of *E. gomphocephala* roots; (a) control treatment without root lesions, (b) *P. multivora* (control) showing black necrotic roots (arrow), (c) seedlings inoculated with *Pisolithus albus* MURU 7353 and *P. multivora*, (d) seedlings inoculated with *Scleroderma areolatum* MURU 7271 and *P. multivora*. White arrow indicates necrotic roots.
A close up of necrotic roots of a seedling with \textit{P. multivora} inoculation is shown in Fig. 3.20.

![Image of necrotic roots](image)

Fig. 3.20. Fine root and root tip necrosis of seedling roots following inoculation with \textit{P. multivora}. White arrow indicates necrotic roots.

Non-parametric Kruskal-Wallis analysis of variance found that where plants were exposed to \textit{P. multivora}, no difference in the incidence of root tip necrosis occurred between seedlings without ECM fungal inoculation and seedlings inoculated with ECM fungi (\textit{Pisolithus albus} MURU 7353 and \textit{Scleroderma areolatum} MURU 7271) (Chi-square; df (2) = 3.27, \( P = 0.1947 \)).

Roots infected by \textit{P. multivora}

\textit{Phytophthora multivora} was not recovered from root segments plated from the control treatment (without \textit{P. multivora} inoculation) or from the ECM inoculation only treatment. By contrast, the pathogen was recovered from root segments plated from the treatments inoculated with \textit{P. multivora}, both with and without ECM fungi inoculation. Univariate results indicated that ECM fungi inoculation had no significant effect on the percentage of root segments infected by \textit{P. multivora}, both for root segments plated with surface sterilization (\( F_{(2, 15)} = 0.337 \ P = 0.719 \)) and
those without surface sterilization \( (F_{2, 15} = 0.381, \ P = 0.669) \) (Fig. 3.21).

Morphological structures (hyphae and oospores) of \textit{P. multivora} recovered from the Petri dishes where the root segments were plated are illustrated (Fig. 3.22).

![Graph showing percentage of root segments infected by \textit{P. multivora} without and with ECM fungi (Pisolithus albus MURU 7353 and Scleroderma areolatum MURU 7271) inoculation. Root segments were surface sterilized before plating. Values are means (n = 6) ± SE.]

Fig. 3.21. Percentage of root segments of seedlings infected by \textit{P. multivora} without and with ECM fungi (\textit{Pisolithus albus} MURU 7353 and \textit{Scleroderma areolatum} MURU 7271) inoculation. Root segments were surface sterilized before plating. Values are means (n = 6) ± SE.

![Images of morphological structures of \textit{P. multivora} recovered from root segments of seedlings with \textit{P. multivora} infestation; (a) hyphae, and (b) oospores (white arrows).]

Fig. 3.22. Morphological structures of \textit{P. multivora} recovered from root segments of seedlings with \textit{P. multivora} infestation; (a) hyphae, and (b) oospores (white arrows).
3.4. Discussion

3.4.1 Interaction between *P. multivora* and ECM fungi *in vitro*

The study examined the interaction between three species of ECM fungi and *P. multivora* through secondary metabolites, direct antagonism (shared plates) and volatile compounds (taping two cultures together). The results of interaction tests between ECM fungi and *P. multivora* through secondary metabolites demonstrated that growth of *P. multivora* mycelia was partially inhibited by ECM fungi, particularly by *Pi. albus* and *Pisolithus* sp. whereas, growth of *P. multivora* was not affected by *S. areolatum*. In culture where ECM fungi were grown on the media containing secondary metabolites of *P. multivora*, the ECM fungi did not grow well. This may have indicated that *P. multivora* and the ECM tested inhibited each other, which was possibly due to the release and accumulation of secondary metabolites, i.e. antifungal compounds or antibiosis in the growing media.

Confrontation tests between ECM fungi and *P. multivora* through direct antagonism (shared plates) on PDA medium showed that the growth of *P. albus* was slightly inhibited by *P. multivora* at 5 days after challenge, but *S. areolatum* and *Pisolithus* sp. were not affected. On the other hand, growth of *P. multivora* was inhibited by these three ECM fungi. This trend was quite obvious when the paired cultures were left for longer periods of time. This could be explained as ECM fungi were relatively slow in their growth compared to *P. multivora*. Thus, ECM fungi may need a longer time to exhibit inhibition, and the effect might be different between the species of ECM fungi. *Pisolithus albus* continued to grow after the diameter measurement was taken and started to grow over the *P. multivora* mycelia. The mycelia of *P. multivora* were inhibited and ceased to grow when they were in close proximity to *Pi. albus*, *S. areolatum*, and *Pisolithus* sp., indicating that the ECM tested were capable of
suppressing growth of *P. multivora* in vitro. Since this study did not examine the interaction in detail, future studies should investigate the morphology of hyphal tips as these can change when they come in contact with antagonistic fungi.

Results of the interaction between ECM fungi and *P. multivora* by taping the two cultures together (volatile compounds) showed that both *Pisolithus* species were slightly inhibited by *P. multivora*, whilst *S. areolatum* was not affected. Of the three ECM fungi tested, only *Pisolithus* sp. appeared to inhibit growth of *P. multivora* mycelia. Furthermore, although *P. albus* and *Pisolithus* sp. did not significantly inhibit the diameter growth of *P. multivora*, *Pi. albus* and *Pisolithus* sp. might have affected the morphology of *P. multivora* mycelia as the mycelia of *P. multivora* in paired culture were slightly thinner when compared with control cultures. The same condition was also observed when *P. multivora* was exposed to *S. areolatum*. The change in hyphal morphology of *P. multivora* mycelia may have indicated that damage had occurred.

In general, on consideration of all the interactions (antibiosis/secondary metabolites and volatile compounds), the results of this study showed that *P. multivora* was negatively affected in vitro by ECM fungi, although the ECM fungi were also slightly impacted by *P. multivora*. The mechanisms by which ECM fungi could modulate growth of pathogenic fungi in vitro included the production of anti-fungal compounds i.e. antibiosis (Perrin and Garbaye 1982; Duchesne et al. 1988), or through the effect of antagonism. Antagonism by parasitism of ECM on the growth of pathogens was previously observed and reported by Zengpu et al. (1995).

It is likely that in the interaction study between *P. multivora* and ECM fungi many complex reactions might have occurred between the fungi. In the case of antagonism by secondary metabolites, it would be important to analyse the secondary metabolites
released by the ECM fungi and *P. multivora* in future studies. In a shared container system (both shared plates and taping two cultures together) respiration products or accumulation of carbon dioxide or other gases released by the fungi may affect fungal growth. Since this study did not examine this interaction in detail, future studies should measure the volatile components released by the fungi by gas chromatography.

The result showed that the species of ECM tested differed in their antagonism activity against *P. multivora*. Marx (1972) suggested that although ECM are capable to modulate growth of the pathogens, the capability between a species in genera and between strains in the same species of ECM might be different due to variability in the production of antibiotics. Accordingly, the interaction between ECM fungi and soil-borne pathogens may vary with each host-symbiont-pathogen combination, where disease severity or pathogen activity may be increased, decreased or be unaffected (Perrin 1990). For example, Duchesne et al. (1988) reported suppression of *Fusarium* by *Paxillus involutus* on *Pinus resinosa* (red pine), and Pinto et al. (2006) found that the inhibitory effect of ECM on *Fusarium in vitro and in situ* on *Pinus nigra* (black pine) seedlings varied between the four ECM tested. In contrast, Perrin and Garbaye (1982) found that *P. involutus* associated with *Fagus* increased the infectivity of *Pythium* in soil.

### 3.4.2 Effect of ECM inoculation on seedling growth

Ectomycorrhizal fungi inoculation did not increase shoot growth of *E. gomphocephala* seedlings, but inoculation significantly increased root growth of seedlings compared to the non-inoculated control seedlings. There are some plausible explanations for this result. Firstly, ECM formation has the potential to enhance plant growth, however, species and isolates of ECM may differ in their effect on plants.
due to their variable ability to acquire nutrients and promote growth (Smith and Read 2008), and therefore growth of the root and shoot (Marx 1972). Some fungi can promote the production of shoot biomass over non-inoculated controls, whilst others may induce the production of roots. The variability in host growth response due to ECM inoculation has been previously reported. Working with four different fungi (Hebeloma crustuliniforme, Laccaria laccata, Pisolithus tinctorius and Thelephora terrestris) on three species of conifers, namely Tsuga heterophylla (hemlock), Pinus ponderosa (blackjack pine) and Pseudotsuga menziesii (Douglas fir), Trappe (1977) found variable responses of the host species to inoculation. The four ECM fungi stimulated growth of roots more than shoots of T. heterophylla seedlings, whilst they had no effect on blackjack pine or Douglas fir, and inhibition by Thelephora terrestris occurred on both these hosts. Furthermore, L. laccata induced more top biomass on blackjack pine and Douglas fir, whilst Pi. tinctorius and T. terrestris enhanced root growth rather than shoot biomass of Douglas fir seedlings.

Secondly, the enhanced effect of ECM fungi on root growth might also be related to carbohydrate allocation. In mycorrhizal plants, a substantial proportion of carbohydrate is allocated to the roots for fungal growth, maintenance and nutrient uptake (Smith and Read 2008). Possibly, the carbohydrate demand by the fungus was high during the early stages of seedling development when the fungus was growing rapidly, and large quantities of carbohydrate may have been transferred from the roots to the mycorrhizal fungi. Lastly, mycorrhizal fungi may affect plant nutrient status, however they may not directly affect the distribution of dry matter between roots and shoots (Bougher et al. 1990).

Unsurprisingly, the two ECM types used in this study differed in their ability to promote root growth of E. gomphocephala seedlings. Seedlings inoculated with Pi.
albus MURU 7353 had better root growth than the control treatment and the seedlings inoculated with *S. areolatum* MURU 7271. As the *Pisolithus* isolate used was a fast growing fungus, it could colonize the roots faster than *Scleroderma*, which was relatively slow growing, resulting in better root growth of the seedlings. Accordingly, although the outcome of the association is influenced by the ability of ECM fungi to colonize the plants, the responsiveness of the host plant to different ECM fungi might have a larger contribution to the overall biomass (Smith and Read 2008).

3.4.3 Effect of *P. multivora* infestation on *E. gomphocephala* growth

The study found that *P. multivora* inoculation increased shoot and root growth of *E. gomphocephala* seedlings. In contrast, a previous study by Scott et al. (2011) showed that *P. multivora* infestation resulted in fine root loss of *E. gomphocephala* seedlings. There are a number of possible explanations for this contrasting result including differences in the inoculum used to introduce *P. multivora*, the growing medium used to grow the seedlings, fertilizer application, and time for growing the seedlings. Probably, among the most important of these were the inoculum substrate used for *P. multivora* to colonize and the growing medium. Millet seeds as an inoculum source to infest soil has been successfully used in previous studies dealing with pathogenicity of *Phytophthora* on some tree species (Hardy and Sivasithamparam 1991; Yulia 2012). Possibly, the suggested dose of millet seed inoculum (1% of the growth medium weight), which worked well in previous studies, was too high considering the medium only contained 500 g of sand. It is likely that the millet seeds provided a small and temporary nutrient source.

In addition to the putative fertilizer effect of the millet seeds, the soil environment in the containers used to grow *E. gomphocephala* seedlings might not have been
favourable for the growth of *P. multivora*. The growing medium for the seedlings was dense and it was possible that the reduced aeration surrounding the millet inoculum may not have been conducive for the development of sporangia and release of zoospores by *P. multivora*. Nevertheless, root damage from *P. multivora* was evident. However, the above-ground tissues remained healthy throughout the experiment. This raises a question as to whether the same condition would occur if the experiment was run longer with a declining nutrient source and more competition for space in the rhizosphere. As millet seeds could play a temporary role as a nutrient source, future studies should include an additional control treatment of millet seed colonized by *P. multivora* and autoclaved prior to its application to the pots. Alternatively, other inoculum sources as well as reduced quantities of millet seeds should be considered in future studies.

**3.4.4 Interaction between ECM fungi inoculation and *P. multivora***

Seedlings inoculated with ECM fungi had more ECM tips and more vigorous root growth compared to the non-inoculated seedlings. Unpredictably, *P. multivora* inoculation significantly increased the root tips colonized by ECM fungi, which was possibly due to the nutrient effect of the millet seeds used to introduce *P. multivora*. It seemed that the millet seeds had a positive impact on the growth of ECM fungi.

Results also showed that some roots of *E. gomphocephala* seedlings inoculated with *P. multivora* were necrotic. It was not certain whether the necrotic roots resulted from *P. multivora* infection or were due to other factors such as the level of porosity in sands in the container. However, as *P. multivora* was recovered from the root segments plating onto NARPH medium, this indicated that *P. multivora* colonized and survived in the seedling roots. Thus, as mentioned earlier, it is very likely that the necrosis was due to *P. multivora* infection.
Statistical analysis revealed that the incidence of root necrosis and percentage of root segments infected by *P. multivora* were not different between the ECM fungi inoculated seedlings and the non-ECM inoculated seedlings. Thus, it is likely that ECM fungi inoculation did not limit infection and root necrosis by *P. multivora* on *E. gomphocephala* seedlings. Reasons for this could include a lack of protection of roots by ECM to *P. multivora*, or increased production of roots from the millet inoculum. In this study, seedlings were 30% and 29% colonized by *Pi. albus* and *S. areolatum*, respectively. This may imply that ECM fungi colonization had already reduced the amount of susceptible root tissue exposed to *P. multivora*. However, the number of unprotected fine roots still remained high. In addition, since the millet seed inoculum seemed to enhance the growth of the roots, the rapid growth of the whole root system may have exceeded the growth of the ECM fungi and reduced their subsequent level of protection against *P. multivora*. Ross and Marx (1972) examined the susceptibility of Ocala and Choctawhatchee races of *Pinus clausa* (sand pine) to *P. cinnamomi* with and without inoculation with *Pisolithus tinctorius*. The authors found that the ectomycorrhizal roots were not penetrated by the pathogen, and the mycorrhizal seedlings of the Ocala race were better protected against *P. cinnamomi* compared with the mycorrhizal seedlings of the Choctawhatchee races which had more vigorous root systems. Root protection by ECM fungi was postulated to be the result of a barrier effect caused by the fungal mantle around roots, nutrient competition in the rhizosphere and antimicrobial substances produced either by the ECM fungi or by the host plants (Marx 1972). All these factors may act simultaneously and synergistically to suppress disease.

To conclude, growth of ECM fungi *in vitro* was not or was only slightly reduced by *P. multivora*, and ECM colonization on *E. gomphocephala* seedlings *in vivo* was not
negatively impacted by *P. multivora*, thus the first hypothesis was confirmed. Furthermore, although some roots were necrotic possibly due to *P. multivora* infestation, ECM colonization was high and *E. gomphocephala* seedlings grew well, indicating that ECM may have provided protection to the seedlings from *P. multivora*. Thus the second hypothesis was confirmed. The third hypothesis that ECM fungi differ in their ability to promote growth of *E. gomphocephala* seedlings was also confirmed as the result showed that *Pi. albus* promoted growth of *E. gomphocephala* seedlings more than *S. areolatum*.

Given that *P. multivora* did not kill *E. gomphocephala* seedlings and there was no direct evidence that *P. multivora* could kill adult trees in the field, the remainder of this thesis will now focus on mycorrhizal fungi and tree decline.
Chapter 4

Diversity and abundance of arbuscular mycorrhizal fungi and relationship with *Eucalyptus gomphocephala* health

4.1 Introduction

The results from the preceding chapter showed that although *P. multivora* inoculation resulted in root necrosis in *E. gomphocephala* seedlings, no seedling death occurred during the trial. This indicated that *P. multivora* might not be an aggressive pathogen to *E. gomphocephala* under the conditions provided during the inoculation trial, and may not be the primary cause of *E. gomphocephala* decline in the field. Additionally, the results also showed that growth of ECM fungi was not negatively impacted by *P. multivora*. Therefore, the main focus of this chapter was on mycorrhizal fungi. Mycorrhizal fungi may be involved in preventing *E. gomphocephala* decline due to their important role in many ecosystems processes (Chapter 2). These include C transport and cycling (Hobbie and Hobbie 2006), plant absorption of P and various other nutrients from soil (Marschner and Dell 1994; Clark and Zeto 2000; He et al. 2003; Plassard and Dell 2010), and formation of soil aggregates (Tisdall 1991; Bethlenfalvay et al. 1999; Muchane et al. 2013).

Eucalypts predominantly form ECM associations (Chilvers and Pryor 1965). However, they can also have associations with AM fungi, especially as seedlings under glasshouse conditions (Lapeyrie and Chilvers 1985; Chen et al. 2000a) but also in some native woodlands (Reddell and Malajczuk 1984; Adams et al. 2006). Arbuscular mycorrhiza also appear to be common on eucalypts grown outside their natural range (Santos et al. 2001; Campos et al. 2011) where the diversity of compatible ECM fungi is low (Dell et al. 2002a).
Although AM fungi are not generally considered to be host specific, the species composition and population levels of AM fungi are highly variable and are influenced by environmental factors such as the prevailing climate, soil chemical and physical properties, and variety and age of host plant species (Smith and Read 2008). Rhizosphere-associated microorganisms, including mycorrhizal fungi, are known to support plant growth (van der Heijden et al. 1998b; van Bruggen and Semenov 2000), thus any adverse change in the functioning of this group of organisms may alter the response of plants to disturbance (Rillig et al. 2002). Damage to vegetation and soils resulting from natural processes and human intervention might adversely affect mycorrhizal fungi (Brundrett 1991; Amaranthus 1998; Erland and Taylor 2002). However, the resilience of mycorrhizal fungal communities to disturbance may vary depending on the species (Smith and Read 2008) and the initial composition of the community (Erland and Taylor 2002; Hart and Reader 2004).

Some studies have reported that fine roots of declining trees have less mycorrhiza than healthy trees (Ellis and Pennington 1992; Causin et al. 1996; Coughland et al. 2000; Montecchio et al. 2004; Peter et al. 2008). Indeed, in previous studies a loss in soil microbial function (Cai et al. 2010), and reduced density of fine roots (Scott et al. 2011) and abundance of ECM fungal mats (Scott et al. 2013a) were related to the decline in health of *E. gomphocephala*. However, so far no study has evaluated whether there is any relationship between AM fungi and *E. gomphocephala* decline. Therefore, the objectives of the current study were to:

- Determine whether AM fungal spore density and species richness vary with canopy health of *E. gomphocephala*,
- Evaluate whether soil chemical properties are related to canopy health of *E. gomphocephala* and abundance and diversity of AM fungi, and
• Enhance our knowledge of the importance of AM fungi to the health of *E. gomphocephala*.

The following hypotheses were examined:

H4. The presence of AM fungal spores is not related to the health of *E. gomphocephala*,

H5. Soil chemical properties influence the health of *E. gomphocephala* and,

H6. Soil chemical properties influence AM fungi spore density and species richness.

In order to achieve the above objectives, the approach used was to collect soil samples from 12 field study sites representative of the *E. gomphocephala* decline phenomenon and used in previous studies (Cai et al. 2010). *Eucalyptus gomphocephala* woodland/forest in the Yalgorup National Park (YNP) was chosen for this study since this species is endemic to Western Australia and it is experiencing serious decline. This study forms part of ongoing integrated research into the cause and management of tuart decline (Tuart Health Research Group) funded by the Australian Research Council.

4.2 Materials and methods

4.2.1 Study site

The study area was in YNP, located between Mandurah and Bunbury on the western edge of the Swan Coastal Plain, southwestern Australia (Fig. 4.1). *Eucalyptus gomphocephala* is the dominant tree species and has been listed as category II (National Park; protected area managed mainly for ecosystem conservation and recreation) by the International Union for the Conservation of Nature (IUCN). The study area has twelve sites (Fig. 4.1) with different levels of decline ranging from
healthy trees to trees with advanced decline as described by previous workers (Legault 2005; Cai et al. 2010). These twelve study sites are part of a long-term monitoring study and had previously been selected according to their vegetation trend class over a 15-year period (1990-2005) derived from Landsat Thematic Mapper satellite data (Cacceta et al. 2000).

Fig. 4.1. Maps showing the region where the study was undertaken in southwestern Australia and the location of the twelve sites where soil samples were collected.

Most of the study sites were comprised of *E. gomphocephala* as overstorey except sites 10 and 12, which consisted of mixed eucalypt species (*E. gomphocephala*, *E. marginata* and *Corymbia calophylla*). The understorey of most sites (1-9 and 11) consisted of *Agonis flexuosa*, *Melaleuca acerosa* (Myrtaceae); *Allocasuarina fraseriana* (Casuarinaceae); *Banksia attenuata*, *B. grandis*, *B. littoralis* (Proteaceae); *Acacia pulchella*, *A. saligna*, *Jacksonia sternbergiana* (Fabaceae) and *Hibbertia*
*hypericoides* (Dilleniaceae). Sites 10 and 12 contained *B. attenuata*, *A. flexuosa* and *A. fraseriana* as understorey (Portlock et al. 1993; Keighery 2002).

In southwestern Australia, it is standard practice for fuel loads to be reduced by autumn or spring fires (McCaw et al. 2002). However, the study sites have not experienced intense wildfire or prescribed burning for many decades.

The region has a Mediterranean type climate with hot, virtually dry summers and cool winters. The average annual rainfall in the last decade (2002-2011) from the nearest weather station (Mandurah, station No. 009977) was 660.8 mm, of which around 76% fell between May and September. The average annual maximum and minimum temperatures based on monthly mean temperatures were 23.1 and 14.6°C, respectively (www.bom.gov.au/climate) (Figs. 4.2 and 4.3). The soils were predominantly calcareous sands underlain by limestone which occasionally outcroped near ridges (Portlock et al. 1993; Moore 1998).

![Fig. 4.2](image-url) Yearly values for annual rainfall (a) and maximum and minimum temperatures (b) from the Mandurah weather station (009977) online data. Source: Bureau of Meteorology, Australian Government, http://www.bom.gov.au/climate, accessed on 12 July 2012.
4.2.2 Tree selection and crown health measurement

In each site, four individual *E. gomphocephala* trees were randomly selected and located by a global positioning system (GPS) and assessed for crown health. These four trees were the same trees where soil samples were collected for a bioassay trial (Chapter 5). The diameter at breast height of *E. gomphocephala* across the sites ranged from approximately 30 cm to well in excess of 100 cm. It is estimated that the age of the trees ranged from less than 40 years to over 150 years.

Crown health indices were measured and included crown density (CD), foliage transparency (FT), crown dieback ratio (CDR) and epicormic index (EI) (Grimes 1978; USDA 2005). Crown density is the amount of crown branches, foliage and reproductive structures that blocks light visibility through the crown. Crown density estimates crown condition in relation to a typical healthy tree of the species and for the region where it is found, which also serves as an indicator of expected growth in the near future. Foliage transparency is the amount of light visible through the live, normally foliated portion of the crown, excluding the non-photosynthetic tissues. Crown density and foliage transparency were assessed according to previously published methods (USDA 2005). A crown density/foliage transparency card was used as guide to assess each individual tree. Each individual tree was assessed twice at 90° to each other at the same time and the average of the assessment calculated.

Crown dieback reflects the severity of recent stresses on a tree (USDA 2005). The crown dieback ratio was estimated as the proportion of live crown area remaining of the estimated original crown area, expressed as a percentage (to the nearest 10% category). Epicormic buds, which lie dormant beneath the bark, are very common on eucalypts. Under stressed conditions when there is damage from insect attack, fire or other stress factors the epicormic buds can develop into active shoots allowing for
the vegetative regeneration of branches from their trunks (Florence 1996). The epicormic index was estimated as the proportion of crown comprised of growth of epicormic origin (to the nearest 10% category) (Stone and Haywood 2006). All four crown health indices were acquired at a distance from the tree equivalent to or more than the tree height.

Classification of the health status of the trees within each site was calculated based on a combination of all four indices described above, and labeled the total crown health index (TCHI) (Evans et al. 2012). The TCHI is defined as: $TCHI = (C+F+D+I)/4$ where $C =$ Crown Density, $F = 100 - $ Foliage Transparency, $D = 100 - $ Crown Dieback Ratio, and $I = 100 - $ Epicormic Index. The semi-quantitative measure TCHI was used by Evans et al. (2012), in combination with spectral and textural remotely-sensed metrics, to develop a model sensitive enough to accurately predict small variations in crown health of $E. gomphocephala$ in the same sites used for the present study. The TCHI measure was therefore utilized in the present study as a reliable, positively correlated measure of variation in crown health of $E. gomphocephala$.

**4.2.3 Soil collection**

At each site, two soil cores were collected at the end of summer (4 March 2010) at locations 5 m north and south from each tree trunk. Soil samples, approximately 500 g each, were collected from the twelve sites to a depth of 20 cm using a 4 cm diameter stainless steel soil corer. A total of 96 soil core samples were collected (12 sites x 4 trees x 2 positions). The soil samples collected were for AM fungi spore density assessment and soil analysis. This sampling depth was chosen as it contained the main near-surface feeder root system of the trees where mycorrhizas are known to form. The soil samples were taken adjacent to the intact soil cores collected for the
bioassay trial (Chapter 5). Samples were placed in a paper bag enclosed in a plastic bag and stored at 4°C in a cold room for up to 1 month until they were processed for AM fungi spore assessment.

Soil samples (250 g) were sent to a commercial laboratory (CSBP Limited, Bibra Lake, Western Australia) for determining chemical soil properties. The pH and electrical conductivity were determined in H₂O and CaCl₂ (Rayment and Higginson 1992), ammonium (NH₄) using KCl extractant (Searle 1984), organic carbon (C) by the Walkley Black method (Walkley and Black 1934), sulfur (S) using the KCl-40 method (Blair et al. 1991), and P and K using the Colwell method (Colwell 1965). Micronutrients Fe, Cu, Zn and Mn were extracted with diethylen-triamine-penta-acetic acid (DTPA) (Rayment and Higginson 1992), exchangeable cations aluminium (Al), Ca, magnesium (Mg) and sodium (Na) were extracted with NH₄Cl/BaCl₂ (Rayment and Higginson 1992), and boron (B) was measured as hot water extractable B (Rayment and Higginson 1992). The chemical characteristics of these soils are given in Appendix 2.

4.2.4 Extraction of AM fungi spores

Spores of AM fungi were extracted from soil samples using wet-sieving and sucrose methods (Tommerup 1988; Brundrett et al. 1996). Soils samples (50 g) were suspended in 250 ml water, stirred with a magnetic stirrer for 5 minutes and decanted through 700, 160 and 45 µm sieves. This washing and decanting process was repeated three or four times until the water was clear. Roots and coarse debris were collected on a coarse screen (700 µm), while spores were captured on finer screens (160 and 45 µm). Soils from these fine screens were poured into a 50 ml tube with the aid of water to wash the screen to get a total volume of 45 ml, and centrifuged for 5 minutes at 2500 x g to remove organic matter. The supernatant and floating debris
were discarded, and the pellets were resuspended in 50% sucrose by shaking the tubes vigorously for a few seconds. The samples were then centrifuged again for 1 min at 2500 x g to separate spores from the denser soil components. Immediately after centrifugation, the spores in the supernatant were carefully poured onto a 45 µm sieve and washed with water to remove the sucrose. After rinsing, the spores were washed onto a pre-wetted Whatman filter paper (No.1) and then vacuumed in a Buchner funnel. The filter papers with spores were placed in Petri- dishes, covered with plastic food wrap and stored in a cold room at 4°C. The spores were collected from the filter paper using a fine paint brush under a stereomicroscope (Zeiss Stemi SV 11) and placed on numbered pieces of moist filter paper in a Petri dish. The collected spores were then stored in a cold room at 4°C. The AM spores were distinguished from other fungal spores mainly from their size, colour and brightness.

4.2.5 AM fungi spore density and diversity

Total AM fungi spore number was determined under a stereomicroscope. Spore density for each site was an average of 8 replicates (4 trees x 2 sampling positions). Spores were sorted into morphotypes based on colour, size and shape. For identification to genus, spores of each morphotype were crushed and mounted in polyvinyl alcohol-lacto-glycerol (PVLG) and PVLG mixed 1:1 with Melzer’s reagent (Morton 1988; Brundrett et al. 1996). Identification was based on spore features as described by Brundrett et al. (1996) and INVAM (www.invam.caf.wvu.edu/methods), under a compound microscope (Olympus BX 51) and imaged using an Olympus CX 31 digital camera. Spores with similar features were grouped and then 4 - 8 spores were selected for measurement (size and cell wall). Spores were measured vertically and horizontally and then size averaged.
The slides and images of the spores were stored as a reference collection in the laboratory.

Ecological measurement of diversity used to describe the structure of AM fungi communities included spore density, relative abundance and species richness (Table 4.1). Spore density (SD) was expressed as total number of spores occurring in 100 g of soil. Species richness (SR) reflects the number of species present in an ecosystem. The more species present in a sample, the richer the sample. Relative abundance (RA) was defined as the percentage of spore numbers of a species, which reflects different sporulation ability of different species of AM.

Table 4.1. Diversity measures used to describe AMF communities in air-dry soil.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore density (SD)</td>
<td>The number of spores in 100 g soil</td>
</tr>
<tr>
<td>Species richness (SR)</td>
<td>The number of morphotypes in each site / total number</td>
</tr>
<tr>
<td>Relative abundance (RA)</td>
<td>(spore number of a morphotype in each site / total spore in each site) x 100%</td>
</tr>
</tbody>
</table>

4.2.6 Statistical analysis

*Crown health at different sites*

Analysis of variance (ANOVA) was carried out to determine differences in TCHI between the 12 sites. Sites were classified into three groups namely healthy, moderately healthy and declining sites based on the TCHI using k-means clustering. This analysis places sites into groups in such a way that the distance between members of different groups is consistently greater than the distance between members of the same group. The analysis first places sites into groups at random and then, in successive iterations, moves sites to the group that has the closest cluster mean. The iterations cease when individual sites are no longer jumping between
clusters. These analyses were conducted using SPSS software packages version 17.0 developed by SPSS Inc., Chicago, USA.

Soil properties at the different sites

Differences in soil chemical properties at each of the sites were assessed visually with cluster analysis based on the Euclidean distance measure and the unweighed pair-group average (UPGMA) clustering algorithm. The end result is a dendrogram that uses the similarities between sites, based on the 17 soil variables measured, to group sites into clusters. This was followed by analysis of similarity (ANOSIM) to test for significant differences between sites, and where ANOSIM was significant, a similarity percentage (SIMPER) was used to determine which soil variables contributed most to the significant result. The soil data were referred to determine differences in canopy health of *E. gomphocephala* between the sites. These analyses were conducted using PAST (Hammer et al. 2001).

ANOSIM is a non-parametric technique for comparing multivariate data across two or more groups. The test is analogous to ANOVA, because it involves assessing the relative amount of distance in the data that can be ascribed to differences between groups as opposed to differences within groups. An R statistic indicates the degree of dissimilarity between groups. As R approaches 1, groups become increasingly dissimilar. The R values near 0 indicate very similar groups. The statistical significance of R is determined by repeated sampling permutation tests based on group membership (Hammer et al. 2001). If sample sizes are large, it is possible for small R values to be statistically significant. Therefore, Clarke and Warwick (2001) recommend considering R and P values together when interpreting results and not placing great weight on a significant P if the R is low (indicating statistical significance of a small dissimilarity across groups).
Differences in soil composition between sites were assessed with a one-way ANOSIM based on the Bray-Curtis distance measure. If ANOSIM revealed statistically significant differences between sites, SIMPER (Clark 1993) was used to reveal which variables were mainly responsible for the differences. The contribution of each variable to the difference is interpretable most readily if expressed as a cumulative percentage, indicating the amount of variation explained as successive variables are added in order from the largest contributor to the smallest.

*Predicting tree crown health and AM fungi from soil chemical properties*

Multiple regression was applied to determine whether canopy health (based on the four random trees at each of the 12 sites) and AM fungi (spore density and species richness) could be predicted from the 17 soil characteristics measured. The analysis was exploratory, so a forward stepwise approach was applied (Field 2009). A separate forward stepwise multiple regression (the soil characteristics as independent variable and the TCHI value, spore density and species richness of AM as dependent variables) was run for each dependent variable. Spore density was log_{10} transformed before analysis to improve the fit to the normal distribution. These analyses were conducted using STATISTICA software packages version 7.

*Spore density and diversity of AM fungi and their correlation with crown health*

Nested analyses of variance (ANOVA) were carried out to determine differences in spore density and species richness between the 12 sites. Soil came from two dependent cores per tree in the field, so tree was nested inside site for these analyses, following the example and logic of Scheiner (2001). In the process of analysis, logarithm transformation of spore density and square root of species richness were used to satisfy homogeneity of variance assumptions and normal distribution. Pearson’s correlation coefficient was employed to determine the correlation between
AM fungi (spore density and species richness) and crown health indices. These analyses were conducted using SPSS software packages version 17.0 developed by SPSS Inc., Chicago, USA.

4.3 Results

4.3.1 Crown health

The health status of the 12 sites was determined based on the TCHI value (Evans et al. 2012). Analysis of variance indicated that the TCHI differed significantly ($F_{11,36} = 9.15, P < 0.0001$) between the sites. The lowest TCHI was at site 4 (30.31 ± 6.97), whilst the highest TCHI were at sites 11 (80.62 ± 2.07) and 10 (80.31 ± 5.1) (Fig. 4.3). K-means cluster analysis grouped sites 1, 2, 3, 8, 10 and 11 into group 1. This group had TCHI values ranging from 68.4 - 80.6, and the sites within this group were considered to be the healthiest sites, hereafter called healthy. The second group contained sites 5 and 12, TCHI values ranged from 53.1 - 56.6, and these were considered to be intermediate sites with regards to tree health, hereafter called moderate. The third group consisted of sites 4, 6, 7 and 9, had TCHI values ranging from 30.3 - 45, and was considered to be the declining sites.

![Fig. 4.3. Total crown health index (TCHI) of twelve sites in 2009. Values are means (n = 4) ± SE.](image-url)
4.3.2 Soil chemical properties

Soil chemical characteristics of the study sites are presented in Appendix 2. Cluster analysis revealed five broad groupings (Fig. 4.4). Site 9 separated from the others at the first step, representing a soil composition quite distinct from the others. The next division in the dendrogram separated sites 1, 2, 3 and 10 in a tight cluster, forming a second group. Site 6 then separated to form a third group, with sites 4, 5, and 7 forming a fourth group and sites 8, 11 and 12 in a fifth group.

Fig. 4.4. Cluster analysis of the twelve sites based on soil chemical properties.

ANOSIM supported the cluster analysis (Fig. 4.4). The P and R values of ANOSIM are presented in Table 4.2. Site 9 was significantly different from all other sites and also had R values of 0.54 or greater for paired comparisons with all other sites. This indicates substantial dissimilarity between site 9 and the other sites. Site 1 was not different from sites 2, 3 or 10 (the other sites placed alongside it in the cluster
analysis in Fig. 4.4). Sites 8, 11 and 12 were not different in their soil composition, nor were sites 4, 5 and 7.

SIMPER based on differences across all sites was remarkable for the very similar contributions of all variables. DTPA extractable Fe and conductivity made the largest contribution, but that was only 8.9 and 8.4%, respectively. The smallest contribution was pH (H$_2$O), and contributed 2.6% (Table 4.3).

Table 4.2. ANOSIM P and R values for pair-wise comparisons between different sites using soil variables measured. Significantly different sites are in bold. P values are in the upper half of the table in normal font. R values for the same analysis are shown in the lower half of the table in italics. R values corresponding to significant P values are underlined.

<table>
<thead>
<tr>
<th>Site</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P and R values</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.108</td>
<td>0.170</td>
<td>0.029</td>
<td>0.027</td>
<td>0.085</td>
<td>0.054</td>
<td>0.028</td>
<td>0.028</td>
<td>0.058</td>
<td>0.120</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.417</td>
<td>0.027</td>
<td>0.026</td>
<td>0.027</td>
<td>0.030</td>
<td>0.031</td>
<td>0.028</td>
<td>0.026</td>
<td>0.028</td>
<td>0.056</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.146</td>
<td>0.552</td>
<td>0.029</td>
<td>0.030</td>
<td>0.083</td>
<td>0.027</td>
<td>0.028</td>
<td>0.031</td>
<td>0.039</td>
<td>0.031</td>
<td>0.029</td>
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</tr>
<tr>
<td>4</td>
<td>0.865</td>
<td>0.990</td>
<td>0.917</td>
<td>0.180</td>
<td>0.026</td>
<td>0.141</td>
<td>0.025</td>
<td>0.031</td>
<td>0.029</td>
<td>0.027</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.865</td>
<td>0.792</td>
<td>0.604</td>
<td>0.240</td>
<td>0</td>
<td>0.029</td>
<td>0.292</td>
<td>0.028</td>
<td>0.027</td>
<td>0.030</td>
<td>0.028</td>
<td>0.027</td>
</tr>
<tr>
<td>6</td>
<td>0.365</td>
<td>0.729</td>
<td>0.292</td>
<td>0.75</td>
<td>0.510</td>
<td>0</td>
<td>0.03</td>
<td>0.030</td>
<td>0.027</td>
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<td>7</td>
<td>0.469</td>
<td>0.531</td>
<td>0.531</td>
<td>0.271</td>
<td>0.094</td>
<td>0.042</td>
<td>0</td>
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<td>0.031</td>
<td>0.030</td>
<td>0.145</td>
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<td>0.029</td>
<td>0.145</td>
<td>0.287</td>
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<td>0.740</td>
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<td>1</td>
<td>0.615</td>
<td>0.719</td>
<td>0.917</td>
<td>0</td>
<td>0.031</td>
<td>0.027</td>
<td>0.028</td>
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<tr>
<td>10</td>
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<td>1</td>
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<td>0.312</td>
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<td>0.594</td>
<td>0.375</td>
<td>0.792</td>
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<td>0.208</td>
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<td>0.823</td>
<td>0.510</td>
<td>0.281</td>
<td>0.146</td>
<td>0.885</td>
<td>0.688</td>
<td>0.271</td>
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</tbody>
</table>

4.3.3 Predicting crown health from soil chemical properties

Forward stepwise multiple regression showed that tree crown health could be predicted from soil chemical characteristics (F$_{(12, 35)}$ = 3.38, P = 0.002). Increased tree health was related to increased pH (CaCl$_2$), exchangeable Ca, exchangeable Na, DTPA Mn, DTPA Cu, and DTPA Zn, but declined with increasing P, K and S (Table 4.4).
Table 4.3. SIMPER analysis showing the percentage contribution of each soil variable to the differences between sites. The higher the percentage contribution for an individual variable, the more it contributes to the overall differences between sites.

<table>
<thead>
<tr>
<th>Soil variable</th>
<th>Contribution</th>
<th>Contribution (%)</th>
<th>Cumulative (%)</th>
<th>Mean abund.1</th>
<th>Mean abund.2</th>
<th>Mean abund.3</th>
<th>Mean abund.4</th>
<th>Mean abund.5</th>
<th>Mean abund.6</th>
<th>Mean abund.7</th>
<th>Mean abund.8</th>
<th>Mean abund.9</th>
<th>Mean abund.10</th>
<th>Mean abund.11</th>
<th>Mean abund.12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>2.993</td>
<td>8.869</td>
<td>8.87</td>
<td>0.596</td>
<td>0.402</td>
<td>0.517</td>
<td>0.462</td>
<td>0.253</td>
<td>0.321</td>
<td>0.123</td>
<td>0.047</td>
<td>0.260</td>
<td>0.188</td>
<td>0.691</td>
<td>0.835</td>
</tr>
<tr>
<td>Conductivity</td>
<td>2.817</td>
<td>8.347</td>
<td>17.21</td>
<td>0.656</td>
<td>0.474</td>
<td>0.341</td>
<td>0.043</td>
<td>0.614</td>
<td>0.482</td>
<td>0.101</td>
<td>0.129</td>
<td>0.285</td>
<td>0.178</td>
<td>0.106</td>
<td>0.410</td>
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<tr>
<td>Exc. Ca</td>
<td>2.643</td>
<td>7.831</td>
<td>25.05</td>
<td>0.614</td>
<td>0.418</td>
<td>0.191</td>
<td>0.066</td>
<td>0.398</td>
<td>0.263</td>
<td>0.071</td>
<td>0.099</td>
<td>0.451</td>
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<td>Cu</td>
<td>2.555</td>
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<td>0.731</td>
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<td>0.344</td>
<td>0.366</td>
<td>0.372</td>
<td>0.791</td>
<td>0.600</td>
<td>0.653</td>
<td>0.528</td>
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<tr>
<td>Zn</td>
<td>2.329</td>
<td>6.901</td>
<td>39.52</td>
<td>0.481</td>
<td>0.081</td>
<td>0.169</td>
<td>0.106</td>
<td>0.338</td>
<td>0.425</td>
<td>0.313</td>
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<td>0.356</td>
<td>0.169</td>
<td>0.306</td>
<td>0.119</td>
</tr>
<tr>
<td>Exc. Na</td>
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<td>46.36</td>
<td>0.396</td>
<td>0.212</td>
<td>0.458</td>
<td>0.137</td>
<td>0.269</td>
<td>0.585</td>
<td>0.104</td>
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<td>0.189</td>
<td>0.075</td>
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<td>Mn</td>
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<td>53.06</td>
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<td>0.281</td>
<td>0.205</td>
<td>0.127</td>
<td>0.190</td>
<td>0.264</td>
<td>0.472</td>
<td>0.230</td>
<td>0.432</td>
<td>0.296</td>
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<tr>
<td>N (NH₄)</td>
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<td>6.285</td>
<td>59.34</td>
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<td>0.143</td>
<td>0.429</td>
<td>0.357</td>
<td>0.036</td>
<td>0.214</td>
<td>0.071</td>
<td>0.214</td>
<td>0.286</td>
<td>0.179</td>
<td>0.250</td>
<td>0.643</td>
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<tr>
<td>B</td>
<td>2.040</td>
<td>6.045</td>
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<td>0.519</td>
<td>0.249</td>
<td>0.174</td>
<td>0.075</td>
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<td>0.089</td>
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<td>0.143</td>
<td>0.603</td>
</tr>
<tr>
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<td>5.772</td>
<td>71.16</td>
<td>0.399</td>
<td>0.328</td>
<td>0.353</td>
<td>0.116</td>
<td>0.347</td>
<td>0.405</td>
<td>0.055</td>
<td>0.134</td>
<td>0.132</td>
<td>0.158</td>
<td>0.161</td>
<td>0.361</td>
</tr>
<tr>
<td>Exc. Mg</td>
<td>1.762</td>
<td>5.221</td>
<td>76.38</td>
<td>0.312</td>
<td>0.162</td>
<td>0.215</td>
<td>0.091</td>
<td>0.162</td>
<td>0.329</td>
<td>0.066</td>
<td>0.138</td>
<td>0.308</td>
<td>0.131</td>
<td>0.188</td>
<td>0.646</td>
</tr>
<tr>
<td>Org.C.</td>
<td>1.613</td>
<td>4.779</td>
<td>81.16</td>
<td>0.331</td>
<td>0.188</td>
<td>0.122</td>
<td>0.120</td>
<td>0.145</td>
<td>0.159</td>
<td>0.020</td>
<td>0.031</td>
<td>0.312</td>
<td>0.139</td>
<td>0.143</td>
<td>0.547</td>
</tr>
<tr>
<td>P</td>
<td>1.551</td>
<td>4.595</td>
<td>85.75</td>
<td>0.208</td>
<td>0.052</td>
<td>0.208</td>
<td>0.021</td>
<td>0.073</td>
<td>0.297</td>
<td>0.094</td>
<td>0.109</td>
<td>0.536</td>
<td>0.130</td>
<td>0.130</td>
<td>0.130</td>
</tr>
<tr>
<td>Exc. K</td>
<td>1.548</td>
<td>4.587</td>
<td>90.34</td>
<td>0.299</td>
<td>0.152</td>
<td>0.171</td>
<td>0.116</td>
<td>0.171</td>
<td>0.329</td>
<td>0.110</td>
<td>0.122</td>
<td>0.250</td>
<td>0.091</td>
<td>0.098</td>
<td>0.640</td>
</tr>
<tr>
<td>K</td>
<td>1.494</td>
<td>4.427</td>
<td>94.77</td>
<td>0.277</td>
<td>0.125</td>
<td>0.140</td>
<td>0.102</td>
<td>0.349</td>
<td>0.300</td>
<td>0.085</td>
<td>0.101</td>
<td>0.224</td>
<td>0.065</td>
<td>0.082</td>
<td>0.614</td>
</tr>
<tr>
<td>pH Ca</td>
<td>0.884</td>
<td>2.619</td>
<td>97.39</td>
<td>0.291</td>
<td>0.257</td>
<td>0.166</td>
<td>0.071</td>
<td>0.307</td>
<td>0.264</td>
<td>0.226</td>
<td>0.206</td>
<td>0.216</td>
<td>0.240</td>
<td>0.101</td>
<td>0.213</td>
</tr>
<tr>
<td>pH H₂O</td>
<td>0.881</td>
<td>2.610</td>
<td>100</td>
<td>0.982</td>
<td>0.939</td>
<td>0.832</td>
<td>0.775</td>
<td>1.020</td>
<td>0.979</td>
<td>0.939</td>
<td>0.911</td>
<td>0.936</td>
<td>0.936</td>
<td>0.832</td>
<td>0.911</td>
</tr>
</tbody>
</table>
Table 4.4. Soil chemical properties as predictors for crown health using forward stepwise multiple regression. The soil variables included in the most parsimonious models in each case are shown, together with an indication of which variables are significant.

<table>
<thead>
<tr>
<th>Soil variables measured</th>
<th>Soil variables in the final model for crown health (TCHI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (NH₄)</td>
<td>N (β¹ = 0.15)</td>
</tr>
<tr>
<td>P</td>
<td>P (β = -0.49)*</td>
</tr>
<tr>
<td>K</td>
<td>K (β = -1.5)*</td>
</tr>
<tr>
<td>S</td>
<td>S (β = -0.95)*</td>
</tr>
<tr>
<td>Organic-C</td>
<td>pH CaCl₂ (β = 2.02)*</td>
</tr>
<tr>
<td>Conductivity</td>
<td>pH H₂O (β = -0.82)</td>
</tr>
<tr>
<td>pH (CaCl₂)</td>
<td>Exc. Ca (β = 0.361)*</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>Exc. Na (β = 0.969)*</td>
</tr>
<tr>
<td>DTPA Cu</td>
<td>DTPA Mn (β = 0.499)*</td>
</tr>
<tr>
<td>DTPA Fe</td>
<td>DTPA Cu (β = 0.634)*</td>
</tr>
<tr>
<td>DTPA Mn</td>
<td>DTPA Zn (β = 0.337)*</td>
</tr>
<tr>
<td>Exc. Ca</td>
<td></td>
</tr>
<tr>
<td>Exc. Mg</td>
<td></td>
</tr>
<tr>
<td>Exc. K</td>
<td></td>
</tr>
<tr>
<td>Exc. Na</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
</tbody>
</table>

* significant predictor ¹ β = standardised regression coefficient, which is a measure of how strongly each predictor variable influences the dependent variable.

4.3.4 Spore density of AM fungi between sites

Nested ANOVA indicated that AM fungi spore density differed significantly (F (11, 48) = 3.47, P < 0.0013) between sites (Fig. 4.5). Furthermore, nested analysis indicated that the spore density between the four trees within each site were not significantly different (F (36, 48) = 0.79, P = 0.76). The highest spore density was at site 1 followed by sites 3 and 4. Sites 1 and 3 were considered to be healthy sites, whereas site 4 was a declining site. The lowest spore density was at site 8 followed by sites 6, 2 and 11. Sites 2, 8 and 11 were considered as healthy sites, whilst site 6 as a declining site.
4.3.5 Abundance and species richness of AM fungi spores

Eight spore morphotypes (Table 4.5) were found in the field soil and relative abundance (RA) of each morphotype can be seen at Table 4.6. The abundance of morphotypes varied among the sites. Interestingly, two morphotypes, namely M1 and M2 had a wide distribution as they occurred in all sites. Morphotype M1 was dominant at site 6 contributing 57.1% of RA compared with its RA at site 3 which was only 2.4%. Morphotype M2 was more abundant at site 8 (53.2%), but had low RA at site 2 (7.1%).

The average species richness ranged from 0.34 ± 0.06 (site 7) to 0.65 ± 0.11 (site 12) (Fig. 4.6). Nested ANOVA indicated that species richness of AM fungi was not significantly ($F_{(11, 48)} = 1.64, P = 0.12$) different between sites. Furthermore, species richness between the four trees within each site was not significantly ($F_{(36, 48)} = 0.90, P = 0.62$) different.

Fig. 4.5. Spore density of arbuscular mycorrhizal (AM) fungi at twelve sites. Values are means ($n = 8$) ± SE.
Table 4.5. Description of the eight AM fungal spore morphotypes observed.

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>Identification</th>
<th>Colour</th>
<th>Shape</th>
<th>Size (µm)*</th>
<th>Number of wall layers**</th>
<th>Thickness of wall (µm)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td><em>Glomus</em> sp. 1</td>
<td>yellow</td>
<td>globose/oval</td>
<td>65.9 - 251.2</td>
<td>3</td>
<td>5.4</td>
</tr>
<tr>
<td>M2</td>
<td><em>Glomus</em> sp. 2</td>
<td>brown</td>
<td>globose</td>
<td>66.8 - 209.7</td>
<td>3</td>
<td>5.9</td>
</tr>
<tr>
<td>M3</td>
<td><em>Glomus</em> sp. 3</td>
<td>golden</td>
<td>globose</td>
<td>256.3 - 341.8</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>M4</td>
<td><em>Glomus</em> sp. 4</td>
<td>light yellow</td>
<td>globose</td>
<td>102.5 - 171.1</td>
<td>3</td>
<td>6.9</td>
</tr>
<tr>
<td>M5</td>
<td><em>Glomus</em> sp. 5</td>
<td>golden</td>
<td>globose</td>
<td>143.8 - 204.1</td>
<td>3</td>
<td>5.4</td>
</tr>
<tr>
<td>M6</td>
<td><em>Glomus</em> sp. 6</td>
<td>black</td>
<td>globose</td>
<td>88.9 - 144.9</td>
<td>3</td>
<td>2.7</td>
</tr>
<tr>
<td>M7</td>
<td><em>Acaulospora</em> sp. 1</td>
<td>yellow/orange</td>
<td>globose</td>
<td>55.2 - 75.2</td>
<td>2</td>
<td>8.6</td>
</tr>
<tr>
<td>M8</td>
<td><em>Acaulospora</em> sp. 2</td>
<td>red</td>
<td>globose</td>
<td>69 - 92.8</td>
<td>2</td>
<td>9.1</td>
</tr>
</tbody>
</table>

*4 - 8 spores measured
**4 - 5 spores observed

Table 4.6. Spore number (100 g soil) and relative abundance (in brackets) of AM fungal morphotypes in field soil at twelve sites.

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
<th>S10</th>
<th>S11</th>
<th>S12</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>40 (3.7)*</td>
<td>50 (25.2)</td>
<td>14 (2.4)</td>
<td>40 (10.2)</td>
<td>96 (32.9)</td>
<td>80 (57.1)</td>
<td>78 (35.8)</td>
<td>20 (16.1)</td>
<td>32 (11.4)</td>
<td>38 (11.8)</td>
<td>18 (10.1)</td>
<td>82 (26.8)</td>
</tr>
<tr>
<td>M2</td>
<td>480 (44.3)</td>
<td>14 (7.1)</td>
<td>74 (13.0)</td>
<td>54 (13.8)</td>
<td>70 (24.0)</td>
<td>44 (31.4)</td>
<td>80 (36.7)</td>
<td>66 (53.2)</td>
<td>14 (0.1)</td>
<td>96 (29.8)</td>
<td>14 (7.9)</td>
<td>38 (12.4)</td>
</tr>
<tr>
<td>M3</td>
<td>4 (0.004)</td>
<td>2 (1.0)</td>
<td>16 (2.8)</td>
<td>6 (1.5)</td>
<td>0 (0)</td>
<td>4 (2.9)</td>
<td>0 (0)</td>
<td>4 (3.2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>M4</td>
<td>134 (11.4)</td>
<td>0 (0)</td>
<td>78 (13.7)</td>
<td>122 (31.1)</td>
<td>84 (28.8)</td>
<td>1 (1.4)</td>
<td>10 (4.6)</td>
<td>6 (4.8)</td>
<td>0 (0)</td>
<td>8 (2.5)</td>
<td>2 (1.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>M5</td>
<td>0 (0)</td>
<td>2 (0.3)</td>
<td>2 (0.5)</td>
<td>24 (8.2)</td>
<td>6 (4.3)</td>
<td>50 (22.9)</td>
<td>2 (1.6)</td>
<td>0 (0)</td>
<td>2 (0.6)</td>
<td>4 (2.2)</td>
<td>10 (3.3)</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>0 (0)</td>
<td>26 (13.1)</td>
<td>0 (0)</td>
<td>4 (1.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (1.6)</td>
<td>10 (3.6)</td>
<td>0 (0)</td>
<td>60 (33.7)</td>
<td>4 (1.3)</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>430 (39.7)</td>
<td>76 (38.4)</td>
<td>68 (11.9)</td>
<td>64 (16.3)</td>
<td>0 (0)</td>
<td>4 (2.9)</td>
<td>0 (0)</td>
<td>22 (17.7)</td>
<td>120 (42.9)</td>
<td>130 (40.4)</td>
<td>80 (44.9)</td>
<td>58 (18.9)</td>
</tr>
<tr>
<td>M8</td>
<td>6 (0.5)</td>
<td>30 (15.1)</td>
<td>318 (55.8)</td>
<td>100 (25.5)</td>
<td>18 (6.2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (1.6)</td>
<td>104 (37.1)</td>
<td>48 (14.9)</td>
<td>0 (0)</td>
<td>110 (35.9)</td>
</tr>
</tbody>
</table>

**Total spore number:** 1084 198 570 392 292 140 218 124 280 322 178 306

*relative abundance was calculated as: (spore number of a morphotype in each site / total spore number in each site) x 100%
4.3.6 Correlation between spore density and species richness of AM fungi

Spore density was positively correlated with species richness. However, the correlation was weak \( (P = 0.05, r = 0.299, n = 48) \) (Fig. 4.7).

4.3.7 Predicting spore density and species richness of AM fungi from soil chemical characteristics

The spore density and species richness of AM fungi in the field could be predicted from soil chemical characteristics \( F_{(4, 43)} = 3.85, P < 0.009; F_{(5, 42)} = 3.42, P < 0.01, \)
respectively). The spore density was more abundant with lower DTPA Cu, whilst species richness increased in association with higher DTPA Zn (Table 4.7).

Table 4.7. Soil chemical properties as predictors for AM fungal spore density and species richness using forward stepwise multiple regression. The soil variables included in the most parsimonious models in each case are shown, together with an indication of which variables are significant.

<table>
<thead>
<tr>
<th>Soil variables measured</th>
<th>Soil variables in the final model for spore density</th>
<th>Soil variables in the final model for species richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (NH₄)</td>
<td>N (β₁ = 0.37)</td>
<td>P (β = -0.27)</td>
</tr>
<tr>
<td>P</td>
<td>Exc. Na (β = -0.24)</td>
<td>pH CaCl₂ (β = - 0.25)</td>
</tr>
<tr>
<td>K</td>
<td>DTPA Mn (β = -0.16)</td>
<td>DTPA Mn (β = - 0.22)</td>
</tr>
<tr>
<td>S</td>
<td>DTPA Cu (β = -0.59)*</td>
<td>DTPA Zn (β = 0.362)*</td>
</tr>
<tr>
<td>Organic-C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conductivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (CaCl₂)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA Cu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA Fe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA Mn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA Zn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. Ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. Mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* significant predictor  
₁ β = standardized regression coefficient, which is a measure of how strongly each predictor variable influences the dependent variable

4.3.8 Correlation between AM fungi (spore density and species richness) and crown health

There was no significant correlation between spore density of AM fungi and all crown health indices. Similarly, no correlation was found between species richness and crown health indices.

4.4 Discussion

4.4.1 Soil chemical properties of study sites

The sites were quite different based on their soil chemical composition. Interestingly, from the six sites where trees were mostly healthy, four of them clustered into one
group. This clearly suggested that these healthy sites had similar soil chemical properties. Sites 9 and 6 were quite different in their soil chemical composition, and at these sites trees were declining. Sites 4, 5 and 7 were similar in their soil chemical composition, and trees had poor growth at sites 4 and 7, but were moderately healthy at site 5. The most probable explanation for the differences in soil chemical composition between the sites included geological origin, fire and past land-use history (pasture and fertilizer), and the structure of the understorey, that in turn might affect litter quantity, quality and heterogeneity. Changes in soil condition, nutrient cycles and vegetation structure resulting from exclusion of fire have been hypothesized to be involved in forest/tree decline in Australia (Landsberg 1990; Ellis and Pennington 1992; Jurkis 2005) including *E. gomphocephala* decline (Close et al. 2009; Archibald et al. 2010). More recently, Close et al. (2011), in comparing the effects of prescribed fire and no prescribed fire related to *E. gomphocephala* decline at YNP, reported that some soil nutrients such as exchangeable Zn, Mn and Mg were higher on prescribed fire sites than no prescribed, long unburnt sites. In conventional forest management in the region, prescribed fire may be used as frequently as 7 year intervals, but the period may be much longer in conservation forest. However, the same site had not experienced fire for several decades, so the link to fire cannot be substantiated.

Fe was the highest contributor (8.9%) to the differences between the sites, whilst the lowest (2.6%) was pH (H\textsubscript{2}O). However, it seemed that the contribution of each soil variable to the differences between the sites was quite similar, and no single soil variable appeared to be a dominant factor. Therefore, no firm conclusion can be drawn from the current study on the relative importance of soil chemical properties. This was possibly due to the low number of soil samples used for multiple
regression. A total of 48 soil samples (four samples from each site) were used to predict 17 soil chemical properties, which is slightly lower than has been suggested for multiple regression analysis (Field, 2009).

4.4.2 Soil chemical properties and crown health

Soil chemical properties can be used to predict crown condition (TCHI) of *E. gomphocephala* in the field. In particular, improved tree health was associated with increasing pH (CaCl₂), exchangeable Ca, exchangeable Na, Mn, Cu and Zn, but declined with increasing P, K and S. It is not known whether these relationships are causal. Thus, future studies are required to establish the exact role these relationships play in the health of *E. gomphocephala*. *Eucalyptus gomphocephala* might be classified as a calcicole plant (Chapter 2) as it is largely confined to calcareous soil profiles. Thus, it is likely that the species could be impacted when the soil pH changes due to natural or human disturbances. The crown condition of *E. gomphocephala* tended to improve with increasing DTPA extractable levels of Mn, Cu and Zn. This might be explained by the lower availability of these micronutrients in calcareous soil (Bell and Dell 2008).

There was a tendency for the crown condition of *E. gomphocephala* to decline with increasing P, K and S. It is not known whether the high level of these nutrients comes from natural nutrient pools, or has been caused by humans or is a consequence of ecosystem decline. The high levels of these elements may result in an imbalance in the availability and uptake of other nutrients (Brady 1990), which may subsequently affect the health of *E. gomphocephala*.

There are numerous studies that have revealed similar findings about the relationship between edaphic factors and tree decline. For instance, deficiencies and imbalances of various nutrients such as N, P, K, Mg, Mn and Ca have been implicated with
sugar maple decline in the northern hemisphere (Horsley et al. 2000; Schaberg et al. 2006). In addition, adverse soil chemical properties have also been related to eucalypt decline (Czerniakowski et al. 2006; Parsons and Uren 2007; Grigg et al. 2009; Horton et al. 2013) in Australia. More recently, application of Ca to ameliorate soil chemical properties was shown to improve the health status of sugar maple (Hugget et al. 2007) and mycorrhizal colonization (Juice et al. 2006).

4.4.3 AM fungi abundance and diversity between sites

Spore density was significantly different between the sites; however species richness did not differ between the sites. Moreover, soil chemical properties were related to the AM spore density and species richness. Brundrett (1991) suggested that a number of factors might affect the species composition and population of AM fungi including soil chemical and physical properties and species of host plant. A previous study by Oliveira and Oliveira (2005) on two different fruit-tree species with repeated soil sampling found that soil chemical factors and soil moisture were correlated to AM distribution and colonization. The effects of edaphic conditions on the formation and function of AM fungi has been reviewed by Entry et al. (2002).

Although soil chemical properties could be used to predict spore density and species richness of AM fungi in the field, this relationship might be more complex as it might also be related to the site condition as an environmental factor itself. For example, variety and density of under-storey and/or other herbaceous plants species between the sites might directly or indirectly affect AM fungi. *Eucalyptus gomphocephala* in YNP occurs as a dominant species in multi-species communities, and these species might or might not be potential hosts of AM fungi. Van der Heijden et al. (1998b) suggested that diversity and distribution of AM fungi depended on the community structure and characteristics of ecosystems. Beside their
possible role as hosts, the variety and density of other plants species between the sites may also indirectly affect AM fungi through changes in soil conditions (e.g. the quality, quantity and heterogeneity of litter).

The relative abundance of AM morphotypes varied between sites. Morphological identification indicated *Glomus* as the dominant genus found in this study. In particular, two *Glomus* morphotypes (M1 and M2) might be considered as generalists as they had a wide geographical range and were found on all sites. Whether the ability of these two morphotypes to spread widely is related to wide host preference remains to be established. Some AM morphotypes, on the other hand, had narrow geographical ranges as they were restricted to a few sites. A previous study reported that *Glomus* was the most prevalent genus found in Mediterranean ecosystems (Sanchez-Castro et al. 2012). Molecular analysis is desirable to identify the taxa to species level. This was not pursued as greater emphasis was placed on ectomycorrhization in the following chapter.

The spore density was positively correlated to species richness. This might have indicated that the higher the spore density the higher AM species richness in the site. However, it might not always be the case as the species richness between sites was not statistically different, although the spore density differed between sites.

**4.4.4 AM fungi (abundance and diversity) and crown health**

Although AM spore density was significantly different between sites, there was no correlation between spore density and crown health of *E. gomphocephala*. The AM species richness was not different between sites, and the correlation with crown health of *E. gomphocephala* was also not significant. The lack of relationship between AM fungi and crown health of *E. gomphocephala* might be because the assessment of AM fungi abundance and diversity was only based on one soil
sampling time, which was in late summer. Low-sporulating species as well as species whose sporulation is restricted to one season, were probably not detected. In other words, not all the fungi present may be sporulating at the time of sampling, therefore, the abundance and species richness may have not reflected the entire AM fungal community. Jasper et al. (1991) found that hyphae can retain infectivity in dry soil in the absence of actively growing roots. However, their infectivity may depend on the life cycle stage that is reached at the time of the onset of soil drying (Jasper et al. 1993). In some fungi such as *Acaulospora laevis*, the infectivity of hyphae decreased when sporulation commenced, whilst for other fungi such a *Glomus* species the hyphae remained infective after sporulation (Abbott and Robson 1981).

There was little published research on AM communities and diversity in eucalypt forests or woodlands, however, with regard to other plant species, it is well known that the species composition and population levels of AM fungi were highly variable and influenced by many factors. For example, seasonal fluctuation of AM fungi has been reported in several plant species and communities in tropical ecosystems (Muthukumar and Udayan 2002; Moreira-Souza et al. 2003; Oliveira and Oliveira 2005). A recent study by Oliveira and Oliveira (2010) on seasonal dynamics of AM fungi in the rhizosphere of two fruit species in a tropical climate found that although the sporulation of AM fungi was related to climatic and edaphic factors, the influence of these factors on AM fungi tended to vary with the season and host plant species.

Since the study was only undertaken once, future studies should include repeated soil sampling across different seasons to obtain more robust information on AM spore density and diversity.
To conclude, soil chemistry was an important factor that might contribute to *E. gomphocephala* decline. The influence of soil factors could well be much more complex as it might directly affect both the trees and their associated fungi. Since the assessment of AM fungi through counting spores from soil samples collected from the field provided poor information on the relationship between mycorrhizal fungi and health of *E. gomphocephala*, a more robust method needs to be explored in order to determine whether mycorrhizal fungi play an important role in the fitness of *E. gomphocephala*. This will be explored in Chapter 5.
Chapter 5

Soil inoculum potential of mycorrhizal fungi related to the health status of *Eucalyptus gomphocephala*

5.1 Introduction

The preceding chapter looked at AM fungi spores in soil at the end of summer and found no relationship between AM fungi and *E. gomphocephala* health. However, a previous study on beneficial fungi and *E. gomphocephala* health in YNP, the same area of this study, reported that ECM fungi were more frequently observed in healthy than in declining *E. gomphocephala* (Legault 2005). More recently, Scott (2013a) briefly explored the relationship between crown health, fine roots and ECM of declining *E. gomphocephala* in YNP. By using an Air-spade to blast sand off roots, the author observed fewer ECM mats in declining sites and suggested that loss of ECM mats might be related to *E. gomphocephala* decline. If loss of ECM mats occurred at declining sites, sporocarp production was likely to be reduced leading to fewer ECM spores being dispersed. This could have resulted in reduced inoculum potential of ECM fungi, which in turn could have affected the success of *E. gomphocephala* seedling recruitment (seedling surviving to some later life stage). Despite this possibility, no detailed study has evaluated the ECM status of *E. gomphocephala* roots in the field in its natural habitat. In addition, whether mycorrhiza may be sensitive to or change with the health status of the forest remains to be elucidated.

Colonization of roots by mycorrhizal fungi arises from a source of inoculum. Inoculum potential was defined as the growth of fungi at the surface of its host, which was a consequence of the numbers of active fungal propagules and their
nutritional status (Brundrett et al. 1996). Sources of AM fungal inocula have been shown to include spores, dead root fragments and other colonized organic matter, and the network of hyphae in soil, while inocula of ECM fungi included networks of mycelia strands, old mycorrhizal roots, sclerotia and spores (Brundrett 1991; Smith and Read 2008). Three main methods have been employed to estimate the inoculum potential of mycorrhizal fungi. These included counting propagules such as spores, measuring mycorrhizal formation after serial dilution of soils, and growing bait plants in intact soil cores (Brundrett et al. 1996). Of these three methods, the bioassay method using bait plants grown in intact cores has been widely used to assess the infectivity of both AM and ECM fungi as it provided better prediction of mycorrhizal inoculum potential (Jasper et al. 1991; Brundrett and Abbott 1994; Brundrett et al. 1995; Adams et al. 2006). In the bioassay method, the percentage of colonization of bait plant roots was assessed after a period of time under conditions that were usually optimized for bait plant growth.

Soil chemical properties have also been shown to be important factors that might affect both forest health and mycorrhizal fungi (Chapter 2). Two previous studies have reported that soil chemical properties might be related to the health of *E. gomphocephala* (Cai et al. 2010; Scott et al. 2013b). Furthermore, results from the preceding study (Chapter 4) indicated that soil chemical properties were related to both health of *E. gomphocephala* and AM fungi. Therefore, relationship between soil chemical properties and mycorrhizal colonization were also explored in this Chapter.

Eucalypts are well known to be colonized by both AM and ECM fungi (Oliveira et al. 1997; Santos et al. 2001; Pagano and Scotti 2008), and many studies have shown that ECM were more dominant in eucalypt forests in Australia (Lapeyrie and Chilvers 1985; Chen et al. 2000a; Adams et al. 2006). However, no such information
was available for *E. gomphocephala*. Therefore, the types of mycorrhizas formed and how important these are to the health of *E. gomphocephala* remain to be ascertained. The aim of this study was to determine if the soil inoculum potential of ectomycorrhiza and arbuscular mycorrhiza changed with the health status of *E. gomphocephala*.

The hypotheses tested were:

H7. The type of mycorrhiza (ECM/AM) formed by seedlings is related to the crown health of *E. gomphocephala*,

H8. *Eucalyptus gomphocephala* preferentially forms ECM, but roots can also form AM, and

H9. Soil chemical characteristics are related to mycorrhizal colonization.

5.2 Materials and methods

5.2.1 General approach

Intact soils were collected from the study sites in YNP described in Chapter 4 and used for the *E. gomphocephala* bioassay trial. Seedlings were grown in the cores in an evaporatively cooled glasshouse at Murdoch University, from late March 2010 until October 2010.

5.2.2 Crown health assessment

For details of crown health, see Chapter 4. The four individual *E. gomphocephala* trees at each site were the same trees from which the soil core samples were collected for AM fungi assessment (see Chapter 4).
5.2.3 Soil collection

*Intact soil cores for bioassay*

Relatively undisturbed soil samples were collected using a metal cylindrical soil corer (20 cm deep and 12 cm diameter). This method was based on a previous study that assayed mycorrhizal propagules in undisturbed soil from a wide range of different habitats (Scheltema et al. 1987; Brundrett and Abbott 1991). At each site, two intact soil cores were collected at locations 5 m north and south from the trunk of each of four randomly selected trees. Each intact soil core was carefully placed into a labelled polyurethane pot (17 cm height, 12 cm width) lined with a polyethylene bag (35 cm height, 36 cm width) and transferred to the glasshouse. To check for any contaminating mycorrhizal fungi in the bioassay, four intact soils were collected from around four healthy trees (site 3), wetted and then autoclaved for two consecutive days for 30 minutes at 121°C and used as a control in the glasshouse.

*Soil chemical analysis*

The soil samples used for chemical analysis were the same samples as those assessed in Chapter 4. The soils were collected adjacent to each of the above cores.

5.2.4 Bioassay trial

*Experimental design*

The bioassay experiment was set up as a single factor randomized complete block design. The factor was the site and 12 levels of site, each with 8 replicates, were tested within each site.
Seed

Seeds of *E. gomphocephala* were obtained from more than 20 healthy trees selected at random in YNP. Seeds were surface sterilized by dipping them for 5 seconds in 70% ethanol, then they were soaked and agitated in a solution of 3% sodium hypochlorite (diluted from 12.5% commercial bleach) for 5 minutes, and washed three times in sterile distilled water before germinating. The effectiveness of the surface sterilization was confirmed by incubating 10 surface sterilized seeds in Petri-dishes containing 0.75% (w/v) water agar for 7 days in a 25°C room. The surface sterilized seeds were then germinated in trays (43 cm length, 31 cm width, 12 cm height) containing yellow sand that had been autoclaved at 121°C for 30 minutes on two consecutive days. The seeds were sown approximately 1 cm deep. The trays were covered with brown paper to reduce contamination and incubated in an evaporatively cooled glasshouse. The brown paper was removed 3 days after sowing when the seedlings had started to emerge. The seedlings were maintained for another 7 days and watered daily by hand, until transplanting.

Transplanting

Prior to transplanting, 150 ml of water was added into the soil in each pot and left overnight. However, since almost all the soils appeared to be hydrophobic, another 150 ml of water, which consisted of 100 ml water and 50 ml soil wetting agent (Easy Wetta, Western Australia), was added to each pot. The polyethylene bags were then sealed and the pots were turned up-side down and left over night. The next day, three 10-day-old seedlings of relatively uniform size were transferred into each pot and sown at approximately 1 cm depth. Holes were made into the base of polyethylene bags to allow for drainage.
Maintenance

The pots were maintained on open benches in an evaporatively cooled glasshouse with mean temperatures of 25.8°C/9.2°C (maximum/minimum) and natural sunlight of 600-800 µmol m⁻²s⁻¹. The seedlings were watered for 5 minutes twice daily with an automatic overhead watering system. The watering was reduced to once daily when the temperature started to decline during the autumn and winter as the seedlings showed intolerance to excessive water. Due to oedemas developing on leaves on some plants, seedlings were watered by hand from 4 months old (late July) until harvesting to avoid watering the foliage, and 50 ml of water was applied daily to each pot. A complete liquid fertilizer of low nutrient content (Australian Native Focus, Trade name) at half the recommended dose (2.5 ml/L) was applied to the seedlings every two weeks for the second month and weekly thereafter until 2 weeks prior to harvest. The composition of the fertilizer used was: 500 µM N, 10.9 µM P, 214.8 µM K, 55 µM Ca, 19.5 µM Mg, 81.8 µM S, 0.63 µM Fe, 0.3 µM Mn, 0.6 µM B, 0.1 µM Zn, 0.05 µM Cu and 0.02 µM Mo. The fertilizer regime was based on that developed for mycorrhizal eucalypts in forest nurseries (Dell and Malajczuk 1995). The fertilizer was applied since the soils differed in fertility and many seedlings started to show nutrient deficiency symptoms (Dell et al. 1995). Seedlings were thinned to two plants per pot when they were 2.5 months old.

Harvest

When the seedlings were 6 months old, two soil cores were taken midway between the two seedlings in each pot using a 2 cm diameter and 20 cm deep corer for spore assessment. The soils were placed in a paper bag, inserted in a plastic bag and stored at 4°C in a cold room for spore density and species richness assessment (Chapter 4).
Seedlings were harvested when they were 7 months old. Before harvesting, the ECM mats on the side walls of the soil were observed by gently removed the seedlings from the pots, and then photographs were taken. Some samples of the ECM mats were collected and observed under a microscope. The identification of mat types was based on colour, texture and aroma. The aroma was assessed by nose.

To avoid rotten roots being examined for mycorrhizal assessment by harvesting all pots at the same time and not being able to process all the plants immediately, plants were harvested sequentially across the sites (1 replicate from each site at a time). The shoots were cut at the soil line, put in an oven at 65°C for three consecutive days and dry weights taken. Roots were gently washed from the soil over a 5 mm screen with tap water, immersed for approximately 5 minutes in a bucket of water and agitated gently to remove most of the organic matter and soil particles from the roots. The cleaned roots were then carefully washed again with tap water over a 2 mm screen. The coarse and fine roots caught on the screen were collected and kept separately. These clean roots were then put into a 2 L container, with 700 ml of water added, and the roots were gently massaged by hand for approximately 10 seconds and shaken to separate the fragmented fine roots from the whole root system (Burgess 1995). The coarse roots were removed from the container, placed into a moist zipped plastic bag, and kept in a 4°C room. The remainder of the root samples were sieved over a 1mm nylon sieve to collect the fine roots. The fine roots were cleaned manually at three times magnification under a stereomicroscope (Zeiss Stemi SV 11) to remove soil particles and dead roots. Some fresh ECM root tips were collected for photographic records, and morphotype characterization. The ECM morphotype was characterized based on colour and hyphae of the ECM tips observed. The ECM root tips were then fixed in 3% gluteraldehyde in 0.025M phosphate buffer, pH 7 for
Hartig net assessment. The rest of the fine roots were gently blotted dry with paper towel, the fresh weight recorded, the sample mixed well and then divided into three subsamples based on fresh weight. The first subsample, approximately 20%, was processed for root infection assessment, the second subsample, approximately 20%, was oven dried as for the shoots, and the remainders of the roots (ca. 60%) were retained for DNA analysis of the tips. Root samples for mycorhizal assessment were fixed in 50% ethanol (EtOH) while 100% EtOH was used as fixative for molecular analysis of root tips (Chapter 6).

5.2.5 Mycorrhizal assessment

AM spore assessment

Diversity measures used to describe AMF communities in the bioassay soil included spore density, species richness and relative abundance. Spore density for each site was an average of 8 replicates (4 trees x 2 sampling positions). Spore density was measured as the total number of spores occurring in 100 g of soil. Species richness was defined as the number of species present in an ecosystem, whilst relative abundance (RA) was defined as the percentage of spore numbers of a species. Details are given in Chapter 4.

AM/ECM colonization

Assessment of colonization of roots by mycorrhizal fungi was based on the protocols described by Brundrett et al. (1996). Briefly, root samples (1 - 2 g) fixed in 50% EtOH were rinsed with water, cleared by heating in 10% (w/v) KOH in an autoclave, stained with 0.05% (w/v) trypan blue, and then destained with lactoglycerol. Details of clearing, staining and destaining process of the roots are given in Chapter 3.
Ectomycorrhizal assessment was based on the proportion of ECM root tips over the total number of root tips (see Chapter 3). Arbuscular mycorrhizal assessment was based on the proportion of roots colonized by AM fungi. This was determined by using the gridline intersect method (INVAM; Giovanetti and Mosse 1980; Brundrett et al. 1996) under both stereo (Zeiss Stemi SV 11) and compound (Olympus BX 51) microscopes. Around 25 - 30 randomly stained roots (ca. 1 cm length) were taken and spread onto a 9 cm Petri dish that had gridlines (0.5 cm, 100 grids in total) attached on the base. The assessment was repeated two times and averaged for each sample. In order to confirm the presence of AM fungi, root pieces from the samples were examined with a compound microscope (Olympus BX 51) at 100 and/or 200 times magnification. Arbuscular mycorrhizas were defined by the presence of arbuscules and vesicles within the root.

5.2.6 Plating for Phytophthora

Root fragments were collected from the soil cores taken from the bioassay seedlings. The roots were washed with tap water, surface sterilized with 70% ethanol for a few seconds, and then rinsed 3 times in sterile distilled water. The sterile roots were cut into around 0.5 cm length pieces and plated onto PDA. The plates were observed under a compound microscope 2-4 four days after plating for mycelium growth. Any mycelium that was suspected to be Phytophthora was transferred onto NARPH selective medium (Hüberli et al. 2000) to confirm its identification.

5.2.7 Statistical analysis

Seedling growth, spore density and mycorrhizal colonization in soil from different sites

Nested analyses of variance (ANOVA) were carried out to determine differences in seedling growth (shoot biomass), spore density, species richness and AM/ECM
abundance in seedlings between the 12 sites. Soil came from two dependent cores per tree in the field, so tree was nested inside site for these analyses, following the example and logic of Scheiner (2001). Two seedlings were grown from each of these dependent cores in the glasshouse, so the values for shoot biomass from these seedlings were averaged to avoid issues of dependence. A single value for spore density and a single value for mycorrhizal colonization were recorded for each pot. Post hoc tests were not done because the sites did not correspond to experimental treatments. Spore density and mycorrhizal colonization were log\(_{10}\) transformed before analysis to improve the fit to the normal distribution. Differences in canopy condition between sites were tested using ANOVA, because there were no dependent multiple measurements on individual trees. Pearson’s correlation coefficients were applied to determine the relationship between seedling biomass and canopy condition, AM spore density/species richness and canopy condition, and the relationship between AM and ECM colonization. These analyses were conducted using SPSS for Windows version 17.0 (SPSS Inc.).

*Predicting mycorrhizal colonization from soil chemical properties*

This analysis was exploratory, so forward stepwise multiple regression was applied (Field 2009) to evaluate whether the mycorrhizal colonization at each of the 12 sites could be predicted from the 17 soil characteristics measured (Chapter 4). A separate analysis was undertaken for each dependent variable (AM spore density and species richness, AM and ECM colonization) with the soil characteristics as the independent variables. These analyses were conducted using STATISTICA software package version 7.
Predicting tree crown health from mycorrhizal colonization

This analysis was also exploratory, so a forward stepwise multiple regression was applied to predict tree crown health (based on four random trees at each of the 12 sites) from mycorrhizal colonization. Mycorrhizal colonization (AM and ECM) were placed as independent variables, whilst the tree crown health (TCHI) was the dependent variable. Linear regression analysis was applied to determine the relationship between TCHI and mycorrhizal colonization. The TCHI was log$_{10}$ transformed before analysis to improve the fit to the normal distribution.

5.3 Results

5.3.1 Seedling growth

Excluding the control, nested ANOVA showed that seedling growth (measured as shoot dry weight) differed significantly ($F_{(11, 48)} = 7.53, P < 0.0001$) between the sites (Fig. 5.1). Trees were not significantly different ($F_{(36, 48)} = 1.208, P = 0.27$) within each site. In general, seedlings growth in soils collected from healthy sites (sites 1, 2, 3, 8, 10 and 11) and moderately healthy sites (sites 5 and 12) were variable.

However, excluding site 4, seedlings in soil collected from declining sites (sites 4, 6, 7 and 9) grew quite well. Although shoot dry weight was significantly different between sites, crown health in the field could not be predicted by seedling growth as Pearson’s correlation coefficient was not significant ($r = -0.23, n = 48$).
5.3.2 AM fungi spore density

Nested ANOVA revealed that the spore density of AM fungi in soil collected from the bioassay trial at 6 months differed significantly \((F_{11, 48} = 2.86, P = 0.006)\) between sites (Fig. 5.2). Trees were not significantly different \((F_{36, 48} = 1.22, P = 0.26)\) within each site. The highest spore density was in soil taken from site 9 followed by site 12, whilst the lowest was in soil taken from site 11 followed by sites 2 and 10. Site 9 was considered as declining site and site 12 as a moderately healthy. Sites 2, 10 and 11 were considered as healthy sites.

Compared to the spore density in the field soil at the time of collection, the AM fungi spore density tended to increase in soils collected from unhealthy sites, but, tended to decrease in soils from healthy sites in the bioassay trial (Table 5.1). The highest increase in spore density (291%) occurred in soil collected from site 6 (declining site), whilst the highest decrease in spore density (66%) was in soil collected from site 1 (healthy site) (Table 5.1).
Fig. 5.2. Spore density of arbuscular mycorrhizal (AM) fungi in bioassay soil collected from twelve sites. Values are means (n = 8) ± SE (sampled when seedlings were 6 months old).

Table 5.1. Spore density and change in spore density of AM fungi at twelve sites. Values are means (n = 8) ± SE.

<table>
<thead>
<tr>
<th>Site</th>
<th>Crown health rating</th>
<th>TCHI</th>
<th>Spore density (field soil, Chapter 3)</th>
<th>Spore density (bioassay soil at 6 months old seedlings)</th>
<th>Change in spore density (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Healthy</td>
<td>70.0 ± 8.8</td>
<td>135.5 ± 46.2</td>
<td>45.5 ± 3.2</td>
<td>- 66</td>
</tr>
<tr>
<td>2</td>
<td>Healthy</td>
<td>69.1 ± 6.7</td>
<td>24.7 ± 4.5</td>
<td>17.7 ± 3.8</td>
<td>- 28</td>
</tr>
<tr>
<td>3</td>
<td>Healthy</td>
<td>73.4 ± 3.0</td>
<td>71.2 ± 23.9</td>
<td>68.2 ± 16.3</td>
<td>- 4</td>
</tr>
<tr>
<td>4</td>
<td>Declining</td>
<td>30.3 ± 7.0</td>
<td>52.2 ± 13.7</td>
<td>59.7 ± 13.0</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Moderately healthy</td>
<td>53.1 ± 8.7</td>
<td>36.5 ± 6.4</td>
<td>69.0 ± 13.3</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>Declining</td>
<td>40.3 ± 2.2</td>
<td>17.5 ± 4.9</td>
<td>68.5 ± 12.4</td>
<td>291</td>
</tr>
<tr>
<td>7</td>
<td>Declining</td>
<td>41.9 ± 2.3</td>
<td>27.2 ± 4.2</td>
<td>54.5 ± 13.7</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Healthy</td>
<td>68.4 ± 3.0</td>
<td>15.5 ± 4.1</td>
<td>50.2 ± 25.6</td>
<td>224</td>
</tr>
<tr>
<td>9</td>
<td>Declining</td>
<td>45.0 ± 6.7</td>
<td>35.0 ± 13.0</td>
<td>86.2 ± 50.3</td>
<td>146</td>
</tr>
<tr>
<td>10</td>
<td>Healthy</td>
<td>80.3 ± 5.1</td>
<td>40.2 ± 10.4</td>
<td>15.0 ± 3.2</td>
<td>- 63</td>
</tr>
<tr>
<td>11</td>
<td>Healthy</td>
<td>80.6 ± 2.1</td>
<td>22.2 ± 6.9</td>
<td>13.7 ± 5.3</td>
<td>- 38</td>
</tr>
<tr>
<td>12</td>
<td>Moderately healthy</td>
<td>56.6 ± 4.6</td>
<td>38.2 ± 9.9</td>
<td>71.5 ± 21.5</td>
<td>87</td>
</tr>
</tbody>
</table>
5.3.3 Abundance and diversity of AM fungi spores

There were 19 spore morphotypes (Table 5.2) in the bioassay soil compared to 8 morphotypes in the field soil (Chapter 4). Relative abundance (RA) of each morphotype is given in Table 5.3. The abundance of morphotypes varied between the sites. Similar to spore distribution of field soil where morphotypes M1 and M2 were present across all sites (Chapter 4), these morphotypes were also present across all sites in the bioassay soil. In particular, in the bioassay soil, M1 was dominant at sites 6 and 7, having RA of 36.5% and 37.6% respectively, but the RA of M1 was low at site 9 (8.7%). By comparison, M2 was dominant at site 6 (33.9% of RA) and low at site 9 (2.3% of RA).

In contrast to field soil where species richness was not significantly different between the sites (Chapter 4), Nested ANOVA for the bioassay soil revealed that species richness was significantly \( F_{(11, 48)} = 2.05, P = 0.04 \) different between sites (Fig. 5.3). Tree was not significantly \( F_{(36, 48)} = 0.63, P = 0.93 \) different within each site.

![Species richness of arbuscular mycorrhizal (AM) fungi in bioassay soil collected from twelve sites. Values are means (n = 8) ± SE.](image)

Fig. 5.3. Species richness of arbuscular mycorrhizal (AM) fungi in bioassay soil collected from twelve sites. Values are means (n = 8) ± SE.
The highest species richness was in soils taken from sites 3 and 4 followed by sites 5 and 12. Site 3 was classified as a healthy site, sites 5 and 12 as moderately healthy, and site 4 as a declining site. The lowest species richness was in soil taken from site 11 followed by sites 2 and 10. These 3 sites were classified as healthy sites.

5.3.4 Correlation between spore density and species richness of AM fungi

Pearson’s correlation coefficient analysis indicated that in the bioassay soil, spore density of AM fungi was strongly correlated ($r = 0.639$) with species richness (Fig. 5.4). This correlation was much stronger than in the field soil, where the $r$ value was only 0.299 (Chapter 4).

![Fig. 5.4. Correlation between spore density and species richness of arbuscular mycorrhizal (AM) fungi.](image-url)
Table 5.2. Description of AM fungal spore morphotypes

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>Identification</th>
<th>Colour</th>
<th>Shape</th>
<th>Size (µm)</th>
<th>Number of wall layers</th>
<th>Thickness of wall layers (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1*</td>
<td><em>Glomus sp. 1</em></td>
<td>yellow</td>
<td>globose/oval</td>
<td>65.9 - 251.2</td>
<td>3</td>
<td>5.4</td>
</tr>
<tr>
<td>M2*</td>
<td><em>Glomus sp. 2</em></td>
<td>brown</td>
<td>globose</td>
<td>66.8 - 209.7</td>
<td>3</td>
<td>5.9</td>
</tr>
<tr>
<td>M3*</td>
<td><em>Glomus sp. 3</em></td>
<td>golden</td>
<td>globose</td>
<td>256.3 - 341.8</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>M4*</td>
<td><em>Glomus sp. 4</em></td>
<td>light yellow</td>
<td>globose</td>
<td>102.5 - 171.1</td>
<td>3</td>
<td>6.9</td>
</tr>
<tr>
<td>M5*</td>
<td><em>Glomus sp. 5</em></td>
<td>golden</td>
<td>globose</td>
<td>143.8 - 204.1</td>
<td>3</td>
<td>5.4</td>
</tr>
<tr>
<td>M6*</td>
<td><em>Glomus sp. 6</em></td>
<td>black</td>
<td>globose</td>
<td>88.9 - 144.9</td>
<td>3</td>
<td>2.7</td>
</tr>
<tr>
<td>M7*</td>
<td><em>Glomus sp. 7</em></td>
<td>yellow</td>
<td>oval</td>
<td>109.7 - 153.6</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>M8*</td>
<td><em>Glomus sp. 8</em></td>
<td>brown</td>
<td>Globose (cluster)</td>
<td>91.5 - 96.1</td>
<td>4</td>
<td>10.7</td>
</tr>
<tr>
<td>M7*</td>
<td><em>Acaulospora sp. 1</em></td>
<td>yellow/orange</td>
<td>globose</td>
<td>55.2 - 75.2</td>
<td>2</td>
<td>8.6</td>
</tr>
<tr>
<td>M8*</td>
<td><em>Acaulospora sp. 2</em></td>
<td>red</td>
<td>globose</td>
<td>69.0 - 92.8</td>
<td>2</td>
<td>9.1</td>
</tr>
<tr>
<td>M9</td>
<td><em>Acaulospora sp. 3</em></td>
<td>dark red</td>
<td>globose</td>
<td>78.9 - 82.1</td>
<td>3</td>
<td>7.5</td>
</tr>
<tr>
<td>M10</td>
<td><em>Acaulospora sp. 4</em></td>
<td>yellow whitish</td>
<td>globose</td>
<td>99.3 - 02.8</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>M11</td>
<td><em>Gigaspora sp. 1</em></td>
<td>yellow</td>
<td>oblong</td>
<td>201.2 - 248.4</td>
<td>3</td>
<td>12.9</td>
</tr>
<tr>
<td>M12</td>
<td><em>Gigaspora sp. 2</em></td>
<td>brown</td>
<td>oval</td>
<td>240.4 - 303.6</td>
<td>4</td>
<td>11.8</td>
</tr>
<tr>
<td>M13</td>
<td><em>Gigaspora sp. 3</em></td>
<td>brown</td>
<td>globose</td>
<td>262.3 - 299.8</td>
<td>3</td>
<td>3.2</td>
</tr>
<tr>
<td>M14</td>
<td>Unidentified 1</td>
<td>golden</td>
<td>oval</td>
<td>161.5 - 189.9</td>
<td>2</td>
<td>3.2</td>
</tr>
<tr>
<td>M15</td>
<td>Unidentified 2</td>
<td>white</td>
<td>globose</td>
<td>106.3 - 149.6</td>
<td>4</td>
<td>10.2</td>
</tr>
<tr>
<td>M16</td>
<td>Unidentified 3</td>
<td>grey</td>
<td>globose</td>
<td>88.5 - 137.4</td>
<td>3</td>
<td>2.1</td>
</tr>
<tr>
<td>M17</td>
<td>Unidentified 4</td>
<td>golden</td>
<td>globose (cluster)</td>
<td>82.8 - 86.2</td>
<td>3</td>
<td>4.8</td>
</tr>
</tbody>
</table>

*M1 to M8 are described previously in Table 4.5*
Table 5.3. Spore number and relative abundance (in brackets) of AM fungal morphotypes at twelve sites (bioassay soil).

<table>
<thead>
<tr>
<th>Morphotype</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
<th>S9</th>
<th>S10</th>
<th>S11</th>
<th>S12</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>100 (27.5)*</td>
<td>16 (11.3)</td>
<td>116 (21.2)</td>
<td>168 (35.1)</td>
<td>124 (22.5)</td>
<td>200 (36.5)</td>
<td>164 (37.6)</td>
<td>98 (24.4)</td>
<td>60 (8.7)</td>
<td>22 (18.3)</td>
<td>20 (18.2)</td>
<td>74 (12.9)</td>
</tr>
<tr>
<td>M2</td>
<td>66 (18.1)</td>
<td>30 (21.1)</td>
<td>14 (2.6)</td>
<td>12 (2.5)</td>
<td>72 (13)</td>
<td>186 (33.9)</td>
<td>72 (16.5)</td>
<td>88 (21.9)</td>
<td>16 (2.3)</td>
<td>4 (6.7)</td>
<td>4 (3.6)</td>
<td>62 (10.8)</td>
</tr>
<tr>
<td>M3</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>48 (8.7)</td>
<td>2 (0.4)</td>
<td>12 (2.8)</td>
<td>14 (3.5)</td>
<td>4 (0.6)</td>
<td>2 (1.7)</td>
<td>0 (0)</td>
<td>6 (1.0)</td>
</tr>
<tr>
<td>M4</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>8 (1.5)</td>
<td>0 (0)</td>
<td>8 (1.4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>30 (7.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>M5</td>
<td>6 (1.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>10 (2.1)</td>
<td>0 (0)</td>
<td>8 (1.5)</td>
<td>78 (17.9)</td>
<td>22 (5.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (1.8)</td>
<td>10 (1.7)</td>
</tr>
<tr>
<td>M6</td>
<td>10 (2.7)</td>
<td>4 (2.8)</td>
<td>12 (2.2)</td>
<td>0 (0)</td>
<td>2 (0.4)</td>
<td>10 (1.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (1.7)</td>
<td>0 (0)</td>
<td>10 (1.7)</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>58 (15.9)</td>
<td>58 (40.8)</td>
<td>138 (25.3)</td>
<td>126 (26.4)</td>
<td>20 (3.6)</td>
<td>0 (0)</td>
<td>4 (0.9)</td>
<td>0 (0)</td>
<td>82 (11.9)</td>
<td>6 (5)</td>
<td>8 (7.3)</td>
<td>2 (0.3)</td>
</tr>
<tr>
<td>M8</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>136 (24.9)</td>
<td>62 (13)</td>
<td>38 (6.9)</td>
<td>8 (1.5)</td>
<td>0 (0)</td>
<td>6 (1.5)</td>
<td>430 (62.3)</td>
<td>54 (45)</td>
<td>40 (36.4)</td>
<td>166 (29)</td>
</tr>
<tr>
<td>M9</td>
<td>0 (0)</td>
<td>4 (2.8)</td>
<td>24 (4.4)</td>
<td>4 (0.8)</td>
<td>4 (0.7)</td>
<td>2 (0.4)</td>
<td>4 (0.9)</td>
<td>2 (0.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>12 (2.1)</td>
<td></td>
</tr>
<tr>
<td>M10</td>
<td>18 (4.9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>84 (15.2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>34 (8.5)</td>
<td>0 (0)</td>
<td>4 (3.3)</td>
<td>10 (9.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>M11</td>
<td>10 (2.7)</td>
<td>6 (4.2)</td>
<td>10 (1.8)</td>
<td>26 (5.4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (0.3)</td>
<td></td>
</tr>
<tr>
<td>M12</td>
<td>8 (2.2)</td>
<td>0 (0)</td>
<td>6 (1.1)</td>
<td>0 (0)</td>
<td>102 (20.3)</td>
<td>0 (0)</td>
<td>10 (2.3)</td>
<td>92 (22.9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (1.8)</td>
<td>180 (31.5)</td>
</tr>
<tr>
<td>M13</td>
<td>0 (0)</td>
<td>2 (1.4)</td>
<td>2 (0.4)</td>
<td>4 (0.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (1.8)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>M14</td>
<td>0 (0)</td>
<td>2 (1.4)</td>
<td>6 (1.1)</td>
<td>0 (0)</td>
<td>2 (0.4)</td>
<td>12 (2.2)</td>
<td>0 (0)</td>
<td>2 (0.5)</td>
<td>26 (3.8)</td>
<td>2 (1.7)</td>
<td>2 (1.8)</td>
<td>12 (2.1)</td>
</tr>
<tr>
<td>M15</td>
<td>2 (0.5)</td>
<td>4 (2.8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (0.4)</td>
<td>0 (0)</td>
<td>4 (0.9)</td>
<td>2 (0.5)</td>
<td>4 (0.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>26 (4.5)</td>
</tr>
<tr>
<td>M16</td>
<td>10 (2.7)</td>
<td>2 (1.4)</td>
<td>2 (0.4)</td>
<td>20 (4.2)</td>
<td>6 (1.1)</td>
<td>26 (4.7)</td>
<td>10 (2.3)</td>
<td>2 (0.5)</td>
<td>38 (5.5)</td>
<td>20 (16.7)</td>
<td>20 (18.2)</td>
<td>8 (1.4)</td>
</tr>
<tr>
<td>M17</td>
<td>12 (3.3)</td>
<td>14 (9.9)</td>
<td>6 (1.1)</td>
<td>12 (2.5)</td>
<td>2 (0.4)</td>
<td>26 (4.7)</td>
<td>6 (1.4)</td>
<td>2 (0.5)</td>
<td>2 (0.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>M18</td>
<td>64 (17.6)</td>
<td>0 (0)</td>
<td>66 (12.1)</td>
<td>34 (7.1)</td>
<td>28 (5.1)</td>
<td>42 (7.7)</td>
<td>72 (16.5)</td>
<td>8 (2.0)</td>
<td>28 (4.1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>M19</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>26 (4.7)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*relative abundance was calculated as: (spore number of a morphotype in each site / total spore number in each site) x 100%
5.3.5 Predicting spore density and species richness of AM fungi from soil chemical properties

The spore density and species richness of AM fungi in the bioassay soil could be predicted from soil chemical (Chapter 4) characteristics ($F_{(4, 43)} = 3.85$, $P < 0.009$; $F_{(7, 40)} = 3.98$, $P < 0.002$, respectively). Spore density was more abundant in association with higher P and K, and with lower exchangeable Na, whereas species richness was more abundant in association with higher P and K, and with lower and exchangeable Ca and DTPA Cu (Table 5.4).

Table 5.4. Soil chemical properties as predictors for AM fungal spore density and species richness using forward stepwise multiple regression. The soil variables included in the most parsimonious models in each case are shown, together with an indication of which variables are significant.

<table>
<thead>
<tr>
<th>Soil variables measured</th>
<th>Soil variables in the final model for spore density</th>
<th>Soil variables in the final model for species richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (NH$_4$)</td>
<td>N ($\beta^1 = 0.39$)</td>
<td>P ($\beta = 0.45$) *</td>
</tr>
<tr>
<td>P</td>
<td>P ($\beta = 0.35$)*</td>
<td>K ($\beta = 0.77$) *</td>
</tr>
<tr>
<td>K</td>
<td>S ($\beta = -0.08$)</td>
<td>S ($\beta = 0.44$)</td>
</tr>
<tr>
<td>S</td>
<td>K ($\beta = 0.77$) *</td>
<td>DTPA Cu ($\beta = -0.52$)*</td>
</tr>
<tr>
<td>Organic-C</td>
<td>Org.C ($\beta = -0.58$)</td>
<td>DTPA Zn ($\beta = -0.3$)</td>
</tr>
<tr>
<td>Conductivity</td>
<td>DTPA Cu ($\beta = -0.18$)</td>
<td>Exc. Ca ($\beta = -1.0$) *</td>
</tr>
<tr>
<td>pH (CaCl$_2$)</td>
<td>Exc. Ca ($\beta = -0.77$)</td>
<td>Exc. Na ($\beta = -0.61$)</td>
</tr>
<tr>
<td>pH (H$_2$O)</td>
<td>Exc. Na ($\beta = -1.0$)*</td>
<td></td>
</tr>
<tr>
<td>DTPA Cu</td>
<td>B ($\beta = 0.89$)</td>
<td></td>
</tr>
<tr>
<td>DTPA Fe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA Mn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA Zn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. Ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. Mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exc. Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* significant predictor, $^1\beta$ = standardised regression coefficient, which is a measure of how strongly each predictor variable influences the dependent variable

Note that the soil chemical data used were that given in Chapter 4
5.3.6 Correlation between spore density of AM fungi and crown health

Pearson’s correlation coefficient analysis revealed that in the bioassay soil, spore density was significantly correlated to canopy health with a moderate r value. Spore density was negatively correlated to TCHI ($P = 0.01$, $r = -0.479$, $n = 48$) and to crown density ($P = 0.01$, $r = -0.47$, $n = 48$), but it was positively correlated to crown dieback ratio ($P = 0.01$, $r = 0.434$, $n = 48$) and epicormic index ($P = 0.01$, $r = 0.481$, $n = 48$) (Fig. 5.5.). However, no correlation was found between spore density and foliage transparency.

Fig. 5.5. Correlation between spore density of arbuscular mycorrhizal (AM) fungi from pot bioassays soils collected from 12 sites and (a) total crown health index, (b) crown density, (c) crown dieback ratio, and (d) epicormic index.
5.3.7 Correlation between species richness of AM fungi and crown health

In the bioassay soil, species richness was significantly correlated to crown health, although the correlation was weak. Species richness was negatively correlated to TCHI and crown density ($P = 0.05$, $r = -0.327$, $n = 48$, and $P = 0.05$, $r = -0.360$, $n = 48$, respectively), whereas, it was positively correlated to crown dieback ratio ($P = 0.05$, $r = 0.299$, $n = 48$) and epicormic index ($P = 0.05$, $r = 0.298$, $n = 48$). No correlation was found between species richness and foliage transparency.

5.3.8 Arbuscular mycorrhizal colonization

Nested ANOVA indicated that the proportion of roots colonized by AM fungi differed significantly ($F_{(11, 48)} = 3.71$, $P = 0.0007$) between the sites (Fig. 5.6). Trees were not significantly ($F_{(36, 48)} = 0.59$, $P = 0.94$) different within each site. The highest proportion of roots colonized by AM fungi was in soil taken from site 6 followed by sites 7, 9, and 12. Based on mean cluster analysis (Chapter 4), sites 6, 7 and 9 were classified as declining, while site 12 was moderately healthy. The lowest AM fungi colonization was in soil taken from site 11 followed by sites 2, 5, 8 and 10. Site 5 was classified as moderately healthy, whereas sites 2, 8, 10 and 11 were healthy. No AM fungi colonization was found in the control treatment. An example of AM fungi colonization in *E. gomphocephala* seedling roots is presented in Fig. 5.7.
Fig. 5.6. Proportion of root length colonized by arbuscular mycorrhizal (AM) fungi in bioassay soil collected from twelve sites and a control treatment in sterilized soil collected from healthy site. Values are means (n = 8) ± SE.

Fig. 5.7. Arbuscular mycorrhizal (AM) colonization in roots of *E. gomphocephala* seedlings; (a) arbuscul and (b) vesicle. Soil collected from site 6.

### 5.3.9 Ectomycorrhizal colonization

Nested ANOVA showed that the proportion of ECM tips differed significantly (F (11, 48) = 6.33, P < 0.000002) with site (Fig. 5.8). Trees were not significantly (F (36, 48) = 1.1, P = 0.37) different within each site. The highest ECM colonization was in soil taken from sites 1, 2 and 10. By contrast, the lowest ECM colonization was in soil taken from site 6 followed by sites 4, 7 and 9. Based on mean cluster analysis
(Chapter 4), sites 1, 2 and 10 were classified as healthy sites, whereas, sites 4, 6, 7 and 9 were classified as declining sites.

Fig. 5.8. Proportion of root tips colonized by ectomycorrhizal (ECM) fungi in bioassay soil collected from twelve sites and a control treatment in sterilized soil collected from a healthy site. Values are means (n = 8) ± SE.

Some contamination occurred in the control treatment in which approximately seven percent of root tips were colonized by ECM fungi. Extramatrical mycelia formed by ECM in this bioassay trial were observed (Fig. 5.9). Some ECM morphotypes observed in the bioassay trial are illustrated in Fig. 5.10.

Fig. 5.9. Ectomycorrhizal mats formed on the side walls of the pots of *E. gomphocephala* seedlings grown in intact soil cores; (a) *Hysterangium*, site 10, (b) unknown mat, site 9, and (c) *Scleroderma*, site 5. The identification of mat types was based on colour, texture and aroma.
Fig. 5.10. Examples of ectomycorrhizal (ECM) morphotypes formed on seedlings of *E. gomphocephala* grown in the bioassay trial; (a) smooth black, (b) *Cenococcum*-like, (c) smooth white, (d) smooth brown, (e) brown, (f) black brown, (g) dark brown, (h) *Cenococcum*-like, and (i) thick black. Characterization of ECM morphotype was based on ECM tips (colour and hyphae).

### 5.3.10 Correlation between AM and ECM colonization

Comparing the dual colonization of AM and ECM in roots of *E. gomphocephala* seedlings, Pearson’s correlation coefficient analysis indicated that ECM colonization was negatively correlated with AM colonization (*P* = 0.01, *r* = -0.43, *n* = 96) (Fig. 5.11).
5.3.11 Correlation between seedlings and crown health

Although the seedling biomass differed between the sites, there was no correlation between seedling growth (seedling height and shoot dry weight) and all the canopy indices (TCHI, crown density, foliage transparency, crown dieback and epicormic index).

5.3.12 Correlation between AM/ECM colonization and crown health

Pearson’s correlation coefficient analysis showed that the proportion of roots colonized by AM fungi was negatively correlated to TCHI (P = 0.05, r = -0.447, n = 48), and crown density (P = 0.05, r = -0.335, n = 48), but positively correlated to crown dieback (P = 0.01, r = 0.335, n = 48) and epicormic index (P = 0.01, r = 0.449, n = 48) (Fig. 5.12). No correlation was found between AM colonization and foliage transparency.

Fig. 5.11. Correlation between ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) colonization.
Correlation between arbuscular mycorrhiza (AM) colonization and; (a) total crown health index, (b) crown density, (c) foliage transparency, (d) crown dieback ratio, and (e) epicormic index.

In contrast to AM fungi, a strong positive correlation occurred between the proportion of ECM tips and TCHI (P = 0.01, r = 0.578, n = 48) and with crown density (P = 0.01, r = 0.5, n = 48), whilst there were significant negative correlations with foliage transparency (P = 0.05, r = -0.438, n = 48), crown dieback (P = 0.01, r = -0.434, n = 48), and epicormic index (P = 0.01, r = -0.606, n = 48) (Fig. 5.13).
Correlation between ectomycorrhiza (ECM) colonization and:
(a) total crown health index, (b) crown density, (c) foliage transparency, (d) crown dieback ratio, and (e) epicormic index.

**5.3.13 Predicting tree crown health from mycorrhizal colonization**

In addition to correlation analysis, forward stepwise multiple regression showed that tree crown health could be predicted from mycorrhizal colonization \(F(2, 45) = 8.06, P = 0.001\). Tree health improved with increasing proportion of ECM root tips and lower AM \(\beta = 0.421, P = 0.001\). Model of linear regression showed that ECM root tips was positively related to TCHI \(F(1, 46) = 14.975, \beta = 0.784, P < 0.0001\), but
negatively related to AM colonization ($F_{(1,46)} = 7.357, \beta = -0.237, P < 0.009$) (Fig. 5.14).

Fig. 5.14. Relationship between total crown health index and (a) ectomycorrhizal ( ECM), and (b) arbuscular mycorrhizal ( AM) colonization.

5.3.14 Predicting AM/ECM colonization from soil chemical properties

Forward stepwise multiple regression showed that the AM and ECM colonization could be predicted from soil chemical properties ($F_{(10,37)} = 4.36, P < 0.001; F_{(8,39)} = 5.26, P < 0.001$, respectively) given in the Chapter 4 of field soil. The AM were more abundant in association with higher P, S, and exchangeable K, and with lower exchangeable Na, pH (H$_2$O), DTPA Zn, DTPA Cu, B, K and conductivity. Whereas, the ECM were more abundant in association with higher conductivity, pH (CaCl$_2$) and DTPA Fe, and with lower DTPA Cu, DTPA Mn, B, exchangeable Ca and pH (H$_2$O) (Table 5.5).
Table 5.5. Soil chemical properties as predictors for seedlings growth and AM/ECM colonization using forward stepwise multiple regression. The soil variables included in the most parsimonious models in each case are shown, together with an indication of which variables are significant.

<table>
<thead>
<tr>
<th>Soil variables measured</th>
<th>Soil variables in the final model for AM colonization</th>
<th>Soil variables in the final model for ECM colonization</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (NH₄)</td>
<td>P (β = 0.61)*</td>
<td>DTPA Cu (β = - 0.41)*</td>
</tr>
<tr>
<td>P</td>
<td>S (β = 0.689)*</td>
<td>Conductivity (β = 0.4)</td>
</tr>
<tr>
<td>K</td>
<td>Exc. K (β = 4.52)*</td>
<td>pH CaCl₂ (β = 0.985)*</td>
</tr>
<tr>
<td>S</td>
<td>Exc. Na (β = -1.4)*</td>
<td>pH H₂O (β = - 0.82)*</td>
</tr>
<tr>
<td>Organic-C</td>
<td>B (β = - 0.58)</td>
<td>DTPA Mn (β = - 0.16)</td>
</tr>
<tr>
<td>Conductivity</td>
<td>K (β = - 3.2)</td>
<td>DTPA Mn (β = 0.469)*</td>
</tr>
<tr>
<td>pH (CaCl₂)</td>
<td>pH H₂O (β = - 0.25)</td>
<td>Exc. Ca (β = - 0.43)</td>
</tr>
<tr>
<td>pH (H₂O)</td>
<td>Conductivity (β = - 0.33)</td>
<td>B (β = - 0.38)</td>
</tr>
<tr>
<td>DTPA Cu</td>
<td>DTPA Cu (β = - 0.41)*</td>
<td></td>
</tr>
<tr>
<td>DTPA Fe</td>
<td>DTPA Zn (β = - 0.18)</td>
<td></td>
</tr>
<tr>
<td>DTPA Mn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTPA Zn</td>
<td></td>
<td></td>
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<tr>
<td>Exc. Ca</td>
<td></td>
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<tr>
<td>Exc. Mg</td>
<td></td>
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<tr>
<td>Exc. K</td>
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<tr>
<td>Exc. Na</td>
<td></td>
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<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* significant predictor, † β = standardised regression coefficient, which is a measure of how strongly each predictor variable influences the dependent variable.

5.3 Discussion

5.4.1 Mycorrhizal colonization and crown health of *E. gomphocephala*

The gross type of mycorrhiza formed on *E. gomphocephala* seedlings grown in soil cores taken from the field predicted the canopy condition of *E. gomphocephala* at the sites where the cores were taken. In particular, ECM dominated seedling roots produced in soils taken from under healthy canopies whereas AM dominated seedling roots in soils taken from under trees with declining canopies. This suggested that ECM may have been more important than AM for maintaining the health of *E. gomphocephala* in natural ecosystems. Were the changes in ECM/AM status of seedling roots to be reflected in roots in the field, it is likely to have consequences for seedling recruitment as well as the ongoing health of the *E. gomphocephala* overstorey.
Some studies have reported similar findings on the relationship between mycorrhizal fungi and tree decline, particularly for forest species that are strongly dependent on ECM fungi. For instance, Causin et al. (1996) reported that declining *Quercus robur* stands had a decreased ECM status compared to healthy stands. In other studies, a lower ECM diversity was reported in declining *Q. ilex* (Montecchio et al. 2004) and *Picea abies* stands (Peter et al. 2008) than in healthy forests. Recently, decline of *E. delegatensis* in south-eastern Australia has been related to altered ECM communities mediated by soil chemistry (Horton et al. 2013).

Many ECM fungi are known to be important for the growth of trees in nutrient-poor soil (Marschner and Dell 1994; Plassard and Dell 2010). Furthermore, where compatible ECM fungi were absent, inoculation with some ECM fungi in the nursery significantly improved the growth of outplanted eucalypts in plantations (Malajczuk et al. 1994a; Chen et al. 2000b; Xu et al. 2001; Chen et al. 2006a). In the absence of compatible ECM fungi, eucalypts became colonized by AM fungi (Zambolim and Barros 1982; Oliveira et al. 1997; Santos et al. 2001; Pagano and Scotti 2008; Campos et al. 2011).

It was anticipated that *E. gomphocephala* seedlings in containers would have initially been colonized by AM fungi and that new roots would increasingly have become colonized by ECM fungi over time if suitable inocula were present in the soil cores. For this reason the plants were harvested after 7 months to allow adequate time for ECM colonization to occur. This succession in containerized seedlings has been described previously (Chen et al. 2000a; Egerton-Warburton and Allen 2001; Santos et al. 2001). The lack of ECM in seedlings grown in cores taken from under trees with declining canopies may result from a lack of suitable ECM fungi inoculum at these sites.
Factors that may have influenced ECM fungi inoculum loads in forest soil included spore dispersal and longevity, distribution of host plants across sites, and the abundance of sporocarps above and below ground. Whilst seasonal sampling for viable spore loads of mycorrhizal fungi was not conducted, it is known that ECM fungi such as *Pisolithus* and *Scleroderma*, which were common in the region, persisted in soil for several years and remained viable (unpublished data). The distribution of host plants did not seem to be a factor that may have influenced ECM inoculum load as the understorey of healthy and declining sites contained species within the Myrtaceae, Casuarinaceae and Fabaceae that were known to form ECM (Brundrett 1991).

*Phytophthora multivora* was isolated from declining *E. gomphocephala* in the same region as this study (Scott et al. 2009), and shown to cause loss of fine roots in *E. gomphocephala* seedlings in containers (Scott et al. 2011). Recently, fewer ECM fungal mats were observed in declining *E. gomphocephala* stands in the field (Scott et al. 2013a). In this study, root samples from seedlings were plated onto *Phytophthora* selective NARPH agar (Hüberli et al. 2000) but *P. multivora* and *P. cinnamomi* which were widespread in south-western Australia were not detected. It is not known whether the distribution and abundance of ECM fungi can be differentially affected by *P. multivora* in the field as shown for *Castanea sativa* (Blom et al. 2009; Scattolin et al. 2012). However, the results from an in vitro trial as well as a glasshouse trial (Chapter 3) may have indicated that *P. multivora* may have not been a very aggressive pathogen to *E. gomphocephala*, and the ECM fungi were unlikely to be impacted by *P. multivora*. Also, the canopy condition of *E. gomphocephala* in the field may have impacted on spore production by mycorrhizal fungi. It is generally assumed that ECM associations obtain more energy from host
plants than AM associations (Smith and Read 2008) and the amount of carbon allocated below ground can influence populations of mycorrhizal fungi (Peter et al. 2008).

AM colonization was high in seedlings grown in intact soil collected from declining sites. This was supported by a negative correlation between spore density in the bioassay soil and TCHI and crown density, as well as a negative relationship between tree health and AM colonization. In natural ecosystems, plant communities are associated with communities of AM fungi. These communities might be made up of genotypes either mutualists or cheaters. The position of AM along the continuum from parasitism to mutualism will depend on the plant symbiont (van der Heijden et al. 1998a) and edaphic factors (Johnson 1993). The outcome of symbiosis for the plants depends on the balance between net cost (C loss to the fungus) and net benefits (additional nutrient supply via the fungus) (Smith and Read 2008). Where net costs exceed net benefits, plant growth depression occurred, and thus it might be assumed that the fungus was a cheater as it exploited its host by obtaining C but providing no or little contribution.

Colonization by ECM fungi was low in seedlings grown in intact soils from declining sites, but was quite high in those from moderately healthy sites. Swaty et al. (2004) suggested that ECM colonization might be related to the stress level of the tree. These authors observed that ectomycorrhizal colonization rate of Pinus edulis exposed to drought was low at the most stressed sites. However, trees with an intermediate level of stress did not differ significantly between trees at high and low levels of the stress gradient. Thus, increasing stress may be associated with increasing investment of ECM mutualists until a threshold level is reached. Once this
point is reached, further stress may prevent the allocation of plant resources to ECM fungi, leading to decreased ECM colonization (Swaty et al. 2004).

5.4.2 Soil chemical properties and mycorrhizal colonization

In the bioassay study, AM colonization was more extensive in *E. gomphocephala* seedlings grown in soil with higher availability of P, S and exchangeable K. It is not known whether a causal relationship was present. However, it is possible that some AM fungi were less sensitive than ECM to changes in soil chemical composition. Soil conditions have been suggested to be important factors affecting mycorrhizal fungi (Erland and Taylor 2002; Toljander et al. 2006; Scattolin et al. 2008; Alzetta et al. 2012; Newbound et al. 2012; Horton et al. 2013), and some studies have found that adverse soil conditions such as extreme pH, and inorganic nutrient availability may impact detrimentally on AM species (Entry et al. 2002; Schmidt et al. 2010). On the other hand, a study by Bhadalung et al. (2005) found that some AM fungi persisted with increasing soil fertility. Furthermore, a few studies found that changes in soil chemical composition resulting from fertilization may have had no impact on ECM fungi. For instance, long-term fertilization in two arctic tundra ecosystems increased ECM abundance in *Betula* (Clemmensen et al. 2006), and in south-western Australia, ECM fungi readily invaded new eucalypt plantations on agricultural lands that had a long history of N, P and K additions (Lu et al. 1999). However, a number of studies have reported a negative impact of increased soil fertility on ECM fungi (Pampolina et al. 2002; Treseder 2004; Nehls et al. 2010). Recently, Karlinski et al. (2010) investigated poplar (*Populus* sp.), a dual AM/ECM genus, and found that AM fungi predominated over ECM fungi particularly in polluted soil which was high in carbon, N, P and K. These authors suggested that the proportion of the two
mycorrhizal types was strongly influenced by environmental conditions particularly
site and soil.

Shoot dry weights of seedlings differed significantly between the sites, but there was
no correlation with crown health in the field. This might have happened because
mineral nutrients were added to the seedlings. A minimal nutrient regime was
applied to make sure that carbon was not a limitation for mycorrhizal colonization as
the main point of this study was to trap mycorrhizal fungi. The level of fertilizer
added was based on levels that promoted ECM formation in container nurseries (Dell
and Malajczuk 1995). The greater shoot growth in control seedlings is likely to have
been due to nutrient release from soil organic matter when the soil was autoclaved.

There were more AM morphotypes in the bioassay soil (glasshouse) than in the field
soil (Chapter 3). This might have happened because the volume of intact soil
collected for the bioassay trial (12 cm diameter, 20 cm deep corer) was bigger than
the volume of field soil taken for spore assessment (4 cm diameter, 20 cm deep
corer). Consequently, the intact soil might have contained more AM propagules than
the smaller soil core. In addition, the conditions in the glasshouse might have been
more favourable for the multiplication of rare species than in the field, especially
during late summer when the soil was collected.

There was a small amount of contamination (7%) from ECM fungi in the control
treatment. This was possibly due to air-borne spores (the glasshouse is adjacent to
bush).

The type of mycorrhiza present was shown to predict crown health of *E. gomphocephala*. However, it was difficult to determine whether a decrease in ECM
was the cause of the decline in canopy health or it was caused by the decline in
crown health or both. Previous studies in other forest systems have demonstrated that defoliation might affect ECM fungi through carbon allocation to roots (Markkola et al. 2004; Peter et al. 2008). However, the situation is more complex as ECM fungi may contribute to forest resilience, recovery and vigor (Amaranthus 1998) as well as plant biodiversity and productivity in natural ecosystems (van der Heijden et al. 1998b; van der Heijden and van Straalen 2008).

To sum up, the results clearly indicated that the type of mycorrhiza was related to the canopy condition of *E. gomphocephala*. In particular, ECM dominated seedling roots produced in soils taken from under healthy canopies whereas AM dominated seedling roots in soils taken from under trees with declining canopies. suggesting that *E. gomphocephala* preferentially formed ECM except when compatible ECM were absent. Thus the proposed hypotheses (hypotheses 7 and 8) were accepted. The lack of ECM in seedlings grown in cores taken from under trees with declining canopies may have resulted from a lack of suitable ECM fungi inoculum at these sites. The result also showed that soil chemical properties were important factors related to health of *E. gomphocephala*. They were not only related to the canopy condition of the trees but also related to mycorrhizal colonization. This established the hypothesis that soil chemical properties were related to mycorrhizal colonization.

Following this, there is a great deal of interest to further investigate the ECM communities that colonized the seedlings. A range of ECM morphotypes were observed in the study but were unidentifiable, and it is unknown whether these ECM communities were also related to *E. gomphocephala* decline. Therefore, detailed molecular characterization is required in order to identify those ECM species that might be sensitive to canopy decline of *E. gomphocephala*. This will be explored in Chapter 6. Considering the trend of ECM and AM colonization observed, it is
hypothesized that ECM and AM communities are related to crown health, and the ECM fungi communities are dominant for seedling roots grown in soil collected from healthy site and *vice versa* for AM fungi communities.
Chapter 6

Mycorrhizal communities associated with *Eucalyptus gomphocephala* seedlings grown in soil collected from healthy, moderately healthy and declining sites

6.1 Introduction

Studies on the biodiversity of mycorrhizal fungi are dependent on their clear identification. In the previous chapters (Chapter 4 and Chapter 5), the method to determine the category of mycorrhiza was entirely based on morphological descriptions of the associations (morphotyping). This included microscopical examination of mycorrhizas and description of species based on the shape, colour and appearance of different fungal tissues or propagules, for example as described by Agerer (1987-98) and Goodman et al. (1996). For AM fungi, morphological taxonomy has relied mainly on the asexual spores. Species have been identified by their spore wall structure and substructures, whilst families and genera were mostly distinguished by the type of hyphal attachment (Sieverding 1991; Brundrett et al. 1996). Studies on ECM fungi communities mainly focused on the presence of symbiotic root tips, and the associated sporocarp (Horton and Bruns 2001).

Although morphological and anatomical descriptions have provided useful data for the identification of mycorrhizal fungi, most AM and ECM species have not been described. This might be due to diversity in sporulation condition (i.e. variation in environmental conditions that affect sporulation), sporulation ability and colonization ability of AM fungi (Sharmah et al. 2010), and for ECM fungi, this might be related to the complexity of visual classification of root tips, the large
number of fungal species in natural ecosystems, and the high spatial and temporal variation of the distribution of each species (Martin 2007).

Modern techniques for studying mycorrhizal communities are based on molecular identification. The molecular techniques generally have two major advantages including the possibility to identify fungi with reasonable certainty, and to evaluate the variability of both inter- and intra specific taxa (Dahlberg 2001). Additionally, molecular techniques allow for high throughput studies and to track the diversity of communities in more depth, especially in detecting cryptic species, which are difficult to describe by morphotyping (Anderson and Cairney 2004; Martin 2007).

Molecular techniques are based primarily on the polymerase chain reaction (PCR) amplification of regions of the fungal ribosomal DNA (rDNA). Through targeted amplification of specific regions of the fungal genome via PCR, fungal species present within a community can be identified quickly and accurately (Horton and Bruns 2001). For mycorrhizal fungi, molecular characterization is mostly achieved by PCR amplification of DNA of host plant roots or soil samples. As the majority of DNA extracted from colonized roots or soil samples is of plant origin or non-target organisms, specific PCR primers (a short strand of nucleic acid complement to the target sequence), which bind at the beginning and end of the chosen sequence to start the amplification, must be used in order to obtain fungal DNA fragments (Sharmah et al. 2010). Many molecular techniques result in the identification of operational taxonomic units (OTU’s), which are groups of fungi that have similar molecular profiles or sequences. The OTU’s are often used as a proxy for a single species, but they are different from a species unit as their morphological or sexual compatibilities are generally not considered.
Ribosomal DNA genes occur in multiple copies and contain conserved coding parts including small subunits (SSU) and large subunits (LSU) as well as non-coding parts (internal transcribed spacers, ITS), which separate the SSU and LSU. The ITS region has been widely used for molecular identification of fungi. The ITS region is highly conserved within species but exhibits enough variation between species allowing taxonomic resolution often to the species level (Nilsson et al. 2009; Ryberg et al. 2009). Some methods utilizing the ITS for fungal identification from environmental samples include restriction fragment length polymorphism (RFLP) (Karen et al. 1997; Klamer et al. 2002), terminal restriction fragment length polymorphism (T-RFLP) (Burkea et al. 2005), denaturing gradient gel electrophoresis (DGGE) (Landeweert et al. 2005) and cloning combined with sequencing (Chen and Cairney 2002). Although these methods have been successfully applied to describe fungal diversity and community, identification of all individual species present in a given sample remains a challenge (Ovaskainen et al. 2010).

A recent development in molecular technology is the next generation sequencing (NGS) (e.g. 454 pyrosequencing methodology developed by 454 Life Sciences/Roche Applied Science – the 454 Sequencing™). This technology enables large scale analysis of fungal communities, that can not only provide qualitative information about the fungal communities but may also provide quantitative information on fungal abundance in the mycorrhizosphere (Tedessoo et al. 2010; Kauserud et al. 2012). Further, as the technology analyses huge amounts of sequence simultaneously, it may allow more possibilities to detect infrequent taxa, which might not be detected by screened clones using traditional cloning methods (Buée et al. 2009; Öpik et al. 2009). Although the length of sequence obtained from the 454 sequencing is considerably shorter than those obtained from the Sanger technique, it
has been shown that short sequences can accurately describe community structure if the amplicon location in the genome is carefully selected to provide nucleotide variation among the taxa (Liu et al. 2007). Since pyrosequencing provides an extremely large amount of information, bioinformatic methods are required to analyze the data.

The results from the preceding chapter showed that mycorrhizal fungi were related to the canopy condition of *E. gomphocephala*. Colonization by ECM fungi was high in seedling roots grown in soil collected from under healthy trees, whilst colonization by AM fungi was dominant in plants grown in soils collected from under declining trees. Microscopic observation showed that there was a range of ECM morphotypes on the seedling roots (Chapter 5), but the fungi were not able to be identified. Similarly, spores of AM fungi were also observed (Chapters 4 and 5); however, little is known about the AM fungi communities colonizing the seedling roots. Furthermore, whether the ECM and AM fungi communities are related to *E. gomphocephala* decline, as previously found in terms of their colonization (Chapter 5), needs to be proved. Based on the above, the main aims of this chapter were to determine the associated ECM and AM communities colonizing the seedling roots and to evaluate if the mycorrhizal communities changed with the health status of *E. gomphocephala*.

The hypotheses tested were:

H10. The mycorrhizal fungal communities associated with seedlings are related to crown health of *E. gomphocephala*, and

H11. ECM fungal communities are dominant in seedling roots grown in soil collected from healthy sites, and *vice versa* for AM fungal communities at declining sites.
6.2 Materials and methods

Root samples (96 samples) used for this study were obtained from the bioassay trial (Chapter 5). The roots were preserved in 100% EtOH and placed in 25 ml containers for approximately one year before use. A mixture of root fragments and root tips were randomly transferred into a 1.5 ml eppendorf tube and the roots were allowed to dry in a laminar flow overnight by leaving the eppendorf tube open. The roots were then freeze dried for 3 days (Hetosicc CD 4), and kept at -80°C before DNA extraction.

6.2.1 DNA extraction

The roots were manually ground before DNA extraction. To grind the roots, the 1.5 ml eppendorf tube containing root samples was dipped into liquid nitrogen until frozen, and the roots were ground manually with a sterile fine spatula into a fine powder. The DNA was extracted according to the manufacturer’s instruction manual of ZR Fungal/Bacterial DNA MiniPrep™ Catalog No. D6005 as follows: ten mg of dry root samples was transferred into 900 µl PCR water placed in A ZR BashingBead™ lysis tube, and then suspended. After that, 750 µl of the lysis solution was added into the tube, secured in a bead beater fitted with a 2 ml tube holder, and processed with a cell disrupter (Tissue lyser Qiagen) at a frequency of 30 cycles/sec for 2 minutes. The ZR BashingBead™ lysis tube was then centrifuged at 10,000 x g for 1 min, 400 µl of supernatant was then transferred to a Zymo-SpinTM IV spin filter in a collection tube, and centrifuged at 7,000 x g for 1 min. Afterwards, 1,200 µl of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tube, 800 µl of this mixture was then transferred to a Zymo-SpinTM IIC column in a collection tube and centrifuged at 10,000 x g for 1 min. The flow through from the collection tube was discarded, and another 800 µl of the mixture
was transferred to this collection tube, centrifuged at 10,000 x g for 1 min, and the flow through from this collection tube discarded. Then, 200 µl DNA pre-washed buffer was added to the Zymo-SpinTM IIC column in a new collection tube, centrifuged at 10,000 x g for 1 min, and 500 µl Fungal/Bacterial DNA wash buffer was added to this Zymo-SpinTM IIC column followed by centrifuging at 10,000 x g for 1 min. The Zymo-SpinTM IIC column was transferred to a clean 1.5 ml microcentrifuge tube, 100 µl DNA elution buffer was directly added into the column matrix, and then centrifuged at 10,000 x g for 30 seconds to elute the DNA.

The pure DNA both as full DNA and diluted DNA (1:10 dilution) was sent to Australian Genome Research Facility Ltd (AGRF) Perth for 454 sequencing analysis. 454 pyrosequencing was chosen to identify both the ECM and AM communities associated with roots system of *E. gomphocephala* seedlings since this technology has been used to analyse fungal diversity including mycorrhiza (Öpik et al. 2009; Blaalid et al. 2012; Kauserud et al. 2012).

### 6.2.2. DNA sequence

The genomic DNA samples were amplified separately in a two step PCR using the fungal primer pair ITS1F (5’-CTT GGT CAT TTA GAG GTA A-3’) and ITS2 (5’-GCT GCG TTC TTC ATC GAT GC-3’) to generate PCR ITS rRNA fragments of c. 400 base pairs. In the second round of PCR, the primers were modified to include adaptors for pyrosequencing with ITS1F primer individually barcoded for each sample. The sequence was run on a Genome Sequencer FLX 454 System by AGRF (www.agrf.org.au).

After the sequencing run, results were run through Roche’s standard amplicon analysis pipeline. This is a very stringent pipeline which results in only the long high
quality reads remaining. Demultiplexing of the results based on the barcode assigned to a sample was performed. Each sample underwent the bioinformatic processing for the diversity profiling report, and ITS samples were processed under the UNITE database (http://greengenes.secondgenome.com/). The composition of species diversity in high-throughput amplicon sequencing data was carried out using the Quantitative Insights Into Microbial Ecology (QIIME) software package version (http://qiime.org). A BLAST search was undertaken to compare ITS sequences with known fungal species in GenBank.

6.2.3 Statistical analysis

Diversity (richness) of AM and ECM fungi at each site were determined, and Pearson’s correlation coefficient was employed to determine the correlation between the occurrence of ECM/AM fungi and crown health. Richness is defined as the number of operational taxonomic units (OTU’s) at the family or species level found at each site.

6.3 Results

6.3.1 Fungal community

The DNA sequence analysis detected six Phyla of fungi associated with the seedling roots, namely Ascomycota, Basidiomycota, Glomeromycota, Chytridiomycota, Blastocladiomycota and Zygomycota. The Ascomycota contributed 85.9% of the total DNA sequences detected in the roots in soil from 12 sites, whilst Basidiomycota and Glomeromycota contributed 11 and 2.1%, respectively. The Chytridiomycota contributed 0.9% whereas the Blastocladiomycota and Zygomycota contributed less than 0.1% each (Fig. 6.1).
The highest proportion of the Ascomycota was found for seedling roots grown in soil collected from the moderately healthy sites (90.6%) compared with seedling roots grown in soil collected from the healthy (85.4%) and declining sites (84.4%). The phylum Basidiomycota was mostly detected from seedling roots in soil collected from the healthy sites (13.5%) and a smaller proportion was found for seedling roots grown in soil collected from the moderately healthy (9.23%) and declining sites (8.21%). A higher proportion of the Glomeromycota was identified for roots in soil from the declining sites (5.3%). On the other hand, only 0.65% and 0.11% of this phylum were detected for the seedling roots grown in soil collected from the healthy and moderately healthy sites, respectively (Table 6.1).

At the genus level, all the fungi which might be ecologically important and related to the health of E. gomphocephala were included in the calculation. The fungi were grouped into pathogen (leaf and bark pathogens), saprophyte, endophyte, root rot, ECM and AM fungi (Table 6.2). Some examples of putative or potential pathogens were Botryosphaeria, Mycosphaerella, Teratosphaeria and Cercosphora. Some species of Botryosphaeria are potential pathogens. In this study, fungi which were
neutral were grouped as endophytes. The genera of the Tricholomataceae family (Ascomycota) such as *Aspergillus* and *Eupenicillium*, and some genera of Basidiomycota such as *Cryptococcus* and *Rhodotorula* were considered as saprophytes. The endophytic fungal genera such as *Bartalinia* and *Preussia* and root rot organisms such as *Thielaviopsis* and *Phlebia* were included in the calculation.

The proportion of these six fungal groups in the seedling roots grown in soil collected from healthy, moderately healthy and declining sites is presented (Fig. 6.2).

Table 6.1. Proportion of fungal phyla associated with *E. gomphocephala* seedling roots grown in soil collected from healthy, moderately healthy and declining sites.

<table>
<thead>
<tr>
<th>Phyla</th>
<th>Site type where the soil was collected</th>
<th>Healthy</th>
<th>Moderately healthy</th>
<th>Declining</th>
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<tr>
<td>Ascomycota</td>
<td></td>
<td>85.39</td>
<td>90.59</td>
<td>84.39</td>
</tr>
<tr>
<td>Basidiomycota</td>
<td></td>
<td>13.5</td>
<td>9.23</td>
<td>8.2</td>
</tr>
<tr>
<td>Blastocladiomycota</td>
<td></td>
<td>0.16</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Chytridiomycota</td>
<td></td>
<td>0.3</td>
<td>0.07</td>
<td>2.13</td>
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<td>Glomeromycota</td>
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<td>0.11</td>
<td>5.26</td>
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<td>Zygomycota</td>
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<td>0</td>
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</table>

Table 6.2. Proportion of fungal groups associated with *E. gomphocephala* seedling roots grown in soil collected from healthy (H), moderately healthy (M) and declining (D) sites at the genus level.

<table>
<thead>
<tr>
<th>Fungal group</th>
<th>Site</th>
<th>S1 (H)</th>
<th>S2 (H)</th>
<th>S3 (H)</th>
<th>S8 (H)</th>
<th>S10 (H)</th>
<th>S11 (H)</th>
<th>S5 (M)</th>
<th>S12 (M)</th>
<th>S4 (D)</th>
<th>S6 (D)</th>
<th>S7 (D)</th>
<th>S9 (D)</th>
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<tr>
<td>Pathogen</td>
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<td>21.9</td>
<td>9.9</td>
<td>34.2</td>
<td>6.7</td>
<td>24.6</td>
<td>16.5</td>
<td>14.98</td>
<td>6.6</td>
<td>34.7</td>
<td>24.1</td>
<td>29.7</td>
<td>15.9</td>
</tr>
<tr>
<td>Root rot</td>
<td></td>
<td>13.7</td>
<td>10.7</td>
<td>0.8</td>
<td>2.3</td>
<td>10.3</td>
<td>1.6</td>
<td>3.8</td>
<td>0.1</td>
<td>3.9</td>
<td>1.5</td>
<td>7.1</td>
<td>29.9</td>
</tr>
<tr>
<td>Saprophyte</td>
<td></td>
<td>52.1</td>
<td>65.7</td>
<td>55.6</td>
<td>91.1</td>
<td>56.8</td>
<td>55.2</td>
<td>70.1</td>
<td>88.3</td>
<td>61.1</td>
<td>20.5</td>
<td>45.1</td>
<td>52.7</td>
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<td>Endophyte</td>
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<td>1.9</td>
<td>1.2</td>
<td>3.4</td>
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<td>0.02</td>
<td>2.5</td>
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<td>ECM</td>
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<td>12.4</td>
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<td>5.8</td>
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<td>12.1</td>
<td>9.4</td>
<td>1.5</td>
</tr>
<tr>
<td>AM</td>
<td></td>
<td>4.3</td>
<td>0</td>
<td>1.2</td>
<td>0</td>
<td>0.03</td>
<td>0.3</td>
<td>5.3</td>
<td>0</td>
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<td>30.9</td>
<td>8.3</td>
<td>0</td>
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<tr>
<td>Total</td>
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Fig. 6.2. Proportional distribution of fungal groups at the genus level colonizing the seedling roots grown in soil collected from; (a) healthy, (b) moderately healthy, and (c) declining sites.

There were some interesting trends in fungal distribution particularly regarding saprophytic, pathogenic, root rot and mycorrhizal fungi between the sites. The proportion of saprophytes was higher for the seedling roots grown in soil collected from the moderately healthy and the healthy sites, contributing 79.2 and 62.8%, respectively, compared with those seedling roots grown in soil collected from the declining sites (44.8%). On the other hand, the pathogenic and root rot fungi were more prevalent with seedlings roots grown in soil collected from the declining sites contributing 26.1 and 10.6%, respectively, than those found associated with seedling roots grown in soil collected from the moderately healthy sites (10.7 and 1.9%),
respectively) and the healthy sites (18.9 and 6.9%, respectively). The proportion of ECM to AM fungi was high for seedling roots grown in soil collected from the healthy and moderately sites, whilst the proportion of AM to ECM fungi was high for seedling roots grown in soil collected from the declining sites. The proportion of endophytes was relatively similar in the seedlings roots grown in soil collected from the healthy and moderately healthy sites, but higher in the seedling roots grown in soil collected from the declining sites (Fig. 6.2).

6.3.2 Mycorrhizal community

ECM fungi richness

The DNA sequence analysis identified six ECM families associated with seedlings roots, namely Cortinariaceae, Rhizopogonaceae, Sclerodermataceae, Sebacinaceae, Thelephoraceae, and Tuberaceae (Table 6.3).

Table 6.3. Richness of ECM fungi associated with seedling roots grown in soil collected from healthy (H), moderately healthy (M) and declining (D) sites at the family level.

<table>
<thead>
<tr>
<th>ECM Family</th>
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<td>S3 (H)</td>
<td>S8 (H)</td>
<td>S10 (H)</td>
<td>S11 (H)</td>
<td>S12 (M)</td>
<td>S4 (D)</td>
<td>S6 (D)</td>
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Occurrence

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<td>Occurrence</td>
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<td>2</td>
<td>4</td>
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</tbody>
</table>

0 = absence, 1 = present
*The site number refers to sites described in Chapter 4

All six families were found in the seedlings roots in soil collected from the healthy sites, whereas only two families (Cortinariaceae and Tuberaceae) and four families (Sclerodermataceae, Sebacinaceae, Thelephoraceae, and Tuberaceae) were detected
for roots in soil from the moderately healthy and declining sites, respectively (Table 6.3). Not many ECM fungal communities associated with seedling roots were detected by molecular analysis at the species level, and only sixteen ECM OTU’s were found (Table 6.4).

Table 6.4. Richness of ECM fungi associated with seedling roots grown in soil collected from healthy (H), moderately healthy (M) and declining (D) sites at the species level.

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Richness

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*= Basidiomycota; *= Ascomycota

Pearson’s correlation analysis revealed that the number of ECM fungi present at the species level was not significantly correlated with canopy condition of *E. gomphocephala* in the field; however, there was a trend for more ECM fungi OTU’s for seedlings roots grown in soil collected from the healthy site than in the declining site. Thirteen of the 16 ECM fungi OTU’s were associated with seedling roots grown in soil collected from the healthy sites, whilst only four and eight ECM fungi
OTU’s were found for seedling roots grown in soil collected from the moderately healthy and declining sites, respectively (Table 6.4).

**AM fungi richness**

There were four AM fungi families associated with the roots. These were Diversisporaceae, Claroideoglomeraceae, Glomeraceae and unidentified Glomeraceae in which Glomeraceae appeared as the most prevalent family detected in the roots. All four AM families were found for seedling roots grown in soil collected from the declining sites, whereas only two AM families (Diversisporaceae and Glomeraceae) and one family (Diversisporaceae) were identified for seedling roots grown in soil collected from the healthy and moderately healthy sites, respectively (Table 6.5).

<table>
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<th>S11 (H)</th>
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<th>S12 (D)</th>
<th>S4 (D)</th>
<th>S6 (D)</th>
<th>S7 (D)</th>
<th>S9 (D)</th>
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<td></td>
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</tbody>
</table>

At the species level, a total of 14 AM fungi OTU’s were found in the roots grown in soil collected from the 12 sites. *Glomus* appeared as the dominant AM genus present in the roots followed by the genus *Rhizophagus*. Pearson’s correlation analysis revealed that the number of AM fungi OTU’s was significantly ($r = -0.41$) negatively correlated to canopy health of *E. gomphocephala*. Eleven AM fungi OTU’s out of 14 were found for seedling roots grown in soil collected from the declining sites.
compared with three and six OTU’s for roots in soil collected from the moderately
and healthy sites, respectively (Table 6.6).

Table 6.6. Richness of AM fungi associated with seedling roots grown in soil
collected from healthy (H), moderately healthy (M) and declining (D) sites at species
level.

<table>
<thead>
<tr>
<th>Putative AM fungal species/morphotype</th>
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<th>Site</th>
<th>Site</th>
<th>Site</th>
<th>Site</th>
<th>Site</th>
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<tbody>
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<td></td>
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<td>S2</td>
<td>S3</td>
<td>S8</td>
<td>S10</td>
<td>S11</td>
<td>S12</td>
<td>S4</td>
<td>S6</td>
</tr>
<tr>
<td></td>
<td>(H)</td>
<td>(H)</td>
<td>(H)</td>
<td>(H)</td>
<td>(H)</td>
<td>(H)</td>
<td>(M)</td>
<td>(M)</td>
<td>(D)</td>
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<table>
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</thead>
<tbody>
<tr>
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*Proportion of ECM/AM fungi within the roots*

For the calculation of the proportional distribution of mycorrhizal fungi associated
with the seedling roots, only the ECM and AM fungi were included. The proportion
of ECM and AM fungi OTU’s for seedling roots grown in soil collected from the 12
sites is presented in Table 6.7.
Table 6.7. Proportion of ECM and AM fungi associated with *E. gomphocephala* seedling roots grown in soil collected from healthy (H), moderately healthy (M) and declining (D) sites.

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Ectomycorrhizal fungi contributed 75% of the total proportion of mycorrhizal fungi colonizing the seedling roots grown in soil collected from the healthy sites, whilst
the AM fungi only contributed 25%. On the other hand, seedling roots grown in soil collected from the declining sites were dominantly colonized by AM fungi. The AM fungi contributed 69.4% of the total proportion, whilst the ECM fungi were only 30.6%. A quite different feature was observed for mycorrhizal fungi colonizing the seedling roots grown in soil collected from the moderately healthy sites. A very high proportion (98.6%) of the ECM fungi was found in the root from these sites, whilst the AM proportion was very low (1.4%) (Fig. 6.3).

Figs. 6.3. Proportional distribution of ECM and AM fungi colonizing seedling roots grown in soil collected from; (a) healthy, (b) moderately healthy, and (c) declining sites.
6.4 Discussion

The results showed that the proportion of ECM fungi was very high for the seedling roots grown in soil collected from the healthy sites and the AM fungi proportion was high for the seedling roots grown in soil collected from the declining sites. Although the number of ECM fungi OTU’s was not significantly correlated with crown health of *E. gomphocephala*, there was a trend for more OTU’s to be present for seedling roots grown in soil collected from the healthy sites. The AM fungi OTU’s, however, were negatively correlated with the crown health of *E. gomphocephala*. These results for the ECM dominance at healthy sites and the AM dominance at the declining sites support the key finding in the bioassay trial (Chapter 5).

6.4.1. Fungal community

The majority phylum of fungal sequences recovered belonged to the Ascomycota followed by the Basidiomycota and Glomeromycota. There was a trend for a higher proportion of Basidiomycota to be associated with seedling roots grown in soil collected from the healthy sites, whilst a higher proportion of Glomeromycota was found with roots grown in soil collected from the declining sites.

Some considerations were taken into account when grouping the fungi. For instance, putative pathogens such as *Botryosphaeria, Mycosphaerella* and *Teratosphaeria*, and fungal genera known as major plant pathogens such as *Alternaria, Cytospora*, and *Phaeosphaeriopsis* were grouped as pathogens. Basidiomycota which are neither mycorrhizal nor root rot fungi were grouped as saprophytes. The endophyte group was considered as neutral endophytic fungi. Pathogenic endophytic fungi, such as the genus *Diaporthe*, were grouped with pathogens. Some common genera such as *Fusarium, Cylindrocarpon* and *Rhizoctonia* were detected by molecular analysis.
Some species of *Fusarium* were grouped as saprophyte, whilst some species of *Cylindrocarpon* were grouped as root rot, and one species of *Rhizoctonia* was grouped as pathogen.

Interestingly, the proportion of fungal groups associated with seedling roots indicated some trends. For example, the saprophytic fungi were high for seedling roots grown in soil collected from the healthy and moderately healthy sites, but low for the seedling roots grown in soil collected from the declining sites. Some saprophytes might be ecologically important for organic matter decomposition, contributing to nutrient release into the soil. Whether the lower proportion of saprophytes in the declining sites is related to tree health at these sites is not known. However, it has been reported that a decrease in utilization of carbohydrates, carboxylic acid, amino acids and amines by the soil bacteria community was correlated to poor *E. gomphocephala* crown health indicating a strong relationship between the bacterial community and *E. gomphocephala* decline (Cai et al. 2010).

The proportion of pathogen and root rot groups was higher for the seedling roots grown in soil collected from the declining sites compared with seedling roots grown in soil collected from the moderately healthy and healthy sites. The occurrence of these fungi with roots is not impossible. As the soils used to grow the seedlings were collected from the field, these pathogens possibly could have been introduced with the soil. It is unlikely that pathogens and other endophytes were passaged through the seeds, but this can not be completely ruled out as the seeds were not dissected. There is quite a lot of discussion by plant pathologists about some putative pathogens such as *Botryosphaeria*, *Mycosphaerella* and *Teratosphaeria* in *E. gomphocephala* and other native woody plants such as *Acacia cochlearis*, *A. rostellifera*, *Allocasuarina fraseriana*, *Agonis flexuosa*, *Banksia grandis* and
*Eucalyptus marginata* which may grow in association with *E. gomphocephala* (Barber et al. 2006; Hunter et al. 2009; Taylor et al. 2009; Hunter et al. 2011). Whether the pathogen and root rot groups found in this study behave as opportunistic fungi or they are real pathogens to *E. gomphocephala* is not known. However, it is widely accepted that some opportunistic fungi may become pathogenic when the environmental conditions are favourable and trees are under stress. It is possible that there is a higher diversity and inoculum load of putative pathogens in soil from declining sites and this might be a contributing factor to the declining health of trees. Spores of these species could have been brought into these sites by wind, rain, air currents or other factors. Brown and Hovemeller (2002) suggested that fungal spore dispersal by the wind can spread plant pathogens over long distances. Furthermore, the global movement/gene flow of some pathogens causing canker and leaf spot in eucalypts has been reported (Burgess et al. 2008; Hunter et al. 2008).

For the mycorrhizal groups, the proportion of ECM fungi was high for seedlings roots grown in soil collected from healthy and moderately healthy sites, whilst the AM proportion was high for seedling roots grown in soil collected from declining sites. This finding was in agreement with the results from other previous studies on the relationship between ECM colonization and tree decline (Causin et al. 1996; Swaty et al. 2004; Scott et al. 2013a).

### 6.4.2 Mycorrhizal community

The results showed that ECM fungi were more diverse for seedling roots grown in soil collected from the healthy sites, but it was low for roots of seedlings grown in the soil from the declining sites. At the species level, 16 ECM fungi OTU’s in total were found in the roots, 13 for roots in soil from healthy sites, and 8 for roots in soil from declining sites. In contrast, from the total of 14 AM fungi OTU’s, 11 were for
roots grown in soil collected from declining sites and 6 for roots grown in soil collected from healthy sites.

Thus, ECM fungal communities were dominant for roots grown in soil collected from healthy sites and were low for seedlings grown in soil collected from the declining sites. These findings support some earlier studies dealing with the relationship between ECM fungi communities and tree decline in the other forest systems (Perrin and Estivalet 1989; Causin et al. 1996; Montecchio et al. 2004; Peter et al. 2008; Horton et al. 2013). Cross reference is made to information on page 20.

So far, not many studies have been undertaken on ECM and AM fungal communities in eucalypt forests in Australia, either in natural forest or in plantations. In general, it is estimated that there are 7,000 - 10,000 ECM fungi species present worldwide (Taylor and Alexander 2005), and Australia in particular has a high diversity of ECM fungi with an estimation of 6,500 species. Most major groups of Basidiomycota, Ascomycota and Zygomyccota forming ECM are well represented in Australia, and truffle-like fungi, mostly ECM fungi, are more abundant and diverse in Australia than anywhere else in the world. Despite this diversity, it is assumed that only about 10% have been formally described (Bougher 1995). Recently, Trappe et al. (2006) studied the effect of prescribed burning on the production of hypogeous fungal fruit bodies in northern Australia, and reported that 31 of the 38 hypogeous species found were new to science.

There was no significant correlation between ECM fungal communities (diversity) and crown health of *E. gomphocephala*. This might be due to a relatively small number of ECM fungal communities detected by DNA sequence analysis. In the present study, a key ECM family, the Russulaceae, and some key genera such as *Pisolithus, Russula, Cortinarius, Laccaria* and *Cenococcum* are missing from the
molecular analysis. Indeed, some of the mat-forming ECM fungi like *Hysterangium* were not also detected. Recently, Horton et al. (2013) investigated the relationship between ECM fungi communities, soil chemistry and *E. delegatensis* decline in Tasmania. Using ECM root tips coupled with sporocarps collected from the field to extract DNA for molecular analysis, the authors showed that ECM fungi richness decreased in a declining site, and ECM community composition differed between the declining and the moderately declining sites. In their study, many important key families such as the Cortinariaceae, Russulaceae and Thelephoraceae, and key genera such as *Cortinarius*, *Russula*, and *Lactarius* were successfully detected through molecular analysis.

The smaller number of ECM taxa detected compared to findings by Horton et al. (2013) could be due to large differences in ecosystems between the two regions such as climate and soil. *Eucalyptus delegatensis* is a high-altitude, high-rainfall, lower temperature species with a very closed canopy and rich organic soil, whereas *E. gomphocephala* forest is much lower in altitude, hotter and drier with sandy soil. However, there are other possibilities that might be relevant in explaining the smaller number of ECM taxa detected by molecular analysis in the current study. For instance, it is not known whether the plants only trapped a limited number of ECM fungi in the soil, or whether some fungi did not persist under glasshouse conditions. However, as written earlier, not all morphotypes observed in Chapter 5 were detected in the molecular study. For example, *Cenococcum* was observed in Chapter 5, but was not detected by molecular analysis. Similarly, distinctive spores of the AM genera including *Gigaspora* and *Acaulospora* were observed, but were not detected in the molecular analysis.
Kauserud et al. (2012) compared 454 sequencing and the Sanger sequencing approach for their ability to characterize fungal communities of the ECM plant *Bistorta vivipora*, and reported that 454 performed poorly in detecting *Cenococcum* and Thelephoraceae, although the method had a higher affinity to detect Glomales than the cloning/Sanger approach. A similar study by Tedersoo (2010) reported that several taxa were not captured by Cloning/Sanger and some taxa were poorly detected using 454. Sakakibara (2002) on the other hand suggested that morphological techniques in conjunction with the use of molecular techniques may provide more powerful information on mycorrhizal communities.

There has been an issue regarding taxonomic reclassification in the *Glomeromycota* including the taxonomy of *Glomus intradices* and *Rhizophagus irregularis*. Recently, *Glomus intradices* was transferred to *Rhizophagus irregularis* (Krüger et al. 2012). The disparity in the Blast data that were used to generate fungal taxa, are in the online record for both *G. intradices* and *R. irregularis*. Presently, this cannot be resolved and the two names are retained in Table 6.6. Data available online (GenBank) show some differences in the DNA sequences and without a full study of the species boundaries it is not clear whether the *G. intradices* data set should be included in the *R. irregularis* data set, or in a separate and distinct taxon, or whether there has been an error in identification at the time the accession was given.

Surprisingly, *Tuber indicum* was detected by molecular analysis. Apart from introduced *Tuber* species into commercial tuber farms, as yet there is no record of a true tuber species in Australia. *Tuber indicum* is known to be native to Asia but it is widely dispersed as food around the world including Melbourne (Australia) (Wang et al. 2006; Bonito et al. 2011). The fact that *Rhizopogon* was detected by molecular analysis was quite interesting. *Rhizopogon* is usually associated with conifers such as
Pinus species, and only formed surface colonization with a loose mantle in associations with eucalypts (Malajczuk et al. 1994b). Introduced Rhizopogon is one of the ECM fungi commonly associated with Pinus in Western Australia (Dunstan et al. 1998) and is used commercially in pine nurseries in the region. Since there are Pinus plantations near the Yalgorup National Park, it is possible that the Rhizopogon spores could have been blown into this area.

When only ECM and AM fungi were considered in the calculation of proportional fungal distribution in the roots, the ECM fungi were extremely high in the seedling roots grown in soil collected from moderately healthy sites. It is not known whether this is due to fewer samples (2 sites) or high abundance of propagules in the field. Despite the high proportion of ECM fungi, the diversity of ECM OTU’s detected in the roots were lower (4 ECM OTU’s) than those found for seedling roots grown in soil collected from the healthy site (13 ECM OTU’s).

In conclusion, although not many ECM taxa were detected by molecular analysis, this chapter confirmed that ECM fungi were dominant and more diverse for seedling roots grown in soil collected from healthy sites than for seedlings grown in soil collected from declining sites. In Chapter 7, this trend will be further investigated by examining the development of AM and ECM fungi colonization on E. gomphocephala seedlings over time.
Chapter 7

Mycorrhizal colonization of young *Eucalyptus gomphocephala* seedlings in soil collected from a healthy and a declining site

7.1 Introduction

Previous studies on dual AM and ECM colonization on seedling eucalypts have revealed that some AM fungi can rapidly colonize seedlings, and over time new roots may develop associations with ECM fungi (Lapeyrie and Chilvers 1985; Chen et al. 2000a; Santos et al. 2001; Adams et al. 2006). The results from Chapter 5 showed that *E. gomphocephala* seedlings can also form associations with AM and ECM fungi. However, one of the limitations in Chapter 5 was that temporal colonization by mycorrhizal fungi was not examined because there was only one harvest after seedlings were grown for 7 months.

With regard to the canopy condition of *E. gomphocephala*, it was observed that AM fungi spore density differed between the study sites (Chapters 4 and 5), and was negatively correlated to the canopy health of *E. gomphocephala* (Chapter 5). Furthermore, the bioassay study (Chapter 5) revealed that ECM colonization was higher than AM colonization in seedlings grown in soils collected from healthy sites, and *vice versa* in soils collected from declining sites. Whether ECM inoculum potential is low in declining sites requires confirmation. In order to reassess this possibility and to gain information on the colonization pattern, as seedlings grow, the two extreme site conditions, the healthiest and the most declining sites, will be examined further. Thus the aim of this study was to examine the pattern of development of AM and ECM fungi at the healthiest and the most declining sites over time.
The hypotheses tested were:

H12. AM fungi colonize *E. gomphocephala* seedlings early in growth and are replaced by ECM fungi over time, and

H13. ECM colonization is dominant in seedlings grown in soil collected from the healthy site, and *vice versa* for AM colonization in seedlings grown in soil collected from the declining site.

7.2 Materials and methods

7.2.1 Experimental design

A glasshouse trial was undertaken to investigate the sequence of development of AM and ECM over time in *E. gomphocephala* seedlings grown in soil collected from the healthiest and the most declining sites identified in Chapter 4. There were four treatments in this trial as follows: 1) soil collected from a healthy site, 2) soil collected from a declining site, 3) control 1 (treated soil collected from the healthy site), and 4) control 2 (treated soil collected from the declining site) with 20 replicate pots per treatment. For the controls, soil was treated by wetting and autoclaving at 121°C for 30 minutes on two consecutive days. Four replicates of each treatment were harvested at each harvest, and there were four harvests in total during the trial.

7.2.2 Soil

The soils were collected at the end of summer (5 March 2011) from the healthiest site (site 11) and the most declining site (site 4) (Chapter 4). At each site, soils (approximately 20 cm depth) were collected approximately 5 m from the trunk of each of four randomly selected trees. The soil was placed into polyethylene plastic
bags and transferred to a 4°C cold room before use. The soils collected from four
trees at each site were bulked and mixed well before use.

Germination

Seeds of *E. gomphocephala* were collected from Yalgorup National Park, provided
by the State Centre of Excellence for Climate Change, Woodland and Forest Health
(Murdoch University) as for Chapter 5. Seeds were surface sterilized by dipping
them briefly (5 seconds) in 70% ethanol, soaked in a solution of 3% sodium
hypochlorite (5 minutes), and then washed three times in sterile distilled water
(Chapter 4). The sterilization of the seed was confirmed by incubating a sample of
some surface sterilized seeds in Petri-dishes containing 0.75% (w/v) water agar for 7
days at 25°C. The surface sterilized seeds were then germinated in trays (43 cm
length, 31 cm width, 12 cm height) containing yellow sand that had been autoclaved
at 121°C for 30 minutes on two consecutive days. The seeds were sown at
approximately 1 cm depth. The trays were covered with brown paper to reduce
contamination and incubated in an evaporatively cooled glasshouse. The brown
paper was removed 3 days after sowing when the seedlings had started to emerge.
The seedlings were maintained for 7 days and watered overhead daily by hand, until
transplanting.

7.2.3 Planting

Five hundred grams of soil were placed into each pot (9 cm height and 10 cm
diameter) lined with a polyethylene bag (12 cm height and 15 cm diameter). Two 8-
day-old seedlings of relatively uniform size were transferred into each pot and sown
around 2 cm depth. Holes were made into the base of polyethylene bags to allow for
drainage. In order to avoid any contamination (i.e. air-borne spores), sterile Perlite
(Aquaponic WA) that had been washed three times with distilled water was spread on the pot to cover the soil surface to a depth of approximately 1 cm.

**Maintenance**

Seedlings were maintained in an evaporatively cooled glasshouse with average daily temperatures ranging from 12.2°C minimum to 23.4°C maximum. Every two days the seedlings were watered overhead to container capacity (20 ml). A complete liquid fertilizer of low nutrient content (Australian Native Focus) at half the recommended dose (2.5 ml/L) was applied once to the seedlings (20 ml/pot) when the seedlings were 4-weeks-old, and then no more fertilizers added until harvest. The composition of the fertilizer used was: 500 µM N, 10.9 µM P, 214.8 µM K, 55 µM Ca, 19.5 µM Mg, 81.8 µM S, 0.63 µM Fe, 0.3 µM Mn, 0.6 µM B, 0.1 µM Zn, 0.05 µM Cu and 0.02 µM Mo. The fertilizer regime was based on that developed for mycorrhizal eucalypts in forest nurseries (Dell and Malajczuk 1995).

**Harvest**

Seedlings were harvested at 5, 7, 9 and 11 weeks after transplanting. Prior to harvest, seedling height was measured. At harvest, the shoots were cut at the soil line, and fresh weights taken. Roots were gently washed from the soil over a 2.8 mm screen with tap water, immersed for approximately 5 minutes in a bucket of water and agitated gently to remove most of the organic matter and soil particles from the roots. Detail to clean the roots and separate the root tips from the whole root system was explained in Chapter 3. Some fresh ECM root tips were collected for photographic records. For mycorrhizal assessment, approximately 50% of the roots (the coarse and fine roots) were sub-sampled by vertically partitioning the roots from two sectors of the root ball, and then mixed with the collected root tips.
At the final harvest, due to the dense root system, approximately 25% of roots were subjected to intensive cleaning before being sub-sampled for mycorrhizal assessment. First, after cleaning the whole root system with tap water and blotting dry with clean paper towel, root fresh weight was measured. Then, 25% of the root system was sub-sampled by vertically cutting a root sector, this sector was then cleaned under a microscope to remove dead roots and soil particles, and fresh weight was recorded.

At the first harvest, some roots were plated onto NARPH, a selective medium for *Phytophthora* as described in Chapter 5. At the last harvest, soil samples from the healthy and declining sites were collected when washing the roots and used for baiting for *Phytophthora* (Hüberli et al. 2000).

7.2.4 AM/ECM assessment

Mycorrhizal root assessment of *E. gomphocephala* was based on the protocols described by Brundrett et al. (1996) as described in Chapter 5.

7.2.5 Phytophthora assessment

*Plating for Phytophthora*

Plating for *Phytophthora* was undertaken at the first harvest. For plating, random root fragments were taken from the roots remaining after sub-sampling for mycorrhizal assessment. These roots were blotted dry with paper towel, cut into pieces ca. 0.5 cm in length. The root fragments were plated and observed for mycelium growth characteristic of *Phytophthora* as described previously (Chapter 5).
Baiting for Phytophthora

Soils used for baiting *Phytophthora* were collected at the last harvest prior to washing the roots. Two soil samples each from declining and healthy sites were used for baiting. The soils were placed into transparent takeaway containers to a depth of approximately one third of the container, and filled with deionised water. Fresh petals of *Hibbertia* and leaves of *Eucalyptus sieberi* and *Scholtzia involucrata* were floated on the surface as baits (Aghighi et al. 2012; Crone 2012). The baits were observed daily for one week. Since there was no sign of infection on the baits (brown or water-soaked lesions), water in the container was discarded, the soil was air dried for one day and then baiting was repeated. Those baits that appeared to be infected were plated on NARPH selective medium and observed for the presence of *Phytophthora*.

7.2.5 Data analysis

The effects of site and harvest for mycorrhizal development were investigated using a two-way MANOVA with factors of site (declining and healthy) and harvest (first, second, third and fourth harvest) and dependent variables of height, root fresh weight and shoot fresh weight. This was followed by Univariate analysis of each dependent variable. A Tukey post-hoc test was conducted to evaluate differences between the treatments. The control treatments were not included in the analysis as the main aim of the control was to check for contamination in the glasshouse. Data conformed to all assumptions of the analysis, so no transformations were applied. These analyses were conducted using STATISTICA 8 (Stat Soft. Inc. USA).
7.3 Results

Multivariate analysis revealed significant interaction between site and harvest \( (F_{(5, 55)} = 2.98, P = 0.001) \) on seedling height, shoot fresh weight, root fresh weight, and AM and ECM colonization. Univariate analysis was then conducted for each dependent variable.

7.3.1. Effect of site and harvest on seedling growth

Univariate analysis showed that seedling height significantly increased with time of harvest \( (F_{(3, 24)} = 2.98, P = 0.001) \), but site and the interaction between site and harvest was not significant for seedling height \( F_{(3, 24)} = 0.61, P = 0.44; F_{(3, 24)} = 2.0, P = 0.14, \) respectively) (Fig. 7.1a).

Single factor of harvest and interaction between site and harvest were significant for shoot fresh weight \( F_{(3, 24)} = 29.73, P = 0.0001; F_{(3, 24)} = 3.97, P = 0.02, \) respectively), but site was not significant. Shoot fresh weight increased with time of harvest, with the highest shoot biomass at the final harvest (11 weeks). The shoot fresh weight was also influenced by the interaction between site and harvest. The seedlings grown in soil collected from the healthy site had lower shoot fresh weight than seedlings in soil from the declining site at the first harvest. However, shoot fresh weight increased with time of harvest, and at the final harvest, the seedlings grown in soil collected from the healthy site had slightly greater shoot fresh weight than seedlings grown in soil collected from the declining site (Fig. 7.1b).
Fig. 7.1. Mean (n = 4) height (a) and shoot fresh weight (b) over time of seedlings grown in soil collected from healthy and declining sites. Small letters denote the results of the *post hoc* test (Tukey HSD) and values with the same letters are not significantly (P ≤ 0.05) different.

Root fresh weight was significant for time of harvest (F (3, 24) = 44.33, P = 0.0001) and site (F (3, 24) = 6.97, P = 0.01), but the interaction was not significant (F (3, 24) = 0.75, P = 0.53). Root fresh weight increased with time of harvest. The root fresh weight was also influenced by the site factor effect. The seedlings grown in soil collected from the declining site had higher root fresh weight than seedlings grown in soil collected from the healthy site (Fig.7.2).

Fig. 7.2. Mean (n = 4) root fresh weight over time of seedling roots grown in soil collected from healthy and declining sites. Small letters denote the results of the *post hoc* test (Tukey HSD) and values with the same letters are not significantly (P ≤ 0.05) different.
7.3.2 Effect of site and harvest on mycorrhizal colonization

Arbuscular mycorrhizal colonization was significant for harvest and site ($F_{(3, 24)} = 5.78, P = 0.004$; $F_{(1, 24)} = 135.41, P < 0.00001$, respectively), and the interaction was also significant ($F_{(3, 24)} = 8.86, P = 0.0001$). The AM colonization increased with time of harvest, particularly in seedling roots grown in soil collected from the declining site. The AM colonization in seedling roots grown in soil collected from the declining site was significantly higher than in seedling roots grown in soil collected from the healthy site. This condition was observed from the first to the last harvest. At the last harvest, the proportion of seedlings root colonized by AM fungi were 19.2%, compared to 2.7% in those seedlings grown in soil collected from the healthy site. In contrast to the declining site where AM colonization increased steadily over time, the AM colonization at the healthy site decreased over time (Fig. 7.3).

![Fig. 7.3. Mean (n = 4) proportion of arbuscular mycorrhizal (AM) colonization over time in seedling roots grown in soil collected from healthy and declining sites. Small letters denote the results of the post hoc test (Tukey HSD) and values with the same letters are not significantly (P ≤ 0.05) different.](image)

Univariate analysis revealed that ECM colonization was significant for harvest and site ($F_{(3, 24)} = 11.05, P = 0.00009$; $F_{(1, 24)} = 92.66, P < 0.00001$, respectively), but the
interaction was not significant ($F_{(3, 24)} = 1.23$, $P = 0.32$). The ECM colonization increased with time, especially in seedling roots grown in soil collected from the healthy site. The dominance of ECM in soils collected from the healthy site was observed from the first to the last harvest. At the first harvest, the roots in soil collected from the healthy site were 24% colonized by ECM fungi and increased to 38.5% at the final harvest. At the declining site, roots were 2.9% colonized by ECM fungi at the first harvest, and increased to 16.9% at the final harvest (Fig. 7.4).

Some specific ECM morphotypes observed between the healthy and declining sites are illustrated in Fig. 7.5.

![Graph](image_url)

Fig. 7.4. Mean ($n = 4$) proportion of ectomycorrhizal (ECM) root tips over time in seedling roots grown in soil collected from healthy and declining sites. Small letters denote the results of the post hoc test (Tukey HSD) and values with the same letters are not significantly ($P \leq 0.05$) different.
Fig. 7.5. The main ectomycorrhizal (ECM) morphotypes formed on seedlings of *E. gomphocephala* grown in soils collected from the healthy site; (a) smooth brown, and (b) smooth white; and from the declining site (c) brown, and (d) dark brown.

### 7.3.3 Colonization by contaminant fungi in control treatments

Ectomycorrhizal contamination increased during this study in both control treatments. No ECM contamination was observed at the first harvest in control treatment 1 (control healthy site), and very little contamination (0.8%) occurred in the control treatment 2 (control declining site). The contamination in control treatment 1 increased from 4% at week 7 to 12.8% and 16.3% at weeks 9 and 11, respectively. In control treatment 2 the contamination was 2.5% at week 7, and it increased to 9.6% and 10.7% at weeks 9 and 11, respectively. In contrast to ECM, the AM contamination in both control treatments was low. The trend of AM and ECM contamination in the control treatment compared to the untreated field soils are shown in Figs. 7.7a and 7.7b.
Fig 7.7. Trend of ectomycorrhizal (ECM) colonization (a) and arbuscular mycorrhizal (AM) colonization (b) on roots of seedlings grown in soil from the healthy and the declining sites compared to the corresponding control treatments. Values are mean of four replicates.

Two contaminant ECM fungi (Fig. 7.5), yellow and grey morphotypes, were observed in this study. These two morphotypes occurred in both autoclaved soils.

Fig. 7.5. The main contaminants colonizing seedlings of *E. gomphocephala* grown in the control soils; (a) yellow, and (b) grey ectomycorrhizas.
7.4 Discussion

7.4.1 Site and mycorrhizal colonization

ECM colonization was high in seedling roots grown in soil collected from the healthy site, and AM colonization was dominant in seedling roots grown in soil collected from the declining site. These results for early colonization in young seedlings supported the key finding in the bioassay trial (Chapter 5), and also the trends observed for the molecular results of mycorrhizal fungi associated with roots of 7-month old seedlings (Chapter 6).

The dominance and early colonization of ECM fungi in seedlings at the healthy site was quite surprising. The seedlings were considered to be firstly colonized by AM fungi and then replaced by ECM fungi, and ECM fungi formation would take longer than 5 weeks to occur. Surprisingly, the time taken for ECM formation on *E. gomphocephala* seedlings grown in soil collected from the healthy site was quite fast compared with some other glasshouse studies with eucalypts (Table 7.1).

Chen et al. (2000a) evaluated the effect of ECM and AM alone and in competition on root colonization of two species of eucalypt seedlings, and found that *Laccaria* applied as spores was quite slow to establish two months after inoculation. Similarly, Lapeyrie and Chilvers (1985) reported that at 2 months, the ECM fungi colonization on *E. dumosa* seedlings was low and the seedlings were dominantly colonized by AM fungi. The ECM became dominant when the seedlings were 5 months old. On the other hand, *in vitro* trials on mycorrhizal formation showed that some ECM fungi can form mycorrhizas very quickly.
For instance, Malajczuk et al. (1990), using two different mycelial isolates of *P. tinctorius* to observe mycorrhizal development in *E. urophylla* found that the eucalypt isolate began to form mycorrhizas at 2 days after incubation, and the pine isolate developed ectomycorrhizas at 7 days after incubation. Similarly, Burgess et al. (1994) reported early ECM formation in *E. grandis in vitro*. Using mycelium to synthesize ECM, the authors examined the ability of various isolates of *Pisolithus* sp. to form mycorrhizas, and found that ECM were fully developed at 9 days after

<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Inoculum</th>
<th>Time</th>
<th>Location</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eucalyptus st. johnii</em> seedlings inoculated with natural soil</td>
<td>soil</td>
<td>ECM colonization increased after 8 weeks</td>
<td>Glasshouse pots</td>
<td>(Chilvers and Gust 1982)</td>
</tr>
<tr>
<td><em>Eucalyptus dumosa</em> seedlings inoculated with natural soil</td>
<td>soil</td>
<td>AM present at 2 months, ECM abundant at 5 months</td>
<td>Glasshouse pots</td>
<td>(Lapeyrie and Chilvers 1985)</td>
</tr>
<tr>
<td><em>Eucalyptus urophylla</em> placed on <em>Pisolithus tinctorius</em> mycelium</td>
<td>mycelium</td>
<td>Eucalypt isolate formed ECM at 2 days after contact, Pine isolate at 7 days</td>
<td><em>In vitro</em></td>
<td>(Malajczuk et al. 1990)</td>
</tr>
<tr>
<td><em>Eucalyptus grandis</em> placed on <em>Pisolithus</em> mycelium</td>
<td>mycelium</td>
<td>ECM formed within 9 days of contact</td>
<td><em>In vitro</em></td>
<td>(Burgess et al. 1994)</td>
</tr>
<tr>
<td><em>Eucalyptus globulus</em> seedlings inoculated with various ECM fungi</td>
<td>spore</td>
<td>ECM present 65 days after inoculation</td>
<td>Glasshouse pots</td>
<td>(Lu et al. 1998)</td>
</tr>
<tr>
<td><em>Eucalyptus globulus</em> and <em>E. urophylla</em> inoculated with AM fungi (<em>Glomus</em>, <em>Acaulospora</em>, and <em>Scutellospora</em>) and ECM fungi (<em>Laccaria lateritia</em>) alone and in combination</td>
<td>spore</td>
<td>ECM colonization increased after 2-3 months</td>
<td>Glasshouse pots</td>
<td>(Chen et al. 2000a)</td>
</tr>
<tr>
<td><em>Eucalyptus globulus</em>, <em>E. urophylla</em>, <em>Pinus elliottii</em> and <em>P. radiata</em> inoculated with <em>Scleroderma</em> spore</td>
<td>ECM present at 12 weeks after inoculation</td>
<td>Nursery containers</td>
<td>(Chen et al. 2006b)</td>
<td></td>
</tr>
</tbody>
</table>
contact between the mycelium and the root tips. The isolates varied in their rate and extent to form mycorrhizas, and the more aggressive isolates were able to more rapidly colonize the seedlings than the less aggressive isolates.

Comparing the findings of these previous studies, it seems that the ECM developed much faster from mycelium as inoculum sources than from spores. Additionally, the time taken for ECM fungi to colonize could be different between the ECM species or isolate. It is evident that in the healthy site, there were forms of ECM inocula which were able to rapidly colonize the roots, whilst in the declining site this appeared not to be the case as early colonization by ECM fungi was quite low. Whether the differences were due to greater abundance of inoculum in the healthy site than in the declining site or to differences in the composition of the ECM species between the sites is unknown. Recently, Scott et al. (2013a) reported that ECM extramatrical networks were less abundant in declining E. gomphocephala sites compared to healthy sites, but no attempt was made to identify the fungal species contributing to the mycelia mats.

The high AM fungi colonization in seedlings grown in soil collected from the declining site may result from high inoculum potential in the soil. Observation on AM spore density and diversity in the soil suggested that AM fungi were more abundant in soil collected from the declining site (Chapter 5). Additionally, molecular characterization of AM fungi associated with roots of 7-month old seedlings also showed that there were more AM fungi colonizing roots grown in soil collected from the declining site (Chapter 6).

Time was not available to investigate changes in fungal colonization over time between weeks 5 and 11 using molecular analysis. However, observation of ECM morphotypes suggested that the diversity of ECM colonizing the seedlings grown in
soil collected from the declining site was low and there were no obvious changes in the morphotypes over time (data not presented).

An unexpected feature of this study was the fact that there was an increase in the level of contamination in the two control treatments, particularly at weeks 9 and 11. The level of contamination in the treated control 1 (soil collected from the healthy site) increased from 12.8% to 16.3% at weeks 9 and 11, respectively, and in the treated control soil 2 (soil collected from the declining site), the contamination increased from 9.6% to 10.7% at weeks 9 and 11, respectively. The colonization of seedling roots by contaminating fungi occurs in many nurseries. For example, control Quercus rubra (red oak) nursery seedlings grown in containers filled with soil that had been fumigated with methyl bromide were entirely colonized by Thelephora terrestris (Le Tacon et al. 1997). Brundrett et al. (2005) reported contaminant fungi formed ECM in the non-inoculated control in a eucalypt nursery where the containerised seedlings were inoculated with spores and mycelial inocula of various ECM fungi. In an E. urophylla nursery inoculated with spores of Scleroderma, Chen et al. (2006a) reported on ECM colonization by contaminating fungi. The contamination was less than 10% in inoculated seedlings, but the contaminating species colonized a relatively larger proportion of short roots in seedlings of un-inoculated controls in non-sterile soil. The contamination also occurred in the autoclaved control soil.

In the current study, the contaminants probably arrived in the form of air-borne spores. Ectomycorrhizal colonization by contaminating fungi has been reported previously in glasshouse studies at Murdoch University (Dell, Personal communication).
Phytophthora multivora was not recovered when the roots of seedlings were plated and the soils were baited.

7.4.2 Seedling performance

In general, seedlings grown in the soil collected from the healthy site and from the declining site had better shoot and root growth compared to the control treatment. Further, the seedlings grown in soil collected from the healthy site seemed to have better shoot growth than the seedlings grown in soil collected from the declining site. This could be explained by the role of mycorrhizal fungi to increase nutrient uptake of the host plant. There have been innumerable reports of growth enhancement of seedlings as a result of mycorrhizal colonization (Lu et al. 1998; Chen et al. 2000b; Chen et al. 2006b; Plassard and Dell 2010). However, other factors such as soil fertility and soil microbial diversity cannot be excluded.

The seedlings grown in soil collected from the declining site seemed to have greater biomass below ground than the seedlings grown in soil collected from the healthy site. In mycorrhizal plants, root growth might be related to carbohydrate allocation. A substantial proportion of carbohydrate is allocated to the roots for fungal growth, maintenance and nutrient uptake (Smith and Read 2008). At the declining site, the seedlings were colonized by AM fungi (19%) with approximately 17% colonized by ECM fungi. It is possible that the carbohydrate demand by the fungi was high and larger quantities of carbohydrate were allocated to the roots than to the shoot. Alternatively, root development of the seedlings dominantly colonized by AM fungi might be different from their root development when dominantly colonized by ECM fungi. With regard to crop species, it has been reported that growth rate of root apices was slowed down after colonization by a Glomus species, but this was followed by an increase in initiation of lateral roots. It was hypothesized that the
production of new lateral roots was stimulated by the loss in growth rate of root apices after AM colonization (Barker et al. 1998). In contrast to AM colonization, root morphology of seedlings grown in soil collected from the healthy site might be less dense due to a reduction in lateral root growth due to ECM colonization.

Although mycorrhizal colonization might affect root growth, it is difficult to determine the direct effect from mycorrhizas. This is because root growth and development depend on a number of factors including plant species, age of plants and the interaction between the soil environment and the shoot (Marschner 1995). Furthermore, it has been suggested that although mycorrhizal fungi may affect plant nutrient status, they may not directly affect the distribution of dry matter between roots and shoots (Bougher et al. 1990).

To sum up, the results of this study clearly showed that the ECM colonization was rapid and high in seedling roots grown in soil collected from the healthy site, and was low in seedling roots grown in soil collected from the declining site, suggesting a possibility of low inoculum potential in the declining site. Whether differences in the level of ECM colonization and time for ECM formation between the healthy and the declining sites will have a consequence for seedling recruitment and stand health is not known, and this will be discussed further in the next Chapter.
Chapter 8

General Discussion

This is the first study to explore the relationship between mycorrhizal fungi and *E. gomphocephala* decline in Western Australia. Although this research was focused on *E. gomphocephala*, these findings will help managers and scientists to understand aspects of declines in other eucalypt/tree species in Western Australia, in other parts of Australia or elsewhere. For example, many native trees in WA such as *E. wandoo*, *E. rudis* and *Corymbia calophylla* are in decline, but the cause(s) have not yet been thoroughly studied. Whether the decrease in ECM observed in this study also occurs in the other tree decline syndromes has yet to be investigated. However, a number of studies in other parts of the world have reported some relationship between ECM fungi and tree decline (Causin et al. 1996; Montecchio et al. 2004; Mosca et al. 2007), and there is one finding for south-eastern Australia (Horton et al. 2013).

8.1. Hypotheses and findings

The research was based on a number of hypotheses. The hypotheses proposed in the study and the findings that relate to these hypotheses are summarized in Table 8.1.

Table 8.1. Hypotheses and results obtained in the study

<table>
<thead>
<tr>
<th>No.</th>
<th>Hypothesis</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td><em>Phytophthora multivora</em> is antagonistic to ECM in vitro and on seedlings</td>
<td>Growth of ECM fungi in vitro was not or was slightly reduced, and ECM colonization on seedlings in vivo was not affected in the presence of <em>P. multivora</em></td>
</tr>
<tr>
<td>H2</td>
<td>ECM inoculation provides protection to <em>E. gomphocephala</em> seedlings from <em>P. multivora</em></td>
<td>Although some roots were necrotic due to <em>P. multivora</em> infestation, ECM colonization was high and <em>E. gomphocephala</em> seedlings grew well in the presence of <em>P. multivora</em></td>
</tr>
<tr>
<td>H3</td>
<td>ECM fungi differ in their ability to promote growth of <em>E. gomphocephala</em> seedlings</td>
<td><em>Pisolithus albus</em> promoted growth of <em>E. gomphocephala</em> seedlings more than <em>Scleroderma areolatum</em></td>
</tr>
</tbody>
</table>
The presence of AM fungal spores is not related to the health status of *E. gomphocephala*.

AM fungal spore density and species richness were not related to the health status of *E. gomphocephala*.

Soil chemical properties influence the health of *E. gomphocephala*.

Nine soil chemical properties were significantly related to *E. gomphocephala* crown health.

Soil chemical properties influence AM fungi spore density and species richness.

One soil chemical property was significantly related to AM fungal spore density and another to species richness.

The type of mycorrhiza (ECM/AM) formed by seedlings is related to crown health of *E. gomphocephala*.

The proportion of ECM root tips was positively related to crown health whilst the proportion of root colonized by AM fungi was negatively related to crown health.

*Eucalyptus gomphocephala* preferentially forms ECM but roots can also form AM.

ECM dominated seedling roots formed in soil taken from under healthy canopies, whereas AM dominated seedling roots formed in soil taken from declining sites.

Soil chemical characteristics are related to mycorrhizal colonization.

Soil chemical properties were significantly related to AM (five properties) and ECM (four properties) colonization.

The mycorrhizal fungal communities associated with seedlings is related to crown health of *E. gomphocephala*.

DNA analysis of roots showed that ECM fungal communities were poorly related to crown health of *E. gomphocephala*, but AM fungal communities were negatively related to crown health of *E. gomphocephala*.

ECM fungal communities are dominant in seedling roots grown in soil collected from healthy sites, and vice versa for AM fungal communities at declining sites.

DNA analysis of roots indicated that ECM fungal communities were dominant and more diverse for seedling roots grown in soil collected from healthy sites whilst AM fungal communities dominated seedling roots grown in soil collected from declining sites.

AM fungi colonize *E. gomphocephala* seedlings early in growth and are replaced by ECM fungi over time.

ECM fungi colonized *E. gomphocephala* seedling roots grown in soil collected from healthy site and were not replaced by AM fungi over time.

ECM colonization is dominant in seedlings grown in soil collected from the healthy site, and vice versa for AM colonization in seedlings grown in soil collected from the declining site.

ECM fungi colonization was dominant in seedlings grown in soil collected from a healthy site, whilst AM colonization was dominant in seedlings grown in soil collected from a declining site.

The most important finding of the study was the relationship between the type of mycorrhiza (arbuscular or ectomycorrhizal) formed in containerised seedlings and the crown condition of *E. gomphocephala* at the sites where the soil cores were...
taken. Ectomycorrhizas were relatively abundant in seedlings grown in soils taken from under healthy crowns, and positively related to the crown condition of *E. gomphocephala* in the field. By contrast, arbuscular mycorrhizas were abundant in seedlings grown in soils taken from under declining crowns, and this was negatively related to the crown condition of *E. gomphocephala*. This finding was consistent throughout the study, and confirmed in a second glasshouse trial using soil collected from one healthy and one declining site. Furthermore, molecular analysis used to evaluate fungal communities associated with seedling roots from the bioassay trial showed similar trends with ECM fungi being dominant and diverse for seedling roots grown in soil collected from healthy sites, whilst the AM fungi dominated the seedling roots grown in soil collected from declining sites.

It is worth noting that the pattern of mycorrhizal colonization in the moderately healthy sites was not always intermediate between the healthy and the declining sites. A consistent result of the dominance of ECM in soil taken from healthy sites and the dominance of AM in soil taken from declining sites may indicate that the ecosystems in these two contrasting sites were more stable than the ecosystems in the moderately healthy sites. On the other hand, the ecosystems in the moderately healthy category were possibly still in a transition process, and thus more dynamic than the ecosystems at the healthy and declining sites. Longer term studies are required to ascertain whether the intermediate sites are undergoing continued decline or are in some stage of recovery.

**8.2 Eucalyptus gomphocephala decline**

Decline is a progressive deterioration of vigour often leading to tree death. When considering the cause of premature decline, Manion (1991) proposed a decline spiral model, grouping factors as predisposing, inciting and contributing (Chapter 2).
Long-term predisposing factors influence trees to short-term inciting factors that weaken trees, and allow contributing factors in combination to kill trees. The cause(s) of decline might be different between natural forests due to differences in natural dynamics and the scale of disturbance between forest types (Bengtsson et al. 2000).

A conceptual model of *E. gomphocephala* decline modified from Manion’s spiral decline is proposed (Fig. 8.1). This model is a synthesis of findings of the study; however, other factors that have been previously reported to be involved in *E. gomphocephala* decline were also taken into account in the model.

![Diagram of factors related to *E. gomphocephala* decline](image_url)

**Fig. 8.1.** Factors related to *E. gomphocephala* decline modified from Manion’s concept of decline (Manion 1991).
8.2.1 Soil abiotic factors

Some soil abiotic factors might be involved in the tree decline syndrome, and a number of soil chemical properties analysed were significantly related to *E. gomphocephala* decline. The study found that there was a positive relationship between tree health and soil pH (CaCl$_2$). *Eucalyptus gomphocephala* is largely confined to calcareous soil profiles, so it is possible that a decrease in soil pH may be a factor predisposing trees to decline.

Furthermore, an imbalance in mineral nutrients may also play a role in the health of *E. gomphocephala*, predisposing trees to decline. The study found that crown health of *E. gomphocephala* was positively related to the micronutrients Mn, Cu and Zn. Nutritional disorders such as deficiency symptoms of Mn, Fe, Zn, and B, and P toxicities have been previously observed on *E. gomphocephala* foliage (Eslick 2005; Dell et al. 2006a). As Mn, Fe, Cu and Zn usually have low availability in calcareous soils (Bell and Dell 2008), it is likely that a limiting supply of these micronutrients could predispose *E. gomphocephala* to decline. Indeed, the application of Zn fertilizer improved *E. gomphocephala* crown health (Scott et al. 2013b).

There are numerous reports on altered soil chemistry associated with forest decline. For instance, in the northern hemisphere, acid rain resulting in loss of base cations from the soil has been implicated in decline (St. Clair et al. 2005; Kogelmann and Sharpe 2006). In eastern Australia, increasing pH and reduced Fe availability have been implicated in a widespread tree disorder known as Mundulla Yellows (Czerniakowski et al. 2006; Parsons and Uren 2007), and changes in soil chemistry have been related to *E. delegatensis* decline (Horton et al. 2013). Furthermore, decline of *E. marginata* and *C. calophylla* in urban parkland in Western Australia...
has been associated with Mn deficiency due to irrigation with high pH bore water (Grigg et al. 2009).

The study found that soil chemical properties were related to both crown condition of *E. gomphocephala* and mycorrhizal colonization. Whether the decline of *E. gomphocephala* is directly affected by change in soil chemistry or the decline is related to mycorrhizal fungi mediated by change in soil chemistry is not known. However, it is evident that altered soil chemistry can result in stress. The stress could occur to both the fungus or the plant, with the consequence of the stress being altered plant C supply to the roots, altered fungal absorption of nutrients from soil, or altered exchange capacity between the Hartig net and host cells (Brunner 2001).

### 8.2.2 Climate and landscape change

Changed climate has been implicated worldwide in forest decline phenomena (Moore and Allard 2008; Allen 2009; Allen et al. 2010). Gradual change in climate has been implicated in the decline in health of several dominant eucalypt species endemic to SWWA including *E. gomphocephala* (Hooper and Sivasithamparam 2005; Cai et al. 2010). Extreme weather events including drought, heat waves and frost in SWWA may trigger eucalypt decline (Matusick et al. 2012; Brouwers et al. 2013a; Evans et al. 2013; Matusick et al. 2013). Clearing of native vegetation resulting in highly fragmented landscapes may also predispose eucalypts to decline in SWWA (Brouwers et al. 2013b) including *E. gomphocephala* (Edwards 2004). Changed climate, on the other hand, potentially could also alter mycorrhizal community dynamics (Swaty et al. 2004; Bellgard and Williams 2011), which may exacerbate stress; thereby predisposing trees to further decline.
8.2.3 Fire regime

Fire regimes can have important effects on the forest ecosystem, influencing vegetation dynamics, diversity pattern and ecosystem process (Bengtsson et al. 2000). Change in fire regime has been implicated in *E. gomphocephala* decline resulting in altered water and nutrient relationships (Close et al. 2009; Close et al. 2011), as have changes in vegetation structure (Archibald et al. 2010), and reduced seedling recruitment (Ruthrof et al. 2002).

8.2.4 Groundwater chemistry change

Increasing groundwater alkalinity (both pH and CaCO$_3$ content) and salinity were found to be correlated with increasing *E. gomphocephala* canopy decline in the Yalgorup region (Edwards 2004). These changes across the range of *E. gomphocephala* were localised to Yalgorup suggesting they may play a role in tree decline. Recently, salinity increase in Lake Clifton within Yalgorup National Park and surrounding groundwater has been proposed to be contributing to tree decline (Warden et al. 2009).

8.2.5 Diseases and pests

Diseases and pests have been considered to be involved in tree decline, acting either as primary or secondary agents. Oomycete pathogens such as *Phytophthora* spp. have been shown to be the cause of devastating diseases in *Quercus* and *Fagus* in Europe and USA (Jung et al. 2005; Grunwald et al. 2012), and of many native plants in Australia (Cahill et al. 2008; Scott et al. 2011; Barber et al. 2013). Some fungal pathogens such as *Botryosphaeria* spp., *Mycosphaerella* spp. and *Teratosphaeria* spp. were detected by the molecular analysis of *E. gomphocephala* seedling roots grown in soil collected from under healthy and declining *E. gomphocephala*. For
example, the DNA of *Mycosphaerella cryptica*, an aggressive foliar pathogen, that has been reported to cause damage to regenerating stands of *E. gomphocephala* in YNP (THRG 2006), was found in the this study. Members from the family Botryosphaeriaceae have been isolated from diseased stems or branches of *E. gomphocephala* and other native understorey plants such as *Banksia grandis*, *Agonis flexuosa*, and *Allocasuarina fraseriana* in *E. gomphocephala* woodland (Taylor et al. 2009; Dakin et al. 2010). *Teratosphaeria nubilosa* has been considered as one of the virulent *Teratosphaeria* spp. causing disease on *Eucalyptus* (Hunter et al. 2009). Some of these organisms may be primary pathogens, and others may occur as opportunistic fungi when the trees are healthy, behaving as pathogens when trees are suffering from stress.

Wood borer infestation has been observed in *E. gomphocephala* at Yalgorup. Although not considered to be the primary cause of the decline, the borer may be involved in the decline when the trees are already under stress from other factors (Longman and Keighery 2002).

**8.2.6 Beneficial fungi**

The ECM fungi were found to be positively related to the crown health of *E. gomphocephala*. A previous study reported that there was a decline in soil microbial function (Cai et al. 2010) in declining *E. gomphocephala* sites, and ECM mat density was less abundant in declining sites (Scott et al. 2013a). Going back to Manion’s concept of decline (Chapter 2), the grouping of beneficial soil microbia including ECM fungi and soil bacteria, into predisposing or inciting factors is currently problematic. They might be included with inciting factors, however, they may have relatively long-term effects on tree health, so they could also be considered as a predisposing factor. Old (2000) suggested that although Manion’s concepts are
useful in attempting to diagnose and manage diseases of complex aetiology, separation between predisposing and inciting factors sometimes is difficult. For instance, chronic annual defoliation by insect herbivores on some species of eucalypt and short-term drought may be grouped into inciting factors. However, due to their long-term effect, they may also be assumed as predisposing factors. Castello et al. (1995) suggested that the precise set of interacting factors depends on the specific decline scenario. For example, pathogens may act as either predisposing, inciting, or contributing factors within a complex interaction that leads to mortality.

The impact of tree decline above ground can be easily recognised, however, very little is known about how a decline syndrome is expressed below ground in the tree’s root system. Approximately 25 to 50% of photosynthates produced daily are allocated below ground, depending upon the plant species and its developmental stage (Marschner 1995), and it is estimated that 30% of net primary production might be used by ECM fungal symbionts (Hobbie and Hobbie 2006). Although it is not known how changes in carbon partitioning above and below ground occur following tree decline, it is obvious that damage to a tree and its loss of vitality could result in a decrease of carbon allocation below ground and hence adversely impact on its root system. This will also impact on the associated microorganisms both in the root, the rhizosphere and in the bulk soil. For instance, Cai et al. (2010) showed a decline in soil microbial function following *E. gomphocephala* decline. In this study, whether the type of mycorrhizal fungi formed in the seedling is directly related to a decrease in carbon allocation below ground is not known. However, the low colonization and diversity of ECM fungi observed in the seedlings grown in soil collected from the declining sites indicated that the ECM were more impacted than AM in these sites. As the C demand for ECM is assumed to be much higher than
AM, it is possible that the ECM would be rapidly impacted when there is a decrease in the carbon supply below ground. Carbon is a key factor for the formation and maintenance of ECM, thus a reduction in C allocation to roots may cause C deficiency in ECM leading to decreased ectomycorrhization, reduced external mycelia and lower production of fruiting bodies (Brunner 2001).

South-west WA is recognised globally as an ecological ‘hotspot’ for unique plant and animal species. *Eucalyptus gomphocephala* woodland, in particular is an important habitat of flora and fauna, with many species endemic to the SWWA. Loss of *E. gomphocephala* woodland could have a serious impact both on ecosystem function and biodiversity. It has been reported that ECM sporocarps were less abundant under declining stands (Legault 2005), and there was a decrease of certain bacterial functional groups (Cai et al. 2010), and change in the abundance of particular faunal groups (Wentzel 2010) following *E. gomphocephala* decline. Australia has a very diverse ECM fungal population either epigeous or hypogeous, and many of them might be endemic (Bougher 1995). Although little information is available about the diversity of ECM fungi in *E. gomphocephala* woodland, it is possible that these ECM fungi could be negatively impacted when trees are declining. Biodiversity is related to ecosystem function, and loss of biodiversity could negatively impact on ecosystem function and forest health. Species diversity may have direct positive effects on ecosystem process such as productivity and nutrient cycling, or may be involved in ecosystem stability such as resistance to perturbation, resilience after disturbance, or more stable ecosystem function overtime. Thus, a more diverse system may be more resistant to perturbation and more resilient than a species-poor system (Bengtsson et al. 2000).
8.3 Future research

Whilst this thesis has discovered significant new findings about the relationship between mycorrhizal fungi and *E. gomphocephala* decline in WA, there are many questions that remain open and much research is required. The following issues are considered to be particularly important for future research in order to obtain a better understanding of the role of mycorrhiza on the health of *E. gomphocephala*, which in turn could provide information for managing declining eucalypts.

- The relationship between the type of mycorrhiza and crown condition of *E. gomphocephala* investigated in the glasshouse should now be confirmed in the field. To evaluate the type of mycorrhizal colonization formed in seedlings, *E. gomphocephala* could be grown in the field in the healthy and declining sites as trap plants. Additionally, the mycorrhizal status of adult trees in the field should be assessed (Horton et al. 2013). If the results from the glasshouse are confirmed in the field, restoration to put *E. gomphocephala* back in the field should include the introduction of the relevant ECM fungi. Future research should involve identifying and assessing the major species involved in the symbiotic relationship. The use of exotic inoculum should be avoided and only inoculum from fungi associated with healthy *E. gomphocephala* growing in areas adjacent to the sites should be used. In any restoration trials of *E. gomphocephala*, seedling recruitment should include mycorrhizal assessment of young plants over time.

- The molecular analysis of mycorrhizal diversity examined in glasshouse seedlings should be extended to the field. It seems most likely that there are more ECM fungi in the field than identified on the seedlings in the glasshouse. The role of other ecologically important fungi particularly
saprophytes, which were more abundant in seedling roots grown in soil collected from healthy sites (Chapter 6), needs to be confirmed in the field. The fungal diversity on *E. gomphocephala* roots and in the rhizosphere from healthy and the declining sites needs to be investigated. As sampling could be an issue in the field, consideration needs to be given to developing robust sampling strategies such as direction, rhizosphere depth and distance from the root collar (Montecchio and Scatollin 2009). For instance, Horton et al. (2013) observed ECM communities by collecting 10 soil cores at each site at 10 cm deep within the canopy drip zone (3 m), and Blom et al. (2009) collected 10 soil cores at each site, 2 m from the trunk base at 15 cm depth. In another study, soil cores were collected at two directions (north and south), with 6 cores for each direction per plant at 30 cm depth with the distance from the stem base being 100, 150 and 200 cm (Alzetta et al. 2012).

- The low ECM colonization and diversity of ECM in seedling roots produced in soil taken from under declining canopies may have indicated that there were reduced ECM fungi inocula in soils at declining sites. Therefore, there is considerable interest to explore factors that may influence beneficial fungi inocula loads in the field. Very little is known about the abundance of sporocarps above and below ground, spore dispersal, spore germination and their survival in the field. Unlike a previous study which reported a decrease of ECM extramatrical networks at a declining site based on a qualitative approach (Scott et al. 2013a), the density of ECM fungal mats across healthy, moderately healthy and declining sites needs to be quantified. Other factors that might be related to abundance and distribution of ECM fungal communities include litter, such as litter cover and litter depth and the
understorey/vegetation structure including species dominance and composition which also need to be examined. Mycophagous animals, which consume fruiting bodies of higher fungi, mostly hypogeous (underground-fruited) fungi, might play an important role in ECM fungi spore dispersal (Vernes and Dunn 2009). It is not known whether there has been a loss of ECM fungi diversity due to less digging and spore dispersal through mycophagy.

- The role of pathogens in the *E. gomphocephala* decline syndrome needs to be further investigated in the field. Unlike a previous study that reported *P. multivora* as a pathogen of *E. gomphocephala* (Scott et al. 2011), the study found the pathogen had little impact on *E. gomphocephala* seedlings in the glasshouse trials. The pathogenicity of oomycetes should be extended on seedlings, saplings and mature trees in the field. Indeed, work by Scott (2013b) showed that declining trees recovered following treatment with phosphite, suggesting the possible involvement of an oomycete pathogen in *E. gomphocephala* decline. Phosphite protection is known to be effective against oomycete such as *Phytophthora* spp. (Guest and Grant 1991). Furthermore, the interaction between ECM fungi and *P. multivora* investigated in the glasshouse should also be confirmed in the field. A pathogenicity trial combined with ECM inoculation on *E. gomphocephala* seedlings would be a preferable approach to examine this relationship.

- A lot of ECM sporocarps were observed in the *E. gomphocephala* woodland. However, little is known about the sensitivity of these fungi and their fructification response to disturbance. Therefore, it is desirable to identify which ECM fungi are sensitive to disturbance. The early loss of specific
ECM fungi when an ecosystem is disturbed could be an early warning indicator for decline. Such an approach could be studied on fungal sporocarps. Examples of such studies include long-term monitoring of macro fungal sporocarps in Europe (Straatsma et al. 2001), in the Pacific Northwest of the United States (Pilz and Molina 2002), and in Australia (Robinson and Williams 2011).

- The positive relationship between some soil nutrients and crown health of *E. gomphocephala* (Chapter 4), supported by previous reports on foliar symptoms of nutrient deficiencies (Eslick 2005; Dell et al. 2006a), suggest further nutritional studies are needed. Given that *E. gomphocephala* recovered with trunk nutrient application (Scott et al. 2013b), it is important to further investigate whether the recovery occurred because soil nutrients were not available for plant uptake, or the density of ECM was low thus reducing nutrient uptake. It is also worth evaluating how fertilizer application, both to the tree and the soil, affects soil microorganisms in the rhizosphere under *E. gomphocephala*. Furthermore, it is not known whether the recovery observed by Scott et al. (2013b) was permanent.

- Lastly, monitoring is a well-established concept in forest management, and integrated monitoring of biodiversity including epigeous macrofungi has been established for the *E. marginata* (jarrah) forest (McCaw et al. 2011; Robinson and Williams 2011) in SWWA. A short-term monitoring study has been undertaken on *E. gomphocephala*, however a long-term integrated ecological study on the health of *E. gomphocephala*, together with the monitoring of biotic factors such as mycorrhizal fungi and abiotic factors
such as climate change could provide information on how to sustainably manage a healthy *E. gomphocephala* forest in the future.

In conclusion, this PhD thesis has provided some insight into the role of mycorrhizal fungi and health of *E. gomphocephala*. It provides a strong basis for future research into the roles that ECM might play in maintaining healthy eucalypt forests.
Appendices

Appendix 1. Media types and ingredients

Table 1.1 MMN medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH$_4$)$_2$HPO$_4$</td>
<td>0.25 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.15 g</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.05 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.025 g</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 g</td>
</tr>
<tr>
<td>Malt extract</td>
<td>3 g</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.0001 g</td>
</tr>
<tr>
<td>Agar</td>
<td>18 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 L</td>
</tr>
<tr>
<td>Adjusted to pH 5.8</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 NARPH (a selective medium for Phytophthora).

<table>
<thead>
<tr>
<th>Ingredient</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Cornmeal Agar</td>
<td>17 g</td>
</tr>
<tr>
<td>Nystatin (Nilstat)</td>
<td>1 mL</td>
</tr>
<tr>
<td>Ampicillin sodium</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Rifampicin (Rifadin)</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Hymexazol (Tachigaren)</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Deionised water</td>
<td>1 L</td>
</tr>
</tbody>
</table>
Appendix 2. Soil chemical properties

Table 2.1. Average soil chemical properties

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<tr>
<th>Site</th>
<th>NH$_4$-N (mg/kg)</th>
<th>P (mg/kg)</th>
<th>K (mg/kg)</th>
<th>S (mg/kg)</th>
<th>Organic C (%)</th>
<th>Conductivity (ds/m)</th>
<th>pH (CaCl$_2$)</th>
<th>pH (H$_2$O)</th>
<th>Micronutrients, measured as DTPA (mg/kg)</th>
<th>Exc. Cations (mg/kg)</th>
<th>B (mg/kg)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.75</td>
<td>12</td>
<td>60.5</td>
<td>7.05</td>
<td>2.318</td>
<td>0.149</td>
<td>7.25</td>
<td>7.88</td>
<td>0.138 22.795 2.41 0.243 &lt; 0.001</td>
<td>15.143 1.398 0.153</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>5.5</td>
<td>39.5</td>
<td>6.425</td>
<td>1.525</td>
<td>0.141</td>
<td>7.375</td>
<td>8.15</td>
<td>0.223 10.528 1.898 0.185 &lt; 0.001</td>
<td>10.65 0.87 0.1</td>
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<td>3</td>
<td>0.75</td>
<td>16.25</td>
<td>64.25</td>
<td>7.125</td>
<td>1.585</td>
<td>0.117</td>
<td>7.05</td>
<td>7.85</td>
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<tr>
<td>4</td>
<td>0.25</td>
<td>6.5</td>
<td>29</td>
<td>2.95</td>
<td>0.995</td>
<td>0.047</td>
<td>6.775</td>
<td>7.58</td>
<td>0.303 5.895 2.9 0.175 &lt; 0.001</td>
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<td>0.5</td>
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<td>1.043</td>
<td>0.052</td>
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<td>7.38</td>
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<td>4.465 0.788 0.08</td>
<td>0.15</td>
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<td>51.75</td>
<td>3.875</td>
<td>2.238</td>
<td>0.081</td>
<td>6.7</td>
<td>7.55</td>
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<td>0.061</td>
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<td>7.55</td>
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<td>8.25</td>
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<td>1.518</td>
<td>0.048</td>
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<td>6.83</td>
<td>0.533 26.193 3.14 0.173 &lt; 0.001</td>
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<td>1.5</td>
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<td>115.75</td>
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<td>6.675</td>
<td>7.38</td>
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<tr>
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<td>4.5</td>
<td>35.5</td>
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<td>0.115</td>
<td>7</td>
<td>7.58</td>
<td>0.533 15.875 2.473 0.083 &lt; 0.001</td>
<td>11.07 0.87 0.093</td>
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<tr>
<td>11</td>
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<td>38</td>
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<td>6.83</td>
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<td>3.763 0.62 0.078</td>
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