The role of *Phytophthora multivora* in *Eucalyptus gomphocephala* (tuart) woodland decline

By

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The thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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September 2011
Declaration

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 To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged.

Peter Scott

September 2011
Abstract

Since the 1990’s *Eucalyptus gomphocephala* (tuart) has been suffering a significant decline in Yalgorup National Park, approximately 100 km south of Perth Western Australia. Symptoms range from chronic deterioration to sudden mass collapse. The role of *Phytophthora* pathogens was investigated because the progressive canopy thinning, dieback and heterogeneous distribution of the decline were similar to other forest declines caused by a range of *Phytophthora* species which are widespread throughout south-west Western Australia and worldwide.

In combination with sampling for *Phytophthora* isolation, an initial diagnostic trial tested the effect of trunk applied phosphite, nutrients and combined phosphite and nutrients on natural stands of declining *E. gomphocephala*. Phosphite injection was used as a diagnostic tool to identify the possible role of *Phytophthora* pathogens because the chemical specifically suppresses *Phytophthora* pathogens and has no known direct fertilizer effect on the host. A range of nutrient treatments was also applied as a diagnostic tool to indicate what nutrient deficiencies may be involved in the decline. Initially no *Phytophthora* species were isolated from the treatment sites. However, individual and combined injection treatments of phosphite and nutrients improved the crown health over four years with the greatest improvement from treatments of phosphite and zinc sulphide. In combination with further rhizosphere sampling for *Phytophthora* species, the response of declining trees to phosphite application was further investigated in a second injection trial. Phosphite concentrations from 75 to 375 g phosphite/L improved crown health compared to the control, with the best improvement at 150 g phosphite/L. The positive response of declining trees to phosphite injection implicated a *Phytophthora* pathogen, despite no *Phytophthora* species being isolated at this time. Consequently further work was undertaken to determine the involvement of *Phytophthora* species.
Concurrently to both injection trials, several seedling bioassays were conducted. The first bioassay tested the effect of pasteurising soil from a declining site within Yalgorup and healthy sites outside the Yalgorup woodland on *E. gomphocephala* seedlings grown *ex situ*. Seedling growth *ex situ* was not significantly reduced in non-pasteurised soil compared to the pasteurised soils from declining sites, and no *Phytophthora* species was isolated.

To further investigate the disease the fine root and ectomycorrhizal systems of the largest main lateral root of 18 declining *E. gomphocephala* trees within Yalgorup were exposed using an air spade. Necrotic roots were sampled and the crown, fine root and ectomycorrhizal health were assessed. No *Phytophthora* species was isolated from necrotic roots; however, crown health of the declining trees was significantly correlated with the fine root and ectomycorrhizae density, suggesting that below ground damage could be involved in the decline. The relationship between the above and below ground health of the air spaded trees was investigated further using an *in situ* and *ex situ* seedling bioassay. In the *in situ* bioassay, seedlings were planted within the exposed root mats of the air spaded trees. In the *ex situ* bioassay, seedlings were grown within a glasshouse in pasteurised and non-pasteurised soil collected from the air spaded root mats. No *Phytophthora* species was identified in these bioassays, and seedlings grown *ex situ* in non-pasteurised soil showed no clear decline symptoms, but the health of the woodland trees was significantly correlated with seedling survival, foliar health and height of the seedlings.

An additional 32 sites throughout the *E. gomphocephala* range were sampled for the presence of *Phytophthora* pathogens using a modified sampling and isolation procedure. From this survey a new *Phytophthora* species was isolated from five sites from the roots of declining *E. gomphocephala*, *E. marginata* and *Agonis flexuosa* at Yalgorup National Park. Morphologically similar to *P. citricola*, the new *Phytophthora*
species is unique based on phylogenetic analysis of the ITS and Cox1 gene regions and was named *P. multivora*. *Phytophthora multivora* has subsequently been isolated from all experimental sites showing tuart decline.

Two experiments tested the pathogenicity of *P. multivora* to *E. gomphocephala* and *E. marginata*. The first experiment examined *ex situ* the pathogenicity of five *P. multivora* isolates and one *P. cinnamomi* isolate on the root systems of *E. gomphocephala* and one *P. multivora* isolate on the root system of *E. marginata*. In the second experiment, the pathogenicity of *P. multivora* to *E. gomphocephala* and *E. marginata* saplings was measured *in situ* using under-bark stem inoculation. *Phytophthora multivora* isolates caused significant fine root loss and lesion extension in under-bark inoculated stems of both *E. gomphocephala* and *E. marginata*. *Phytophthora multivora* was also reisolated from necrotic fine roots and lesions of inoculated saplings of both *E. gomphocephala* and *E. marginata*, thus satisfying Koch’s postulates. No seedlings died in these pathogenicity trials and *P. multivora* was not reisolated from beyond the fine roots.

There was evidence that *P. multivora* significantly contributes to *E. gomphocephala* decline by episodically causing fine root damage leading to chronic deterioration of the trees.
Acknowledgments

I am incredibly honoured to have been given the chance to study the natural environment and work in the beautiful *Eucalyptus gomphocephala* woodland of south-west Western Australia. This project has been one of the greatest joys of my life and I would like to thank everyone who cares for the natural world and assists in its conservation.

Many people have vitally assisted in making this PhD possible and a pleasure and I will be eternally grateful to you all. Personally I would like to especially thank my supervisor, Professor Giles Hardy, for this wonderful opportunity. Thank you for all your uplifting comments, patient understanding, time and energy. You are a true and kind friend, an inspirational and passionate scientist and a tireless and dedicated mentor. You have always encouraged me to freely explore my scientific endeavours and strive beyond my self-expectations. For these gifts no words could ever express my gratitude. You tirelessly help so many and I hope you always remember how much you are valued.

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made a real difference in my life. I will always value your example and think of your poetry with fondness.

To Dr. Thomas Jung, certainly my PhD would not have been the same without your assistance. I thoroughly enjoyed all my time working with you and our long discussions. Thank you for all your efforts in helping to conserve the world’s forests and understanding the importance of *Phytophthora* pathogens.

I would like to especially thank all the staff and students at Murdoch University, the Centre of Phytophthora Science and Management, the Tuart health Research Group and the Centre of Excellence in Climate Change, Woodland and Forest Health, who helped with my experiments and training. To Dr. Treena Burgess, thank you for your friendship and you invaluable help with molecular biology. To Professor Jen McComb, thank you for discussing my project, your encouragement and reviewing my manuscripts. To Professor Bernie Dell, thank you for your enlightening discussions and reviewing of my manuscripts. To Harry Eslick, thank you for sharing my PhD journey and our productive discussions. I will always remember our adventures in the forest and the millions of ticks we collected. To Tan Dang, thank you for your warm kind personality and sharp clear intellect. It has been such a pleasure to work with you and your help on my project was invaluable. I hope to work with you again throughout both our careers. Thank you dearly to Trudy Paap, Bill Dunstan, Chid Gilovitz, Robert Archibald, Paul Drake, Diane White, Niels Brouwers, Sarah Jackson and Jennet Box for all your assistance and helping with my training. I would like to thank all the administration staff at Murdoch University and in the School of Biological Sciences and Biotechnology for all your help over the years.

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I respectfully acknowledge the Noongar people who are the past and present traditional owners and custodians of the land on which I completed this study.

At last, but by no means least I would like to thank all my family and friends for their love, prayers and support throughout the years with a special thanks to my dear mother, father and sister for all their encouragement and care. A huge thank you to my beautiful dog Molly for all your love, endless licks and keeping my feet warm during the long endless hours. I would especially like to thank my dear wife Daisy, for all her understanding, support and love, which has been invaluable throughout my project. Thank you for standing next to me and seeing me to the end.
List of publications

The results of experimental chapters 2, 4 and 5 have been published or submitted as indicated below. Only minor changes have been made to these publications in the thesis. Publication of thesis chapters as papers has necessitated some repetition, particularly in the introduction and methods sections.

Chapter 2 submitted as follows:
Scott PM, Barber PA, Shearer BL, Dell B, Calver M, Eslick H, Hardy GESStJ (2011) Phosphite and nutrient applications as explorative tools to determine the possible causes of Eucalyptus gomphocephala (tuart) decline in south-western Australia. European Journal of Plant Pathology.

Chapter 3 submitted as follows:
Scott PM, Barber PA, Shearer BL, Hardy GESStJ (2011) Relationship between the crown health and fine root and ectomycorrhizae density of declining Eucalyptus gomphocephala trees and seedlings growth. Australasian Plant Pathology.

Chapter 4 published as follows:
Chapter 5 submitted as follows:

Scott PM, Jung T, Shearer BL, Barber PA, Calver MC, Hardy GESkJ (2011) Pathogenicity of *Phytophthora multivora* to *Eucalyptus gomphocephala* and *E. marginata*. *Forest Pathology.*

**Conference presentations**

Scott PM, Shearer BL, Barber PA, Hardy GESkJ (2007) Pythiaceous fine feeder root pathogens associated with *Eucalyptus gomphocephala* (tuart) decline in south-west Western Australia. In: 4th IUFRO Phytophthoras in Forests & Natural Ecosystems, August, Monterey, California, United States of America.


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Thesis style, the style of this thesis regarding figure, tables and referencing, has been based on Australasian Plant Pathology, Commonwealth Scientific and Industrial Research Organisation (Springer).
Chapter 1. General introduction

1.1 Woodland and forest decline, worldwide

Woodlands and forest ecosystems are being rapidly cleared and modified world-wide by an expanding human population for housing and agriculture, while remnant ecosystems are suffering degradation and decline (Contreras-Hermosilla 2000). Tree decline significantly affect natural ecosystem function and biodiversity and have been attributed to a range of interacting biotic and abiotic factors resulting in complex aetiologies. They may be caused by climate change (Allen et al. 2010), introduced or native fungal or bacterial pathogens (Loo 2009), insects (Gandhi and Herms 2010), altered fire regimes and fire suppression (Jurskis 2005), pollution and plant community succession (Tables 1.1, 1.2, 1.3, 1.4 and 1.5). Tree decline may also affect human society by effecting timber and watershed protection and recreational, aesthetic, and spiritual benefits (Allen et al. 2010). Ayres and Lombardo (2000) estimate insect herbivores and fungal pathogens alone may be responsible for 20 million ha of declining forest in North America, with an estimated cost of US $1 billion annually. As vegetation is lost, remnant trees are increasingly important keystone structures and biological legacies in modified environments. Remnant trees provide important ecosystem functions and services including distinct microclimates, structural complexity, genetic material for future restoration, increased soil nutrients and habitat for flora and fauna (Manning et al. 2006).

Significant native tree declines in Australia for which the associated abiotic and biotic factors are known are shown in Table 1.1 and for Europe and Northern America in Table 1.2. A range of tree declines specifically involving fungal pathogens for Australia, Europe, Northern America and Asia are given in Table 1.3. Significant
native tree declines associated with *Phytophthora* species that are primarily damaging below or above ground plant parts are indicated in Tables 1.4 and 1.5, respectively.

Vegetation declines are considered deleterious if they reduce species diversity and alter ecosystem function (Shearer et al. 2009); however, forest declines can also have economic implications (Hansen 1999).

1.1.1 Tree decline concepts

Processes involved in tree declines have been characterised into predisposing, inciting and contributing factors by Manion, (1981), and may include:

- Predisposing factors such as climate, site, age or genetic predisposition that are long-term, resulting in permanent stress on trees. They may not lead to obvious problems, but predispose trees to:

- Inciting factors that are typically short-term and may be abiotic, such as drought, or biotic, such as insect defoliation. If not for predisposing factors, trees would recover quickly, but predisposed trees go into rapid decline and are vulnerable to:

- Contributing factors, which include leaf parasites, stem borers, canker fungi and root pathogens. These are opportunistic organisms that may kill the tree, but would not normally do so unless the tree was already declining.

However, there have been exceptions where significant devastating declines have occurred in native ecosystems without predisposing or contributing factors, especially following the introduction of alien pests and pathogens including the introduction of *Cryphonectria parasitica*, *Ophiostoma* species to Europe and North America (Table 1.3) and *Phytophthora cinnamomi* to south-west Western Australia (Table 1.4).
### Table 1.1. Some significant tree declines in native ecosystems in Australia, and associated abiotic and biotic factors excluding fungal and Oomycete pathogens as primary causal agents.

<table>
<thead>
<tr>
<th>Location</th>
<th>‘Syndrome’ and/or tree species</th>
<th>Climate</th>
<th>Factors associated with decline</th>
<th>Other Abiotic</th>
<th>Biotic</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>South-west Western Australia</td>
<td><em>Eucalyptus wandoo</em> woodland</td>
<td>Climate change and prolonged drought</td>
<td></td>
<td></td>
<td>Borer infestation and decay causing fungi</td>
<td>Hooper and Sivasithamparam (2005) and Hooper (2009)</td>
</tr>
<tr>
<td>Australia, throughout</td>
<td><em>Eucalyptus</em> species</td>
<td></td>
<td>Altered fire regime (suppression) and site degradation</td>
<td></td>
<td>Understorey competition</td>
<td>Jurskis (2005)</td>
</tr>
<tr>
<td>Western Australia, Swan Coastal Plain</td>
<td><em>E. gomphocephala</em> woodland</td>
<td>Altered climate</td>
<td>Altered fire regime (suppression) and site degradation</td>
<td></td>
<td>Understorey competition</td>
<td>Archibald (2006), Close et al. (2009), Archibald et al. (2010) and Close et al. (2011)</td>
</tr>
<tr>
<td>Tasmania</td>
<td><em>E. delegatensis</em> forest</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Australia, Western Australia, New South Wales, Australian Capital Territory, Victoria, Tasmania, Queensland and Northern Territory</td>
<td>‘Mundulla yellows’ – Over 70 species including <em>Eucalyptus</em> and other species</td>
<td>Unknown aetiology</td>
<td>Unknown aetiology and disturbance</td>
<td></td>
<td>Unknown aetiology and unknown contagion</td>
<td>Hanold et al. (2006)</td>
</tr>
<tr>
<td>Tasmania, above 800 m</td>
<td>‘High altitude dieback and regrowth dieback’ – <em>E. delegatensis</em></td>
<td></td>
<td>Altered fire regime (suppression)</td>
<td></td>
<td>Vegetation succession</td>
<td>Ellis et al. (1980) and Ellis and Pennington (1992)</td>
</tr>
</tbody>
</table>
Table 1.2. Some significant tree declines in native ecosystems in Europe and North America and associated abiotic and biotic factors, excluding fungal and Oomycete pathogens as primary causal agents.

<table>
<thead>
<tr>
<th>Tree species</th>
<th>Climate</th>
<th>Factors associated with decline</th>
<th>Other Abiotic</th>
<th>Biotic</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Europe</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Europe, Bavaria, West Germany</td>
<td><em>Picea abies</em></td>
<td>Air pollution deposition of sulphur, nitrate and ammonium</td>
<td></td>
<td></td>
<td>Schulze (1989)</td>
</tr>
<tr>
<td>• Central and eastern Europe, Carpathian mountains</td>
<td><em>P. abies</em> and <em>Fagus sylvatica</em></td>
<td>Air pollution ozone, nitrogen dioxide and sulphur dioxide</td>
<td></td>
<td></td>
<td>Muzika <em>et al.</em> (2004)</td>
</tr>
<tr>
<td>• Central Europe</td>
<td><em>Quercus</em> species</td>
<td>Climatic extremes including winter frost and summer drought</td>
<td>Air pollution, nitrogen eutrophication, and soil chemical stress</td>
<td>Defoliating insects, borer insects, pathogenic fungi, microorganisms and <em>Phytophthora</em> species</td>
<td>Thomas <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><strong>North America</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• North America widespread, Rocky Mountain region</td>
<td><em>Populus tremuloides</em></td>
<td>Altered fire regime (suppression)</td>
<td>Vegetation succession</td>
<td></td>
<td>Rogers (2002)</td>
</tr>
<tr>
<td>• United States, Pennsylvania</td>
<td><em>Q. rubra</em></td>
<td>Drought</td>
<td>Nutrient deficiency and soil acidity</td>
<td></td>
<td>Demchik and Sharpe (2000)</td>
</tr>
<tr>
<td>• United States, Ozark highlands of Missouri, Arkansas and Oklahoma</td>
<td><em>Quercus</em> species in particular red oak group species (<em>Quercus</em> section <em>Lobatae</em>)</td>
<td>Periodic drought, frost, ice and wind damage</td>
<td>Nutrient-deficient soils</td>
<td>Stand density, population dynamics and senescence</td>
<td>Kabrick <em>et al.</em> (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Periodic drought</td>
<td></td>
<td><em>Armillaria</em> root damage, oak borers and senescence</td>
<td>Fan <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>• United States, north-eastern and mid-western, and Canada eastern, Quebec southern</td>
<td><em>Acer saccharum</em></td>
<td>Extreme weather, late spring frost, mid-winter thaw/freeze, glaze damage and atmospheric deposition</td>
<td>Soil moisture deficiency or excess and injury from management</td>
<td>Defoliating insects, sugar maple borer (<em>Glycobius speciosus</em>) and <em>Armillaria</em> root disease</td>
<td>Horsley <em>et al.</em> (2002) and Payette <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>• United States, north-eastern</td>
<td><em>Ac. saccharum</em></td>
<td>Cool temperatures during <em>Malacosoma disstria</em> outbreak</td>
<td>Low soil moisture during outbreaks</td>
<td>Malacosoma disstria (forest tent caterpillar)</td>
<td>Wood <em>et al.</em> (2009)</td>
</tr>
<tr>
<td>• Canada, southern east of Lake Superior, eastern United States</td>
<td><em>Quercus</em> species (preferred) and hundreds of different shrubs and both hardwood and conifer trees</td>
<td>Climate change including warming temperatures increasing the range of <em>Lymantria dispar</em> (gypsy moth)</td>
<td></td>
<td><em>Lymantria dispar</em></td>
<td>Canada (Régnière <em>et al.</em> 2009), United Sates (Davidson <em>et al.</em> 1999)</td>
</tr>
</tbody>
</table>
Table 1.3. Some significant global tree declines where fungal pathogens have been identified as primary factors associated with decline.

<table>
<thead>
<tr>
<th>Location</th>
<th>‘Syndrome’ – Tree species</th>
<th>Factors associated with decline</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South-west Western Australia</td>
<td><em>Corymbia calophylla</em> and <em>C. ficifolia</em> in woodland/forest/urban areas</td>
<td><em>Quambalaria</em> species including <em>Quambalaria coyrecusp</em> (a native pathogen) causing canker and foliar and shoot damage</td>
<td>Paap (2006) and Paap et al. (2008)</td>
</tr>
<tr>
<td>South-west Western Australia</td>
<td><em>E. wandoo</em> woodland</td>
<td><em>Armillaria luteobubalina</em> causing stem and basal lesions, resulting in high mortality and discrete discontinuous disease</td>
<td>Shearer et al. (1997)</td>
</tr>
<tr>
<td>Europe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Europe, mountainous areas of southern Europe, southern foothills of the Alps, Italy to Hungary and along the Black Sea</td>
<td>‘Chestnut blight’ – <em>Castanea sativa</em>, <em>Ca. species</em> and <em>Quercus</em> species</td>
<td><em>Cryphonectria parasitica</em> (formerly <em>Endothia parasitica</em>)</td>
<td>Heiniger and Rigling (1994)</td>
</tr>
<tr>
<td>Europe, widespread to south-west and central Asia</td>
<td>‘Dutch elm disease’ – <em>Ulmus</em> species (all European species)</td>
<td>Fungal agents include <em>Ophiostoma ulmi</em> and <em>O. novo-ulmi</em>. Insect vectors include <em>Scolytus multistriatus</em> (European elm bark beetle) and <em>S. scolytus</em> (large elm bark beetle)</td>
<td>Brasier and Mehotra (1995), Brasier (2000) and Webber (2000)</td>
</tr>
<tr>
<td>North America</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>United States and Canada</td>
<td>‘Chestnut blight’ – <em>Ca. dentate</em> and <em>Ca. pumila</em></td>
<td><em>C. parasitica</em> (formerly <em>Endothia parasitica</em>)</td>
<td>Anagnostakis (1987)</td>
</tr>
<tr>
<td>United States and Canada</td>
<td>‘Dutch elm disease’ – <em>Ulmaceae</em> species including <em>U. americana</em></td>
<td>Fungal agents include <em>O. ulmi</em> and <em>O. novo-ulmi</em>. Insect vector <em>Hylurgopinus rufipes</em> (North American elm bark beetle), <em>S. multistriatus</em> (European elm bark beetle) and <em>S. schevyrewi</em> (banded elm bark beetle)</td>
<td>Brasier and Mehotra (1995), Brasier (2000) and Webber (2000)</td>
</tr>
<tr>
<td>United States and Canada, central hardwood forests</td>
<td>‘Butternut canker’ – <em>Juglans cinerea</em> and some <em>Juglans</em> and <em>Carya</em> species</td>
<td><em>Sirococcus clavigignenti-juglandacearum</em></td>
<td>Ostry and Woeste (2004)</td>
</tr>
<tr>
<td>United States and Canada</td>
<td><em>Pinus subgenus</em> <em>Strobus</em></td>
<td><em>Cronartium ribicola</em>.</td>
<td>Kinloch (2003)</td>
</tr>
<tr>
<td>Asia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Himalayas, northern Himachal Pradesh</td>
<td>‘Dutch elm disease’ – <em>Ulmus wallichiana</em></td>
<td>Fungal agents include <em>O. ulmi</em> and <em>O. novo-ulmi</em>. Insect vector <em>Scolytid</em> larvae (probably <em>S. kashmirensis</em>)</td>
<td>Brasier and Mehotra (1995) and Brasier (2000)</td>
</tr>
</tbody>
</table>
Table 1.4. Some significant global tree declines associated with *Phytophthora* pathogens primarily causing damage below ground.

<table>
<thead>
<tr>
<th>Phytophthora species</th>
<th>Location</th>
<th>Tree species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. cinnamomi</em> (some collar damage)</td>
<td>• Western Australia, south-west</td>
<td><em>Eucalyptus marginata</em> woodland/forest</td>
<td>Podger <em>et al.</em> (1965), Shearer and Tippett (1989) and Davison (1994)</td>
</tr>
<tr>
<td></td>
<td>• Western Australia, south-west</td>
<td>An estimated 2282 susceptible and 800 highly susceptible species (trees and shrubs)</td>
<td>Shearer <em>et al.</em> (2004a)</td>
</tr>
<tr>
<td></td>
<td>• Eastern Australia, including Brisbane, New South Wales and Victoria (East Gippsland)</td>
<td>Sclerophyll forest vegetation species including <em>E. sieberi</em> and <em>E. obliqua</em></td>
<td>Weste and Marks (1987) and Wilson (2003)</td>
</tr>
<tr>
<td></td>
<td>• Tasmania, mainly on sites with mean annual temperature &gt; 7.5°C and rainfall &gt; 600mm</td>
<td>Range of species including 136 species from a range of families including the <em>Epacridaceae, Fabaceae, Myrtaceae, Proteaceae</em> and <em>Dilleniaceae</em></td>
<td>Podger <em>et al.</em> (1990) and Barker and Wardlaw (1995)</td>
</tr>
<tr>
<td></td>
<td>• New Zealand</td>
<td><em>Pinus radiata, Pseudotsuga mensiesii</em> and <em>Cupressus macrocarpa</em></td>
<td>Newhook (1959; 1970) and Newhook and Podger (1972)</td>
</tr>
<tr>
<td></td>
<td>• Mediterranean Europe</td>
<td><em>Quercus suber</em> and <em>Q. ilex</em></td>
<td>Robin <em>et al.</em> (1998; 2001)</td>
</tr>
<tr>
<td></td>
<td>• Spain and Portugal</td>
<td><em>Q. suber</em> and <em>Q. ilex</em></td>
<td>Brasier <em>et al.</em> (1993)</td>
</tr>
<tr>
<td></td>
<td>• Europe and the United States</td>
<td><em>Castanea sativa</em></td>
<td>Petri (1917) and Crandall <em>et al.</em> (1945)</td>
</tr>
<tr>
<td></td>
<td>• North America</td>
<td><em>Q. rubra</em></td>
<td>Robin <em>et al.</em> (1994)</td>
</tr>
<tr>
<td></td>
<td>• United States, southern Ohio</td>
<td><em>Q. alba</em></td>
<td>Balci <em>et al.</em> (2007) and Nagle <em>et al.</em> (2010)</td>
</tr>
<tr>
<td><em>P. elongata</em></td>
<td>• Western Australia, northern jarrah forest</td>
<td><em>E. marginata</em> and <em>Corymbia calophylla</em> (rehabilitated bauxite mine pits)</td>
<td>Rea <em>et al.</em> (2010)</td>
</tr>
<tr>
<td></td>
<td>• Western Australia, northern and southern jarrah forest</td>
<td><em>Banksia grandis, Leucopogon propinquus, Dryandra squarrosa, Andersonia</em> species, <em>Xanthorrhoea preissii, X. gracilis</em> and <em>Patersonia xanthina</em></td>
<td></td>
</tr>
</tbody>
</table>
### Table 1.4 (continued). Some significant global tree declines associated with *Phytophthora* pathogens primarily causing damage below ground.

<table>
<thead>
<tr>
<th><em>Phytophthora</em> species</th>
<th>Location</th>
<th>Tree species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. plurivora</em> from re-evaluation of <em>P. citricola</em> (Some collar and aerial cankers)</td>
<td>• Europe, forests, parks and nurseries</td>
<td>From rhizosphere soil samples of 39 species of mature and young trees</td>
<td>Jung and Burgess (2009)</td>
</tr>
<tr>
<td></td>
<td>• Germany, Austria, Italy and Switzerland</td>
<td>From necrotic bark of Alnus glutinosa, Al. incana, Aesculus hippocastanum and Tsuga canadensis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Germany</td>
<td>From fine roots of Q. robur and Q. petraea</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Canada, Montreal, Mount Royal</td>
<td>From fine roots of Acer saccharum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Czech Republic</td>
<td>Rhododendron species, Pieris floribunda, Vaccinium species and Azalea species</td>
<td>Mrázková <em>et al.</em> (2011)</td>
</tr>
<tr>
<td><em>P. quercina</em></td>
<td>Europe from southern Sweden (north) to Turkey (south east) and the United Kingdom in the west</td>
<td>Quercus species</td>
<td>Jung <em>et al.</em> (1999; 2000), Balci and Halmschlager (2003), Brasier and Jung (2003) and Jónsson <em>et al.</em> (2003)</td>
</tr>
<tr>
<td><em>P. alni</em></td>
<td>Europe including southern Britain and Italy</td>
<td>Alnus species including A. glutinosa, A. incana and A. cordata</td>
<td>Brasier and Kirk (2001), Brasier (2003), Brasier <em>et al.</em> (2004b) and Stenlid <em>et al.</em> (2011)</td>
</tr>
<tr>
<td><em>P. gallica</em></td>
<td>• North-east France</td>
<td>Quercus species</td>
<td>Jung and Nechwatal (2008)</td>
</tr>
<tr>
<td></td>
<td>• South-west Germany, Lake Constance</td>
<td><em>Phragmites australis</em></td>
<td></td>
</tr>
<tr>
<td><em>P. lateralis</em> (Root damage from zoospores in water and chlamydospores in transported mud)</td>
<td>North America, Pacific Northwest</td>
<td><em>Chamaecyparis lawsoniana</em> and <em>Taxus brevifolia</em></td>
<td>Hansen <em>et al.</em> (2000)</td>
</tr>
</tbody>
</table>
Table 1.5. Some significant global tree declines associated with *Phytophthora* pathogens primarily causing damage above ground.

<table>
<thead>
<tr>
<th>Phytophthora species</th>
<th>Location</th>
<th>Tree species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• South-west Britain, Cornwall</td>
<td><em>Rhododendron</em> species, <em>Viburnum</em> species, <em>Q. cerris</em>, <em>Q. ilex</em>, <em>Fagus sylvatica</em>, <em>Nothofagus obliqua</em>, <em>Aesculus hippocastanum</em>, <em>Castanea sativa</em> and <em>Drymis winteri</em></td>
<td>Brasier <em>et al.</em> (2004a)</td>
</tr>
<tr>
<td></td>
<td>• France</td>
<td><em>Rhododendron</em> species</td>
<td>Delatour <em>et al.</em> (2002)</td>
</tr>
<tr>
<td></td>
<td>• United Kingdom</td>
<td><em>V. tinus</em></td>
<td>Lane <em>et al.</em> (2003)</td>
</tr>
<tr>
<td></td>
<td>• Belgium</td>
<td><em>V. bodnantense</em></td>
<td>De Merlier <em>et al.</em> (2003)</td>
</tr>
<tr>
<td></td>
<td>• Southern Britain, Sussex</td>
<td><em>Q. falcata</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Netherlands</td>
<td><em>Q. rubra</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Germany and the Netherlands</td>
<td><em>Rhododendron</em> and <em>Viburnum</em> species</td>
<td>Werres (2001)</td>
</tr>
<tr>
<td></td>
<td>• Europe</td>
<td><em>Larix kaempferi</em> and secondary infections of <em>F. sylvatica</em>, <em>N. obliqua</em>, <em>C. sativa</em>, <em>B. pendula</em>, <em>R. ponticum</em>, <em>Tsuga heterophylla</em> and <em>Pseudotsuga menziesii</em></td>
<td>Webber <em>et al.</em> (2011)</td>
</tr>
<tr>
<td><em>P. pinifolia</em></td>
<td>Chile, including Arauco province – (Daño Foliar del Pino)</td>
<td><em>Pinus radiata</em></td>
<td>Durán <em>et al.</em> (2008)</td>
</tr>
<tr>
<td><em>P. kernoviae</em></td>
<td>South-west England, Cornwall</td>
<td><em>F. sylvatica</em> and <em>R. ponticum</em></td>
<td>Brasier <em>et al.</em> (2005)</td>
</tr>
</tbody>
</table>
1.1.2 Climate change

Climate has always shaped the world’s forests (Bhatti et al. 2006). The world’s climate has recently become warmer and will change further at an unprecedented rate (Pachauri and Reisinger 2007). Allen et al. (2010) identified 88 examples of forest mortality from around the world that were driven by climate induced water and heat stress since 1970. The deaths were across a broad gradient of woody ecosystems with mean precipitation from 400 to 3000 mm/year. Climate change is expected to affect the health and biodiversity of native forests and plantations, and the impact of exotic pests and pathogens in Western Australia (Robinson 2008).

Changes in climate may increase tree mortality through: climate induced physiological stress, including heat stress, drought and flooding; and climate mediated processes such as insect outbreaks and wildfires (Allen et al. 2010). The mechanisms by which climate change is and may potentially influence plant pathogen interactions including the plant and pathogen vector response to climate change, are discussed by Garret et al. (2006). From a review of climate change and forest diseases, Sturrock et al. (2011), made the following predictions:

- Climate change will affect pests, pathogens and hosts, resulting in changes in disease impacts.
- Changes in interactions between biotic and abiotic stressors may represent the most substantial drivers of disease outbreaks.
- The distribution of pests, pathogens and hosts will change. Models frequently predict reductions in geographic distribution of trees (Rehfeldt et al. 2009). Pests and pathogens may play a key role in reducing the range of forest trees.
- Most pests and pathogens will be able to migrate to climate-suitable locations faster than their long lived hosts and cause greater disease outbreaks and disturbance. Modest climate change will have rapid impacts on the distribution
and abundance of many forest insects and pathogens because of their short life cycles, mobility, reproductive potential, and physiological sensitivity to temperature (Ayres and Lombardero 2000).

- Pests and pathogens affecting water stressed hosts will have an increased impact on forest regions where precipitation is reduced.

### 1.1.3 Climate change, tree decline feedbacks

Forests influence climate through physical, chemical, and biological feedback processes that influence hydrological cycles and atmospheric composition and help mitigate climate change (Bonan 2008). Climate warming may increase insect outbreaks in boreal forests, which would tend to increase forest fires and exacerbate further climate warming by releasing carbon stores from boreal ecosystems (Ayres and Lombardero 2000).

Human induced land cover changes have been associated with climate change through bio-geophysical impacts on climate at regional and global scales (Lawrence and Chase 2010). The impact of land cover change on the micrometeorology of south-west Western Australia has been reviewed by Pitman et al. (2004) and demonstrates a strong relationship between recent land cover change and observed temperature increases, reductions in rainfall over south-west Western Australia and an increase in rainfall inland.

### 1.1.4 Pests and pathogens in native ecosystems

Tree loss is a component of healthy ecosystem regeneration, where weak or unfit trees are removed, opening up resources for further growth (Manion 1981). Through removing weak or unfit trees, indigenous pathogens are crucial for healthy natural ecosystems and influence species diversity, evenness and help redistribute limited
resources such as light (Manion 1981; Castello et al. 1995; Hansen 1999; Burgess and Wingfield 2002). Indigenous pathogens are less likely to significantly affect plant communities because evolution acts on populations ensuring the survival of plants and pathogens (Hansen 1999).

Native pathogens have, however, been associated with significant diseases. In south-west Western Australia the native pathogen *Armillaria luteobubalina* causes stem and basal lesions on *E. wandoo* resulting in high mortality and discrete discontinuous disease (Shearer et al. 1997, Table 1.3) and the native fungus *Quambalaria coyrecup* causes cankers and decline of *Corymbia calophylla* and *C. ficifolia* (Paap 2008, Table 1.3). Climate change has been identified as a driving force in modifying host susceptibility and pathogenicity of indigenous pathogens (Boland et al. 2004; Dukes et al. 2009). For example, climate change has been implicated in increased needle blight incidence and mortality of *Pinus contorta* var. *latifolia* in the forests of north-western British Columbia, Canada, caused by the native pathogen *Dothistroma septosporum* (Welsh et al. 2009).

Pathogen impacts are most significant when highly successful invading pathogens attack keystone species, setting in motion a long-lasting cascade of effects on the host and associated species (Loo 2009). Examples of recently introduced insect pests and pathogens having significant effects on native trees and vegetation ecosystems include:

- *Malacosoma disstria* (forest tent caterpillar) affecting *Acer saccharum* (sugar maple) and *Lymantria dispar* (gypsy moth) affecting *Quercus* species (oaks) and hardwood and conifer forests in northern America (Table 1.2);
- Devastating fungal pathogens introduced to Europe and northern America (Table 1.3); and
- *Phytophthora* pathogens including *P. cinnamomi* (Table 1.4) and *P. ramorum* (Table 1.5), which have been introduced to many ecosystems worldwide.
1.1.5 Spatial and temporal influences of biotic agents

Temporal and spatial patterns and processes in natural systems are more complex than these in agro-ecosystem because of the genetic, numeric, temporal and spatial variation of forest populations (Burdon et al. 1989; Real and McElhany 1996). For example, the consequences of tree mortality differ in mixed species stands and in natural or planted monocultures and there are different effects in early or late successional forests (Hansen 1999). Shearer et al. (2010) found that patterns of inoculum occurrence in *P. cinnamomi* disease centres were influenced by the creation of dynamic spatiotemporal niche refuges favourable to the pathogen, through ecosystem engineering by host and pathogen.

1.1.6 *Phytophthora* pathogens associated with native tree declines

*Phytophthora* is a major genus of plant pathogens within the phylum Oomycota and includes primary invaders of healthy plant tissue with limited saprotrophic ability that affect leaves, stems and roots (Cooke et al. 2000). Many *Phytophthora* species are benign in co-evolved plant communities, but cause significant epidemics when introduced to new hosts in new environments or when given the opportunity for dispersal or unexpected sexual recombination (Hansen 2008).

Many species are responsible for serious disease and damage to economically important crops (Gregory 1983), and natural plant communities within Australia and worldwide (Erwin and Ribeiro, Tables 1.4 and 1.5). Tree declines associated with *Phytophthora* pathogens include: *Quercus* species (oak) declines in northern (Jönsson 2006), central (Jung et al. 2000) and southern (Brasier et al. 1993) Europe; *Castanea* species (chestnut) decline in the United States of America (Crandall et al. 1945); and *E. marginata* decline in south-western Australia (Shearer and Tippett 1989; Hardy...
2004). Typically these declines caused by pests and pathogens, including *Phytophthora* pathogens, involve conducive site conditions and secondary pathogens. For example, decline of *Fagus sylvatica* (European beech) in central Europe has been shown to involve complex interactions between climate change and excessive rainfall and drought, triggering disease caused by a range of *Phytophthora* species, leading to bark necrosis and facilitating entry of secondary bark pathogens, including *Nectria coccinea*, and wood decay fungi (Jung 2009).

An ecological, temporal and spatial approach to understanding epidemic development is fundamental to integrated and sustainable methods of management for diseases caused by *Phytophthora* species (Ristaino and Gumpertz 2000; Benson et al. 2006).

### 1.1.7 Quantifying soilborne *Phytophthora* disease

Root diseases may be more difficult to diagnose than canker or leaf and shoot diseases, because they are usually expressed via non-specific aerial symptoms of branch or crown dieback, which are often associated with other pathogens or insects (Hansen and Delatour 1999). It is also difficult to quantify how fine feeder root damage can result in above ground damage (Dell and Wallace 1983; Davison 1994; Jönsson 2006). The role of *Phytophthora* species in native ecosystem declines has typically been studied by measuring disease expression in seedlings grown *ex situ* (Jung et al. 1996; Jung et al. 1999; Jönsson et al. 2003; Jönsson 2004), rather than testing the role of causal organisms by determining Koch’s postulates *in situ* (Podger 1972; Podger 1973) or measuring disease and pathogen dynamics *in situ* (Shearer et al. 2010).
1.1.8 Phosphite as a means to control *Phytophthora* pathogens

Phosphite has been used to control many plant diseases caused by Oomycete pathogens (Cohen and Coffey 1986). It has been successfully used to protect endangered plant species and threatened ecological communities from *P. cinnamomi* in south-west Western Australia (Barrett 2001; Hardy *et al.* 2001), and oaks in Europe (Fernandez-Escobar *et al.* 1999) and the United States of America (Garbelotto *et al.* 2007). Phosphite application is one of the only cost effective methods for controlling tree decline associated with *Phytophthora* species (Smith 2001). The chemical occurs as the anionic form of phosphonic acid (HPO$_3^{2-}$). It is a systemic fungicide that is readily translocated in both the xylem and phloem, in a source-sink relationship (Saindrenan *et al.* 1988b; Ouimette and Coffey 1989; Guest and Grant 1991). Phosphite also has a complex mode of action involving both directly and indirect effects on oomycete pathogens (Guest and Grant 1991).

1.1.9 Phosphite as a diagnostic tool for *Phytophthora* pathogens

Given the specificity of phosphite for Oomycete species, phosphite treatment could be used as a diagnostic tool to determine the role of *Phytophthora* pathogens in tree decline. Phosphite application may also help indicate the role of *Phytophthora* pathogens in native declines, where it is practically difficult and/or biologically irresponsible to measure Koch’s postulates in native ecosystems. Paul *et al.* (1989) and Newsham *et al.* (1994) proposed the use of fungicides with known effects to study native tree declines. Improvements in crown health of trees treated with phosphite has shown the importance of *Phytophthora* pathogens in *Quercus ilex* (holm oak) and *Q. suber* (cork oak), as part of Iberian oak decline (Fernandez-Escobar *et al.* 1999).

Phosphite treatment is a useful diagnostic tool to determine the role of *Phytophthora* species in plant declines, because phosphite (a) has a specific effect on
Oomycetes; (b) controls *Phytophthora* organisms *in planta* at concentrations that only partially inhibit pathogen growth *in vitro* (Guest and Bompeix 1984; Smille *et al.* 1989; Guest and Grant 1991; Hardy *et al.* 2001; Wilkinson *et al.* 2001), (c) at effective application rates it has extremely low toxicity to invertebrates, aquatic organisms, or animals, including humans (Hardy *et al.* 2001), and (d) is not directly metabolized by plants and does not have any direct beneficial or deleterious fertilizer effects on plant growth, as a source of phosphate (Guest and Grant 1991; Carswell *et al.* 1996; Thao and Yamakawa 2009). Phosphite may be metabolized and converted to plant available phosphate, via soil bacteria (White and Metcalf 2007), although the conversion to plant available phosphate is slow (Adams and Conrad 1953; McDonald *et al.* 2001), and would therefore not negate the use of phosphite as a diagnostic tool. Phosphite may, however, exacerbate symptoms of phosphate deficiency, by suppressing the typical molecular and developmental responses of plants to phosphate deficiency (Carswell *et al.* 1996; Carswell *et al.* 1997; McDonald *et al.* 2001; Ticconi *et al.* 2001; Abel *et al.* 2002; Varadarjan *et al.* 2002).

1.1.10 Ectomycorrhizal fungi

Micronutrient deficiencies may be associated with tree declines, as they are typically limiting in native vegetation and their uptake is associated with rhizosphere mediated soil biology (Daroub and Snyder 2007). Symbiosis with ectomycorrhizal fungi plays an important role in nutrient acquisition of *Eucalyptus* species (Marschner and Dell 1994) including *E. gomphocephala* (Malajczuk *et al.* 1982). Ectomycorrhizae improve drought resistance and nutrient uptake during dry periods (Bowen and Theodorou 1973; Bolan 1991). Ectomycorrhizae may also give plants a competitive advantage in exploiting nutrient resources from non-ectomycorrhizal plants (Moorhead *et al.* 1998). On *E. marginata* in the jarrah forest, ectomycorrhizal development and diversity
were highest on sites from which fire had been excluded and lowest on sites with more frequent fires, which reduce leaf litter and organic layers associated with mycorrhizal concentration and developments (Harvey et al. 1978; Malajczuk and Hingston 1981).

1.2 *Eucalyptus gomphocephala* woodland decline

*Eucalyptus gomphocephala* decline has been observed since 1920 (Baird 1967) and since 1921 in the Ludlow tuart forest (Forests Department 1979). Sporadic episodes of unexplained deaths and defoliation were observed at Bold Park in 1975 (Longman and Keighery 2002). However, since the 1990’s, the forest has suffered severe and extensive decline. A major dieback event was observed in 1997 near Preston Beach, Yalgorup National Park, with more than 90% mortality in some areas and with the decline spreading north and south (Mitchell, D., Department of Conservation and Land Management, personal communication). In Yalgorup, *E. gomphocephala* has been declining since the 1990’s (Longman and Keighery 2002; Edwards 2004), and in the same area *E. marginata* has been declining since 2000 (Edwards 2004) and *Agonis flexuosa* (peppermint) since 2007 (Taylor et al. 2009).

*Eucalyptus gomphocephala* decline is characterised by canopy contraction, a decrease in canopy density, the death of small to large branches, the progressive dominance of epicormic growth and finally death (Edwards 2004). Branch deaths occurs predominantly from August to May (Longman and Keighery 2002). There is also concern about the lack of *E. gomphocephala* recruitment in urban remnants (Fox and Curry 1980), at Ludlow (Keene and Cracknell 1972) and at Yalgorup (Backshall 1983). Declining adult tree health and failure of seedling regeneration may result in the permanent loss of some *E. gomphocephala* woodlands in the absence of human intervention (Archibald 2006).

Some factors identified as potentially contributing to *E. gomphocephala* decline...
include population changes and succession, biotic factors including fungal pathogens and changing insect and beneficial fungi populations, and abiotic factors including fire, drought, fragmentation, frost, hydrology and nutrient changes (Longman and Keighery 2002). The roles of some of these factors have been examined further and are indicated below.

1.2.1 *Eucalyptus gomphocephala* woodlands


Since European settlement in 1829, the area of *E. gomphocephala* has been reduced from 111,600 ha to approximately 38,800 ha in 2003 (Tuart Response Group 2003), and land adjoining and throughout its range has been highly modified for urban and agricultural use (Seddon 1972). The Yalgorup National Park comprises the largest remnant section of *E. gomphocephala* woodland and this has been significantly disturbed. As early as the 1850’s, the Old Coast Road, south of Mandurah passed through most of the *E. gomphocephala* woodland, running adjacent to the current Yalgorup National Park boundaries. Regions of the park have been used for grazing up to the 1970’s, resulting in floristic modifications, including the removal of grasses unsuitable for grazing and plants poisonous to livestock such as *Gastrolobium bilobum* (Powell and Emberson 1981).

*Eucalyptus gomphocephala* is most commonly distributed along Aeolian-derived Spearwood Dune Systems (Ruthrof *et al.* 2002), is the dominant canopy species associated with soils high in calcium carbonate (Semeniuk and Glassford 1989;
McArthur 1991), and is one of the few *Eucalyptus* species adapted to highly alkaline calcareous soils (Eldridge *et al.* 1994).

### 1.2.2 Tree declines in south-west Western Australia

Laurance *et al.* (2011) have identified the Mediterranean ecosystems of south-western Australia as one of the 10 major groups of terrestrial and marine ecosystems in Australia most vulnerable to tipping points, in which modest environmental changes can cause disproportionately large changes in ecosystem properties. These Mediterranean ecosystems includes the dry sclerophyll forests, woodlands, and heathlands which are near important thresholds of temperature and rainfall (Abbott and Le Maitre 2010), are geographically restricted, rely on vital ‘framework’ species of one or more locally dominant tree species, have suffered losses of key fauna including mycophagous marsupials (Garkaklis *et al.* 2004), and are prone to positive feedbacks between weed invasions and destructively intense fires (Laurance *et al.* 2011).

Presently there is no formal forest health surveillance program in Western Australia, except for surveillance and mapping by the Department of Environment and Conservation of *Phytophthora* dieback of *E. marginata* in the jarrah forest, *Armillaria* root disease survey in *E. diversicolor* (karri) forest, surveillance of *Sirex noctilio* (sirex) wasp and *Hylotrupes bajulus* (European house borer) in pine plantations, and general pest surveillance in blue gum plantations by private industry (Robinson 2008). However, recent noticeable declines in different forest, woodland and rural tree species in south-west Western Australia are a major concern and are an important community issue (Robinson 2008). These species include *Agonis flexuosa* (Dakin *et al.* 2010), *E. marginata* (Podger *et al.* 1965; Shearer and Tippett 1989; Davison 1994), *E. rudis* (Gibson 2001; Wallace *et al.* 2006), *E. wandoo* (Hooper and Sivasithamparam 2005; Hooper 2009), *E. diversicolor* (Robinson 2008) and *C. calophylla* (Paap 2006; Paap *et al.*
These declines have been attributed to a range of biotic and abiotic factors including: *P. cinnamomi*, *Armillaria luteobubalina*, foliar, stem and canker pathogens, drought, climate change, nutrient deficiencies and salinity.

*Agonis flexuosa* decline, especially in Yalgorup where it grows in close proximity to declining *E. gomphocephala*, has suffered canker damage from opportunistic endophytic fungi including *Neofusicoccum australe* (Dakin et al. 2010), which has also been shown to cause branch cankers on *E. gomphocephala* (Taylor et al. 2009).

*Eucalyptus marginata* has undergone significant decline throughout its distribution, noted as early as 1921, and this has been associated with the soilborne pathogen *Phytophthora cinnamomi* (Podger et al. 1965; Podger 1972) and has since been shown to have a wide host range and a large impact in south-west Western Australia (Shearer et al. 2004a). Water gaining sites and site disturbances have since been shown to be significant inciting and contributing factors associated with *E. marginata* mortality caused by *P. cinnamomi* (Shearer and Tippett 1989; Davison 1994). Shearer and Tippett (1989) provide a comprehensive epidemiology and review of the role of *P. cinnamomi* in *E. marginata* decline.

*Corymbia calophylla* has been shown to have stem cankers since the 1960’s (MacNish 1963), and has suffered a significant decline since the 1970’s, associated with the canker-forming fungus *Sporotricium destructor* (Kimber 1980). *Corymbia calophylla* decline is widespread, being more prevalent on cleared sites and is associated with *Quambalaria* species, which have also been shown to damage *C. ficifolia* (red flowering gum) (Paap 2006; Paap et al. 2008).

*Eucalyptus wandoo* has undergone a series of decline events since the 1960’s (Podger 1963) with the most recent events starting around the 1990’s (Mercer 2003). The decline pattern is variable (Wills et al. 2001) and discontinuous (Mercer 2003) across the landscape, with very few unaffected stands remaining (Hooper and
Sivasithamparam 2005). Symptoms involve cycles of foliage chlorosis, death of small branches, flushes of epicormic growth that subsequently die and severe canopy decline (Hooper and Sivasithamparam 2005). *Eucalyptus wandoo* crown decline and branch cankers have been associated with borer infestation and decay causing fungi, in association with climate change and several drought periods (Hooper and Sivasithamparam 2005; Hooper 2009).

### 1.2.3 Climate change

In south-west Western Australia a sharp drop in winter rains of around 15-20 % (which was only partially offset by an increase in spring rains since the 1970’s), and a warming trend since the middle of last century, (Indian Ocean Climate Initiative 2002), may be associated with *E. gomphocephala* decline (Longman and Keighery 2002).

Bates *et al.* (2008) have attribute deaths of *E. gomphocephala* forest in southern south-west Western Australia and the *E. wandoo* woodland in eastern south-west Western Australia to apparent water stress. As a result of rainfall decline in south-west Western Australia there has been a reduction in surface water available for storage. The time series of May to April inflows to the region’s Integrated Water Supply System up to the 16th of August 2011 is shown in Fig. 1.1. The average inflow over the period 1911 to 1974 was 338 gigalitre (GL) which is almost twice the average of 177 GL over the subsequent 22-year period (1975 to 2000). Average inflow from 2001 to 2005 was 92.4 GL, and for 2006 to 2010 was 57.7 GL, with, 26.7 GL recorded up to August 2011.

Hughes (2003) provides a review of climate change in Australia over the last few decades and consistent with global trends, Australia has warmed by approximately 0.8°C since 1910 over the last century with minimum temperatures increasing faster than maxima, with the greatest warming inland, particularly in Queensland and the southern half of Western Australia, while south-west Western Australia has become 25% drier in winter. The sharpest rainfall decline occurred between 1960 and 1972. Observed changes have been attributed to the enhanced greenhouse effect (Indian Ocean Climate Initiative 2002) and land cover changes, including clearing and deforestation, which have accounted for up to 50% of the observed warming (Pitman et al. 2004). Drake (2008) provides a detailed review of the response of *E. gomphocephala* to drought conditions.

### 1.2.4 Modified fire regimes

Modifications to the frequency and impact of fires have also been associated with
E. gomphocephala decline and reduced regeneration (Archibald 2006). Less frequent fires have been linked to competition-mediated decline by favouring a dense understory (Ward 2000), including A. flexuosa which may have allelopathic effects on E. gomphocephala (Bradshaw 2000). Eucalyptus gomphocephala decline has been associated with reduced fire and change in vegetation structure including increased midstory vegetation and litter resulting in reducing availability of soil water and locking up and reducing access to nutrients (Close et al. 2009). Compared to frequently burnt E. gomphocephala sites (burnt every 5–15 years for the past 6 decades and within 10 since 2010), long unburnt sites (not burnt since 1972-2011) had greater understory cover, higher water use efficiency indicating reduced soil water availability, and significantly lower foliar copper and zinc (Close et al. 2011). However, Archibald (2006) indicates that it may be difficult to determine whether the understory changes in the E. gomphocephala forest are a cause, or a reaction to, decreased E. gomphocephala cover.

Fire scars on Xanthorrhoea species (grass tree) (Ward et al. 2001) and E. marginata trees (Burrows and Wardell-Johnson 2003), and historical fire accounts (Ward et al. 2001; Abbott 2003; Archibald et al. 2005), suggest a decrease in fire frequency in south-west Western Australia, since European settlement.

Greater fire frequency in the form of successive wildfires has been linked to direct damage, facilitated damage from borers including Phoracantha species, pathogens entering trees via fire scars (Fox and Curry 1980) and reduced regenerative capacity by depletion of storage carbohydrates and/or destruction of epicormic shoots (Archibald et al. 2005). Archibald (2006) provides a comprehensive review of the role of fire in E. gomphocephala decline and has identified the following important interactions:

- Eucalyptus gomphocephala health was poorest at the longest unburnt sites and also in the sites burnt frequently;
• There is a significant negative correlation between *A. flexuosa* density and *E. gomphocephala* health;

• *Eucalyptus gomphocephala* may respond better than *A. flexuosa* to fire, with *E. gomphocephala* surviving better on ashbeds compared to *A. flexuosa*, which was attributed to a greater root to shoot ratio and a better response to increased nutrient availability; and

• *Eucalyptus gomphocephala* suffered greater insect and foliar pathogen damage under the influence of high *A. flexuosa* density.

In the absence of seed damage associated with *Haplonyx tibialis* (bud weevil) (Fox and Curry 1980), fire is the dominant influence on natural *E. gomphocephala* regeneration (Ruthrof et al. 2002). Archibald (2006) confirms that at sites with infrequent fires there is less regeneration of *E. gomphocephala* seedlings. In the jarrah forest, frequent fires have also been associated with *E. marginata* regeneration through reducing competition and increasing the availability of nutrients which are returned to the soil from litter and woody debris following combustion (Grove et al. 1986).

Fire may, however, have negative effects on soil structure, biodiversity, and nutrient concentration through removal of organic matter, deterioration of both structure and porosity, decreased wetting, considerable loss of nutrients through volatilisation, ash entrapment in smoke columns, leaching and erosion, and a marked alteration of both the quantity and specific composition of microbial and soil-dwelling invertebrate communities (Certini 2005). From a global study of the extent to which fire determines global vegetation patterns, Bond et al. (2005) predicted that without fire, closed forests would double from 27% to 56% of the world’s surface. After fire, nutrients loss through erosion is likely to be greater on wetter, more easily eroded sites (Thomas et al. 1999). Archibald (2006) and Archibald et al. (2010) recommend caution with the
excessive use of fires to manage *E. gomphocephala* decline, because declining trees are more likely to suffer greater damage from fire than healthy trees.

### 1.2.5 Air pollution

Air pollution from the Kwinana industrial area has been associated with *E. gomphocephala* decline (Chilcot 1992); however, the prevalence of decline throughout the *E. gomphocephala* range suggests that air pollution is not linked to decline outside the metropolitan area (Chilcot 1992; Longman and Keighery 2002; Edwards 2004).

### 1.2.6 Zinc deficiency

Moore (2004), Eslick (2005) and Close *et al.* (2009; 2011) have identified zinc deficiency as a factor contributing to *E. gomphocephala* decline. *Eucalyptus gomphocephala* is most commonly distributed along Aeolian-derived Spearwood Dune Systems (Ruthrof *et al.* 2002), and is the dominant canopy species associated with soils high in calcium carbonate (Semeniuk and Glassford 1989). It is one of the few *Eucalyptus* species adapted to highly alkaline calcareous soils (Eldridge *et al.* 1994). In acid soils, the availability of zinc for plant uptake is reduced by the addition of calcium carbonate (Lucas and Knezek 1972). The availability of zinc on calcareous soils is believed to be reduced as the alkaline pH limits the availability of compounds of low solubility (Clarke and Graham 1968; Saeed and Fox 1977) or the zinc is absorbed by carbonates (Udo *et al.* 1970). Zinc deficiencies in *E. gomphocephala* may contribute to the woodland decline by either increasing the susceptibility of *E. gomphocephala* trees to stress factors including plant pathogens, and/or exacerbating the influence of stress factors on tree health. Many Australian soils are naturally deficient in zinc for crop and pasture production (Donald and Prescott 1975; Brennan 1990). Disease symptoms of
Pinus plantations grown on calcareous soils within the *E. gomphocephala* forest north of Ludlow, were amended via zinc application (Kessell and Stoate 1936; Kessell and Stoate 1938). Zinc deficiencies have been observed for *Eucalyptus* plantations grown on calcareous soil and disturbed sites (Dell and Wilson 1985; Haridasan 1985; Wallace *et al.* 1986; Dell and Wilson 1989).

On calcareous sites, *E. gomphocephala* may have a competitive advantage over other *Eucalyptus* species and large trees, through its ability to absorb and tolerate low levels of zinc. A range of ectomycorrhizal fungi are associated with *E. gomphocephala* and these are believed to be both affected by and involved in *E. gomphocephala* decline (Legault 2005). Arbuscular mycorrhizae have been shown to significantly increase zinc uptake in *Trifolium* species (Chena *et al.* 2002) and *Cajanus cajan* (Wellings *et al.* 1991) on calcareous soils. Zinc deficiency associated with *E. gomphocephala* decline may have resulted from multiple factors including an interaction between *Phytophthora* root pathogens and the loss of fine roots and associated beneficial mycorrhizae that facilitate zinc uptake, amongst other elements.

1.2.7 Insect damage

*Phoracantha impavida* (tuart borer or longhorn borer), *P. semipunctata* and *Haplonyx tibialis* (bud weevil) have long been associated with *E. gomphocephala* decline, although they are regarded as secondary rather than primary decline agents, but the predisposing conditions are not fully understood (Fox and Curry 1980; Fox 1981). *Phoracantha impavida* was considered a contributing factor associated with *E. gomphocephala* decline in metropolitan areas in the 1980’s in response to reduced tree vigour from factors including *Haplonyx tibialis* (Fox and Curry 1980).
1.2.8 Root pathogens

The root pathogen *Armillaria luteobubalina* has been isolated from *E. gomphocephala* (Kile *et al.* 1983; Dunne *et al.* 2002); however, *E. gomphocephala* was shown to be resistant to being killed by the pathogen in natural environments (Shearer *et al.* 1998). In contrast, in urban environments *E. gomphocephala* is killed by *A. luteobubalina*, as mentioned in the discussion of Shearer *et al.* (1998).

1.2.9 *Phytophthora* pathogens

The progressive canopy thinning, dieback and the heterogeneous distribution of *E. gomphocephala* decline are similar to *E. marginata* (jarrah) dieback (Podger 1972; Podger 1973; Shearer and Tippett 1989), the declines of woodland trees such as *Pinus radiata*, *Pseudotsuga mensiesii* and *Cupressus macrocarpa* in New Zealand (Newhook 1959; Newhook and Podger 1972), and *Quercus* species throughout Europe and the America’s caused by a range of *Phytophthora* species that reduce the fine root density of diseased trees (Brasier *et al.* 1993; Jung *et al.* 1996; Jung *et al.* 2000; Rizzo and Fichtner 2009). Prior to the current study, there were no records of any *Phytophthora* species, including *P. cinnamomi*, being isolated from any plant species in the *E. gomphocephala* woodland. It is unlikely that *P. cinnamomi* poses a threat to *E. gomphocephala*, which grows predominantly on the calcaric Spearwood and Quindalup soils (Ruthrof *et al.* 2002) which are not conducive to *P. cinnamomi* infestation (Shearer and Dillon 1996). There would have been many opportunities for *P. cinnamomi* to be introduced to the *E. gomphocephala* woodland, which is situated near active, known infections in a developed urban and agricultural region. *Phytophthora cinnamomi* has been reported near Yalgorup National Park in areas of State forest east of the Old Coast Road, on Karrakatta and Bassendean soil complexes (Portlock *et al.* 1993), which are conducive to *P. cinnamomi* infestation and high disease.
impact (Hill et al. 1994; Shearer and Dillon 1996). However, it is unlikely that *P. cinnamomi* is contributing to *E. gomphocephala* decline in the Yalgorup woodland, since I have observed that highly susceptible species including *B. grandis* and *Xanthorrhoea* species, which are used as ‘indicator species’ to map *P. cinnamomi* distribution (Strelein et al. 2006), do not exhibit typical symptoms associated with *P. cinnamomi* infection.

### 1.3 Aims of the study and structure of the thesis

The aim of the studies reported in the experimental chapters, 2 to 5, was to identify the cause of *E. gomphocephala* decline. Fig. 1.2 shows research chapters within the thesis, with bold lines showing the progressive linkages between research aims and the development of the research focus. Initially, the role of soil nutrients and *Phytophthora* pathogens was investigated. In response to progressive findings, research focused on determining the role of soilborne *Phytophthora* pathogens.
**Fig. 1.2.** Research chapters of the thesis, with bold lines showing the linkages between research aims and the development of the research focus.
Chapter 2. Use of phosphite and nutrient applications as explorative tools to determine the possible causes of *Eucalyptus gomphocephala* decline in south-western Australia

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Chapter 2 Abstract

Robust tools are needed to unravel the complexity of factors contributing to tree declines around the world. Since the 1990’s *Eucalyptus gomphocephala* (tuart) has suffered a significant decline in the Yalgorup National Park, approximately 100 km south of Perth Western Australia. The effects of trunk injected phosphite, nutrients and combined phosphite and nutrients were tested on natural stands of *E. gomphocephala* that had decline symptoms. Treatment efficacy was used as an explorative tool to help determine the cause of disease and to develop management option to mitigate crown decline. In Experiment 1 the efficacy of combined treatments of trunk injections of different phosphite concentrations, trunk nutrient implants of different compositions and combined phosphite and nutrient treatments was assessed. In Experiment 2 the efficacy of different phosphite concentrations was assessed. In Experiment 1, treatments with different phosphite nutrient concentrations, increased the crown health score (CHS) by 21% over 4 years, with the greatest improvements 6 months after injection. The greatest increase in CHS occurred after injection of 25 g phosphite/L combined with 0.3 g zinc sulphide. In Experiment 2, 150 and 225 g phosphite/L increased the CHS compared to the control blank treatments with the best improvement at 150 g phosphite/L. Foliar analysis for Experiment 2 confirmed a significant uptake of phosphite for all phosphite treatments. Phosphite injection did not affect foliar nutrient concentrations. Increase in the CHS and significant flushes in new growth resulted from individual phosphite treatments and phosphite combined with a zinc sulphide and a combined (complete) nutrient treatment. This is evidence of the possible involvement of *Phytophthora* species and Zn deficiency in the decline, which was subsequently confirmed. Further work on combined phosphite and nutrient injections, with a particular emphasis on zinc, is required to help understand and potentially mitigate the *E. gomphocephala* decline.
Chapter 2. Phosphite and nutrient applications as explorative tools to determine the possible causes of *Eucalyptus gomphocephala* decline in south-western Australia

2.1 Introduction

Tree declines are malfunction in stands of trees, often leading to protracted death due to the persistent action of damaging factors or to relatively sudden trees deaths (Podger 1981; Jurskis 2005). Tree declines significantly affect natural ecosystems, plantations and urban environments, worldwide. Examples include oak declines throughout Europe (Thomas *et al.* 2002), *Eucalyptus* declines within Australia (Jurskis 2005) and declines of many other species (Ciesla and Donaubauer 1994; Jurskis 2005). Significant urban tree declines have been associated with a range of factors including insect damage (Poland and McCullough 2006) and climate stress (Yang 2009). Internationally the recent impacts of climate change including abnormal drought, flooding and heat stress have been associated with increased tree mortality, especially with other stress factors that historically may not been associated with decline (Allen *et al.* 2010). Tree declines may result from specific biotic and abiotic modifications to the ecosystem, such as the introduction of pathogens or drought stress; however, declines may involve complex disease syndromes resulting from multiple inciting and contributing factors. *Eucalyptus* declines have been associated with a range of disease syndromes, including declines associated with specific, and/or multiple inciting factors (Jurskis 2005).

Tree declines of *Quercus* species (oak) declines in northern (Jönsson 2006), central (Jung *et al.* 2000) and southern (Brasier *et al.* 1993) Europe associated with *Phytophthora* plant pathogens are typically worse on conducive sites and in the presence of secondary pathogens. Damage caused by *P. cinnamomi* on *Castanea* species (chestnut) in the United States of America (Crandall *et al.* 1945) and *E. marginata* (jarrah) in south-western Australia (Shearer and Tippett 1989; Hardy 2004), varies depending on site hydrology.
As tree declines may involve complex relationships between multiple factors, tools are required to help determine what factors are involved in the decline, and their specific relationship to each other. Fungicides with known effects can be used in studies of indigenous communities to help quantify disease (Paul et al. 1989; Newsham et al. 1994). Stem injections of other chemicals with known biological effects, such as mineral nutrients and insecticides, may also help to clarify the significance of some abiotic and biotic factors in tree decline.

Since the early 1990s, *Eucalyptus gomphocephala* (tuart) health has been steadily declining in the Yalgorup National Park woodlands on the Swan Coastal Plain south of Perth, Western Australia (Longman and Keighery 2002). The Yalgorup *E. gomphocephala* decline is expressed through a range of disease symptoms that include branch deaths and limb falls; however, the most uniform decline symptoms involves a progressive, often uniform thinning and dieback of foliage, followed by a dominance of epicormic growth, and eventual tree death. A range of biotic and abiotic factors including boring insects (Fox and Curry 1980; Fox 1981), foliar and canker pathogens (Taylor et al. 2009), changes in the soil bacterial functional diversity (Cai et al. 2010) and changes to fire regimes (Archibald et al. 2005; Archibald 2006; Close et al. 2009; Archibald et al. 2010) and hydrology (Drake 2008), have been associated with *E. gomphocephala* decline, but causal relationships have not been established. *Eucalyptus gomphocephala* provides the majority of the upper storey canopy structure and has a dominant impact on the floristic and structural complexity of the entire woodland ecosystem (Ruthrof et al. 2002). The *E. gomphocephala* woodland estate has been reduced from 111 600 ha in 1829 to 38 800 ha in 2003, predominantly from urbanization and agriculture (Tuart Response Group 2003). Disturbances such as grazing, timber harvesting and limestone quarrying (Keighery 2002) (McArthur and Bartle 1980) have impacted on ecosystem function and may have contributed to...
E. gomphocephala decline.

The progressive canopy thinning and diebacks typical of some Phytophthora-associated tree declines (Shearer and Tippett 1989; Hansen and Delatour 1999; Brasier et al. 2003b) suggest that Phytophthora species may also contribute to E. gomphocephala decline. There is no record in the literature of phosphite (= phosphonate) being applied to E. gomphocephala, however, phosphite has successfully been used to control the decline of many plant species infected with a range of Oomycetes (Cohen and Coffey 1986), including Phytophthora species (Allen et al. 1980; Saindrenan et al. 1988a; Barrett et al. 2002; Shearer et al. 2004b). The recovery of declining Quercus ilex (holm oak) and Q. suber (cork oak) trees that were later shown to be infected with Phytophthora species (Jung et al. 2000), following trunk injections with potassium phosphonate (Fernandez-Escobar et al. 1999), supports the hypothesis that Iberian oak decline is most probably caused by Phytophthora species.

Phosphite treatment is a useful diagnostic tool to determine the role of Oomycota, which include Phytophthora species, in plant declines, because phosphite: (a) specifically controls Oomycetes; (b) controls Phytophthora organisms in planta at concentrations that only partially inhibit pathogen growth in vitro (Guest and Bompeix 1984; Smille et al. 1989; Guest and Grant 1991; Hardy et al. 2001; Wilkinson et al. 2001), (c) it has extremely low toxicity to invertebrates, aquatic organisms, or animals, including humans at effective application rates (Hardy et al. 2001), and (d) is not directly metabolized by plants and does not have any direct beneficial or deleterious fertilizer effects on plant growth, as a source of phosphate (Guest and Grant 1991; Carswell et al. 1996; Thao and Yamakawa 2009). Phosphite may be metabolized and converted to plant available phosphate via soil bacteria (White and Metcalf 2007), although the conversion to plant available phosphate is slow (Adams and Conrad 1953; McDonald et al. 2001), and would therefore not negate the use of phosphite as a
Chapter 2. Phosphite and nutrient applications as explorative tools to determine the possible causes of *Eucalyptus gomphocephala* decline in south-western Australia

Phosphite may, however, exacerbate symptoms of phosphate deficiency, by suppressing the typical molecular and developmental responses of plants to phosphate deficiency (Carswell *et al.* 1996; Carswell *et al.* 1997; McDonald *et al.* 2001; Ticconi *et al.* 2001; Abel *et al.* 2002; Varadarjan *et al.* 2002). The response of declining *E. gomphocephala* to phosphite treatment may indicate the role of species of Oomycetes, including *Phytophthora* pathogens, in the disease syndrome.

South-western Australia is an ancient, semi-arid land with a diverse native flora long adapted to the nutrient poor soils to which considerable quantities of fertilizers and trace elements are applied for the economic cultivation of crops and pasture (Hodgkin and Hamilton 1993). It is possible that disturbance may have led to nutrient imbalances in mature *E. gomphocephala* trees. Host micronutrient deficiencies are associated with reduced disease resistance, known to involve polygenic mechanisms and diverse biochemical systems (Nelson 1978). Systemic nutrient implants and injections have effectively been used to correct nutrient deficiencies in ornamental and horticultural plants including *Quercus* species (Smith 1978; Harrell *et al.* 1984; Markham 1987), *Pinus* species, *Liquidambar* species (sweet gum), *Magnolia* species, *Photinia villosa* (oriental photinia) (Smith 1978), *Prunus avium* (flowering cherry), *Acer* species (maple) (Kielbaso 1978; Smith 1978; Harrell *et al.* 1984) and *Carya illinoinensis* (pecan) (Worley and Littrell 1978; Worley *et al.* 1980).

The aim of this study was to determine if *Phytophthora* species and nutrient deficiencies are associated with *E. gomphocephala* decline. As there is no record in the literature of phosphite being applied to *E. gomphocephala*, an objective of Experiments 1 and 2 was to determine the sensitivity of *E. gomphocephala* to phosphite.
2.2 Methods

Two experiments were conducted on mature stands of *E. gomphocephala* in declining woodlands. A preliminary trial with twelve *E. gomphocephala* trees found no association with insect bores and subsequently insecticides were not including within this study.

Experiment 1 was conducted as an initial explorative trial. Single trees were trunk injected with phosphite and nutrient implants either alone or in combination, in a cross-classified trial design (Table 2.1) with three replicates for each treatment interaction. Nutrient implants used within this study were selected based on nutrient deficiency symptoms (Eslick 2005) and the availability of systemic nutrient implants. Treatment efficacy was assessed using the crown condition, measured as the crown health score (CHS), foliar nutrient status, and phytotoxicity expressed as chlorotic and necrotic tissue in the foliage.

The aim of Experiment 2 was to clarify the specific role of phosphite injection, demonstrated in Experiment 1. Trees were trunk injected with different phosphite concentrations in a replicated block design with 10 replicates. Treatment efficacy was measured as per Experiment 1, and phosphite uptake was measured (as indicated below).

2.2.1 Experiment 1

Experiment 1 was established in April 2005, near Yalgorup National Park with declining trees based on mortality and poor crown health, with a crown rating scores of less than 3 out of a total of 5.4 (Grimes 1978). *Eucalyptus gomphocephala* trees with circumference over bark ranging from 91 - 399 cm [mean (±SEM) of 221.4 ± 12.7cm] and heights from 9.6 - 35 m [mean (±SEM) of 19.3 ± 0.9 m] were selected at random.
Treatment injections were allocated to individual trees by ranking trees in order of height, and evenly allocating treatments randomly across the range.

Phosphite treatments included three concentrations (25, 50 and 75 g phosphite/L) diluted with deionised water from a Foli-R-fos 400 (UIM Agrochemicals Pty Ltd, Rocklea, Queensland, Australia), a 40% (400 g/L) solution of mono-di potassium phosphite neutralised to pH 6.7 (see later section on phosphite injection) and a no phosphite control. Nutrient treatments included implants of zinc (MEDICAP ZN®), iron (MEDICAP FE®) and a combined treatment with N, P, K, Fe, Mn, Zn (MEDICAP MD®, Complete) (see later section on nutrient application and Table 2.2). The control phosphite injection was distilled H₂O and the control nutrient application were implants with no nutrients. Multiple injections, as described below, of different treatments on the same tree were spaced 20 cm apart vertically but arranged to ensure different treatments did not interfere with each other. Each tree was given a nutrient treatment, applied every 10 cm and a phosphite treatment every 20 cm, giving 1.5 treatments per 10 cm of circumference.

The effects of treatments were, measured as change in the crown health score (CHS), foliar nutrient status, and phytotoxicity expressed as chlorotic and necrotic tissue in the foliage. The dependent variable (CHS) was assessed at the time of injection and at 6 months, 1, 2 and 4 years after injection. Leaf nutrient status and phytotoxicity were measured at the time of injection and after 6 months. At the start of Experiment 1 the crown height, diameter measured at a height of 1.5 m and the original CHS were assessed and used as covariates in the analysis.
Table 2.1. Factorial design for Experiment 1, showing treatment combinations of phosphite (Phi) at 0, 25, 50, 75 g phosphite/L and Control; and nutrient implants, MEDICAP ZN® (Zinc, Zn), MEDICAP FE® (Iron, Fe), MEDICAP MD® (Complete, Com) and Nutrient Control, applied to 48 trees. Chemical composition of nutrient implants is indicated in Table 2.2.

<table>
<thead>
<tr>
<th></th>
<th>Phi 25 g/L</th>
<th>Phi 50 g/L</th>
<th>Phi 75 g/L</th>
<th>Phi 0 g/L (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zn</strong></td>
<td>Trees 1, 17, 33</td>
<td>Trees 2, 18, 34</td>
<td>Trees 3, 19, 35</td>
<td>Trees 4, 20, 36</td>
</tr>
<tr>
<td><strong>Fe</strong></td>
<td>Trees 5, 21, 37</td>
<td>Trees 6, 22, 38</td>
<td>Trees 7, 23, 39</td>
<td>Trees 8, 24, 40</td>
</tr>
<tr>
<td><strong>Com</strong></td>
<td>Trees 9, 25, 41</td>
<td>Trees 10, 26, 42</td>
<td>Trees 11, 27, 43</td>
<td>Trees 12, 28, 44</td>
</tr>
<tr>
<td><strong>No nutrient</strong></td>
<td>Trees 13, 29, 45</td>
<td>Trees 14, 30, 46</td>
<td>Trees 15, 31, 47</td>
<td>Trees 16, 32, 48</td>
</tr>
</tbody>
</table>

2.2.2 Experiment 2

Experiment 2 was established in March 2007 near Yalgorup National Park with declining trees based on mortality and poor crown health, with a crown rating scores of less than 3 out of a total of 5.4 (Grimes 1978). *Eucalyptus gomphocephala* trees of similar size with trunk circumference between 29 - 127 cm [mean (±SEM) of 72.1 ± 4.1 cm] and in height from 9.6 – 35 m [mean (± SEM) of 18.8 ± 1.0 m] and were accessible for clear crown assessment and foliar sampling were selected and measured. Individual trees were injected with one of six concentrations of phosphite (0, 75, 150, 225, 300 and 375 g phosphite/L), the control treatment of 0 g phosphite/L was comprised of distilled H₂O injections. Ten replicate trees for each treatment were randomly distributed across 10 blocks in a replicated blocks design, selected at random among available trees. Replicate blocks were spread north south over 1.9 km. The dependent variable CHS was assessed at the time of treatment injection and 1 year after injection. The dependent variables phosphite uptake and phytoxicity were measured 3 months after injection. Foliar nutrient concentration was determined 3 months after treatment injection on trees...
treated with phosphite at 150 g/L and the control trees without phosphite injection. At the start of Experiment 2 the crown height, diameter measured at a height of 1.5 m, and the original CHS, were assessed and used as covariates in the analysis.

2.2.3 Sites
The vegetation community type for both experiments was Southern *E. gomphocephala* – *A. flexuosa* woodland (Donald and Prescott 1975), dominated by *E. gomphocephala* exhibiting typical decline symptoms observed throughout the Yalgorup region. The location of both experimental sites is indicated in Appendix 1.

Experiment 1 was located 88.5 km south of Perth Western Australia, 32.708143°S and 115.637193°E, covering 0.96 hectares, adjacent to Yalgorup National Park. For Experiment 1, the site was low lying and flat, and situated approximately 1.5 km north of Lake Clifton in an area that appears to be intermediately inundated with water, as indicated by the exposed roots of large trees and a close proximity to a swamp comprised of *Melaleuca* species.

Experiment 2, was located approximately 100 km south of Perth Western Australia, 33.8711°S and 115.7149°E, in State forest comprised of 10 replicate blocks less than 0.5 km in width and spanning 2.5 km in a north south direction. The topography varied throughout the site for Experiment 2: however, the ground was predominantly undulating, with no clear indication of regular flooding.

2.2.4 Sampling and Phytophthora isolation
To determine the presence of *Phytophthora* species, rhizosphere soil was sampled at the start of Experiment 1 in April 2005, and at the start of Experiment 2, in March 2007. For Experiment 1, five randomly selected trees were sampled, while for Experiment 2, each treated tree was sampled. Four soil-root-monoliths of about 10 x 10 x 10 cm were
taken around each tree base sampled, mixed and a 1 litre subsample was randomly collected and tested for the presence of *Phytophthora* species, according to the methods of Marks and Kassaby (1974) and Stukely *et al.* (1997).

In May and June 2007, *Phytophthora* isolates were recovered from the rhizosphere of declining *E. gomphocephala*, *E. marginata* and *A. flexuosa* in Yalgorup National Park using modifications to the baiting and isolation techniques as outlined by Jung *et al.* (1996; 2000), which were then identified and published as the new species *P. multivora* (Scott *et al.* 2009, Chapter 4). Both experiments were then re-sampled in September 2007, and the harvested rhizosphere material was processed for *Phytophthora* species as described in Scott *et al.* (2009, Chapter 4).

### 2.2.4 Phosphite injection

Phosphite was applied at a rate of 1 mL/cm trunk circumference (Shearer *et al.* 2004b; Shearer *et al.* 2006) and injections of 20 mL given at every 20 cm of circumference. Holes were drilled around the trunk 1.5 m above ground level through the outer bark layer into the sapwood at 20 cm intervals with a 6.5 mm drill bit and the phosphite solutions were injected using 20 mL spring-loaded tree syringes that lock tightly into the trees (Chemjet Pty Ltd, Bongaree, Queensland, Australia) (Shearer *et al.* 2004b; Shearer *et al.* 2006). Where required, the thick outer bark layers were removed using a 20 mm diameter spade drill bit.

### 2.2.5 Nutrient application

Three nutrient treatments were included in Experiment 1: zinc, iron, and combined nutrient (iron, manganese, zinc, nitrogen, phosphorus and potassium) MEDICAP® (Creative Sales, Inc., Fremont, Nebraska, United States of America) (Table 2.2). The three nutrient treatments were applied as a capsule injection in accordance with the
manufacturer’s instructions following protocols outlined on the MEDICAP Material Safety Data Sheets. Capsules were applied around the trunk canopy circumference, at a height of between 0.5 and 1 m. Capsule diameters were 0.95 cm (3/8 inch) and were applied as close as possible to the manufacturer’s recommended spacing’s (zinc = 10.16 cm, iron = 10 and combined nutrients = 10 cm) apart around the circumference. Holes were drilled with a 0.95 cm bit approximately 3.2 cm into the cambium, drill shavings were removed and the capsules were manually inserted with a 6 mm diameter section of wooden dowel, until the capsule plug was flush with the cambium.

### Table 2.2. Composition of nutrient and capsules.

<table>
<thead>
<tr>
<th>Nutrient Treatment</th>
<th>Weight per Capsule</th>
<th>Capsule Constituents</th>
<th>Dose (mg/10cm trunk circumference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc - (MEDICAP ZN&lt;sup&gt;®&lt;/sup&gt;)</td>
<td>1.0 g</td>
<td>Zinc sulphide</td>
<td>300</td>
</tr>
<tr>
<td>Iron - (MEDICAP FE&lt;sup&gt;®&lt;/sup&gt;)</td>
<td>1.0 g</td>
<td>Ammonium iron (II) citrate about 28% Fe</td>
<td>448</td>
</tr>
<tr>
<td>Complete 12-4-4 nutrient - (MEDICAP MD&lt;sup&gt;®&lt;/sup&gt;)</td>
<td>0.8 g</td>
<td>Iron</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Manganese</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zinc</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total Nitrogen</td>
<td>960</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ammoniacal Nitrogen</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrate Nitrogen</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urea Nitrogen</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Available Phosphoric Acid</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soluble Potash</td>
<td>32</td>
</tr>
</tbody>
</table>

### 2.2.6 Crown assessment

Crown assessment for each tree was determined to the north of the tree at approximately the same distance as crown height, and from the same position for all assessments. Crown condition was determined by ranking characteristics of the crown condition, against stylised images and a written description of characteristics of crown decline, or dieback (Grimes 1978). Characteristics of crown dieback included the relative crown
position, crown size, crown density, proportion of dead branches, and proportion of epicormic growth. The scale for each crown characteristic was originally determined using *Corymbia maculata* (synonym *E. maculata*), *E. fibrosa* and *E. drepanophylla* (Grimes 1978). The crown condition for each tree was determined by adding the scores for each characteristic for each tree to give the crown health score (CHS). The CHS for each tree was rated on site and verified against digital images captured with a Fujifilm FinePix S500® digital camera, at each assessment. To reduce variation between repeated assessments of each tree, the assessments were done by the same person and in the same order and where possible, assessments were only performed on cloudless days. For Experiment 1, the reliability of the crown assessment method was tested by the same observer assessing the treated trees on a separate occasion within two weeks of the first assessment without reference to the original results. The two assessments were compared using regression analysis and were significantly correlated ($r = 0.70$).

### 2.2.7 Foliar nutrient assessment

Leaf nutrient status was determined for trees in both experiments from twenty youngest fully expanded leaves per tree, selected at random throughout the upper 40% of the crown to ensure leaves were not shaded and had approximately uniform constant light exposure. For Experiment 1, leaves were collected 6 months after treatment, while for Experiment 2 leaves were collected 3 months after treatment. Leaves were removed using a Bigshot® Slingshot (Sherrill, Greensboro, North Carolina, United States of America) to propel a weighted bag with an attached rope over desired branches which were then pulled to the ground. The leaves were handled with powder-free latex gloves, bulked for each tree and transferred into a fan forced oven at 70 °C for 48 hours, to reach a constant mass. The dried leaves were ground in a stainless steel mill and a 4 g subsample was analysed by CSBP Limited (Bibra Lake, Western Australia, Australia).
The samples were ground and microwave digested in concentrated HNO₃. The concentrations of Ca, Cu, Fe, K, Mg, Mn, Na, P, S and Zn were determined using an inductively coupled plasma analyser (Perkin Elmer, Connecticut, United States of America). To determine the concentration of N, finely ground plant material was combusted at 950°C in oxygen using a Leco FP-428 Nitrogen Analyser, that measured the released nitrogen from the sample as it passed through a thermal conductivity cell.

2.2.8 Phosphite uptake

For Experiment 2, the phosphite concentrations within the leaves were determined for each trunk injected tree, from 10 randomly selected, youngest fully expanded leaves harvested 3 months after treatment, at the same time as leaves used for foliar nutrient analysis. Leaves were handled with powder free latex gloves and bulked for each tree. Leaves were then washed in 1% Deconex® 15E phosphate free detergent (In vitro Technologies, Noble Park North, Victoria) solution and rinsed twice in DI water. All samples were dried at 60°C for 4 days, ground to 1 mm and sent to the WA Chemistry Centre (Perth, Western Australia, Australia) for phosphite analysis. To each 0.5 g ground sample, 5 mL of 0.1 M sulphuric acid was added and extractions occurred overnight on a roller-shaker. Following 20 min centrifugation at 6970 g, 100 μL of the clear acid extract was added to 1 mL of 50 μg/mL methyl phosphonic acid in methanol (internal standard solution). A phosphite standard curve was prepared by adding 100 μL of solutions containing from 0.05 to 100 μg/mL phosphite to 11 tubes containing 1 mL of internal standard solution. The solutions were mixed and diazomethane was added to 400 μL of the samples in excess until a persistent yellow colour was observed. Excess diazomethane was neutralised with a few drops of 2% acetic acid, then the dimethyl phosphite content was determined by gas chromatography. A split less injection with a D.B-Wax column (J & W Scientific, Salsam, California, United States of America) and
a phosphorous-specific flame photometric detector (Hewlett Packard, United States of America) were used. The limit of quantitation was 0.5 μg/g dry weights. A replicate sample was taken every 10 samples to provide a control during the analysis. Two control samples of known phosphite content were included in each batch of 40 samples analysed.

2.2.9 Phytotoxicity
Phytotoxicity symptoms were determined on 20 leaves from each tree. Leaves were randomly collected from around the tree, from representative branches removed from the upper 60% of the crown, four weeks after phosphite injection. Phytotoxicity was measured as the percentage area of harvested leaves exhibiting chlorosis or necrosis (Barrett 2001; Hardy et al. 2001; Barrett et al. 2004) on both the abaxial and adaxial surfaces. Symptoms of phytotoxicity were compared to images by Barrett (Barrett 2001)

2.2.10 Statistical analysis
Analyses were carried out in Statistica software package Version 5 (Statsoft 1999). Assumptions of normality were checked by plotting residuals (Clarke and Warwick 2001). For both experiments the CHS for each time in the analysis is expressed as a ratio of CHS at that time to the CHS at time 0 (when the trial began). Changes in the CHS were transformed to square root arcsin values to homogenize the variance. Where appropriate, the Pearson correlation coefficient was calculated for a measure of association between variables. For both Experiments 1 and 2, the crown height, diameter at 1.5 m height, and CHS at the time of treatment application were tested as covariates in the analysis. Significance was determined for both experiments at $P \leq 0.05$. 
Experiment 1, the dependent variable CHS was analysed as a cross classified (factorial) multivariate analysis of variance (MANOVA) with fixed factors of phosphite (concentrations 25, 50 and 75 g/L and phosphite control), nutrients (zinc, iron, complete nutrients and nutrient control) and time (6 months, 1 year, 2 years and 4 years) as the repeat measure factor, and the CHS as the dependent variable. The effect of injection treatments on foliar nutrient concentrations were analysed individually for each measured nutrient as a factorial repeated measures MANOVA with fixed factors of applied phosphite and nutrients injections, and time (at application 0 months and 6 months) as the repeat measure factor. The Bonferroni correction was not applied, in accordance to Moran (Moran 2003).

Experiment 2, the dependent variable CHS was analysed as an ANOVA, measured one year after treatment application with different phosphite concentrations 0 (control) 75, 150, 225, 300 and 375 g/L. Phosphite uptake and phytotoxicity were analysed as an ANOVA, measured 3 months after treatment application for all injection concentration. Foliar nutrient concentration was analysed as an ANOVA, measured 3 months after treatment application for phosphite at 150 g/L and the control trees without phosphite injection.

2.3 Results

No *Phytophthora* species was isolated at the start of Experiment 1 in April 2005 or at the start of Experiment 2 in March 2007. However, in September 2007, *P. multivora* was isolated throughout both sites for Experiment 1 and Experiment 2.

The covariates of crown height, diameter measured at a height of 1.5 m, and CHS at treatment application, were not significantly different between treatment variables for Experiment 1 ($P \leq 0.24$) and Experiment 2 ($P \leq 0.26$).
2.3.1 Experiment 1

The treatment interaction of phosphite, nutrients and time was not significant \( (P = 0.13) \): however, the treatment interaction of phosphite with nutrients had a significant \( (P = 0.05) \) influence on the CHS (Fig 2.1). The greatest improvement of a 56.4% increase in the CHS resulted from phosphite at 25 g/L combined with MEDICAP ZN treatments.

In the treatments without nutrient injections, trees injected with phosphite at 25 and 50 g/L did not significantly vary from the control; however, treatments with phosphite at 75 g/L significantly improved CHS by 40.5% compared to the control. MEDICAP FE treatments did not result in a significant improvement in CHS, compared to the control; however, the average CHS of iron-phosphite treatments did increase when phosphite was increased from 25 g/L to 75 g/L.

In the treatments without phosphite injections MEDICAP ZN and MEDICAP MD treatments did result in a significant improvement of 13.0 and 37.4% CHS, respectively. Analysis of the combined treatments showed that all MEDICAP ZN phosphite treatments resulted in a significant improvement in CHS compared with the control; however, the improvement decreased with increasing phosphite concentration. Complete nutrients together with phosphite at 25 and 75 g/L, significantly improved the CHS by 42.6 and 21.4% respectively compared with the control. The treatments of complete nutrient and phosphite at 50 g/L did not significantly vary from the control. No individual treatment resulted in a significant decrease in the CHS compared to the control (Fig. 2.1).
**Chapter 2.** Phosphite and nutrient applications as explorative tools to determine the possible causes of *Eucalyptus gomphocephala* decline in south-western Australia

![Fig. 2.1](image)

**Fig. 2.1.** Mean (± standard error) for percentage change in the crown health score (CHS) of *Eucalyptus gomphocephala* trees averaged over 4 years after trunk injection with: phosphite (Phi); nutrient ( ); and phosphite plus nutrient treatments. Individual treatments are represented by combinations of Phi 25, 50 and 75 corresponding to phosphite concentrations of 25, 50 and 75 g phosphite/L; and nutrient treatments of MEDICAP ZN® (zinc - Zn), MEDICAP FE® (iron - Fe) and MEDICAP MD® (nitrogen, phosphorus, potassium, iron, manganese, zinc - Com). Crown condition was measured as the CHS (Grimes 1978), and is represented as the percentage change in CHS compared to CHS at the time of treatment, averaged over four years of assessment. Statistics are for one-way ANOVA. Treatment interactions analysed with the post hoc test (Dunnett) showing a comparison with the control group and * indicating significant differences to the control group at $P \leq 0.05$.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>25</th>
<th>50</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrients</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Zn</td>
<td>Fe</td>
<td>Com</td>
<td>Zn</td>
<td>Zn</td>
<td>Zn</td>
<td>Fe</td>
<td>Fe</td>
<td>Com</td>
</tr>
<tr>
<td>Phosphite</td>
<td>Phi</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

All measured foliar nutrient concentrations were not significantly ($P > 0.07$) influenced by the main treatments or interaction of phosphite and nutrient treatments 6 months after injection.

Phytotoxicity was predominantly observed as regions of localized patchy necrosis although some non-necrotic leaf patches were chlorotic. No treatment within Experiment 1 resulted in phytotoxicity symptoms covering more than 5% of the sampled leaf surface. Phytotoxicity was only clearly exhibited on trees treated with 75 g phosphite/L combined with the complete nutrient treatment.
2.3.2 Experiment 2

Phosphite treatments (Fig. 2.2) did not significantly ($P = 0.13$) affect CHS and phosphite concentration within harvested leaves was not correlated ($r^2 = 0.01$) with CHS. However, a *priori* analysis indicates that the combined CHS of all trees trunk injected with different phosphite treatments was significantly ($P = 0.05$) higher than the control treatments.

By 1 year after phosphite injection there was a significant nonlinear relationship between injected phosphite concentration and percentage change in CHS, with greatest improvement of CHS for trees injected with 150 g phosphite/L and least for trees not injected with phosphite: Percentage change CHS = -29.10 + 25.32 Phosphite concentration - 5.29 Phosphite concentration$^2$ + 0.30 Phosphite concentration$^3$ ($r^2 = 0.90$).

**Fig. 2.2.** Mean (± standard error) for percentage change in crown health score (Grimes 1978) of *Eucalyptus gomphocephala* trees, one year after trunk injection with phosphite at concentrations of 0 (control), 75, 150, 225, 300 and 375 g/L phosphite/L at a rate of 1 mL/cm of trunk circumference at 1.5 m height.
Foliar nutrient concentrations were not significantly different \((P = 0.91)\) between control trees without phosphite and trees treated with phosphite at 150 g/L, 3 months after injection.

Phosphite concentrations in leaves from phosphite trunk injected trees were significantly \((P < 0.01)\) greater for all phosphite treatments compared to the control. Phosphite concentration in leaves was significantly positively correlated with injected phosphite concentration: Foliar phosphite concentration \(= 3.51e^{0.87} \) Treatment phosphite concentration \((r^2 = 0.82)\) (Fig. 2.3).

![Graph](image)

**Fig. 2.3.** Mean (± standard error) for phosphite concentrations, measured as phosphorus acid in leaves of *Eucalyptus gomphocephala* trees trunk injected with 0 (control), 75, 150, 225, 300 and 375 g/L phosphite/L at a rate of 1 mL/cm of trunk circumference at 1.5 m height.

Foliar phytotoxicity occurred for phosphite concentrations greater than 225 g/L with the highest measured phytotoxicity of 5% of the foliage exhibiting burn symptoms for 375 g/L. The expressions of phytotoxicity on harvested leaves were similar in both experiments. Phosphite treatment did not have a significant \((P = 0.99)\) effect on the
foliar nutrient concentration.

2.4 Discussion

2.4.1 Phosphte injection

Phosphite trunk injection at concentrations of 75g/L improved the crown health of *E. gomphocephala* trees. This is similar to the concentrations shown to be effective in other species known to be infected with *P. cinnamomi*. Trunk injections of 75, 100 and 200 g phosphite/L, significantly improved the health of *Persea americana* (avocado) trees affected by *Phytophthora* root rot, 22 months after injection treatments (Whiley *et al.* 1991). Trunk injections of 50, 100 and 200 g phosphite/L of naturally grown *B. grandis* and *E. marginata* controlled lesion extension of *P. cinnamomi* in wound inoculated plants (Shearer and Fairman 2007). In addition phosphite concentration in planta was directly related to the injection rate. The improvement in the crown health of *E. gomphocephala* trees following trunk injection with phosphite at concentrations of 75g/L or 150g/L may be evidence that *Phytophthora* pathogens, have contributed to the CHS of *E. gomphocephala*.

2.4.2 *Phytophthora* isolation

No *Phytophthora* species were recovered from either site at the start of Experiment 1 in April 2005, or Experiment 2 in March 2007. However, *P. multivora* was isolated in September 2007 from both sites, using the modified isolation techniques of Jung *et al.* (1996; 2000). In September 2007, *P. multivora* was isolated from five tree selected at random throughout Experiment 1 and from each replicate block, from a bulk sample collected from each tree treated tree, for Experiment 2. *Phytophthora multivora* may not have originally been isolated because of entrenched dormant resting structures.
(Bunny 1996), low population density or problems with the isolation technique.

*Phytophthora multivora*, originally identified as *P. citricola* using morphological techniques, has been episodically isolated from rehabilitated bauxite mine sites since 1992 in the jarrah forest on the Darling Scarp, usually after major rainfall events (Hardy personal communication). Similarly, Bunny (1996) needed to repeatedly flood natural soil samples to isolate a *Phytophthora* species, identified using morphological techniques as *P. citricola*, that was probably *P. multivora*. Only one isolate from this study is available (DEC 236 VHS culture), which has since been identified as *P. multivora* based on molecular re-evaluation (Scott *et al.* 2009, Chapter 4). Bunny (1996) identified rainfall as an important factor influencing the distribution of *P. citricola* (probably *P. multivora*) isolates, with greatest isolation frequency when rainfall was above 800 mm and much lower isolation frequency at rainfall below 500 mm. However, in 2005 when trees in Experiment 1 were originally sampled and no *Phytophthora* species were isolated, the annual rainfall was 915.22 mm (BoM 2011b), compared to 649.40 mm of rain in 2007 (BoM 2011b) when *P. multivora* was isolated from both experiments.

*Phytophthora multivora* may not have been isolated in 2005 because the isolation methods were inadequate: however, the methods of Marks and Kassaby (1974) and Stukely *et al.* (1997) have been successfully used to isolate *Phytophthora* species in south-west Western Australia by the Vegetation Health Service of the Department of Environment and Conservation, as indicated in Burgess *et al.* (2009). Briefly the methods of Jung *et al.* (1996; 2000) varied from those of Marks and Kassaby (1974) and Stukely *et al.* (1997), in that the soil was moistened for 12 hours prior to flooding, surface organic material was removed prior to setting baits, and *Quercus* species baits were used instead of *E. sieberi* cotyledons.
2.4.3 Variation in phosphite effectiveness between sites

Phosphite treatments in Experiments 1 and 2 may have resulted in varying improvements in crown condition because of differences between the sites. Trees in Experiment 1 were taller and had larger circumferences than trees in Experiment 2. Differences in tree age between sites, may account for variation in phosphite effectiveness. Increased host age has been associated with increased sporangia production and disease severity in a range of disease syndromes, including diseases in *Pinus* species caused by *P. cinnamomi* (Newhook 1959; Newhook 1970). Increased host age has also been proposed as a significant contributing factor in the susceptibility of *Quercus* species to *P. quercina* and other *Phytophthora* species (Jung et al. 2000; Jönsson 2006). Trees in Experiment 1 may also have been more susceptible to *Phytophthora* species as they were growing on a site that appeared to be intermittently inundated with water. Experiment 1 was located on a Vasse complex soil type, which is derived from marine, lagoonal and estuarine deposits, associated with low lying topography, susceptible to water logging and associated with wetland species including *Melaluca* species (McArthur and Bartle 1980). Waterlogging has been shown to be associated with increased infection by *P. cinnamomi* (Dawson and Weste 1982; Davison 1994). Experiment 2 was located on the eastern margin of the Spearwood complex dune system, associated with hilly ridges and deep sands, resulting from the leaching of older dunes and through wind accumulation (McArthur and Bettenay 1960; Seddon 1972; McArthur and Bartle 1980; McArthur 1991). Waterlogging was not observed on this site during the experiments.

2.4.4 Phosphite plus nutrient treatments

Improvement in the CHS resulting from phosphite injection may have resulted from the suppression of *Phytophthora* soil pathogens; however, further work is required to
confirm this relationship. Combined treatments of phosphite and MEDICAP ZN resulted in greater improvements in the CHS than individual treatments with MEDICAP ZN. Synergistic treatment interactions may have resulted from a range of direct and indirect effects. MEDICAP ZN application may have improved the CHS by amending an underlying nutrient deficiency within the plant, decreasing the susceptibility to pathogens including *Phytophthora* species, or through a combination of both.

Combined treatments of MEDICAP MD and phosphite at 25 g/L, resulted in greater improvements in the CHS, than phosphite application at higher concentrations. Phosphite concentrations above 25 g/L, in combination with MEDICAP MD may therefore negate the effectiveness of individual MEDICAP MD injections, through mechanisms which may include subclinical stress.

### 2.4.5 Nutrient application

A range of nutrient applications improved crown health suggesting that declining trees are suffering nutrient deficiencies and have reached an eco-physiological limit, which is partially ameliorated by some nutrient implants. Nutrient applications may have increased crown health by ameliorating an underlying nutrient deficiency, indirectly by stimulating fine root/ectomycorrhizal development/function or helping to control an underlying disease or combinations of these factors. Further work is required to resolve how different nutrient applications improve crown health.

All nutrient amendments used within this trial have been shown to have specific associations with disease expression. For example, zinc deficiencies predispose *Carya* species to *Mycosphaerella dendroides* and *Cerospora fusca* pathogens (Moznette 1940) and deficiencies in manganese increase disease expression in *Solanum tuberosum* caused by *P. infestans* (Thompson and Huber 2007). Nutrient amendments may reduce disease expression through either increasing resistance, or increasing tolerance to
pathogens (Graham and Webb 1991).

Many Australian soils are naturally deficient in zinc, for crop and pasture production (Donald and Prescott 1975; Brennan 1990). Disease symptoms of Pinus plantations grown on calcareous soils within the E. gomphocephala forest north of Ludlow, were amended with zinc application (Kessell and Stoate 1936; Kessell and Stoate 1938). Zinc has been shown to be particularly deficient for Eucalyptus plantations grown on calcareous soil and disturbed sites (Dell and Wilson 1985; Haridasan 1985; Wallace et al. 1986; Dell and Wilson 1989). In acid soils, the availability of zinc for plant uptake has been reduced by the addition of calcium carbonate (Lucas and Knezek 1972). The availability of zinc on calcareous soils, such as those on which E. gomphocephala occurs, is believed to be reduced as the alkaline pH limits the availability of compounds of low solubility (Clarke and Graham 1968; Saeed and Fox 1977) or because the zinc is adsorbed by carbonates (Udo et al. 1970). Eucalyptus gomphocephala may have a specific productivity advantage over other eucalypt species via unique mechanisms facilitating the uptake of zinc on the calcareous soils including associations with mycorrhizal fungi (Legault 2005).

Arbuscular mycorrhizae have been shown to significantly increase zinc uptake in Trifolium species on calcareous soils (Chena et al. 2002). Zinc application has also been associated with significant increases in root growth of Triticum species (Dong et al. 1995). The association of Zinc deficiency with E. gomphocephala decline may have resulted from multiple factors including a loss of fine root function and associated beneficial mycorrhizae that facilitate zinc uptake, caused by Phytophthora root pathogens.

2.4.6 Phosphite phytotoxicity

None of the treatments in either experiment, caused levels of phytotoxicity which
significantly reduced the CHS compared to the control trees. Within Australian natural ecosystems, phosphite is typically applied with trunk injections at concentrations between 50 and 200 g/L depending on the sensitivity of the species to phytotoxicity (Hardy et al. 2001). Low phytotoxicity was observed at injected concentrations greater than 225 g phosphite/L, suggesting that *E. gomphocephala* was able to withstand phosphite at high concentrations *in planta* when compared with levels that cause significant toxicity and death in other *Eucalyptus* species (Aberton et al. 1999; Barrett et al. 2004). The low susceptibility of *E. gomphocephala* to phosphite phytotoxicity may be a unique characteristic, as most Myrtaceae are phosphite sensitive (Hardy et al. 2001). It would be useful to determine how the metabolism and translocation of phosphite in *E. gomphocephala* contributes to its low phytotoxicity. Further work is required to determine the exact impact of phosphite phytotoxicity on *E. gomphocephala*. As the improvement in the CHS appears to decrease above phosphite concentrations of 225 g/L; it is likely that the apparent optimal injection rate of 150 g/L will not result in phosphite phytotoxicity that is deleterious to plant growth and health.

### 2.4.7 Future research

The effectiveness of nutrient applications in improving crown health, confirms that declining trees have reached an eco-physiological limit. Further work is required to understand what eco-physiological limits declining *E. gomphocephala* have reached, and how nutrient applications improve crown health. For example, the role of zinc deficiency within *E. gomphocephala* decline may be resolved through further injection trials using Zinc alone, or through soil amendment treatments.

Phosphite *in planta* has been shown to suppress *Phytophthora* species at concentrations that only partially inhibit pathogen growth *in vitro* (Guest and Bompeix
1984; Smille et al. 1989; Guest and Grant 1991; Hardy et al. 2001; Wilkinson et al. 2001). Phosphite itself has no beneficial effect on the growth of healthy plants, and is not a direct P nutrient source (Thao and Yamakawa 2009). The effectiveness of phosphite in controlling *P. multivora* on *E. gomphocephala* needs to be directly measured *in situ* in both under-bark inoculated stems and on naturally infected trees.

An understanding of the mechanisms of phosphite activity on crown condition is required to help determine the cause of *E. gomphocephala* decline. The use of phosphite as an exploratory tool, to help determine the role of *Phytophthora* species, is a new method for assessing disease aetiology.

Further research is required to explain why phosphite application in Experiments 1 and 2 resulted in varying improvements in crown condition.

Changes in crown health were measured using categorical techniques. Repeat assessments of the same tree may not be entirely consistent as the assessors skills would improve and change over time. To improve the repeatability of assessments, changes in crown health should include non-destructive quantitative physiological measurements of plant water relations and photosynthesis.

Since starting this study Koch's postulates have been satisfied as *P. multivora* has been isolated from the rhizosphere of declining *E. gomphocephala* woodland trees suffering significant fine root loss. The rhizosphere of *E. gomphocephala* seedlings grown under controlled conditions was infested with *P. multivora* and subsequently suffered significant fine feeder root loss compared to controls. *Phytophthora multivora* was then reisolated from the rhizosphere and roots of these artificially infected seedlings (Chapter 5). This study is a novel example of using phosphite injections and nutrient implants, to help determine the cause of a disease.
Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth

Submitted as: Scott PM, Shearer BL, Barber PA, Hardy GESJ (2011) Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth. *Australasian Plant Pathology*.

Minor authors’ contributions: Barber PA, Shearer BL and Hardy GESJ (academic) were supervisors.
Chapter 3 Abstract

Two *ex situ* seedling bioassays, one *in situ* woodland tree assay and one *in situ* seedling bioassay were used to examine the biotic causes of *Eucalyptus gomphocephala* (tuart) woodland decline in the Yalgorup region, approximately 100km south of Perth, Western Australia. The initial seedling bioassay compared the influence of soils harvested from healthy and declining *E. gomphocephala* sites on *E. gomphocephala* seedlings *ex situ*. A tree assay measured the relationship between the crown health and the fine root and ectomycorrhizal total density scores (TDS) on 18 *E. gomphocephala* trees in declining sites. The relationship between crown health score and fine root and ectomycorrhizal TDS of these *E. gomphocephala* trees and the health of *E. gomphocephala* seedlings were further assessed *in situ* in the second seedling bioassay and *ex situ* in the third seedling bioassay. No soilborne *Phytophthora* species were isolated from sites in this study in 2005 and 2006 but they were shown to be infected with *P. multivora* in 2007, suggesting that the pathogen was present during/prior to the study but was not isolated because of variable population density or dormancy factors. Crown health was significantly correlated with the fine root and ectomycorrhizal TDS’s and trees with crown decline symptoms had significantly less fine roots and ectomycorrhizae than trees with healthy crowns. Decline symptoms of woodland *E. gomphocephala* trees were not seen in seedlings grown *ex situ* in harvested woodland soil. For seedlings grown *in situ* in association with woodland trees the survival, height and foliar health significantly correlated with the crown health of the adjacent woodland trees and seedling survival was significantly correlated with ectomycorrhizae TDS. Seedling survival was significantly less for those grown in association with trees with crown decline symptoms than those grown in association with trees with healthy crowns. The seedling bioassays indicate that decline symptoms may be associated with biotic
components of soils including root pathogens and/or the absence of ectomycorrhizae. The study demonstrates new techniques for assessing the fine root and ectomycorrhizal densities of large woodland tree species and their relationships to crown health.

3.1 Introduction

*Eucalyptus gomphocephala* (tuart) has undergone a significant decline since the early 1990’s, throughout its distribution along the coastal dune belts of the Swan Coastal Plain, Western Australia, as indicated in chapter 1.

Greater knowledge is required about the health of roots and ectomycorrhizae, and role of plant pathogens. A wide variety of ectomycorrhizal fungi has been associated with *E. gomphocephala* and is believed to be both affected by and involved in *E. gomphocephala* decline (Legault 2005). Ectomycorrhizal fungi, where the fungal hyphae radiate out beyond the root and into soil particles (Harley 1989), play an important role in nutrient acquisition by *Eucalyptus* species (Marschner and Dell 1994) including *E. gomphocephala* (Malajczuk et al. 1982). Micronutrient deficiencies associated with *E. gomphocephala* decline, particularly of zinc (Eslick 2005), may be explained by loss of mycorrhizae and fine roots.

Various *Phytophthora* species have been associated with native woodland trees decline in the south-west Western Australia (Shearer et al. 1987; Burgess et al. 2009; Scott et al. 2009). The introduced multi-host, soilborne plant pathogen, *P. cinnamomi* has caused significant deaths in the south-west Botanical Province of Western Australia with approximately 40% of the 5710 species regarded as susceptible (Shearer et al. 2004a). Prior to the isolation of *P. multivora* in 2007 (Scott et al. 2009, Chapter 4), no *Phytophthora* species had been isolated from *E. gomphocephala*; however, the expression and epidemiology of the decline are similar to other woodland declines.
associated with *Phytophthora* pathogens. The progressive canopy thinning, dieback and the patchy distribution of *E. gomphocephala* decline, are similar to expression of *E. marginata* (jarrah) dieback (Podger 1972; Podger 1973; Shearer and Tippett 1989) and declines of woodland trees in New Zealand (Newhook 1959), and *Quercus* species throughout Europe and the America’s caused by a range of *Phytophthora* species that reduce the fine root density of diseased trees (Brasier et al. 1993; Jung et al. 1996; Jung et al. 2000; Rizzo and Fichtner 2009).

The relationship between the crown, fine roots and ectomycorrhizae of *E. gomphocephala* trees exhibiting decline were studied to determine if tree health is influenced by the biotic components of the woodland soil including *Phytophthora* species. Four experiments were conducted:

Experiment 1 - *Ex situ* seedling bioassay 1 ‘healthy and a declining site’, aimed to determine if site (healthy and declining), depth from which soil samples were collected, and pasteurization affected growth of tuart seedlings, and if *Phytophthora* species were associated with either site.

Experiment 2 - *In situ* woodland tree assay, aimed to determine if the crown health of declining *E. gomphocephala* woodland trees was correlated with density of fine root and ectomycorrhizae and if soilborne *Phytophthora* species were associated with declining *E. gomphocephala* woodland trees.

Experiment 3 - *In situ* seedling bioassay ‘declining sites’, aimed to determine if declining *E. gomphocephala* woodland trees affected the health of *E. gomphocephala* seedlings, planted throughout the fine root mats of declining trees and if soilborne *Phytophthora* species were associated with declining *E. gomphocephala* woodland trees.

Experiment 4 - *Ex situ* seedling bioassay 2 ‘declining sites’, aimed to determine if the health of *E. gomphocephala* seedlings grown *ex situ* in pasteurised or non-
pasteurised soil from trees was associated with symptoms of *E. gomphocephala* woodland trees and if soilborne *Phytophthora* species were associated with declining *E. gomphocephala* woodland trees.

### 3.2 Methods

Experiments were conducted in *E. gomphocephala* dominated woodland, with some *E. marginata* and other woodland trees. Site 1 was located on private land approximately 39 km North of Yalgorup, and sites 2 to 5 were in Yalgorup National Park (Table 3.1, Appendix 1).

**Table 3.1. Decline status and location (Latitude and Longitude) of woodland sites used for Experiments 1, 2.1, 2.2 and 2.3.**

<table>
<thead>
<tr>
<th>Sites</th>
<th>Decline status</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude (S)</td>
<td>Healthy</td>
<td>32.395483°</td>
<td>32.684400°</td>
<td>32.659677°</td>
<td>32.686683°</td>
<td>32.687969°</td>
</tr>
<tr>
<td>Longitude (E)</td>
<td>Declining</td>
<td>115.796986°</td>
<td>115.639203°</td>
<td>115.622919°</td>
<td>115.643597°</td>
<td>115.637025°</td>
</tr>
<tr>
<td>Experiment. 1</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Experiments. 2.1, 2.2 and 2.3</td>
<td>✓</td>
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</tr>
</tbody>
</table>

The region has a Mediterranean climate and receives approximately 649.4 mm rainfall annually, mainly over winter (June–August), and has a mean monthly temperature range from 10.6 to 29.2 °C (2002-2010) (BoM 2011b). Actual annual rainfall for Experiment 1 (2005), was 915.22 mm, and Experiments 2.1, 2.2 and 2.3 was 434.8 mm (2006) (BoM 2011b).

**3.2.1 Experiment 1 - *Ex situ* seedling bioassay 1 ‘healthy and a declining site’**

The aim of Experiment 1 was to determine if site (healthy and declining), depth from which soil samples were collected, and pasteurization affected growth of tuart seedlings, and if *Phytophthora* species were associated with either site.
Independent variables were soils harvested from a healthy (site 1) on declining site (site 2), soil depth (0-15 cm or 15-30 cm), and soil pasteurisation (pasteurised, non-pasteurised) to give a total of eight soil treatments, in a factorial design, representing the following interactions:

1. Healthy site, 0-15 cm depth, Non-pasteurised;
2. Healthy Site, 0-15 cm depth, Pasteurised;
3. Healthy Site, 15-30 cm depth, Non-pasteurised;
4. Healthy Site, 15-30 cm depth, Pasteurised;
5. Declining site, 0-15 cm depth, Non-pasteurised;
6. Declining site, 0-15 cm depth, Pasteurised;
7. Declining Site, 15-30 cm depth, Non-pasteurised; and
8. Declining Site, 15-30 cm depth, Pasteurised.

_Eucalyptus gomphocephala_ seedlings were grown in the eight soil treatments collected and treated in spring (October) 2005. Seedlings were grown in the different soil treatments, which were arranged in a randomized block design with 10 replicate pots per treatment, to give a total of 80 pots. Dependent variables were seedling height and foliar health at 3 and 6 months of age, the presence of _Phytophthora_ species from the rhizosphere soil and roots at the time of collection, and subsequently from seedlings at death or at harvest after 6 months.

Both the healthy and declining site were located on soils classified as Spearwood phase 2a (McArthur and Bettenay 1960). The crown health score of trees at the healthy site (site 1, Table 3.1) had crown health scores of 4-5.4 (Grimes 1978), while trees sampled at the declining site (site 2, Table 3.1) had crown rating scores of less than 3 out of a total of 5.4 (Grimes 1978).
3.2.1(a) Soil treatments

From both the healthy and declining site (Table 3.1), 12 trees were sampled within a 2 km radius. From each tree, a 1 L soil sample was collected from four cardinal points approximately 1 m from the base of the tree at 0-15 cm and 15-30 cm depth. All collection equipment was sprayed with 70% ethanol and dried between soil samples, including at each depth. Soils from each tree at each depth were bulked and mixed and a 0.4 L sub-sample was removed and used to test for the presence of *Phytophthora* species.

To obtain the eight soil treatments, initially the remaining soil samples from each tree was bulked according to if they were sampled from the healthy or declining site and from the 0-15 cm or 15-30 cm depth, and mixed in a cement mixer for 1 minute. The cement mixer was sterilised with 5% sodium hypochlorite, rinsed with deionised water and thoroughly dried between samples. After mixing soils in the cement mixer, a 1 L sub-sample was collected and analysed for soil properties and nutrients. The remainder of the four soils from the cement mixer (healthy or declining site, from 0-15 or 15-30 cm depth) were separately potted into 20, 15-cm diameter free-draining polypropylene pots. Ten of the 20 pots were selected at random and pasteurised, while the remaining 10 pots were not pasteurised, giving 10 replicate pots per treatment.

Soil for pasteurisation was raised to water holding capacity and steam pasteurised at 60-65°C for between 30 to 60 minutes (Brundrett *et al.* 1996; Hardy and Sivasithamparam 2002). The duration and temperature of steam treatment was selected to kill most pathogens, without creating a biological vacuum (Baker and Olsen 1962).

3.2.1(b) Soil nutrient analysis

Soil samples for nutrient analysis were processed by CSBP Limited (Bibra Lake, WA, Australia). Soil texture was measured as a dispersion index, based on soil texture, clay
type, soil organic matter, soil salinity and exchangeable cations, and was determined according to Loveday and Pyle (1973) and Allen and Jeffery (1990).

Soil nitrate and ammonium was determined from a soil extraction made with a 1M potassium chloride solution dissolved for 1 hour at 25°C. After dilution, the resulting soil solution was measured on a Lachat Flow Injection Analyser (Quikchem Pty Ltd automated ion analyser). The concentration of ammonium nitrogen was measured colorimetrically at 420 nm using the indo-phenol blue reaction (Searle 1984). Nitrate was reduced to nitrite through a copperized-cadmium column and measured colorimetrically at 520 nm (Searle 1984).

For phosphorus and potassium analysis, soils were extracted with a 0.5 M sodium bicarbonate solution adjusted to pH 8.5 for 16 hours to achieve a soil: solution ratio of 1:100. The acidified extract was treated with ammonium molybdate/antimony trichloride reagent and the concentration of phosphorus was measured colorimetrically at 880 nm (Colwell 1965). The concentration of potassium was determined using a flame atomic absorption spectrophotometer at 766.5 nm (Rayment and Higginson 1992).

Sulphur concentration within the soils was determined using the method of Blair et al (1991). Soils were extracted at 40°C for 3 hours with a 0.25M potassium chloride extraction solution and extracts were analysed using an inductively coupled plasma analyser (Perkin Elmer, Connecticut, United States of America).

The soil organic carbon content was determined using the methods of Walkley and Black (1934) and Walkley (1947). Concentrated sulfuric acid was added to soil wetted with a dichromate solution. The heat of the acid-based reaction was used to induce oxidation of soil organic material. The resulting chromic ions are proportional to oxidized organic carbon and were measured colorimetrically at 600 nm.
Iron concentration within the soil was determined according to Rayment and Higginson (1992). Soils were tumbled with oxalic acid for 1 hour to achieve a soil: solution ratio of 1:33. The concentration of iron was determined using a flame atomic absorption spectrophotometer at 248.3 nm.

The pH in deionised water and CaCl, and the conductivity were determined according to Rayment and Higginson (1992). Soils were extracted in deionised water for 1 hour to achieve a soil: solution ratio of 1:5. The water pH and electrical conductivity of the extract were measured using a combination pH electrode. The pH in CaCl was determined using a pH electrode after adding a calcium chloride solution to the mixture.

3.2.1(c) Crown Assessment

The crown health score for all trees was assessed from the north of each tree at approximately the same distance from the base as the crown height. The crown health score was determined by ranking different characteristics of the crown, against stylised images and a written description of crown health characteristics (Grimes 1978). Characteristics of crown health include the relative crown position, crown size, crown density, proportion of dead branches and proportion of epicormic growth. The scale for each crown characteristic was originally determined using *C. maculata* (synonym *E. maculata*), *E. fibrosa* and *E. drepanophylla* (Grimes 1978). The crown health score for each tree was determined by averaging the scores for each scaled characteristic for each tree. The crown health score for each tree was rated on site and later verified against digital images captured with a Fujifilm FinePix S500® digital camera, taken during the crown assessment. Where possible, assessments were performed close to midday on cloudless days.
3.2.1(d) *Isolation of Phytophthora species*

Immediately after soil collection and at seedling harvest, rhizosphere soil and root sections were analysed for the presence of *Phytophthora* species. Rhizosphere soil was baited for *Phytophthora* species using the methods of Marks and Kassaby (1974) and Stukely *et al.* (1997) at the vegetation health service (VHS) laboratory of the Western Australia Department of Environment and Conservation, using *E. sieberi* cotyledons, which were plated onto a *Phytophthora* selective medium, NARPH (Hüberli *et al.* 2000). NARPH contained per 1 L deionised water: 17 g Oxoid cornmeal agar (Wade Road, Basingstoke, England), 1 mL nystatin (Nilstat; Wyeth-Ayerst Australia Pty Ltd, Baulkham Hills, New South Wales, Australia), 100 mg ampicillin sodium (Fisons Pty Ltd, Sydney, New South Wales, Australia), 10 mg rifampicin (Rifadin; Hoechst Marion Roussel Australia Pty Ltd, Lane Cove, New South Wales, Australia), 100 mg PCNB (Terraclor; Uniroyal Australia Pty Ltd, Melbourne, Victoria, Australia), and 50 mg hymexazol (Tachigaren; Sankyo Company, Tokyo, Japan). Living roots and stem material were separated into two equal volumes, with one half baited in deionised water as indicated above, and the other half surface-sterilised in 70% ethanol for 10 seconds and rinsed four times in distilled water before being cut into sections between (1.0–2.0 cm) and plated onto NARPH.

3.2.1(e) *Seedlings*

*Eucalyptus gomphocephala* seed was obtained through the Seed Centre of the Forest Products Commission (Manjimup, Western Australia, Australia), collected from Lake Preston Western Australia (collection reference A93009d: MM1513) and germinated in 4 x 4 x 8 cm germination tubes containing pasteurised potting mix (2 parts pine bark, 2 parts course river sand and one part coco peat, Richgro®, Jandakot, Western Australia, Australia). One 4 week-old seedling was transferred to each pot. Pots were arranged in
a completely randomized block design. Bench orientation and position was randomly altered every 2 weeks, to account for environmental variation throughout the glasshouse. Seedlings were hand watered daily to container capacity with deionised water, and grown in an evaporative temperature controlled glasshouse.

3.2.1(f) Seedling assessment

Seedlings were assessed at planting, at 3 months and then 6 months at harvest. Time until death was recorded daily at each watering. At each assessment, seedling height was measured from soil level to the apical meristem. Foliar health of each seedling was estimated on a scale from 5 - 0 (5 = healthy; 0 = dead), based on the yellowing and wilting of leaves and the presence of necrotic leaf spots. Seedlings were processed for the presence of Phytophthora species, when they died or at harvest after 6 months. The majority of the rhizosphere soil was removed from the root ball with a brush and collected. The remaining soil was gently removed in deionised water, whilst fine roots which were lost during washing, were collected with a 1 mm pore size sieve. Leaves and all branches were removed and discarded. The stems were separated from the root butt and the remaining root masses were collected separately. The collected rhizosphere soil, roots and stems were processed for the presence of Phytophthora species as described in section 3.2.1(d).

3.2.2 Experiment 2 – In situ woodland tree assay

The aim of experiment 2 was to assess the relationship between the crown health score and the density of fine roots less than 2 mm in diameter and ectomycorrhizae, and the presence of Phytophthora species, for declining E. gomphocephala woodland trees.

The independent variable was the crown health score. Dependant variables were the fine roots density score for roots less than 2 mm in diameter, the ectomycorrhizal
density score and the isolation of *Phytophthora* species from the rhizosphere soil. The crown health score (CHS) as described in section 3.2.1(c), fine root and ectomycorrhizal density scores of 18 *E. gomphocephala* trees were examined in early spring (September) 2006. Trees were located throughout the Yalgorup National Park at sites 2 - 5 (Table 3.1, Appendix 1), with four trees at sites 2 and 4 and five trees at sites 3 and 5. At each site, all trees were sampled within a 1 km radius and were selected to represent the following decline symptoms and CHS scores: no crown decline symptoms and a CHS of 5.4 to 3.8; mild symptoms and a CHS of 3.8 to 3.2; strong symptoms and a CHS of 3.2 to 2.6; and extreme symptoms and a CHS of 2.6-2.0 and extreme symptoms. At sites 3 and 5, one additional tree with no clear decline symptoms and a CHS of 5.4 and 3.8, respectively, was assessed. All four sites had soils classified as Spearwood phase 2a (McArthur and Bettenay 1960). For each tree the DBH and crown heights were measured.

3.2.2(a) Fine root and ectomycorrhizae excavation

The root system on each of the 18 selected trees was excavated around the base to expose the lateral root mats using an Air-spade® Series 2000 (AIR-SPADE Guard air Corporation, Chicopee, United States of America) connected to a Ingersoll-Rand (Dublin, Ireland) compressor supplying air at 0.8 m$^3$/s and pressure of 0.6 MPa, giving an air stream with a speed of Mach 2. The fine roots and ectomycorrhizal tips of the largest major lateral root of each tree with the largest surface area, was then fully exposed in detail to an approximate depth of 25 cm. Deep sinker roots attached to the lateral were not excavated. Where ectomycorrhizal pads were present, the air spade was not used, and the roots were excavated by hand or using a soft tip brush (Nadezhdina and Cermak 2003), with bristles approximately 4 cm in length. Necrotic fine roots were opportunistically harvested and processed for isolating *Phytophthora* species as
Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth

3.2.1(d) [described in section 3.2.1(d)].

3.2.2(b) Fine root and ectomycorrhizae assessment

A step by step worked example of how the fine root and ectomycorrhizal density was determined is given in Appendix 2. For each tree, the largest major lateral root system emerging from the base of each tree was examined in detail and a separate total density score (TDS) of the fine roots, less than 2 mm in diameter, and visible ectomycorrhizae was determined. Ectomycorrhizae included ectomycorrhizae and hyphal mats which extended out beyond the roots into the organic layer. The fine roots and ectomycorrhizae TDS’s were determined using a modification of the USDA crown assessment method (United States Department of Agriculture 2007) and was determined for the area inside the perimeter of the outer-most root tips, described as the total reach (Fig. 3.1 indicated by dashed line). The main lateral root system was overlayed with a one meter grid (Fig. 3.1) and the fine root and ectomycorrhizal densities scores were determined separately within each grid.

![Fig. 3.1. Schematic representation of a theoretical fine root system (< 2 mm in diameter) or ectomycorrhizal mats (shaded area) attached to the largest main lateral root. The dashed line delineates the area inside the perimeter of the outer root tips defined as the ‘total reach’. The right triangle indicates the region where the fine root assessment was conducted](image)

---

Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth
square represents $1\text{m}^2$.

Initially a separate score was determined visually for the area covered with fine roots and ectomycorrhizal mats, for each grid within the area of the total reach, termed the percentage cover, as indicated in Fig. 3.2.

**Fig. 3.2.** Representations for determining the percentage cover score within each grid. (a) Schematic representation of the fine root (< 2 mm in diameter) or ectomycorrhizal mats, (shaded area) attached to the largest main lateral root. (b) The area covered with fine root or ectomycorrhizal mats within the enlarged grid. (c) Area within each grid within the total reach. The percentage cover score was calculated visually for each grid and is equivalent to area (a) divided by area (b) as a percentage. Therefore, for the highlighted grid, the percentage cover is 65%.

The average density of fine root mats (FRD) and density of ectomycorrhizal mats (ED) within each grid were measured visually against a standard reference scale of images of fine root (Fig. 3.3a) and ectomycorrhizal mats (Fig. 3.3b).
Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth

3.3a Fine root (< 2 mm diameter) reference scale

3.3b Ectomycorrhizal reference scale

**Fig. 3.3.** Reference photographs showing reference scale for the (a) fine root density (FRD) (< 2mm in diameter) of the fine root mats and, (b) ectomycorrhizal (ED), of the ectomycorrhizal mats scored in Experiment 2.

Separate fine root and ectomycorrhizal density scores were determined in each grid (DSG). Fine root and ectomycorrhizal DSG were calculated using an assessment matrix (Table 3.2), which incorporates the percentage cover, for the area covered with fine root and ectomycorrhizal mats, together with the fine root density (FRD) and the ectomycorrhizal density (ED) score within each grid in the area of the total reach (determined using Fig. 3.3).
Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth

Table 3.2. Assessment matrix used to determine the density score within each 1 m\(^2\) grid (DSG) for fine roots (< 2 mm in diameter) and ectomycorrhizal mats. The fine roots DSG was calculated within each grid using the area covered with fine root mats in the area of the total reach as a percentage cover, and the average fine root density (FRD) of the fine root mats (determined using Fig. 3.3a). Similarly, the ectomycorrhizae DSG was calculated within each grid using the area covered with ectomycorrhizal mats in the area of the total reach termed the percentage cover, and the average ectomycorrhizal density (ED) of the ectomycorrhizal mats (determined using Fig. 3.3b).

<table>
<thead>
<tr>
<th>Percentage cover for fine roots or ectomycorrhizae</th>
<th>Fine roots (FRD) within fine root mats or ectomycorrhizal (ED) within ectomycorrhizal mats determined using Fig. 3.3.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>11 -20</td>
</tr>
<tr>
<td>21 -30</td>
<td>31-40</td>
</tr>
<tr>
<td>41-50</td>
<td>51 -60</td>
</tr>
<tr>
<td>61-70</td>
<td>71-80</td>
</tr>
<tr>
<td>81-90</td>
<td>91-100</td>
</tr>
<tr>
<td>5 14 24 33 43 52 62 71 81 90</td>
<td>4 13 21 30 38 47 55 64 72 81</td>
</tr>
<tr>
<td>4 11 19 26 34 41 49 56 64 71</td>
<td>3 10 16 23 29 36 42 49 55 62</td>
</tr>
<tr>
<td>3 8 14 19 25 30 36 41 47 52</td>
<td>2 7 11 16 20 25 29 34 38 43</td>
</tr>
<tr>
<td>2 5 9 12 16 19 23 26 30 33</td>
<td>1 4 6 9 11 14 16 19 21 24</td>
</tr>
<tr>
<td>1 2 4 5 7 8 10 11 13 14</td>
<td>0 1 1 2 2 3 3 4 4 5</td>
</tr>
</tbody>
</table>

The fine root TDS and ectomycorrhizae TDS for each main lateral root was determined by combining the DSG from inside each one meter grid, multiplied by the proportion of each grid inside the total reach of the root system (Ag/Atr), and is represented by,

\[
TDS = \left[ \left( \frac{Ag_1}{Atr} \times DSG1 \right) + \left( \frac{Ag_2}{Atr} \times DSG2 \right) + \left( \frac{Ag_3}{Atr} \times DSG3 \right) + etc \right]
\]

Where:

- **TDS** is the total density score (of fine roots or ectomycorrhizae);
- **Ag**, 1, 2, 3 etc. is the area inside each grid, inside the total reach;
- **Atr** is the area of the total reach across all grids; and
• DSG 1, 2, 3 etc., corresponds to the calculated fine roots and/or ectomycorrhizal density score within each grid.

3.2.3 Experiment 3 - In situ seedling bioassay ‘declining sites’

The aim of Experiment 3 was to determine if decline symptoms of *E. gomphocephala* woodland trees affected the health of *E. gomphocephala* seedlings planted throughout the fine root mats of declining trees and if *Phytophthora* species were associated with declining trees or seedlings. The independent variables for the *in situ* seedling bioassay were the crown health score, fine root TDS and ectomycorrhizal TDS of the *E. gomphocephala* woodland trees measured in Experiment 2. Dependant variables were seedling survival, height and foliar health after 3 and 6 months and the presence of *Phytophthora* species at harvesting after 6 months. Seedlings were planted in early spring (September) 2006.

3.2.3(a) Seedlings

*Eucalyptus gomphocephala* seed was sourced and germinated (as in section 3.2.1e) and grown in 4 x 4 x 8 cm germination tubes containing pasteurised potting mix (2 parts pine bark, 2 parts course river sand and one part coco peat, Richgro®, Jandakot, Western Australia). Seedlings were grown in a temperature controlled glasshouse and watered daily to container capacity with deionised water, for eight weeks before transplanting to the woodland sites.

Ten *E. gomphocephala* seedlings were removed from the tubes and evenly planted throughout the root mats of the major lateral root for each of the 18 trees exposed and examined in Experiment 2, to give a total of 180 seedlings planted. The seedling root balls were initially buried to a depth of 4 cm, so that the surface of the exposed root mats were approximately half way up the seedling root ball, leaving the upper 4 cm of
the root ball exposed. The exposed section of the root ball was then covered with the air spaded soil to carefully cover the entire exposed tree root system, to a depth similar to that prior to excavation. After planting, each seedling was protected from disturbance with a 30 cm high by 20 cm wide perforated plastic sleeve kept in place with bamboo stakes that had been autoclaved at 120 C for 20 minutes. Seedlings were watered with deionised water to soil saturation at planting and then, weekly for 4 weeks.

3.2.3(b) Seedling assessment

Seedling height and foliar health were measured 3 and 6 months after planting as described in section 3.2.1(f). When seedlings had died, or at harvest after 6 months, the seedlings and the surrounding rhizosphere soil was collected and analysed for the presence of *Phytophthora* species, as described in section 3.2.1(d).

3.2.4 Experiment 4 - *Ex situ* seedling bioassay 2 ‘declining sites’

The aim of Experiment 4 was to determine if the health of *E. gomphocephala* seedlings grown *ex situ* in pasteurised or non-pasteurised soil was affected by the health of the trees and if soilborne *Phytophthora* species were associated with decline. Independent variables were the crown, fine root and ectomycorrhizal scores of the sampled woodland trees from Experiment 2 as recorded in section 3.2.2, and the pasteurised or non-pasteurised soil treatments. Dependant variables were the number of leaves, stem length, growth rate, fine root measurements, the area of living and dead leaves and the recovery of *Phytophthora* species from seedlings and baited soil, when seedlings died or at harvesting after 6 months.

Seedlings were grown *ex situ* in pasteurised and non-pasteurised intact soil monoliths collected in early spring (September) 2006 from the lateral root mats of 12 *E. gomphocephala* trees from Experiment 2 (section 3.2.2) using three trees from each
of the four sites, with decline symptoms and a CHS of 3.8 or less. From each tree replicate pairs of adjacent soil monoliths (16 x 12 x 1 cm) were collected with a flat spade head, to keep the soil intact, from the level of the exposed lateral roots (Experiment 2) and placed directly into improvised growth containers, modified from Dobrowolski et al. (2008) (Fig. 3.4). Pairs of adjacent soil monoliths were harvested within 30 cm from where seedlings had been planted in situ in Experiment 3 (section 3.2.3(a)). Sampling equipment was treated with 70% ethanol between soil samples. Of the two adjacent intact soil monoliths, one was pasteurised and the other left non-pasteurised. In total, 10 replicate pairs of adjacent pasteurised or non-pasteurised soil monoliths were collected from each of the 12 trees, to give 120 pasteurised and 120 non-pasteurised soil monoliths. Soil treatments were arranged in a randomized block design.

3.2.4(a) Seedlings

*Eucalyptus gomphocephala* seed, as described in section 3.2.1(e), were germinated on filter paper, moistened with deionised water in 9 cm diameter petri dishes. One, 1-4 day-old *E. gomphocephala* seedling, at the cotyledon stage was transferred to each container. The seedlings were grown in an evaporative temperature controlled glasshouse.

3.2.4(b) Design of the containers in which seedlings were grown

The improvised growth containers were made of two polypropylene take-away container lids taped together on the sides and base, with one short edge cut off to form a thin pocket 12 cm wide, 16 cm high and approximately 1 cm thick (Fig. 3.4). Surfaces exposed to the light were covered with aluminium foil. Holes in the upper corners of each container were used to suspend them from wires stretched across tubs.
approximately 1 m in length, 0.4 m wide and 0.4 m in height. The lower 3 cm of each seedling container was encased in a plastic sleeve that extended 3 cm below the containers, designed to eliminate water exchange between drainage points.

![Fig. 3.4. (a) Growth container used in the in situ seedling bioassay Experiment 3 and (b) growth containers stacked together in polystyrene tubs in the glasshouse.](image)

### 3.2.4(c) Seedling assessment

When seedlings died or at harvest after 6 months, the rhizosphere soil, and root and stem material were collected and analysed for the presence of *Phytophthora* species as described in Experiment 1. The stem and leaves of each seedling were removed at the soil line and scanned on a Canon® Cano 8800FF scanner, so that each leaf was separated with the adaxial side facing down. Leaf surfaces were analysed using the WINRHIZO Pro V 2007d (Reagent Instruments, Québec, Canada) software to determine the area of living and dead tissue. After the rhizosphere soil was initially removed with a brush, as described in Experiment 1, the root balls were washed to remove the remaining attached soil. Root balls were washed for 5 minutes, in three consecutive 5 x 25 x 40 cm trays filled with deionised water to a depth of 2.5 cm that were attached to Richmond Laboratory Shakers SHA116 vibrating at a rate of 20 revolutions per minute and an
orbital motion of 25 mm. All equipment used to wash the roots was thoroughly washed and sterilized with 70% ethanol, before each use. Root length and surface area were measured for five root diameter classes (0-0.5, 0.5-1, 1-2, 2-5, >5 mm) using the software WINRHIZO Pro V 2007d. After scanning, the seedlings were processed for the presence of Phytophthora species as described for Experiment 1.

3.2.5 Statistical analysis

Significance was determined at $P \leq 0.05$. Analyses were carried out in Statistica software package Version 5 (Statsoft 1999). Assumptions of normality were checked by plotting residuals (Clarke and Warwick 2001). Proportional data were square root arcsine transformed, and probabilities were determined for both transformed and non-transformed data. In the cases where both transformed and non-transformed data were significant, correlation coefficients, regression coefficients and linear correlation equations are given for the non-transformed data set. Where appropriate, the Pearson correlation coefficient was calculated for a measure of association between variables. The Bonferroni correction was not applied to correlation coefficients as recommended by Moran (2003).

Multivariate analysis of variance (MANOVA) and repeat measure MANOVA were used to test for significance between the means for treatments with multiple measurements per treatment. Where univariate tests were significant, post hoc Fisher LSD tests were used to identify significantly different variables (Day and Quinn 1989).

Experiment 1 - Ex situ seedling bioassay 1 ‘healthy and a declining site’

Differences between crown health, height and DBH, between healthy and declining sites, were analysed using a MANOVA. The dependent variables of seedling height and foliar health were analysed as a repeated measure factorial MANOVA, with independent
variables including time of growth, and factors of site condition (declining or healthy), soil depth (0-15 or 15-30 cm) and soil treatment (pasteurisation or non-pasteurisation). Seedling height and foliar health were transformed by the natural logarithm to account for assumptions of normality.

**Experiment 2 – In situ woodland tree assay**

Correlation between the crown health, fine root, and ectomycorrhizal scores were performed using a Pearson’s correlation (Quinn and Keough 2002). Differences in the fine root and ectomycorrhizal scores between trees without decline symptoms and a crown health score of above 3.8 out of 5.4 and with decline symptoms and a crown health score of below 3.8 out of 5.4, was determined using a MANOVA.

**Experiment 3 - In situ seedling bioassay ‘declining sites’**

The relationship between the measured dependent variables of the *E. gomphocephala* seedlings grown in situ within the rhizosphere of *E. gomphocephala* trees from Experiment 2 and the crown health, fine root and ectomycorrhizal scores of the *E. gomphocephala* trees was determined using a Pearson’s correlation (Quinn and Keough 2002). Differences in the survival, height and foliar health after three and 6 months of seedlings planted in the rhizosphere of trees without decline symptoms and a crown health score of above 3.8 out of 5.4 and with decline symptoms and a crown health score of below 3.8 out of 5.4, was determined using a repeated measures MANOVA.

**Experiment 4 - Ex situ seedling bioassay 2 ‘declining sites’**

The relationship between the measured dependent variables of the seedlings grown ex situ (Experiment 2) and the crown health score, fine root density and ectomycorrhizal
density of the selected woodland *E. gomphocephala* trees was determined using a Pearson’s correlation (Quinn and Keough 2002).

### 3.3 Results

#### 3.3.1 Experiment 1 - *Ex situ* seedling bioassay 1 ‘healthy and a declining site’

Trees on the ‘healthy’ site were on average 7.1 m taller, but with an average DBH 167.1 cm less, than trees at the ‘declining’ site (Table 3.3). Soil had consistent texture, colour and nitrate concentrations between healthy and declining sites at the 0-15 and 15-30 cm soil depths. The ammonium, phosphorus, potassium and sulphur concentrations were equivalent between healthy and declining sites and in both cases were higher in the top 0-15 cm soil layers than in the lower 15-30 cm layers. Healthy and declining sites had more organic carbon in the upper soil layer and declining sites had more organic carbon in both soil layers compared to the healthy site. The healthy site had more iron in both soil layers than the declining site with the highest iron in the lower soil layers. Iron concentration was similar between both soil layers in the declining site. Conductivity was lowest at the healthy site in the lower soil profile and similar between the upper soil layer healthy site and both soil layers from the declining site. The soil pH was roughly neutral and similar between both sites, with the lower soil profile at the declining site being slightly basic (Table 3.3).
Table 3.3. Experiment 1, (a) site characteristics and (b) nutrient status of the bulk unpasteurised soil sampled from a healthy and a declining *Eucalyptus gomphocephala* site at 0-15 cm and 15 – 30 cm depth. Crown health score assessed as per Grimes (1978). Statistics are for dependant variables of crown health score, height and diameter breast height at 1.5 m (DBH) and shown as a univariate test where the multivariate main effect of site is significant (* P ≤ 0.01). Small letters denote the statistical results of the post hoc test (Fisher LSD) where values with the same letters are not significantly (*P ≤ 0.05) different. Statistics were not determined for soil characteristics for interactions between site and soil depth.

(a)

<table>
<thead>
<tr>
<th>Sites</th>
<th>Healthy site (Site 1)</th>
<th>Declining site (Site 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude (S)</td>
<td>32.395483°</td>
<td>32.684400°</td>
</tr>
<tr>
<td>Longitude (E)</td>
<td>115.796986°</td>
<td>115.639203°</td>
</tr>
<tr>
<td>Crown health score*</td>
<td>4.4 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Mean ± SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crown height (m)*</td>
<td>28.6 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.5 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Mean ± SEM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBH*</td>
<td>86.8 ± 12.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>253.9 ± 37.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(Mean ± SEM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Soil Layer</th>
<th>0 - 15 cm</th>
<th>15 – 30 cm</th>
<th>0 - 15 cm</th>
<th>15 – 30 cm</th>
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</thead>
<tbody>
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<td>Texture</td>
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<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Colour</td>
<td>Brown green</td>
<td>Light brown</td>
<td>Brown green</td>
<td>Brown green</td>
</tr>
<tr>
<td>Nitrate (µm/g)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium (mg/kg)</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Phosphorus (mg/kg)</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Potassium (µm/g)</td>
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<td>23</td>
<td>59</td>
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<tr>
<td>Sulphur (µm/g)</td>
<td>5.5</td>
<td>3.5</td>
<td>5.2</td>
<td>4.5</td>
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<tr>
<td>Organic Carbon (%)</td>
<td>2.9</td>
<td>1.04</td>
<td>4.46</td>
<td>2.18</td>
</tr>
<tr>
<td>Iron (µm/g)</td>
<td>290</td>
<td>411</td>
<td>231</td>
<td>233</td>
</tr>
<tr>
<td>Conductivity (dS/m)</td>
<td>0.088</td>
<td>0.051</td>
<td>0.091</td>
<td>0.095</td>
</tr>
<tr>
<td>pH (CaCl)</td>
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<td>6.5</td>
<td>6.5</td>
<td>7.1</td>
</tr>
<tr>
<td>pH (H&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>7.1</td>
<td>7</td>
<td>6.9</td>
<td>7.4</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean. DBH = diameter at breast height (1.5 m)
The main (effect) interaction of decline status, soil depth and pasteurisation did not significantly affect seedling height (Fig 3.5a) and foliar health (Fig.3.5b).

**Fig. 3.5.** Experiment 1, mean (± standard error bars) for seedling height (a) and foliar health (scored as 0 dead to 5 healthy) (b) after 3 months (clear bars) and 6 months (dark bars) for *Eucalyptus gomphocephala* seedlings grown in eight soil treatments. Soil treatments include non-pasteurised and pasteurised soil collected from a healthy and declining site at 0-15 cm and 15-30 cm soil depths. Statistics are for a repeated measure factorial MANOVA. ns, not
significant.

Seedling height was significantly greater in pasteurised than non-pasteurised soil (Fig. 3.6a), in soil harvested from depth rather than surface soil (Fig. 3.6b), with time (data not shown) and by the treatment interaction of pasteurisation and time (Fig. 3.6c). Seedling height and foliar health was not significantly influenced by the decline status of sites from which soil was harvested.

![Fig. 3.6. Experiment 1, mean (± standard error bars) for significant \( P < 0.001 \) treatment interactions expressed in the height of *Eucalyptus gomphocephala* seedlings in Experiment 1. Soil treatments include non-pasteurised and pasteurised soil collected from a healthy and a declining site at 0-15 cm and 15-30 cm soil depths. Significant treatment interactions include soil pasteurisation (a), soil depth (b) and the treatment interactions between non-pasteurisation (solid line), pasteurisation (dashed line); and time (c). Statistics are for repeated measure MANOVA. \( **P \leq 0.001 \). Small letters denote the statistical results of the post hoc test (Fisher LSD) and bars with the same letters are not significantly \( P \leq 0.05 \) different.

No *Phytophthora* species was isolated from rhizosphere soil collected from the *E. gomphocephala* woodland, or any seedlings grown *ex situ* in Experiment 1, from direct plating plant material or through baiting rhizosphere soil and plant material.

### 3.3.2 Experiment 2 – *In situ* woodland tree assay
Average crown health scores, height and DBH, did not significantly vary between sites (Table 3.4).

**Table 3.4.** Site characteristics for Experiments 2.1, 2.2 and 2.2, as indicated in Table 3.1. Crown health score assessed as per Grimes (1978). Statistics are for dependant variables of crown health score, height and diameter breast height at 1.5 m (DBH) determined using a multivariate analysis of variance with significance determined at ($P \leq 0.05$). Factors of crown health score, height and DBH, were not significantly different between sites.

<table>
<thead>
<tr>
<th>Sites</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude (S)</td>
<td>32.684400°</td>
<td>32.659677°</td>
<td>32.68683°</td>
<td>32.687969°</td>
</tr>
<tr>
<td>Longitude (E)</td>
<td>115.639203°</td>
<td>115.622919°</td>
<td>115.643597°</td>
<td>115.637025°</td>
</tr>
<tr>
<td>Crown health score (Mean ± SEM)</td>
<td>3.4 ± 0.5</td>
<td>2.9 ± 0.6</td>
<td>3.6 ± 0.3</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Crown height (Mean ± SEM)</td>
<td>2.9 ± 0.3</td>
<td>1.8 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>5.1 ± 1.4</td>
</tr>
<tr>
<td>DBH (Mean ± SEM)</td>
<td>33.0 ± 5.9</td>
<td>27.5 ± 6.0</td>
<td>30.0 ± 5.3</td>
<td>29.5 ± 8.3</td>
</tr>
<tr>
<td>Number of trees assessed</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean. DBH = diameter breast height at 1.5 m

_Eucalyptus gomphocephala_ trees with healthier crowns had more fine roots and ectomycorrhizae. The fine root and ectomycorrhizal TDS of _E. gomphocephala_ trees were significantly positively correlated with their crown health scores (Fig. 3.7a and b). Fine root TDS was significantly correlated with ectomycorrhizal TDS (Fig. 3.7c).
Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth

Fig. 3.7. Experiment 2, significant linear relationships between: fine root (less than 2 mm in diameter) total density score (TDS) and crown health score (a); and ectomycorrhizal TDS and crown health index (b); and fine root TDS and ectomycorrhizal TDS (c), for the main lateral root of healthy and declining *Eucalyptus gomphocephala* woodland trees. Crown health score was assessed according to Grimes (1978). Continuous lines are the regression equations fitted to the data. Regression equations for: (a) Crown health score = 1.17 + 0.03 Fine root TDS; (b) Crown health score = 2.02 + 0.03 Ectomycorrhizal TDS and (c) Ectomycorrhizal TDS = -0.65 + 0.73 Fine root TDS.

Trees with declining crowns had significantly less fine roots and ectomycorrhizae than trees with healthy crowns (Table 3.5).

Table 3.5. Experiment 2, mean (± standard error) for fine root and ectomycorrhizal TDS of *Eucalyptus gomphocephala* trees with healthy and declining crowns. Healthy crowns defined as having no decline symptoms and a crown health score of 3.8-5.4 (Grimes 1978) and declining crowns defined as having decline symptoms and a crown health score below 3.8. Statistics are shown for univariate tests where the multivariate main effect is significant (* P ≤ 0.01). Small letters within rows denote the statistical results of the post hoc test (Fisher LSD) where values with the same letters are not significantly (P ≤ 0.05) different.

<table>
<thead>
<tr>
<th>Measured characteristics</th>
<th>Univariate test</th>
<th>Healthy crowns</th>
<th>Declining crowns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine root TDS</td>
<td>*</td>
<td>84 ± 5.04a</td>
<td>62.17 ± 3.19b</td>
</tr>
<tr>
<td>Ectomycorrhizal TDS</td>
<td>*</td>
<td>74.61 ± 2.90a</td>
<td>37.41 ± 4.0b</td>
</tr>
</tbody>
</table>

No *Phytophthora* species was isolated from any seedling in Experiment 2, by direct plating or baiting root sections, or baiting rhizosphere soil.
3.3.3 Experiment 3 - *In situ* seedling bioassay ‘declining site’

The crown health score of the *E. gomphocephala* woodland trees was significantly positively correlated with *in situ* *E. gomphocephala* seedling survival after 3 months, height after three and 6 months and foliar health after 6 months (Table 3.6, Fig. 3.8a-d). The survival of seedlings grown *in situ* after 3 months was also significantly correlated with the ectomycorrhizal TDS of the woodland trees (Table 3.6, Fig. 3.8e). The remaining interactions between *E. gomphocephala* seedlings grown *in situ* and woodland trees were not significant (Table 3.6).

Table 3.6. Experiment 3, relationships between *Eucalyptus gomphocephala* seedlings grown *in situ* within the fine root mats of healthy and declining *E. gomphocephala* woodland trees, and the woodland trees. Correlation coefficients for linear relationships between seedling survival, height and foliar health, and tree crown health score and fine root and ectomycorrhizal total density score (TDS) (assessed in Experiment 2). Significant correlation coefficients (Pearson’s) are indicated in bold at *P* ≤ 0.05, and were determined without using the Bonferroni correction according to Moran (2003).

<table>
<thead>
<tr>
<th><em>In situ</em> seedlings</th>
<th>Time (months)</th>
<th>Correlation coefficients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tree crown health score</td>
<td>Tree fine root TDS</td>
</tr>
<tr>
<td>Survival</td>
<td>3</td>
<td>0.64</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.07</td>
<td>-0.2</td>
</tr>
<tr>
<td>Height</td>
<td>3</td>
<td>0.54</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.64</td>
<td>0.41</td>
</tr>
<tr>
<td>Foliar health</td>
<td>3</td>
<td>0.12</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.5</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Fig. 3.8. Experiment 3, significant linear relationships ($P \leq 0.05$ as shown Table 3.6) between *Eucalyptus gomphocephala* seedlings, grown *in situ* within the fine root mats of healthy and declining *E. gomphocephala* woodland trees assessed in Experiment 2. Relationships include: seedling survival % (3 months) and tree crown health score (CHS) (Grimes 1978) (a); seedling height (3 months) and tree CHS (b); seedling height (6 months) and tree CHS (c); seedling foliar health (6 months) and tree CHS (d); and seedling survival % (3 months) and tree ectomycorrhizal total density score (TDS) (e). Equations for linear relationships were: (a) Seedling survival % (3 months) = 20.47 + 15.94 Tree CHS; (b) Seedling height (3 months) = 0.59 + 0.46 Tree CHS; (c) Seedling height (6 months) = 1.53 + 1.29 Tree CHS; (d) Seedling foliar health (6 months) = 3.11 + 0.20 Tree CHS and seedling survival % (3 months) = 49.86 + 0.49 Tree ectomycorrhizal density.

Seedling survival after 3 months was significantly greater when planted within the rhizosphere of trees with healthy crowns than trees with declining crowns (Table 3.7).
Table 3.7. Experiment 3, mean (± standard error) for survival, height and foliar health of *Eucalyptus gomphocephala* seedlings 3 and 6 months after planting within the rhizosphere of *E. gomphocephala* trees with healthy and declining crowns. Healthy crowns defined as having no decline symptoms and a crown health score of 3.8-5.4 (Grimes 1978) and declining crowns defined as having decline symptoms and a crown health score below 3.8. Statistics are shown for univariate tests where the repeated measures multivariate main effect is significant. *P* ≤ 0.01; ns, not significant. Small letters denote the statistical results of the post hoc test (Fisher LSD) where values with the same letters are not significantly (*P* ≤ 0.05) different.

<table>
<thead>
<tr>
<th>Ex situ seedling</th>
<th>Time (months)</th>
<th>Univariate test</th>
<th>Healthy crowns</th>
<th>Declining crowns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival</td>
<td>3</td>
<td>*</td>
<td>90.00 ± 3.65(^a)</td>
<td>66.67 ± 5.12(^b)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ns</td>
<td>58.33 ± 8.33</td>
<td>60.83 ± 6.09</td>
</tr>
<tr>
<td>Height</td>
<td>3</td>
<td>ns</td>
<td>2.52 ± 0.15</td>
<td>1.95 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ns</td>
<td>6.88 ± 0.28</td>
<td>5.42 ± 0.47</td>
</tr>
<tr>
<td>Foliar health</td>
<td>3</td>
<td>ns</td>
<td>3.90 ± 0.15</td>
<td>4.03 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ns</td>
<td>3.90 ± 0.05</td>
<td>3.73 ± 0.10</td>
</tr>
</tbody>
</table>

No *Phytophthora* species was isolated from any seedlings, grown *in situ* in Experiment 3, from direct plating plant material or through baiting rhizosphere soil and plant material.

3.3.4 Experiment 4 - *Ex situ* seedling bioassay 2 ‘declining sites’

The number of leaves of *E. gomphocephala* seedlings grown *ex situ* in non-pasteurised woodland soil was significantly correlated with the crown health scores of the *E. gomphocephala* woodland trees after 6 months (Table 3.8, Fig. 3.9a). The ectomycorrhizal TDS of the woodland trees was significantly correlated with the height, leaf number and leaf area of seedlings grown in pasteurised soil (Table 3.8, Fig. 3.9b-d). There were no other significant correlations between *E. gomphocephala* seedlings grown *ex situ* and CHS, fine root TDS or ectomycorrhizal TDS (Table 3.8).
Table 3.8. Experiment 4, relationships between *Eucalyptus gomphocephala* seedlings grown *ex situ* within pasteurised and non-pasteurised soil harvested from declining *E. gomphocephala* woodland trees, and the woodland trees. Correlation coefficients for linear relationships between seedling height leaf number, area and area proportion necrotic, root length (diameter 0-5 mm, 0-1, 1-2 mm, 2-5 mm) and root surface (diameters 0-1 mm and 1-2 mm) when grown in pasteurised and non-pasteurised soil, and the crown health score, fine root and ectomycorrhizal total density score (TDS) of the woodland trees (assessed in Experiment 2). Significant correlation coefficients (Pearson’s) are indicated in bold at *P* ≤ 0.05, and were determined without using the Bonferroni correction in accordance with Moran (2003).

<table>
<thead>
<tr>
<th><em>E. gomphocephala</em> seedlings grown <em>ex situ</em></th>
<th></th>
<th>Tree crown health score</th>
<th>Tree fine root TDS</th>
<th>Tree ectomycorrhizal TDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pasturised</td>
<td>Non-pasteurised</td>
<td>Pasturised</td>
<td>Non-pasteurised</td>
</tr>
<tr>
<td>Height</td>
<td>0.4</td>
<td>0.29</td>
<td>-0.52</td>
<td>-0.35</td>
</tr>
<tr>
<td>Leaf</td>
<td>0.38</td>
<td>0.58</td>
<td>-0.47</td>
<td>-0.07</td>
</tr>
<tr>
<td>Number</td>
<td>0.44</td>
<td>0.28</td>
<td>-0.55</td>
<td>-0.37</td>
</tr>
<tr>
<td>Area</td>
<td>0.28</td>
<td>-0.34</td>
<td>0.35</td>
<td>0.15</td>
</tr>
<tr>
<td>Proportion necrotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length (0-5 mm diam.)</td>
<td>0.16</td>
<td>0.46</td>
<td>-0.3</td>
<td>-0.17</td>
</tr>
<tr>
<td>Length (0-1 mm diam.)</td>
<td>0.05</td>
<td>0.49</td>
<td>-0.15</td>
<td>-0.01</td>
</tr>
<tr>
<td>Length (1-2 mm diam.)</td>
<td>0.05</td>
<td>0.39</td>
<td>-0.18</td>
<td>-0.1</td>
</tr>
<tr>
<td>Length (2-5 mm diam.)</td>
<td>0.35</td>
<td>0.4</td>
<td>-0.53</td>
<td>-0.35</td>
</tr>
<tr>
<td>Surface area (0-1 mm diam.)</td>
<td>-0.03</td>
<td>0.45</td>
<td>-0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>Surface area (1-2 mm diam.)</td>
<td>0.03</td>
<td>0.38</td>
<td>-0.13</td>
<td>-0.1</td>
</tr>
</tbody>
</table>
Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth

Fig. 3.9. Experiment 4, significant linear relationships ($P \leq 0.05$ as shown Table 3.8) between *Eucalyptus gomphocephala* seedlings, grown *ex situ* in soil harvested from the rhizosphere of declining *E. gomphocephala* woodland trees that had been pasteurised and non-pasteurised, and the woodland trees measured in Experiment 2. Relationships include: seedling leaf number (No.) (grown in non-pasteurised soils) and tree crown health score (Grimes 1978) (a); seedling height (grown in pasteurised soils) and tree ectomycorrhizal total density scores (TDS) (b); seedling leaf No. (grown in pasteurised soils) and ectomycorrhizal TDS (c); and seedling leaf area (grown in pasteurised soils) and ectomycorrhizal TDS (d). Equations for linear relationships indicated as: (a) Seedling leaf No. (grown in non-pasteurised soils) = 4.49 + 3.72 Tree CHS; (b) Seedling height (grown in pasteurised soils) = 4.97 + 0.18 Tree ectomycorrhizal TDS; (c) Seedling leaf No. (grown in pasteurised soils) = 11.34 + 0.17 Tree ectomycorrhizal TDS and Seedling leaf area (cm²) (grown in pasteurised soils) = 0.12 + 1.07 Tree ectomycorrhizal TDS.

No *Phytophthora* species was isolated from any seedlings, grown *in situ* in Experiment 4, from direct plating plant material or through baiting rhizosphere soil and plant material.
3.4 Discussion

3.4.1 Role of soil organisms in *Eucalyptus gomphocephala* decline

Results provide contradictory information about the role of soil organisms within the decline. In Experiment 4, crown health of the woodland trees significantly correlated with the leaf number of seedlings grown *ex situ* in non-pasteurised soil. This indirectly indicates that soil organisms contribute to tree health. However, in Experiment 1 the height and foliar health of seedlings was not significantly different when they were grown in pasteurized or non-pasteurised soil from healthy or declining sites. This indirectly indicates that soil organisms may not be involved in the decline.

It is unlikely that the soil organisms that influence the *E. gomphocephala* trees would have an equal influence on seedlings grown *ex situ* in non-pasteurised soil. Beneficial and/or deleterious soil organisms may not have been present in the non-pasteurised samples because insufficient soil was sampled, the organisms were killed during the sampling and mixing processes or growth conditions *ex situ* were unfavourable for soil organisms to influence seedlings.

3.4.2 *Phytophthora* isolation

No *Phytophthora* species was isolated from any *E. gomphocephala* trees or seedlings in any of the four experiments in 2005 and 2006. This suggests that *Phytophthora* species may not have been involved in *E. gomphocephala* decline, or were not originally present at the study sites. However, in June 2007, using the sampling and isolation techniques as outlined in Scott *et al.* (2009, Chapter 4), *P. multivora* was isolated from all sites with declining *E. gomphocephala* used in the present study. This suggests that sites 2-5 were probably infested with *P. multivora* at the start of this study, although it was not originally isolated. It is also possible that *P. multivora* is only partially
distributed over the study sites. Further work is required to map the exact distribution of *P. multivora* within the *E. gomphocephala* woodland and south west of Western Australia.

*Phytophthora multivora* was probably not isolated from the sites in 2005 and 2006 due to either dormant resting structures (Bunny 1996), low population density and/or problems with the isolation technique. The pathogen was probably isolated in 2007 (Scott *et al.* 2009, Chapter 4) because environmental conditions prior to sampling coincided with a break in the dormancy of resting structures and to an increase in population density. *Phytophthora multivora* (previously described as *P. citricola* based on morphological identification) has been isolated episodically from rehabilitated bauxite mine sites since 1992 in the jarrah forest on the Darling Scarp, usually after major rainfall events (Hardy personal communication). Similarly, Bunny (1996) needed to repeatedly flood soil samples to isolate *P. citricola*. Only one isolate designated by Bunny (1996) as *P. citricola* (DEC 236 VHS culture) is available and has since been identified as *P. multivora* based on molecular re-evaluation (Scott *et al.* 2009, Chapter 4). Bunny (1996) identified rainfall as an important factor influencing the distribution of *P. citricola* (*P. multivora*) isolates, with greatest isolation frequency of rainfall above 800 mm/annum and falling sharply below 500 mm/annum.

The annual rainfall for 2007, when *P. multivora* was first isolated at the *E. gomphocephala* Yalgorup forest (Chapter 4) was 678.8 mm, which was close to the 2002 - 2010 average of 649.40 mm (BoM 2011b). Rainfall in 2005 for Experiment 1 was 915.22 mm, and for 2006 for Experiment 2, 3 and 4, was 434.8 mm (BoM 2011b). *Phytophthora multivora* was originally isolated in May 2007 (Chapter 4). The combined rainfall for April and May 2007 was 107.2 mm and the average temperature for May was 19.7°C. For Experiment 1, conducted in October 2005, the combined rainfall for September and October was 167.4 mm and the average temperature for October was
18.9°C. For Experiment 2, conducted in September 2006, the combined rainfall for August and September was 129.8 mm and the average temperature for October was 19.5°C. For Experiment 3, when the seedlings were harvested in the February 2007, the combined rainfall for January and February was 23.4 mm and the average temperature for October was 28.2°C. High rainfall in 2005 (Experiment 1) should have favoured the isolation of *P. multivora*. Sites were extensively and carefully sampled in 2006 for Experiments 2 and 3; however, the below average rainfall may have reduced the inoculum density prior to sampling, and the pathogen may have been dormant. *Phytophthora multivora* may not have been isolated from seedlings harvested in February 2007, for Experiment 3, because of the low rainfall and high temperature.

The inability to isolate cryptic, yet potentially important soilborne, pathogens due to temporal and spatial variation in soil population densities in response to environmental conditions, may lead to misdiagnosis of a forest decline and hence inadequate disease management. Further work is required to determine what environmental and biological factors may influence the population density of *P. multivora* over time.

### 3.4.3 Inferring decline aetiology from above and below ground health

*Eucalyptus gomphocephala* crown decline was significantly correlated with fine root and ectomycorrhizal density and trees with declining crowns had significantly less fine roots and ectomycorrhizal mats. This suggests that fine root and ectomycorrhizal loss is associated with crown decline. However, the observed relationships between crown, fine root and ectomycorrhizal health do not directly reflect how decline symptoms have developed within the plant. Specifically results do not provide evidence as to whether the decline is progressing from above or below ground.

According to extrapolation of the line of best fit equation, if *E. gomphocephala*
Chapter 3. Relationship between the crown health, fine roots and ectomycorrhizae of declining *Eucalyptus gomphocephala* trees and *E. gomphocephala* seedling growth

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trees had no fine roots they would have a crown health score of 1.17 out of 5.4, while if trees had no ectomycorrhizae they would have a crown health score of 2.02 out of 5.4. Declining trees may therefore be able to tolerate more ectomycorrhizal loss than fine root loss. Further research is required to resolve the relationship between crown health and fine root and ectomycorrhizal density. The relationship between crown health and fine root or ectomycorrhizal density may vary for trees at different stages of decline. For trees with substantial crown decline, water availability is probably more important than nutrient uptake, especially in Mediterranean ecosystems with hot dry summers. In contrast, trees with healthier crowns are more likely to have sufficient root networks for water uptake, and so nutrient availability is more likely to limit growth. Therefore, for trees with healthier crowns, ectomycorrhizal density will more likely cause changes in crown health, because ectomycorrhizae are directly associated with nutrient uptake.

3.4.4 Inferring decline aetiology from *in situ* seedling growth

Significant correlations between *in situ* seedling health and crown health and ectomycorrhizal TDS of woodland trees suggests that factors responsible for *E. gomphocephala* decline were affecting *E. gomphocephala* seedlings and may include beneficial or pathogenic organisms, such as *P. multivora*. However, *P. multivora* was not initially isolated from the soil used in this trial. Seedling health and survival *in situ* may also be associated with crown health because more intact crowns may protect seedlings from environmental extremes and increase biological diversity and nutrient availability (Callaway and Walker 1997; Christian 2001). Conversely, trees with more established crowns may reduce seedling survival by shading out competing understorey species, or by producing allelopathic chemicals through the leaf litter (May and Ash 1990). Ectomycorrhizal density may affect seedling survival, because ectomycorrhizal mats directly improve the soil environment and increase nutrient and water availability.
and organic decomposition and provided protection from root pathogens to the tree to which they are attached (Lu et al. 1998). Ectomycorrhizal loss associated with the decline may have wide ecological implications beyond E. gomphocephala health, including impacts on seed germination (Malajczuk et al. 1982), mycophagous animals (Bougher 1999; Bougher and Lebel 2001) and orchids (Hollick 2004; Newman 2009).

3.4.5 Evidence for a fine feeder root pathogen driven decline

Decline symptoms which may be explained by a root pathogen include the significant associations between crown health, fine root density, ectomycorrhizae density, in situ seedling height and in situ seedling survival. These symptoms may be explained by the activity of a pathogen of fine and major roots, which episodically damages the roots of large trees and subsequently impairs fine roots, ectomycorrhizae and crown health. Fine feeder root loss has previously been observed in trees suffering gradual chronic deterioration including E. marginata in the jarrah forest in south-west Western Australia caused by P. cinnamomi (Somerford et al. 1987), and Quercus robur and Q. petraea in central Europe caused by several Phytophthora species including P. quercina (Jung et al. 2000).

It may also be difficult to validate the role of fine feeder root pathogens in E. gomphocephala decline, because there is probably a high rate of natural fine root turnover. Kummerow et al. (1978) proposed that Mediterranean vegetation has a high capacity for fine root production and a high rate of fine root turnover in response to seasonal variations in soil moisture and temperature. For example, E. marginata has been shown to have a high rate of turnover in fine roots throughout the year (Shea and Dell 1981), often in rapid response to rainfall events (Dell and Wallace 1983).
3.4.6 Future research

An understanding of the temporal changes in above and below ground tree health is helpful in determining if a decline is originating from above or below ground. Air spading allows detailed measurements of the rhizosphere of large trees. However, it is difficult to measure temporal changes in the rhizosphere of trees that have been exposed and disturbed using the air spade. Air spading may be useful in measuring the relationship between the above and below ground health of trees across a range of decline classes. If declining trees have more fine root than foliar damage, compared to healthier trees, this would suggest a decline is originating from the bottom up. Alternatively, if declining trees have more foliar than fine root damage, compared to healthier trees, this would suggest a decline is originating from the top down. However, it may be difficult to detect differences in above or below ground tree health. Damage originating in one part of the tree may be quickly expressed in co-dependent structures through feedback mechanisms, especially in the fine roots and foliage which are metabolically and hydrologically active.

Changes in below ground tree health may also be assessed using non-destructive techniques, such as the use of the ground penetrating radar (Nadezhdina and Cermak 2003; Zenone et al. 2008). Disease progress may be monitored within trees using non-destructive techniques which measure physiological characteristics. Non-destructive physiological measurements may include assessments of the soil water profile, soil respiration, leaf water potential and photosynthesis (Drake 2008; Bader et al. 2010).

Further Research is required to determine how rainfall and temperature affect the isolation of P. multivora.


3.4.7 Conclusion

The below ground fine root and ectomycorrhizae assessment techniques developed in this study, provide a practical and logical approach to determine the aetiology of large tree declines involving root pathogens. This approach may be expanded upon to determine if large tree declines result from above or below ground damage.

Results show a clear relationship between above and below ground health of declining E. gomphocephala trees, and reduced E. gomphocephala seedling survival and health. The results also confirm the importance of ectomycorrhizae to E. gomphocephala. Any pressures which reduce ectomycorrhizal density, such as Phytophthora root pathogens (Blom et al. 2009) or frequent fires (Malajczuk and Hingston 1981), are therefore likely to reduce plant health. The fine feeder root pathogen *P. multivora* has previously satisfied Koch’s postulates, by being isolated from declining *E. gomphocephala* and *E. marginata* (Scott et al. 2009, Chapter 4) and causing disease after artificial inoculation (Chapter 5). The observed loss of fine roots and ectomycorrhizae in declining trees, and the reduced *in situ* seedling health, may be explained by *P. multivora* episodically damaging the roots of large trees and subsequently impairing ectomycorrhizae and crown health.
Chapter 4. *Phytophthora multivora* sp. nov., a new species recovered from declining *Eucalyptus, Banksia, Agonis* and other plant species in Western Australia

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**Minor authors’ contributions:** Barber PA, Shearer BL and Hardy GEStJ (academic) were supervisors; Burgess TI (academic) assisted with molecular sequence analysis; Stukely MJC (industry) provided isolate information; and Jung T (academic) provided technical assistance on the isolation, identification and description of *Phytophthora* species.
Chapter 4 Abstract

A new *Phytophthora* species, isolated from the rhizosphere soil of declining or dead trees of *Eucalyptus gomphocephala*, *E. marginata*, *Agonis flexuosa* and another 13 plant species, and from fine roots of *E. marginata* and collar lesions of *Banksia attenuata* in Western Australia, is described as *Phytophthora multivora* sp. nov. It is homothallic and produces semipapillate sporangia, smooth walled oogonia containing thick-walled oospores, and paragynous antheridia. Although morphologically similar to *P. citricola*, phylogenetic analyses of the ITS and coxI gene regions demonstrate that *P. multivora* is unique. *P. multivora* is pathogenic to bark and cambium of *E. gomphocephala* and *E. marginata* and is believed to be involved in the decline syndrome of both of these eucalypt species within the *E. gomphocephala* woodland in south-west Western Australia.

4.1 Introduction

The oomycete genus *Phytophthora* includes many well-known species that contribute to and often drive tree declines worldwide. Knowledge about the diversity and significance of *Phytophthora* species in forest ecosystems has significantly increased in recent years as research has focussed on new and devastating tree declines in natural ecosystems in Europe and the Americas ([Jung et al. 1999; Jung et al. 2000; Vetraino et al. 2001; Jung et al. 2002; Rizzo et al. 2002; Vetraino et al. 2002; Jung and Blaschke 2004; Brasier et al. 2005; Balci et al. 2007; Greslebin et al. 2007; Jung and Nechwatal 2008]), and advances in molecular techniques have improved our phylogenetic understanding of the genus ([Cooke et al. 2000; Kroon et al. 2004]). Since the discovery of *P. cinnamomi* in the south-west of Western Australia (WA) ([Podger et al. 1965]), this introduced pathogen has become renowned for its unparalleled impact on flora.
biodiversity with 40% of the 5710 species in the South-west Botanical Province found to be susceptible and 14% highly susceptible (Shearer et al. 2004a).

As a result of the wide scale forest quarantine and management of *P. cinnamomi* in WA, extensive and regular testing of soil and plant tissue samples for *P. cinnamomi* at the Vegetation Health Service (VHS) laboratory of the Department of Environment and Conservation has led to the isolation of a large range of *Phytophthora* species and undescribed *Phytophthora* taxa (Stukely et al. 1997; Stukely et al. 2007a; Stukely et al. 2007b; Burgess et al. 2009). The recovery of *Phytophthora* taxa other than *P. cinnamomi* from some sites with declining vegetation in WA has recently focussed attention onto their role in the decline of these woodland and forest ecosystems (Shearer and Smith 2000).

Across the south-west of Western Australia there are a number of recently observed and significant forest declines occurring. In particular, the declines of *Corymbia calophylla* (Paap et al. 2008), *Eucalyptus wandoo* (Hooper and Sivasithamparam 2005), *E. gomphocephala* (tuart, Fig. 4.1a-b), *E. marginata* (jarrah, Fig. 4.1c-d), *E. rudis, Agonis flexuosa* and *Banksia* species (Fig. 4.1e-f) are causing concern to land managers and the general community. Within the *E. gomphocephala* woodland of Yalgorup National Park on the Swan Coastal Plain south of Perth, Western Australia, a significant decline and substantial numbers of deaths of *E. gomphocephala* have been observed together with localised declines and mortality of *E. marginata* since the 1990’s and *A. flexuosa* since 2006. A range of biotic and abiotic factors has been shown to contribute to *E. gomphocephala* decline (Edwards 2004; Archibald 2006), although as yet, no satisfactory aetiology has been established. The progressive canopy thinning and dieback, and the heterogenous distribution of the decline, are similar to Jarrah dieback (Shearer and Tippett 1989) and suggest the potential involvement of a *Phytophthora* species.
In May and June 2007, five *Phytophthora* isolates were recovered from the rhizosphere of declining *E. gomphocephala, E. marginata* and *A. flexuosa* in Yalgorup National Park. These isolates morphologically resembled *P. citricola*, which has been recovered over the past three decades throughout the south-west of WA by the VHS (Stukely *et al.* 1997). However, recent re-evaluation of the VHS culture collection using molecular techniques has identified most of these isolates as a new taxon (P. sp. 4) in the *P. citricola* complex (Burgess *et al.* 2009). DNA sequence data from the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S gene of the rRNA operon and the mitochondrial *cox1* gene were used in combination with morphological and physiological characteristics to characterise these isolates and compare them to the ex-type isolate of *P. citricola* as described by Sawada (1927). Due to their unique combination of morphological and physiological characters, and ITS and *cox1* sequences, these semipapillate homothallic isolates from the south-west of WA are described here as a new species, *P. multivora* sp. nov.
Fig. 4.1. a. severe dieback and mortality of a forest stand of *Eucalyptus gomphocephala*; b. crown symptoms of a declining *E. gomphocephala* including thinning, clustering of leaves, and dieback of branches and parts of the crown; c. dieback and mortality of a forest stand of *E. marginata*; d. crown symptoms of a declining *E. marginata* including thinning, clustering of leaves and dieback of branches and parts of the crown; e-f. collar rot of *Banksia attenuata* caused by *Phytophthora multivora*; e. sudden wilting and death due to the girdling of the collar; f. tongue-shaped, orange-brown necrosis of the inner bark.
4.2 Methods

4.2.1 Sampling and Phytophthora isolation

Phytophthora isolates were obtained from the *E. gomphocephala* forest using methods of soil sampling, baiting and isolation techniques modified from Jung et al. (1996; 2000). Soils were sampled beneath trees of *E. gomphocephala*, *E. marginata* or *A. flexuosa* from 32 sites (four trees per site). Sites sampled included 24 sites with all stages of crown dieback, and 8 sites without visible signs of canopy decline. From each tree a total of 4 L of soil was collected from 4 points, at a distance of 50–150 cm from the stem base. Soils were sampled below the upper 5 cm organic layer to a depth of 30 cm, mainly along lateral roots. The four subsamples from each tree were bulked, and baited in 35 x 35 cm polypropylene trays. Samples were pre-moistened for 12 hours before flooding with distilled water to 3–4 cm in depth above the soil line. Floating organic material was moved to the side of the baiting tray with flyscreen meshing and any remaining organic material floating on the surface of the baiting water was removed with paper towelling. Juvenile leaves of *Quercus ilex*, *Q. suber* and *Pittosporum undulatum* were floated on the water as baits. Leaves with brownish lesions appearing after 48–96 hours were examined for the presence of *Phytophthora* sporangia using a light microscope. Leaflets with sporangia were blotted dry, and the lesions cut into 1–2 mm² sections and plated onto *Phytophthora* selective PARPNH medium (Jung et al. 2000). Colonies growing from the plated lesion sections were transferred to V8 agar for confirmation as *Phytophthora* isolates.

4.2.2 Phytophthora isolates

In addition to the five semi-papillate *Phytophthora* isolates (WAC13200–WAC13204) collected in the present study, another two isolates were used for morphological and physiological comparisons including a semi-papillate isolate from the VHS collection
(DCE 236, WAC13205) previously isolated from fine roots of a recently dead *E. marginata* in the jarrah forest near Jarrahdale in 1981, and the ex-type isolate of *P. citricola* (IMI 021173) recovered from *Citrus sinensis* fruits in Taiwan (Sawada 1927) (Table 4.1).

Immediately prior to the present study, all isolates maintained in 90 mm petri dishes on V8A media and as 9 mm diam. V8A discs stored in 20 mL sterile water in McCartney bottles, were passaged through juvenile leaves of *Q. suber* used as baits on colonised agar discs flooded with sterile deionised water, and re-isolated using PARPNH selective medium.
Table 4.1. Isolates of *Phytophthora multivora*, *P. citricola* and *P. ‘inflata’* considered in the morphological, physiological and phylogenetic studies

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Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; IMI = CABI Bioscience (Imperial Mycological Institute), UK; WAC = Department of Agriculture and Food Western Australia Plant Pathogen Collection, Perth, Australia; VHS = Vegetation Health Service of the Department of Environment and Conservation, Perth, Australia; DDS, DCE = earlier prefixes of VHS collection. Other isolate names and numbers are as given on GenBank.

Isolates used in the morphological and growth-temperature studies.
4.2.3 Colony morphology, growth rates and cardinal temperatures

Hyphal morphology and colony growth patterns were described from 7-day-old cultures grown at 20°C in the dark on V8A, malt-extract agar (MEA), corn-meal agar and potato-dextrose agar (PDA) (all from BBL, Becton, Dickinson and Co, Sparks MD 21152 United States of America). Colony morphologies were described according to Brasier and Griffin (1979), Erwin and Ribeiro (1996) and Jung et al. (2003). Radial growth rate was recorded 5–7 days after the onset of linear growth along two lines intersecting the centre of the inoculum at right angles (Jung et al. 1999). The growth test was repeated once. For temperature growth studies, all isolates were subcultured onto V8A plates and incubated for 24 hours at 20°C to initiate growth. Three replicate plates for each isolate and temperature were then transferred to incubators set at 10, 15, 17.5, 20, 22.5, 25, 30 and 32.5°C, and radial colony growth was measured as above after 5-7 days.

4.2.4 Morphology of sporangia and gametangia

Sporangia and gametangia were produced on V8A and measurements were made as described by Jung et al. (1999). Sporangia were obtained by flooding 5 x 5 mm square agar discs taken from growing margins of 3-5 day-old colonies with non-sterile soil extract in 90 mm Petri dishes and incubating them in the dark at 18–22°C for 12 to 16 hours. The non-sterile soil extract was obtained by flooding 100ml of commercial composted potting mix (Richgro, Jandakot, WA) with 1 L of deionised water. After 24 hours at 10-25°C, the soil extract was removed from the water surface with a pipette and diluted to 10% with deionised water. Dimensions and characteristic features of 50 mature sporangia chosen at random were determined at x400 magnification (BH-Olympus) for each isolate. For each isolate dimensions and characteristic features of 50 mature oogonia, oospores and antheridia, and diameters of 25 primary hyphae chosen at
random were measured at x400 magnification at the surface of 15 mm discs cut from the centre of 14–22 days old V8A cultures grown in the dark at 20°C. For each isolate the oospore wall index was calculated as the ratio between the volume of the oospore wall and the volume of the entire oospore (Dick 1990).

4.2.5 DNA isolation, amplification and sequencing
The Phytophthora isolates were grown on half strength potato dextrose agar PDA (Becton, Dickinson and Company, Sparks, United States of America, 19.5g PDA, 7.5 g of agar and 1 L of distilled water) at 20°C for 2 weeks and the mycelium was harvested by scraping from the agar surface with a sterile blade and placed in a 1.5 mL sterile Eppendorf® tube. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder and genomic DNA was extracted according to Andjic et al. (2007). The region spanning the internal transcribed spacer (ITS)1-5.8S-ITS2 region of the ribosomal DNA was amplified using the primers ITS-6 (5’ GAA GGT GAA GTC GTA ACA AGG 3’)(Cooke et al. 2000) and ITS-4 (5’TCC TCC GCT TAT TGA TAT GC 3’) (White et al. 1990). The PCR reaction mixture, PCR conditions, the clean-up of products and sequencing were as described by Andjic et al. (2007).

The mitochondrial gene coxI was amplified with primers Fm84 (5’T TT T AAT T TT TAG TGC TTT TGC) and Fm83 (5’CTC CAA TAA AAA ATA ACC AAA AAT G) (Martin and Tooley 2003). The PCR reaction mixture was the same as for the ITS region, but the PCR conditions were as described previously (Martin and Tooley 2003). Templates were sequenced in both directions with primers used in amplification, as well as primers FM 85 (5’AAC TTG ACT AAT AAT ACC AAA) and FM 50 (5’GTT TAC TGT TGG TTT AGA TG) (Martin and Tooley 2003). The clean-up of products and sequencing were the same as for the ITS region.
4.2.6 Phylogenetic analysis

In order to compare *Phytophthora* isolates used in this study with other closely related species (ITS clade 2, Cooke *et al.* 2000), additional sequences were obtained from GenBank (Table 4.1). Sequences were also obtained for species representing other ITS clades (Cooke *et al.* 2000). Sequence data for the ITS region were initially assembled using Sequence Navigator version 1.01 (Perkin Elmer, Connecticut, United States of America) and aligned in Clustal X (Thompson *et al.* 1997). Manual adjustments were made visually by inserting gaps where necessary in Bioedit version 5.0.6 (Hall 2001). There were no gaps in the *coxI* alignment. All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 4.1.

Parsimony analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2003). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis 1992). Branch and branch node support was determined using 1000 bootstrap replicates (Felsenstein 1985).

Bayesian analysis was conducted on the same individual dataset as that used in the parsimony analysis. First, MrModeltest v.2.5 (Nylander, J.A.A. 2004 Program distributed by the author. Evolutionary Biology Centre, Uppsala University) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v. 3.1 (Ronquist and Huelsenbeck 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of
Markov Chain Monte Carlo (MCMC) using 4 chains were run over 1 000 000 generations. Trees were saved each 1 000 generations, resulting in 10 001 trees. Burn-in was set at 101 000 generations (i.e. 101 trees), well after the likelihood values converged to stationary, leaving 9 900 trees from which the consensus trees and posterior probabilities were calculated.

4.2.7 Statistical analysis
Analyses of Variances were carried out using Statistica version 5.1 (Statsoft Inc., Tulsa, Oklahoma) to determine whether physiological and morphological measurements were different between isolates.

4.3 Results

4.3.1 Phylogenetic analysis
The ITS dataset consisted of 894 characters of which 420 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1000 random trees ($P < 0.01, g_1 = -1.38$). Heuristic searches resulted in 10 most parsimonious trees of 848 steps (CI = 0.72, RI = 0.89). The topology of the Bayesian tree was very similar (TreeBASE SN4153) (Fig. 4.2). Several ITS sequences from GenBank are identical to *P. multivora* (EU244846, EU594606, AB367494, EU194425, EU000083, AF228080, AB217685, DQ821185, AM235209, EF153674, EF193230). These sequences were not all included in the phylogenetic analysis, but information on origin and studies they have derived from is given in Table 4.1 and the position of polymorphic nucleotides indicating their similarity to *P. multivora* in Table 4.2. In addition, on GenBank there are several isolates of *P. multivora*, originally designated as P. sp. 4 by Burgess *et al.* (2009).
All isolates of *P. multivora* reside in a strongly supported terminal clade clearly distinct from the ex-type and authentic type of *P. citricola* (IMI 021173 and CBS 195.29) within ITS clade 2 (Cooke *et al.* 2000). Two additional isolates listed on GenBank (Citri-P0713 and BR518) have identical sequences to the ex-type of *P. citricola* (IMI 021173) (Fig. 4.2). There are seven fixed polymorphisms that are different between *P. multivora* and IMI 021173 (Table 4.2). Isolates listed on GenBank as *P. citricola* from the northern hemisphere (CIT7, CIT9, CIT35, P44, MN21HH, UASWS0208, 92-198, P131, IMI031372, 112) differ by at least 10 bp from *P. multivora*. (Fig. 4.2; Table 4.2). Isolates listed on GenBank as *P. ‘inflata’* (6f, P44, InfGaul, InfRhod2, InfVacc, 804, IMI 342898) are dispersed among the northern hemisphere isolates of *P. citricola* (Fig. 4.2; Table 4.2) and it is unclear whether any of these isolates represent the original *P. inflata*. 
**Fig. 4.2.** Bayesian inference tree using rDNA ITS sequences showing phylogenetic relationships between (A) clade 2 species and representative species from other clades and (B) isolates from the *P. 'citricola' complex*. Numbers above branches represent posterior probability based on Bayesian analysis of the dataset. Both trees result from a single analysis as given in TreeBASE (SN4153). For tree A, clades were collapsed to show the relationship between isolates from *P. citricola* complex and other species in clade 2. Tree B shows the finer details within the *P. citricola* complex (node enclosed in circle on tree A) and the relationship between *P. multivora* and other *P. citricola* and *P. 'inflata'* isolates including the ex-type of *P. citricola* (IMI021173).
Table 4.2. Positions of polymorphic nucleotides (bp) from aligned sequence data of the ITS gene region showing the variation between *Phytophthora multivora*, *P. citricola* and *P. ‘inflata’* isolates. Polymorphisms that differ from the type of *P. multivora* (WAC13201) are shaded.

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The **coxI** dataset consisted of 742 characters of which 107 were parsimony informative.
The dataset contained significant phylogenetic signal compared to 1000 random trees \( P < 0.01, g1 = -0.66 \). Heuristic searches resulted in 12 most parsimonious trees of 301 steps (CI = 0.50, RI = 0.67). The topology of the Bayesian tree was very similar (TreeBASE SN4153) (Fig. 4.3). Species from ITS clade 2 group together with strong support. Isolates of \( P. \) multivora were in a separate clade from the ex-type isolate of \( P. \) citricola (IMI 021173) and two European isolates (CIT9 and CIT35) also sequenced in this study, and the isolates listed on GenBank as \( P. \) inflata. There was another single sequence of \( P. \) citricola, P1817, available from the study of Kroon et al. (2004). This sequence was distinct from our sequences for \( P. \) citricola and \( P. \) multivora, all GenBank sequences for \( P. \) inflata and other ITS clade 2 species, and, based on the findings of this study, must be either an incorrectly identified isolate or an incorrect sequence.
Fig. 4.3. Bayesian inference tree using sequences of mitochondrial gene coxl showing phylogenetic relationships between *P. multivora* and *P. citricola*, including the ex-type of *P. citricola* (IMI021173).

### 4.3.2 Taxonomy

*Phytophthora multivora* P. M. Scott and T. Jung, *sp. nov.* Figs. 4.4-4.5. MycoBank no.: MB 512497
Chapter 4. *Phytophthora multivora* sp. nov., a new species recovered from declining *Eucalyptus, Banksia, Agonis* and other plant species in Western Australia

Fig. 4.4. Semipapillate sporangia of *Phytophthora multivora* on V8 agar. Scale bar = 50 µm, applies to a-l; a-i after 12-24 hours flooding with soil extract. a. ovoid, the left sporangium with swollen papilla shortly before release of the already differentiated zoospores; b. ovoid; c. limoniform; d. obpyriform; e. bi-papillate; f. bi-papillate, bilobed; g. limoniform, laterally inserted to the sporangiosphere (arrow); h. ovoid, shortly before release of zoospores; i. limoniform, intercalary inserted, with conspicuous basal plug (arrow) protruding into empty sporangium after release of zoospores; j-l direct germination after 48 hours flooding. j. bipapillate, bilobed with several germ tubes growing from each papilla; k, l. bi-papillate, bell-shaped with one germ tube growing from each papilla.
Chapter 4. Phytophthora multivora sp. nov., a new species recovered from declining Eucalyptus, Banksia, Agonis and other plant species in Western Australia

Fig. 4.5. a-d. Oogonia of Phytophthora multivora with paragynous antheridia and plerotic oospores on V8 agar; scale bars = 25 µm; a. juvenile oogonium with thin-walled oospore and undifferentiated cytoplasm; b-d. mature oogonia with thick-walled oospores and ooplast; b. oogonium on the left side is aborted; e. direct germination of oospores with several germ tubes through the oogonial bases (arrow) after 5 weeks incubation at 20 °C; f. tubular, irregular lateral hyphae.

Sporangia abundantia in cultura liquida, persistentia, terminalia, semi-papillata, ovoidea aut limoniformia, rare distorta vel bipapillata, 53 ± 10.1 x 31.8 ± 6.2 µm, ratio longitudo ad altitudinem 1.7 ± 0.2 µm. Systema sexus homothallica; oogonia globosa vel rare subglobosa, 27.1 ± 2.1 µm. Oosporae fere pleroticae, 23.9 ± 2 µm, paries 2.6 ± 0.5 µm. Antheridia paragynosa, 13 ± 2.2 x 8.8 ± 1.1 µm. Chlamydosporae et inflationes hypharum non observatae. Temperaturae crescentiae in agaro ‘V8A’, optima c. 25 °C et maxima 30–32.5 °C. Coloniae in agaro ‘V8A’ stellatae cum mycelio aerio restricto et margine submersa. Regiones ‘rDNA ITS’ et ‘coxI’ cum unica sequentia (GenBank FJ237508, FJ237521).

Etymology: Name refers to the wide host range (multi Lat = many, -vora Lat = feeding).
**Sporangia** (Fig. 4.4): Sporangia were rarely observed on solid agar but were produced abundantly in non-sterile soil extract. The majority of sporangia for all *P. multivora* isolates and the ex-type of *P. citricola* (IMI 021173) were formed between 7–12 hours after flooding with soil extract. Little variation in sporangial shapes was observed between the *P. multivora* isolates. The majority of sporangia were semipapillate and either ovoid, limoniform, ellipsoid or obpyriform (Fig. 4.4a-d, g-i), sometimes with just a very shallow apical thickening (Fig. 4.4f), non-caducous, occasionally forming a conspicuous basal plug (Fig. 4.4i) that protruded into the empty sporangium. Sporangia with two or three papillae or distorted shapes were occasionally formed by all isolates (Fig. 4.4e-f, j-l). Sporangia were typically borne terminally (Fig. 4.4a-f, j-l) but some were laterally attached (Fig. 4.4g) or intercalary (Fig. 4.4i). External proliferation was regularly observed (Fig. 4.4a-d, j, l), either irregular or in lax or dense sympodia. The majority of sporangia of each isolate had released zoospores between 15–20 hours after flooding. Compared to *P. citricola*, sporangia of *P. multivora* showed a higher proportion of abortion or direct germination (Fig. 4.4j-l) after 24-48 hours within the same soil extract. After 24-48 hours, bell-shaped sporangia were formed by all six isolates of *P. multivora* which germinated directly from two points without prior formation of papillae (Fig. 4.4k-l). The mean sporangial dimensions of the six *P. multivora* isolates were 51.0 ± 10.4 x 30.0 ± 5.1 µm (overall range of 25 – 97 x 13 – 63 µm) with a length/breadth ratio of 1.7 ± 0.22 (overall range 1.3 – 3.3). The mean sporangial dimensions of the ex-type of *P. citricola* (IMI 021173), at 50.9 ± 6.9 x 29.9 ± 5.1 µm (range 39 – 70 x 22 – 40 µm) and a length/breadth ratio of 1.7 ± 0.3 (overall range 1.3 – 2.6), were within the range of the *P. multivora* isolates (Table 4.3). In contrast to *P. multivora*, sporangia of *P. citricola* were generally more variable and often showed distorted shapes including: multiple papillae, curved apices and hyphal
beaks. Twelve percent of sporangia of *P. citricola* were distorted compared to 5% in *P. multivora*. With 9% and 10%, respectively, *P. multivora* and *P. citricola* had a similar proportion of sporangia with lateral attachment to the sporangiophore. Isolate WAC13204 was different from all other *P. multivora* isolates by forming significantly (*P < 0.05*) larger sporangia with a mean size of 62.3 ± 10.8 x 34.0 ± 4.9 µm.
Table 4.3 Morphological dimensions (µm) and temperature-growth relations of *Phytophthora multivora* and *P. citricola*

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>WAC13200</th>
<th>WAC13201</th>
<th>WAC13202</th>
<th>WAC13203</th>
<th>WAC13204</th>
<th>WAC13205</th>
<th>P. citricola</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporangia</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>lxb mean</td>
<td>56.5 ± 7 x</td>
<td>53 ± 10.1 x</td>
<td>44.2 ± 4.4 x</td>
<td>44.5 ± 7.8 x</td>
<td>62.3 ± 10.8 x</td>
<td>45.7 ± 5.2 x</td>
<td>50.9 ± 6.9 x</td>
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<tr>
<td>Range</td>
<td>31.8 ± 4</td>
<td>31.8 ± 6.2</td>
<td>26.2 ± 3.1</td>
<td>28.9 ± 4.2</td>
<td>34.0 ± 4.9</td>
<td>27.9 ± 3.6</td>
<td>29.9 ± 5.1</td>
</tr>
<tr>
<td>Oogonia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean diameter</td>
<td>25.6 ± 1.3</td>
<td>27.1 ± 2.1</td>
<td>25.5 ± 1.4</td>
<td>26.2 ± 1.5</td>
<td>26.8 ± 1.5</td>
<td>27.8 ± 2.5</td>
<td>30.3 ± 2.7</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean diameter</td>
<td>22.7 ± 1.3</td>
<td>23.9 ± 2</td>
<td>22.8 ± 1.4</td>
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<td>27.3 ± 2.6</td>
</tr>
<tr>
<td>wall diameter</td>
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<td>2.6 ± 0.5</td>
<td>2.7 ± 0.4</td>
<td>2.7 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>2.5 ± 0.5</td>
<td>1.9 ± 0.3</td>
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<tr>
<td>Antheridia</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>lxb mean</td>
<td>13.1 ± 1.8 x</td>
<td>13 ± 2.2 x</td>
<td>11.7 ± 1.9 x</td>
<td>13.5 ± 1.6 x</td>
<td>12.4 ± 1.6 x</td>
<td>13.8 ± 1.8 x</td>
<td>13.2 ± 2.5 x</td>
</tr>
<tr>
<td>lxb range</td>
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<td>8.8 ± 1.1</td>
<td>7.9 ± 1</td>
<td>9 ± 1.4</td>
<td>8.7 ± 1.4</td>
<td>8.5 ± 1.2</td>
<td>8.1 ± 1.8</td>
</tr>
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<td>Maximum temperature (°C)</td>
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<td>30–32.5</td>
<td>30–32.5</td>
<td>30–32.5</td>
<td>&gt;32.5</td>
<td>30–32.5</td>
<td>30–32.5</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
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<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>22.5</td>
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<tr>
<td>Growth rate on V8A at optimum (mm/d)</td>
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<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>6.1</td>
<td>5.4</td>
<td>5.7</td>
</tr>
<tr>
<td>V8A</td>
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<td>4.0</td>
<td>4.4</td>
<td>4.5</td>
<td>4.7</td>
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</tr>
<tr>
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<td>3.6</td>
<td>4.7</td>
<td>3.4</td>
<td>4.0</td>
</tr>
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<td>4.5</td>
<td>3.3</td>
<td>3.3</td>
<td>4.5</td>
<td>3.5</td>
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</tr>
<tr>
<td>PDA</td>
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<td>2.9</td>
<td>2.6</td>
<td>2.5</td>
<td>3.7</td>
<td>2.9</td>
<td>2.3</td>
</tr>
</tbody>
</table>

1 For isolate details see Table 4.1. 2 Ex-type isolate.
**Oogonia, oospores and antheridia** (Fig. 4.5a-e): Gametangia were readily produced in pure cultures by all *P. multivora* isolates and the ex-type of *P. citricola* (IMI 021173) on V8A within four days. Oogonia of both *P. multivora* and *P. citricola* were borne terminally, had smooth walls and were globose to slightly subglobose (Fig. 4.5a-d). With a mean diameter of 26.5 ± 1.9 µm (overall range 19–37 µm and range of isolate means 25.5 to 27.8 µm) the oogonia of the six *P. multivora* isolates were on average smaller than those of *P. citricola* (30.3 ± 2.7 µm, range 22–34 µm), although the ranges were broadly overlapping (Table 4.3). Oospores of both *P. multivora* (Fig. 4.5b-d) and *P. citricola* were globose and nearly plerotic. The *P. multivora* isolates produced significantly (*P* < 0.05) thicker oospore walls (2.6 ± 0.5 µm, overall range 1.4–4.6 µm) than *P. citricola* (1.9 ± 0.3 µm, overall range 1.2–2.6). Due to the smaller oospore size of *P. multivora* the oospore wall index was significantly higher (*P* < 0.0001) in *P. multivora* (0.52 ± 0.07) than in *P. citricola* (0.36 ± 0.05). Antheridia of both species were obovoid, club-shaped or irregular, almost exclusively paragynous, diclinous and typically attached close to the oogonial stalk. Intercalary and amphigynous antheridia were only rarely observed. After 4 weeks in V8A at 20 °C, more than 90% of all *P. multivora* oospores had germinated directly. Since the thick inner oospore wall of *Phytophthora* species erodes during the germination process due to enzymatic digestion of its major components, the glucans, (Erwin and Ribeiro 1996) only the thin outer oospore wall surrounded by the thin oogonial wall was left (Fig. 4.5e). No direct germination was observed in cultures of the ex-type of *P. citricola* (IMI 021173) growing under the same conditions.
4.3.3 Colony morphology, growth rates and cardinal temperatures

Colony growth patterns of two isolates of *P. multivora* (WAC13201 and WAC13205) and the ex-type isolate of *P. citricola* (IMI 021173) are shown in Fig. 4.6. All *P. multivora* isolates except isolate WAC13204 produced similar colony growth patterns on the four different types of media. On V8A, CMA and MEA *P. multivora* isolates produced limited aerial mycelium and distinct growth patterns, while isolate WAC13204 formed fluffy to felty, uniform colonies without distinct growth pattern. The colony morphology on V8A and MEA of all *P. multivora* isolates clearly differed from the colony morphology of the ex-type isolate of *P. citricola* (IMI 021173). *Phytophthora multivora* isolates produced stellate growth patterns with a clearly delimited, submerged margin on V8A and faintly stellate to dendroid patterns on MEA while *P. citricola* formed a typical chrysanthemum pattern on both media. On CMA, *P. multivora* isolates formed appressed to submerged colonies with a faintly stellate to petaloid pattern while *P. citricola* produced even sparser submerged colonies with a faintly stellate pattern. On PDA, the *P. multivora* isolates and *P. citricola* produced petaloid felty to fluffy colonies. Diameters of primary hyphae varied from 3.8–4.6 µm. Lateral hyphae of *P. multivora* were often tubular and slightly inflated (Fig. 4.5f). No substantial differences were observed between hyphae of *P. multivora* and *P. citricola*. 
Chapter 4. *Phytophthora multivora* sp. nov., a new species recovered from declining *Eucalyptus, Banksia, Agonis* and other plant species in Western Australia

Temperature growth relations of *P. multivora* and the ex-type isolate of *P. citricola* are shown in Fig. 4.7. The maximum growth temperature for isolates of both *P. multivora* and the ex-type of *P. citricola* (IMI 021173) on V8A was between 30 and 32.5 °C. All isolates of *P. multivora* except isolate WAC13204 were unable to grow at 32.5 °C, but started re-growth within 12 hours when plates that were incubated for 7 days at 32.5 °C were transferred to 25 °C. All six *P. multivora* isolates had a growth optimum at 25 °C with growth rates ranging from 4.7–6.1 mm/d while *P. citricola* showed a broad growth optimum between 22.5 °C (5.7 mm d⁻¹) and 30 °C (5.5 mm/d). Compared to all *P. multivora* isolates the growth rate of *P. citricola* at 20 °C was higher on V8A and CMA and lower on PDA (Table 4.3). On V8A, over the whole temperature range

![Image of colony morphology](image-url)
except at 25 °C, all *P. multivora* isolates were markedly slower growing than *P. citricola* (Fig. 4.7).

![](image.png)

**Fig. 4.7.** Radial growth rates of *Phytophthora multivora* (means and standard errors calculated from six isolates), solid line, and the ex-type isolate of *P. citricola*, dashed line, on V8 agar at different temperatures.

*Specimens examined.* **WESTERN AUSTRALIA,** Yalgorup, from rhizosphere soil of declining *Eucalyptus gomphocephala,* May 2007, T. Jung and P. Scott, WAC13200; from rhizosphere soil of declining *Eucalyptus marginata,* May 2007, P. Scott and T. Jung, holotype MURU 434 (dried culture on V8A, Herbarium of Murdoch University, Western Australia, Australia), culture ex-type WAC13201; from rhizosphere soil of declining *Eucalyptus gomphocephala,* June 2007, P. Scott, WAC13202; from rhizosphere soil of declining *Agonis flexuosa,* June 2007, P. Scott, WAC13203; from rhizosphere soil of declining *Eucalyptus gomphocephala,* June 2007, P. Scott, WAC13204; North Jarrahdale, from rhizosphere soil of declining *Eucalyptus marginata,* 1980, unknown, WAC13205.
4.3.4 Notes

In previous studies *P. multivora* is referred to as *P. citricola* (Shearer et al. 1987; Shearer et al. 1988; Shearer and Tippett 1989; Bunny 1996; Stukely et al. 1997), and more recently as P. sp. 4 (Burgess et al. 2009). Many isolates from a wide range of host species in WA that had been identified as *P. citricola* in the past must be reassigned to *P. multivora*. As indicated above, *P. multivora* has been isolated from the south-west of WA from rhizosphere soil of *E. gomphocephala*, *E. marginata* and *A. flexuosa* in Yalgorup National Park. It has also been recovered by the VHS from soil and root samples collected beneath dying, *Phytophthora*-sensitive “indicator species” in native ecosystems in the south-west of WA by the VHS over the last 30 years, which extends the host list to include *Banksia attenuata*, *B. grandis*, *B. littoralis*, *B. menziesii*, *B. prionotes*, *Conospermum* sp., *Leucopogon verticillatus*, *Xanthorrhoea gracilis*, *Podocarpus drouyniana*, *Patersonia* sp., *Bossiaeae* sp., *Gastrolobium spinosum* and *Pinus radiata* (plantation) (Burgess et al. 2009). *Phytophthora multivora* has also recently been isolated from large girdling stem lesions of *B. attenuata* in Injidup, WA (Hardy personal communication, Fig. 4.1e-f), and from fine roots of declining *E. marginata* in the Jarrah forest near Jarrahdale in 1981 (Stukely personal communication) and near Dwellingup in 2008 (Jung personal communication).

4.4 Discussion

*Phytophthora multivora* was previously identified as *P. citricola* in WA based solely on morphological characters including homothallic breeding behaviour, production of paragynous antheridia, semipapillate persistent sporangia and oogonia with dimensions in the correct range, absence of catenulate hyphal swellings in liquid culture, and similar growth rates at 25°C. Phylogenetic analyses of the ITS and *coxI* gene regions show that
P. multivora is unique and comprises a discrete cluster within the major ITS clade 2 of Cooke et al. (2000) with its present closest relative being P. citricola.

Morphological and molecular studies using a broad range of P. citricola isolates have demonstrated that P. citricola is very diverse (Oudemans et al. 1994; Bhat and Browne 2007; Moralejo et al. 2008), and that many of the differences are associated with host and geography (Bhat and Browne 2007). In the isozyme study of Oudemans et al. (1994) a global collection of 125 isolates of P. citricola clustered into five distinct subgroups suggesting P. citricola is a species complex instead of a single species which is to be expected considering the broad geographic and host range of P. citricola isolates (Fontaneto et al. 2008; O'Malley 2008).

Even though multiple P. citricola sequences have been submitted to GenBank, sequence data for the ex-type of P. citricola (IMI 021173) from Citrus sinensis fruits in Taiwan (Sawada 1927) has not previously been available, and this has led to confusion in the phylogeny. Besides the ex-type culture, an authentic type of P. citricola (CBS 195.29) isolated from Citrus leaves in Japan was submitted to CBS in 1929 by Sawada. The present study is the first to provide sequence data of these isolates, and our results clearly demonstrate that the isolates from WA constitute a new species, P. multivora. Isolates designated as P. ‘inflata’ were distributed through the P. citricola complex demonstrating the difficulty in distinguishing between P. inflata and P. citricola. The original P. inflata ex-type from elm trees in the United States (Caroselli and Tucker 1949) has been lost and it has been suggested that designated isolates of P. inflata from other hosts (Hall et al. 1992) are conspecific with P. citricola (Cooke et al. 2000). Among isolates from the P. citricola complex, isolates now described as P. multivora are the most distant to the ex-type of P. citricola, differing in the ITS region by 7 bp. However, there appear to be many sub-clades within the P. citricola complex which may correspond to additional new taxa. Further study of this important species complex is
required to elucidate the host and geographic range and phylogeny of isolates within the complex and to determine if they constitute new species.

In GenBank, eleven ITS sequences, designated as *P. citricola*, are identical to *P. multivora*. Seven are from unpublished studies in Hungary, Canada, Switzerland, Korea and Japan, and two sequences are from isolates of Moralejo et al. (2008) from ornamental nurseries in Spain, an isolate from *Mangifera indica* in Spain (Zea-Bonilla et al. 2007). In addition, an isolate designated as *P. sojae* in a study from Japan also has identical sequence (Villa et al. 2006). This low number of very recent submissions of *P. multivora* sequences as compared to the high number of other sequences from the *P. citricola* complex indicates that *P. multivora* may have been introduced to these countries. Due to the widespread distribution of *P. multivora* across natural ecosystems in WA, it is likely that WA may be a source of dispersal, possibly via the nursery trade (Brasier 2008). However, in a recent molecular study, *P. multivora* isolates from South African populations were found to be more diverse than isolates from Australia, indicating that *P. multivora* may not be native to Australia but was introduced more recently than in South Africa (Burgess, personal communication) (Chapter 6 section 6.4.4). Further work is required to determine the origins of *P. multivora* and ensure it is not moved into new ecosystems.

In the *coxI* analysis *P. multivora* and the ex-type isolate of *P. citricola* grouped together with other ITS clade 2 species, although the distance between *P. multivora* and *P. citricola* was greater in the *coxI* analysis than in the ITS analysis. In the ITS sequence there was only 1bp difference between all isolates of *P. multivora*. In the *coxI* analysis there were more differences resulting in the formation of sub-clades. The greater phylogenetic variation and presence of sub-clades in the *coxI* analysis reflects the expected faster rate of mitochondrial than genomic DNA evolution (Wallace 2008). The observed variability, however, strongly supports the hypothesis that *P. multivora* in
WA is not a recent clonal introduction, but rather was introduced long ago or is endemic. This is also reflected by the phenotypic variability observed among isolates of *P. multivora*. There was generally some variation in the colony growth patterns and growth rates, and in the dimensions of morphological structures of the different *P. multivora* isolates. However, isolate WAC13204 was particularly different from the other five isolates of *P. multivora*, having significantly larger sporangia, a higher maximum growth temperature and faster growth rates.

A *coxI* sequence for *P. citricola* was available on Genbank from the study of Kroon *et al.* (2004). In their study, this putative *P. citricola* was closest to *P. cryptogea* and they discussed this incongruency, as it was one of the few species that did not fall into the same clades in both the mitochondrial and nuclear gene analysis. With our new sequences for *P. citricola*, including the ex-type isolate, it is clear that the sequence used for *P. citricola* by Kroon *et al.* (2004) was incorrect.

Morphological similarities between taxa, as observed between *P. multivora* and *P. citricola*, are increasingly found in the unravelling of different species complexes within the genus *Phytophthora* using molecular methods (Brasier 2003; Brasier *et al.* 2003a; Brasier *et al.* 2003b; Jung *et al.* 2003). This study therefore highlights the importance of using ex-type cultures where available and the value of using molecular tools to unravel the ambiguity of species previously identified solely on morphological characteristics. Over the last 30 years, in the absence of sufficient molecular techniques, *P. multivora* has been routinely identified in the south-west of WA as *P. citricola* using morphological characteristics. Similar mis-identification of *Phytophthora* species has occurred with the identification of *P. pseudosyringae* isolates as *P. syringae* (Jung *et al.* 2003).

Despite the similarities, there are clear morphological and physiological differences between *P. multivora* and the ex-type isolate of *P. citricola*. If more isolates
of *P. citricola* were to be examined the morphological differences between the two species may be less resolved. *P. multivora* and *P. citricola* produce different colony growth patterns on V8A, MEA and CMA with the most distinct variation observed on V8A. *Phytophthora multivora* has a clear optimum growth temperature of 25 °C while the optimum growth rate of *P. citricola* is at 22.5 °C and decreases by only 0.2 mm/d between 22.5 and 30 °C. Over the whole temperature range, except of the optimum temperature of 25 °C, *P. multivora* isolates are slower growing than *P. citricola*. Sporangial shapes of *P. multivora* are generally more uniform while in *P. citricola* sporangia are more variable and the frequency of distorted shapes is significantly higher. A high variability of sporangial shapes was also found by Zentmyer *et al.* (1974) studying *P. citricola* isolates from *Persea americana* in California. Although most morphological measurements of the ex-type isolate of *P. citricola* fell within the range of *P. multivora* isolates there were clear differences between both species. All six *P. multivora* isolates produced on average significantly smaller oogonia and oospores, and significantly thicker oospore walls than *P. citricola*. This is reflected by the oospore wall index, which is the ratio between the volume of the oospore wall and the volume of the entire oospore (Dick 1990). The oospore wall index of *P. multivora* (0.52 ± 0.07) was almost 50% higher than that of the ex-type isolate of *P. citricola* (0.36 ± 0.05). A calculation of the oospore wall index using the original datasets of Jung *et al.* (1999; 2002; 2003), for *P. europaea* (0.37 ± 0.07), *P. ilicis* (0.41 ± 0.11), *P. pseudosyringae* (0.27 ± 0.09), *P. psychrophila* (0.42 ± 0.06), *P. quercina* (0.45 ± 0.08), *P. syringae* (0.24 ± 0.07) and *P. uliginosa* (0.46 ± 0.09) demonstrated that *P. multivora* had the highest oospore wall index of all nine species examined. The thick oospore wall of *P. multivora* is most likely an adaptation to the seasonally extremely dry soil conditions in WA. This survival mechanism was also suggested for *P. quercina* in
European oak forests (Jung et al. 1999, 2000). After 4 weeks in V8A at 20 °C, in all six *P. multivora* isolates, more than 90% of the oospores had germinated directly. This lack of dormancy had previously been observed for oospores of *P. medicaginis* (Erwin and Ribeiro 1996), however, this does not preclude dormancy occurring under different conditions. No direct germination was observed in cultures of the ex-type isolate of *P. citricola* growing under the same conditions. This result corresponds to the low oospore germination rates observed in European isolates of the *P. citricola* complex (Delcan and Brasier 2001). Whether these differences in germination rates reflect different survival mechanisms of the two species requires further investigation.

*Phytophthora multivora* can easily be distinguished from other homothallic *Phytophthora* species with paragynous antheridia and semipapillate sporangia by its unique combination of morphological and physiological characters, and DNA sequences. *Phytophthora multivora* is separated from *P. syringae* by the absence of hyphal swellings, the occurrence of distorted and bipapillate sporangia, thicker oospore walls, different colony growth patterns on V8A, MEA and CMA, higher optimum and maximum temperatures for growth, and different ITS and coxI sequences (Jung et al. 2003). *Phytophthora multivora* can be distinguished from *P. pseudosyringae* by the absence of hyphal swellings and caducity of sporangia, the occurrence of distorted bipapillate sporangia, thicker oospore walls, different colony growth patterns on V8A, MEA and CMA, higher optimum and maximum temperatures for growth, and different ITS and coxI sequences (Jung et al. 2003). *Phytophthora multivora* is discriminated from the original *P. inflata* of Caroselli and Tucker (1949) by having larger sporangia, markedly smaller oogonia and thinner oospore walls, and by the absence of inflated irregular antheridia which are often twining or twisted around the oogonial stalk in *P. inflata*.

Under the original morphological identification as *P. citricola*, an isolate of
*P. multivora* was used in an under-bark inoculation test, and caused significantly longer lesions on stems of *E. marginata* and *C. calophylla* than *P. cinnamomi* (Shearer *et al.* 1988).

*Phytophthora multivora* has been isolated in WA from natural forest and heath-land stands for the last 30 years from beneath dead and dying plants of 16 species from seven families. *Phytophthora multivora* is very widespread in south-west WA with a distribution similar to that known for *P. cinnamomi*. The VHS uses detection methods developed specifically for *P. cinnamomi* and even under these conditions, *P. multivora* is the next most commonly isolated taxon after *P. cinnamomi*. There is now evidence that in some sites it may be *P. multivora* and not *P. cinnamomi* that is responsible for tree mortality, while the latter is driving the collapse of whole ecosystems known as *Phytophthora* dieback. These findings may have direct implications for forest management and biosecurity, and our study highlights the potential importance of new and yet undescribed *Phytophthora* taxa in natural ecosystems in the south-west of WA (Burgess *et al.* 2009), and the need for continued research.
Chapter 5. Pathogenicity of *Phytophthora multivora* to *Eucalyptus gomphocephala* and *E. marginata*

Submitted as: Scott PM, Jung T, Shearer BL, Barber PA, Calver MC, Hardy GESJ (2011) Pathogenicity of *Phytophthora multivora* to *Eucalyptus gomphocephala* and *E. marginata*. *Forest Pathology.*

Minor authors’ contributions: Shearer BL, Barber PA, and Hardy GESJ (academic) were supervisors; Calver MC (academic), assisted with the data analysis; and Jung T (academic) provided technical assistance *Phytophthora* species.
Chapter 5 Abstract

*Phytophthora multivora* is associated with the rhizosphere of declining *Eucalyptus gomphocephala* (tuart), *E. marginata* (jarrah) and *Agonis flexuosa* (peppermint). Two pathogenicity experiments were conducted. The first examined *ex situ* the pathogenicity of five *P. multivora* isolates and one *P. cinnamomi* isolate on the root systems of *E. gomphocephala*, and one *P. multivora* isolate on the root system of *E. marginata*. In the second experiment, the pathogenicity of *P. multivora* to *E. gomphocephala* and *E. marginata* saplings was measured *in situ* using under-bark stem inoculation. In Experiment 1 the *P. cinnamomi* isolate was more aggressive than all *P. multivora* isolates, causing significant loss of fine roots and plant death. Two *P. multivora* isolates and the *P. cinnamomi* isolate caused significant losses of *E. gomphocephala* fine roots 0-2 mm in diameter and significantly reduced the surface area of roots 0-1 mm in diameter. One *P. multivora* and the *P. cinnamomi* isolate significantly reduced the surface area of roots 1-2 mm in diameter. Two of the *P. multivora* isolates significantly reduced the number of *E. gomphocephala* root tips. The length and surface area of *E. marginata* roots 0-1 mm in diameter and number of root tips were significantly reduced by *P. multivora* infestation. Rhizosphere infestation with the *P. multivora* isolates and *P. cinnamomi* isolate on *E. gomphocephala*, and one *P. multivora* isolate on *E. marginata*, did not significantly influence the foliar nutrient concentrations. In Experiment 2 under-bark stem inoculation with *P. multivora* caused significant lesion extension in *E. gomphocephala* and *E. marginata* saplings, compared to the control. We propose that *P. multivora* is inciting *E. gomphocephala* and *E. marginata* decline by causing fine root loss and subsequently interfering with nutrient uptake in plants. The impact of fine root loss on the physiology of plants in sites infested with *P. multivora* requires further research.
5.1 Introduction

Decline and mortality of *Eucalyptus gomphocephala* (tuart) has occurred throughout its native range in the south-west of Western Australia since the early 1990’s. In particular, the greatest decline has occurred in *E. gomphocephala* and *E. marginata* (jarrah) since 2000 (Edwards 2004) and *Agonis flexuosa* (peppermint) since 2007 (Taylor *et al.* 2009) in the woodlands of the Yalgorup region on the Swan Coastal Plain south of Perth, Western Australia. Although a range of biotic and abiotic factors have been shown to contribute to *E. gomphocephala* decline (Edwards 2004; Archibald 2006; Cai *et al.* 2010), no satisfactory aetiology has been established. The progressive canopy thinning and dieback, and the heterogeneous distribution of the decline, are similar to Phytophthora dieback of *E. marginata* involving fine and coarse root necrosis caused by *Phytophthora cinnamomi* (Shearer and Tippett 1989). In 2007, isolates of the recently described *P. multivora* (Scott *et al.* 2009, Chapter 4) were recovered from rhizosphere soil of declining or dead trees of *E. gomphocephala*, *E. marginata* and *A. flexuosa*. A molecular re-evaluation of isolates from the culture collection of the Department of Environment and Conservation’s Vegetation Health Service in Perth, Western Australia, showed that over the past three decades *P. multivora*, previously identified as *P. citricola* based on morphological characteristics, has been isolated regularly throughout the south-west of Western Australia (Burgess *et al.* 2009) as part of regular soil and plant tissue testing for the wide scale quarantine of *P. cinnamomi* (Dell *et al.* 2005). *Phytophthora multivora* is the first *Phytophthora* species to be found in association with declining *E. gomphocephala* trees.

The pathogenicity of *P. multivora* on *E. gomphocephala* has not been tested, but an isolate of *P. multivora*, designated as *P. citricola* DEC 236 from the VHS culture collection, had previously been used in an *in situ* under-bark pathogenicity experiment on stems of *E. marginata*, *Corymbia calophylla* and *Banksia grandis* (Shearer *et al.*
Isolate DEC 236, now confirmed as *P. multivora* (Scott et al. 2009, Chapter 4), produced significantly larger lesions on stems of *E. marginata* than isolates of *P. cinnamomi*, but did not produce lesions in stems of *B. grandis* (Shearer et al. 1988). These results support the potential role of *P. multivora* in the decline of *E. gomphocephala* in Yalgorup National Park, since *E. marginata* trees are suffering substantial dieback whilst *B. grandis* trees remain comparatively healthy. However, a range of *Botryosphaeriaceae* pathogens have been shown to cause stems and branches dieback of *B. grandis* within Yalgorup (Taylor et al. 2009).

The progressive and gradual nature of crown decline associated with *E. gomphocephala*, *E. marginata* and *A. flexuosa* dieback within the Yalgorup woodland, and the association of *Phytophthora* species in the rhizosphere, suggests that a fine feeder root pathogen may be involved in the decline. Compared to cankers, leaf and shoot diseases of above-ground parts of trees, fine root diseases are more difficult to diagnose because fine root losses are more challenging to measure than aerial symptoms and their impacts are usually expressed via non-specific symptoms of branch or crown dieback, which may also be associated with other pathogens or insects (Hansen and Delatour 1999). In addition, diebacks incited by fine root damage often show a scattered spatial distribution (Jung et al. 1996; Jung et al. 2000). The assessment of fine root parameters has been used successfully in both adult forest trees and artificially infested seedlings to demonstrate the involvement of the fine root pathogen *P. quercina* in European oak decline (Jung et al. 1999; Jung et al. 2000; Jönsson et al. 2003; Jönsson 2004; Jönsson et al. 2005). The association between the reduction of fine feeder roots of *E. marginata* caused by *P. cinnamomi* and dieback has been difficult to substantiate. In the *E. marginata* forest, *P. cinnamomi* only reaches high inoculum densities in the soil for relatively short periods of time (Shea et al. 1979; Shea et al. 1980; Shearer et al. 2010) and *E. marginata* has a large turnover in fine roots throughout the year (Shea and...
Dell 1981), often in rapid response to rainfall events (Dell and Wallace 1983). The high
tolerance of *E. marginata* to root loss was demonstrated by Crombie *et al.* (1987), when
a significant reduction in stomatal conductance only occurred after removal of 50% of
roots arising from the root collar. To explain the resilience in stomatal conductance of
*E. marginata*, to fine root loss, Crombie *et al.* (1987) proposed that because of the
dimorphic structure of *E. marginata*, the deep tap roots are predominantly involved in
water relations and the fine roots have other primary functions including nutrient
cycling. In the jarrah forest, *E. marginata* is predominantly killed by major root and
collar damage (Shearer and Tippett 1989), on sites with lateritic hard pans, which favour
*P. cinnamomi* infection of the major roots followed by rapid mortality in response to
drought (Shea *et al.* 1984). In contrast, more scattered slow declines of *E. marginata*
may not be related to subsurface infection and the death of primary roots and the collar,
but rather to a gradual chronic deterioration of the fine root system (Somerford *et al.*
1987).

Given that many tree species have a great capacity to produce fine roots, a fine
feeder root pathogen may induce a decline only if the tree is weekend by of other factors
such as water logging or severe droughts (Dawson and Weste 1982; Ploetz and Schaffer
1989; Davison 1994; Brasier 1996) or stress caused by insects and other pathogens
(Brasier and Scott 1994). The seasonal variation in fine feeder root necrosis can result
in a net reduction in root volume resulting in resource depletion (Jönsson 2006).
Equally, fine root losses may increase susceptibility to drought, secondary pathogens or
secondary pests like bark beetles (Jung *et al.* 2000). On Western Australia’s highly
nutrient poor soils, fine feeder root loss and associated mycorrhizal loss could result in
nutrient deficiency symptoms, contributing to decline (Podger 1972; Shea and Dell
1981). The condition of the fine root system of declining *E. gomphocephala* trees has
not been assessed and extensive studies of mature trees in the field and pathogenicity
trials are required to understand the aetiology of the decline and the possible association of *P. multivora*.

The aim of this study was to determine if:

- *Phytophthora multivora* can infect and cause fine and small woody root dieback and death in *E. gomphocephala* and *E. marginata* seedlings grown under controlled glasshouse conditions;
- *Phytophthora multivora* can cause necrotic lesions following under-bark inoculation of stems of *E. gomphocephala* and *E. marginata in situ*;
- there is no significant variation in the pathogenicity of different *P. multivora* isolates and *P. cinnamomi* to *E. gomphocephala*; and
- above-ground growth and nutrient concentrations in leaves are affected by the presence of *P. multivora* and *P. cinnamomi* in the soil.

### 5.2 Methods

Two experiments were conducted. In Experiment 1, *ex situ* soil infestation was used to test the pathogenicity of *P. multivora* and *P. cinnamomi* in the root systems of *E. gomphocephala* and *E. marginata* seedlings. In Experiment 2, an *in situ* under-bark inoculation was used to measure the pathogenicity of *P. multivora* in the stems of *E. gomphocephala* and *E. marginata* saplings.

### 5.2.1 Experiment 1 - Soil infestation

Five isolates of *P. multivora* and a single isolate of *P. cinnamomi* were tested for their pathogenicity on 4-month-old seedlings of *E. gomphocephala* in an *ex situ* soil infestation test over 12 months (Table 5.1). One of the five *P. multivora* isolates was also tested against 4-month old *E. marginata* seedlings. Each isolate-host combination
and the control treatments consisted of four replicate pots with six seedlings per pot, giving 24 seedlings for each treatment and a total of 168 *E. gomphocephala* seedlings and 48 *E. marginata* seedlings. Pots were arranged in a randomised block design. The isolates of *P. multivora* had been isolated in 2007 from the rhizosphere of declining *E. gomphocephala*, *E. marginata* and *A. flexuosa* at five sites in the *E. gomphocephala* forest of Yalgorup National Park (Scott et al. 2009, Chapter 4) as shown in Appendix 1.

**Table 5.1. Phytophthora species, isolate numbers, their origin and the pathogenicity Experiments in which they were used.** For Experiments, ✓ indicates host plants included in trials. Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; WAC = Department of Agriculture and Food Western Australia Plant Pathogen Collection; MP = Murdoch University Phytophthora Collection.

<table>
<thead>
<tr>
<th>Species and isolates</th>
<th>Origin of rhizosphere soils</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soil infestation</td>
<td>Under-bark inoculation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Host plant</td>
<td>Host plant</td>
</tr>
<tr>
<td></td>
<td>E. gomphocephala E. marginata</td>
<td>✓ ✓</td>
<td>✓ ✓</td>
</tr>
<tr>
<td><em>P. multivora</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WAC 13201</td>
<td><em>E. gomphocephala</em> and <em>E. marginata</em> – mixed</td>
<td>✓ ✓</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>(CBS 124094)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WAC 13200</td>
<td><em>E. gomphocephala</em></td>
<td>✓ ✓</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>WAC 13202</td>
<td><em>E. gomphocephala</em></td>
<td>✓ ✓</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>WAC 13203</td>
<td><em>Agonis flexuosa</em></td>
<td>✓ ✓</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>WAC 13204</td>
<td><em>E. gomphocephala</em></td>
<td>✓ ✓</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>(CBS 124095)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. cinnamomi</em></td>
<td><em>E. marginata forest</em></td>
<td>✓ ✓</td>
<td>✓ ✓</td>
</tr>
</tbody>
</table>

*Eucalyptus gomphocephala* and *E. marginata* seed were germinated and grown in a steam pasteurised mix (2:2:1) of composted pine bark, coarse river sand and coco peat in 5-cm-deep free-draining seedling punnets. Six-week-old seedlings were then transplanted into 20 cm diameter free-draining polypropylene pots containing 4.3 L of coarse washed river sand (Table 5.2) (Richgro®, Jandakot, Western Australia, Australia) pasteurised twice over two days at 65 ± 5°C for 60 minutes. Samples of the pasteurised river sand were baited with young oak leaflets using the method of Jung et
al. (1996) to confirm the absence of Phytophthora species. No Phytophthora species were recovered. One 12 cm long, 1 cm diameter polypropylene tube was pushed into the container of river sand, adjacent to each of the six seedlings with an additional 2 cm diameter tube placed into the centre of the pot at the time of transplanting. Every 2 weeks the pots were fertilized to container capacity with a water soluble, low phosphate fertilizer specifically formulated for Australian Natives (NPK 28-1.8-10) (Osmocote Plus Native Gardens, Scotts, New South Wales, Australia), at the recommended injection rate of 3.25 g/L, for eight weeks until the soil was infested with the Phytophthora isolates. After infestation, the pots were fertilized every 4 weeks as described above. The plants were grown under controlled conditions in a glasshouse (6 to 27°C) (Murdoch University, Perth, Western Australia, Australia) and watered twice daily to container capacity with deionised water. Air temperature, soil temperature and relative humidity were measured every 15 minutes with a data logger (HOBO®, Onset Computer Corporation).
Table 5.2. Nutrient status of the container medium used in the pathogenicity test (Experiment 1) prior to adding nutrients. Concentrations of base cations Mn, Fe and B were determined by inductively coupled plasma spectroscopy (Perkin Elmer, Connecticut, United States of America) after extraction of 20 g soil in 100 mL 0.1 BaCl₂ for 2 hours. The total concentration of C was determined using an automatic combustion analyzer (LECO corporation instruments).

<table>
<thead>
<tr>
<th>Elements</th>
<th>Concentration</th>
<th>Concentration¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>1 μg/g</td>
<td></td>
</tr>
<tr>
<td>Ammonium</td>
<td>1 μg/g</td>
<td></td>
</tr>
<tr>
<td>Phosphorus</td>
<td>3 μg/g</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>15 μg/g</td>
<td></td>
</tr>
<tr>
<td>Sulphur</td>
<td>1.7 μg/g</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>0.03 μg/g</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>0.18 μg/g</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>0.1 μg/g</td>
<td></td>
</tr>
<tr>
<td>Organic carbon</td>
<td>0.11 %</td>
<td></td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>pH_CaCl₂</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>pH_H₂O</td>
<td>6.7</td>
<td></td>
</tr>
</tbody>
</table>

Two different inoculum sources were produced for each isolate (Table 5.1), colonised *Pinus radiata* (pine) plug inoculum and a colonised mixture of vermiculite, oat grain and vegetable juice (VOV). The pine plug inoculum was modified after Butcher *et al.* (1984). Briefly, live *P. radiata* branches were cut into plugs 1 – 2 cm in diameter and approximately 2 cm long, and the bark was removed. Plugs were soaked overnight in distilled water, rinsed and placed in a 1 L conical flask with 100 plugs per flask. Distilled water was added to the flasks to a depth of 1 cm and flasks were plugged with non-absorbent cotton wool and covered with aluminium foil. Flasks were autoclaved for 20 minutes at 120 kPa then cooled to room temperature for 24 hours and then re-autoclaved on 2 consecutive days. Individual cultures of isolates grown on 8 cm diameter Petri dishes with vegetable juice agar (V8A) [100 mL/L vegetable juice (Campbells V8), 900 mL/L demineralised water, 3 g/L CaCO₃ and 15 g A Grade Agar (Becton, Dickinson and Company, Sparks, United States of America)] for 5 days at 20ºC in the dark were cut into 1 cm squares and the whole culture transferred to the
cooled flask, and incubated for 4 weeks at 20ºC in the dark and shaken periodically. The VOV inoculum was prepared according to Matheron and Mircetich (1985) as modified by Jung et al. (1999). Briefly the VOV inoculum was prepared by inoculating an autoclaved mixture of 250 cm³ vermiculite, 20 cm³ whole oat grains, and 175 mL vegetable juice broth (consisting of 100 mL/L V8 juice, 900 mL/L demineralised water and 3 g/L CaCO₃) with five 1 cm² V8A disks of five-day-old cultures. After 4 weeks incubation at 20ºC in the dark, the inoculum of each isolate was rinsed with demineralised water to remove excess nutrients immediately before soil infestation (Matheron and Mircetich 1985).

Plants were grown in the pots for 8 weeks before soil infestation. For infestation, all seven tubes were removed from each pot. A colonised pine plug was inserted into each hole adjacent to the seedlings, which was left by the removal of the tubes adjacent to each plant. A total of 86.0 cm³ (equivalent to 2% of container river sand volume) of the VOV inoculum was inserted into the central 2 cm hole left by the removal of the tube in the centre of each pot. The holes were then filled with sterile river sand. Control treatments received pine plug inoculum, and rinsed vermiculite, oat grains and vegetable juice broth inoculum that had been colonised with Phytophthora isolates and autoclaved for 20 minutes at 120 kPa and then cooled to room temperature. Sterility of the autoclaved control inoculum was confirmed using the oak leaf baiting method (Jung et al. 1996). In order to stimulate the production of sporangia and the release of zoospores from the inoculum source, the containers were flooded with deionised water to approximately 2 cm above the surface for 24 hours, immediately after inoculation, and once again after 14 days to mimic woodland conditions after a heavy rainfall event. Flooding was achieved by placing each pot in a water-tight bag (Batini and Podger 1968). At each flooding event the water of each pot was baited with oak leaflets to confirm the presence of Phytophthora inoculum, as described above. Sterile pine plugs
placed in pots containing plants similar to inoculated pots served as controls.

Stem length and the above ground condition of the seedlings were measured every four weeks for 12 months, when the trial was harvested. The health of each seedling was based on wilting and estimated on a scale from 5 – 0 (5 = healthy; 0 = dead), assessed daily up to the time of death after which the plant was left in the pot, to avoid disturbing the root systems of surrounding plants. One year after infestation, the remainder of the living plants were harvested. The stems were separated from the roots at the point where the cotyledon had been attached. Leaves were separated from the stem and branches and bagged in separate paper bags for each plant. Leaves and stems were dried at 70°C for 10 days and weighed. The root system of each plant was separated, and the river sand was sieved through a 2 mm sieve to collect any remaining pieces of the root system. Reisolation from the soil and a small subset of root fragments, covering the size classes of 0-2 mm diameter and above 2 mm diameter, was performed to confirm pathogen survival and root infection. Reisolation from the soil was done using the oak leaf baiting method as described previously and root fragments were gently dried with paper towel and plated onto selective PARPNH agar, containing V8 juice \( (\text{Campbell's}^{\text{®}}) \) 100 mL/1, agar 20 g/L, \( \text{CaCO}_3 \) 3 g/L, pimaricin 10 mg/L, ampicillin 200 mg/L, rifampicin 10 mg/L, pentachloronitrobenzene (PCNB) 25 mg/L, nystatin 50 mg/L, hymexazol 50 mg/L modified from (Tsao 1983).

5.2.2 Root analysis

Roots were harvested by submerging the whole root ball from a pot in a water-filled 50 x 50 cm tub, and bubbling air for 12 hours from underneath through the root ball and then removing the loosened river sand by hand. Initially the root balls were completely submerged in deionised water in a polypropylene tub, 50 cm deep by 50 cm wide. Inside the plastic tubs the root balls rested on a polypropylene mesh with 3 cm²
openings, raising the root balls 5 cm above the tub bottom. Beneath the mesh 6 mm diameter tubing was used to gently bubble air underneath the root balls. The bubbling air dislodged the river sand particles, which settled beneath the mesh, while leaving the root structure relatively intact. The root balls from each pot were exposed to the bubbling air for approximately 12 hours. On removal, the residual container substrate was gently removed by hand and the excised root fragments were amalgamated. All equipment was thoroughly washed and sterilized with 70% ethanol before re-use.

After washing, the roots were scanned on an Epson Expression 10 000XL light transmission scanner in a 10 mm deep water bath and root length, surface area and numbers of root tips were measured for five root diameter classes (0-0.5, 0.5-1, 1-2, 2-5, >5 mm) using the software WINRHIZO Pro V 2007d (Reagent Instruments, Québec, Canada). Root samples per plant were then sorted into diameter classes 0-2 mm (fine roots) and >2 mm (mother roots), and dried at 70ºC for 10 days and weighed.

5.2.3 Chemical analysis

*Eucalyptus gomphocephala* leaves for each pot were bulked to give four composite samples per treatment and analysed by CSBP Pty Ltd (Bibra Lake, Western Australia, Australia). To determine the concentration of nitrogen (N), finely ground plant material was digested in nitric acid and combusted at 950ºC in oxygen using a FP-428 Nitrogen Analyser (LECO corporation instruments) that measured the released nitrogen from the sample as it passed through a thermal conductivity cell. The concentrations of calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), boron (B) iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), sulphur (S) and phosphorus (P) were determined using an inductively coupled plasma analyse (Perkin Elmer, Connecticut, United States of America). Nitrate (NO$_3^-$) concentration was analysed by colorimetric measurements using flow injection analysis on a water extraction (Searle 1984).
5.2.4 Experiment 2 - Under-bark inoculation

In autumn (May) 2008, five isolates of P. multivora (Table 5.1) were tested *in situ* for their pathogenicity towards self-seeded E. gomphocephala saplings using an under-bark inoculation method. One of the five P. multivora isolates was also tested on saplings of E. marginata. The pathogenicity experiment was conducted in a mixed E. gomphocephala and E. marginata stand (32.6822°S, 115.6417°E) in Yalgorup National Park, near the original isolation site of the P. multivora isolates (Table 5.1). The region has a Mediterranean climate and the study area receives approximately 649 mm rainfall annually, mainly over winter (June–August), and the mean monthly temperatures range from 10.6 to 29.2 °C (2002-2010) (BoM 2011b). Soils have a sandy texture, are derived from limestone (McArthur and Bartle 1980), and have an average pH of 7.1 pH (H$_2$O) (Cai et al. 2010).

There were 10 replicate plants for each of the five isolates and the non-inoculated control treatments. Inoculation treatments were distributed with a randomised replicate block design. The saplings were between 0.5 to 1.5 m in height with stem diameters of 10-30 mm and were inoculated approximately halfway along the main stem at least 10 cm above ground level. An oblique incision was made with a sterilized, sharp scalpel through the outer bark into the phloem (O’Gara et al. 1996). A 9 mm diameter inoculum plug was removed from a five day-old Phytophthora culture of each isolate grown on V8 agar and placed mycelial surface face down onto the exposed wound. The bark flaps were replaced over the inoculum disc and the wound was covered with a 1 cm$^3$ piece of moist cotton wool, and sealed with Parafilm$^\text{®}$ (Menasha Wisconsin) tape and aluminium foil (Shearer et al. 1988; O’Gara et al. 1996). Plugs of non-colonised V8 agar were used for the control inoculations. Lesion extension was measured after nine weeks. Colonisation above and below any visible lesion was assessed by plating 1 cm
stem sections from 5 cm above and below the visible lesion onto PARPHN agar, giving a total of 10 sections per plant.

5.2.5 Statistical analysis
Data were analysed using parametric tests, and where required, non-parametric data were log transformed to achieve normal distribution and homogeneous variances before analysis. Significance was determined at $P \leq 0.05$. The soil infestation and under-bark inoculation experiments on *E. gomphocephala* and *E. marginata* were analysed separately. Experiment 1 (soil infestation), was analysed as a mixed model nested MANOVA (multivariate analysis of variance), with the higher level factors being fixed (Bennington and Thayne 1994) or model I (Sokal and Rohlf 1995) and the lower level, nested factor of pot being random (Bennington and Thayne 1994) or model II (Sokal and Rohlf 1995). For Experiment 2 (under-bark inoculation), a one-way ANOVA was used to test for significant differences between the means of each treatment. Where treatments were significant, *post hoc* Fisher LSD tests were used to identify significantly different factor levels (Day and Quinn 1989). Analyses were carried out in Statistica software package Version 5 (Statsoft 1999).

5.3 Results

5.3.1 Experiment 1 - Soil infestation

5.3.2 Above ground condition and growth
No *E. gomphocephala* seedlings died in pots infested with *P. multivora* or in the control pots. Six *E. marginata* seedlings died in pots infested with *P. multivora* between 26 and 48 weeks after infestation. Four *E. gomphocephala* seedlings died in pots infested with *P. cinnamomi* between 26 to 34 weeks after infestation. There were no significant differences in the above ground condition, stem length, or weight of dried plant parts
when harvested including the fine roots (diameters 0-2 mm), all roots (diameters > 0 mm), stems, or leaves, between plants inoculated with *P. multivora* or *P. cinnamomi* isolates and the control (data not shown).

### 5.3.3 Reisolation

During each flooding event, both *Phytophthora* species were recovered from the water/soil of *Phytophthora* infested pots. No *Phytophthora* species were isolated from the control pots. *Phytophthora cinnamomi* was isolated by plating onto PARPHN selective medium from the collars of all four *E. gomphocephala* seedlings that died during the infestation experiment. After harvesting, all *Phytophthora* isolates were recovered from washed root material by both baiting and direct plating from the infested pots. *Phytophthora multivora* was recovered from fine root material of *E. gomphocephala* and *E. marginata* seedlings up to 2 mm in diameter, but not from thicker roots, collars or stem. *Phytophthora cinnamomi* was recovered from all root diameters of *E. gomphocephala* and from bark lesions below the collar of the remaining live seedlings. *Phytophthora cinnamomi* was reisolated from the collars of dead *E. gomphocephala* seedlings. No *Phytophthora* was isolated from the soil samples or fine-root fragments of the control seedlings.

### 5.3.4 Root growth

In all inoculated and control plants of *E. gomphocephala* and *E. marginata* the fine feeder roots less than 1 mm in diameter represented 96.9% and 98.2% of the total root length, respectively.

Two of the five *P. multivora* isolates, WAC 13200 and WAC 13201, and the *P. cinnamomi* isolate MP 94-48, caused significant reductions of root lengths compared to the control for all root diameters combined (Fig. 5.1a), root diameters 0-1 mm (Fig.
5.1b), and root diameters 1-2 mm (Fig. 5.1c). The length of *E. gomphocephala* roots between 2-5 mm in diameter was not influenced by any of the *P. multivora* isolates, or the *P. cinnamomi* isolate (data not shown). The *P. multivora* isolates WAC 13200 and 13201 significantly reduced the number of root tips of *E. gomphocephala* (Fig. 5.1d). *Phytophthora multivora* isolates WAC 13200 and 13201 and *P. cinnamomi* isolate MP 94-48 caused significant reductions in the surface area of *E. gomphocephala* roots 0-1 mm in diameter (Fig. 5.1e). *Phytophthora multivora* isolate WAC 13201 and *P. cinnamomi* isolate MP 94-48 also significantly (Fig. 5.1f) reduced the surface area of *E. gomphocephala* roots 1-2 mm in diameter.

In *E. marginata*, the *P. multivora* isolate WAC 13201 significantly reduced the length of roots when all diameter classes were combined (Fig. 5.2a) and for the 0-1 mm diameter range (Fig. 5.2b), but not for the 1-2 mm (Fig. 5.2c) or 2-5 mm root diameters (data not shown) compared to the control. The *P. multivora* isolate WAC 13201 significantly reduced the number of root tips (Fig. 5.2d), and area of 0-1 mm roots (Fig. 5.2e) of *E. marginata* compared to the control. Isolate WAC 13201, did not significantly reduce the area of 1-2 mm diameter roots (Fig. 5.2f) when compared to the control.
Fig. 5.1. Mean root parameters (± standard error) of *Eucalyptus gomphocephala* seedlings after 12 months growth in soil infested with *Phytophthora multivora* isolates (WAC13200 – WAC13204), *P. cinnamomi* isolate MP 94-48, or non-infested control (C) soil. Root parameters include: mean root length for (a) all root diameters combined, and roots 0–1 mm (b) and 1–2 mm (c); number of root tips; and the surface area of diameter root classes 0–1 mm (e) and 1–2 mm (f). Statistics are for univariate tests where the multivariate main effect is significant. *** $P \leq 0.001$. Small letters denote the results of the post hoc test (Fisher LSD) and bars with the same letters are not significantly ($P \leq 0.05$) different.
Fig. 5.2. Mean root parameters (± standard error) of *Eucalyptus marginata* seedlings after 12 months growth in soil infested with *Phytophthora multivora* isolates (WAC13201) or non-infested control (C) soil. Root parameters include: mean root length for (a) all root diameters combined, and roots 0–1 mm (b) and 1–2 mm (c); number of root tips; and the surface area of diameter root classes 0–1 mm (e) and 1–2 mm (f). Statistics are for univariate tests where the multivariate main effect is significant. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant. Small letters denote the results of the post hoc test (Fisher LSD) and bars with the same letters are not significantly ($P \leq 0.05$) different.

5.3.4 Foliar nutrient analysis

There were no significant differences in the concentrations of all measured foliar nutrients between *E. gomphocephala* plants inoculated with the *P. multivora* or *P. cinnamomi* isolates and the control and between *E. marginata* plants inoculated with *P. multivora* and the control.
5.3.5 Experiment 2 – Under-bark inoculation

Under-bark inoculation with *P. multivora* isolates caused significant lesion extension in self-seeded *E. gomphocephala* and *E. marginata* saplings. The greatest extension was recorded on *E. marginata* saplings (Fig. 5.3b) (averaging 90.2 mm), compared to the *E. gomphocephala* saplings (Fig. 5.3a), which ranged from 3.2 to 20.6 mm across all five *P. multivora* isolates tested.

![Fig. 5.3. Mean lesion lengths (± standard error) from under-bark inoculation (a) *Eucalyptus gomphocephala* saplings inoculated with *Phytophthora multivora* isolates WAC13200 – WAC13204 and (b) *E. marginata* saplings inoculated with *P. multivora* isolate WAC13201. C indicates blank inoculated control. Statistics are for one-way ANOVA. ***P ≤ 0.001. Small letters denote the results of the post hoc test (Fisher LSD) and bars with the same letters are not significantly (P ≤ 0.05) different.](image)

5.4 Discussion

*Phytophthora multivora* was pathogenic to *E. gomphocephala* and *E. marginata* in soil and under-bark stem inoculations. It can be concluded that *E. gomphocephala* was susceptible to *P. multivora* based on the evidence that two of the five isolates caused significant fine root loss, with the most substantial loss for roots less than 1 mm in diameter, and all isolates caused significant stem lesions. Soil infestation and under-bark inoculation with *P. multivora* did not, however, kill *E. gomphocephala* seedlings or saplings. In contrast, *E. marginata* was shown to be more susceptible to *P. multivora*
than *E. gomphocephala* because of greater fine root loss and the death of six plants in the soil infestation trial and greater lesion extension in the under-bark stem inoculation trial. Koch's postulates were satisfied as *P. multivora* was isolated from the rhizosphere of declining *E. gomphocephala* woodland trees suffering significant fine root loss. The rhizosphere of *E. gomphocephala* seedlings grown under controlled conditions was infested with *P. multivora* and subsequently suffered significant loss of fine feeder roots. Finally *P. multivora* was reisolated from the rhizosphere and roots of these artificially infested seedlings. *Phytophthora multivora* has not been directly isolated from fine roots *in situ* (Chapter 3).

*Eucalyptus gomphocephala* was more susceptible to infection by the *P. cinnamomi* isolate than the *P. multivora* isolates, as indicated by the death of 17% of plants. However, it is unlikely that *P. cinnamomi* poses a threat to *E. gomphocephala*, which grows predominantly on the calcareic Spearwood and Quindalup soils (Ruthrof *et al.* 2002) which are not conducive to *P. cinnamomi* infestation (Shearer and Dillon 1996). It is also unlikely that *P. cinnamomi* is present throughout the *E. gomphocephala* Yalgorup woodland, since highly susceptible species including *B. grandis* and *Xanthorrhoea* species, which are used as ‘indicator species’ to map *P. cinnamomi* distribution, do not exhibit typical symptoms associated with *P. cinnamomi* infested sites. Previous surveys for *Phytophthora* soilborne pathogens associated with *E. gomphocephala* decline have not isolated *P. cinnamomi* (Scott *et al.* 2009, Chapter 4). However, *P. cinnamomi* has been reported near Yalgorup National Park in areas of State forest east of the Old Coast Road, on Karrakatta and the Bassendean soil complexes (Portlock *et al.* 1993), which are conducive to *P. cinnamomi* infestation and high disease impact (Shearer and Dillon 1996). As it is evident that *E. gomphocephala* is susceptible to *P. cinnamomi* based on the current study, regular monitoring is required to determine if *P. cinnamomi* affects *E. gomphocephala* on these soil types.
Molecular re-evaluation of historic *Phytophthora* isolates (Burgess *et al.* 2009) has found that a species now known to be *P. multivora* has killed *E. marginata* growing in restored mine sites and causes large stem girdling cankers on *E. marginata* growing on restored bauxite mines in the jarrah forest (Hardy, personal communication). Therefore, *P. multivora* is probably also associated with the decline of *E. marginata* at Yalgorup, where *E. marginata* is considered outside of its optimal range on calcareous soils. In the Yalgorup woodland, where *E. marginata* is only found in scattered pockets, it may be more susceptible to *P. multivora* than throughout the remainder of its range where it is often the dominant keystone species and the environment is more suitable for *E. marginata*. Further work is required to determine the role of *P. multivora* in *E. marginata* decline, both in Yalgorup National Park and elsewhere. Conditions for the *ex situ* pathogenicity trial in the glasshouse may not directly relate to natural environments. While some isolates of *P. multivora* and the *P. cinnamomi* isolate were able to induce significant fine root loss *in vitro* it is not known if the observed root damage would result in *E. gomphocephala* decline in natural stands.

Under-bark stem inoculation with a *P. multivora* isolate caused greater lesion extension on *E. marginata* than on *E. gomphocephala*. While the under-bark inoculation experiments demonstrate pathogenicity, the inoculation procedure by-passes early infection processes. Further work is required to determine if under natural conditions in the *E. gomphocephala* woodland, *P. multivora* can invade the bark and phloem of *E. gomphocephala* and *E. marginata* and cause expanding lesions throughout the thicker root systems and stems. The susceptibility of thicker roots and stems to invasion by *P. multivora* may be explored using the unwounded periderm zoospore infestation method of O’Gara *et al.* (1996). *Phytophthora multivora* may only infect the major roots and collar in the presence of specific environmental conditions which enable the pathogen to bypass outer defence mechanisms, as shown for collar rot of mature...
beech trees caused by *P. cambivora* in the presence of boring insects (Jung 2009) or of *E. marginata* seedlings caused by *P. cinnamomi* in the presence of prolonged flooding (O'Gara *et al.* 1996). Infection by *P. multivora* of the collar and major roots, leading to girdling of stems and roots, would likely result in greater damage than if it is only a fine root pathogen.

The fine feeder root damage caused by *P. multivora* on *E. gomphocephala* seedlings suggests that the pathogen is acting as a fine feeder root pathogen. The failure to reisolate *P. multivora* from roots larger than 1 mm diameter demonstrates its specificity as a fine feeder root pathogen. In addition, *P. multivora* caused only small lesions on *E. gomphocephala* in the under-bark stem inoculation experiment, which is in accordance with the absence of bark lesions on stems of *E. gomphocephala* in the field. On *E. marginata*, *P. multivora* caused larger stem lesions than on *E. gomphocephala*. Interestingly, *Phytophthora* isolates, that were later shown to be *P. multivora* based on DNA analysis (Scott *et al.* 2009, Chapter 4) were also shown to be aggressive pathogens of *E. marginata* and *Corymbia calophylla* by Shearer *et al.* (1987) and *E. marginata* by Bunny (1996). A wider molecular re-evaluation of other *Phytophthora* isolates identified as *P. citricola* based on morphological and isozyme identification may likely reveal that *P. multivora* isolates have a wider host range.

Variation in pathogenicity of *P. multivora* isolates within the current study is reflected in variation between the colony morphology, growth rates and sequences of the mitochondrial cox1 gene region of isolates used (Scott *et al.* 2009, Chapter 4). This suggests that *P. multivora* has had a long presence within the south-west of Western Australia leading to diversification, or aspects of the reproduction facilitate diversification, or the population structure has resulted from multiple introductions. In order to ascertain whether *P. multivora* is endemic or introduced, further work is required to determine susceptible hosts, map the distribution and the population
structure. A greater understanding of how the population structure of *P. multivora* is influenced by biological and environmental characteristics, including pH, calcium content and soil hydrology, may improve management.

In general, the pathogenicity of the *P. multivora* isolates in the soil infestation experiment negatively correlated to the pathogenicity observed in the under-bark inoculation experiment. For example, *P. multivora* isolate WAC1302 caused the greatest fine feeder root loss, but caused the smallest stem lesions during the under-bark inoculation experiment, while *P. multivora* isolate WAC 13203 isolated from *A. flexuosa* (Scott et al. 2009, Chapter 4) caused little fine root damage but large stem lesions. As yet pathogenicity of *P. multivora* to *A. flexuosa* remains to be tested. Previous recoveries of *P. multivora*, then designated as P. sp 4, show that *P. multivora* is associated with dying plants from a range of species including *E. marginata*, five *Banksia* species, a *Conospermum* sp., *Leucopogon verticillatus*, *Xanthorrhoea gracilis*, *Podocarpus drouyniana*, a *Patersonia* sp., a *Bossiaea* sp., *Gastrolobium spinosum* and *Pinus radiata* (Burgess et al. 2009). As many of these genera are also found in the Yalgorup National Park, where the decline and/or death of *E. gomphocephala* and *E. marginata* and decline of *A. flexuosa* is widespread, it is likely that *P. multivora* may also be associated with the decline and/or death of other plant species. Future research could screen the pathogenicity of *P. multivora* isolates obtained from specific hosts (including *A. flexuosa*) against other host species to determine if there is any host specificity.

A greater understanding of the aetiology of *E. gomphocephala* decline may be gained by measuring the association between host-pathogen dynamics at different scales. The temporal and spatial inoculum dynamics of *P. cinnamomi* have been measured in the *E. marginata* forest (Shea et al. 1980; Blowes et al. 1982; Shea et al. 1983; Shearer and Shea 1987; Kinal et al. 1993) and between the *Banksia* woodland and
*E. marginata* forest of the Swan Coastal Plain (Shearer et al. 2010). Shea and Dell (1981) and Dell and Wallace (1983) measured the interaction between inoculum levels and the dynamics of *E. marginata* root cycling under natural conditions. Jung et al. (2000) demonstrated highly significant correlations between the presence of *Phytophthora* species in the rhizosphere and fine root degradation and canopy decline of oaks. Similar spatial and temporal approaches are required to determine the aetiology of *E. gomphocephala* and *E. marginata* declines, specifically in the *E. gomphocephala* woodland on calcareous soils. Currently, little is known about the physiology of *E. gomphocephala* roots, and their dynamics and turnover in the presence or absence of pathogens and other damaging factors.

Further work is required to understand how *P. multivora* pathogenicity is influenced by climate and site. Brasier et al. (1993) proposed that in Iberian oak decline, *P. cinnamomi* was a more aggressive pathogen on moist sites which allowed greater fine root infections than on soils that are only seasonally wet, where infestation only resulted in partial loss of roots which would be replaced by vigorous root growth except when combined with drought, flooding or other decline pressures. Brasier et al. (1993) proposed that a similar interaction was responsible for the disease expression of *P. cinnamomi* on *Castanea* species (Chestnut) in the United States of America, which often began on poorly drained sites, and spread onto drier hills causing chronic disease (Crandall et al. 1945). The relationship between unseasonal rainfall and drought periods and *Phytophthora* diseases associated with *Fagus sylvatica* (Beech) decline was also demonstrated in central Europe (Jung 2009). Similar disease and climate interaction may be involved in the distribution of the decline syndrome within the Yalgorup National Park woodland of *E. gomphocephala* and *E. marginata*. The Yalgorup woodland is in an area with a Mediterranean climate and with variable soil drainage. The relationship between disease expression and soil hydrology may help indicate the
role of *P. multivora* in *E. gomphocephala* decline. Increased climatic variability within south-western Australia (Hope *et al.* 2006) may be a significant factor influencing *P. multivora* pathogenicity in the future. In the current study, plant roots were initially flooded with deionised water for 24 hours after inoculation and then again after 14 days, and then continuously kept moist with regular watering. Greater fine root loss may have resulted from treating seedlings with periods of restricted watering intermixed with flooding. Cycles of soil flooding and drying may have increased plant stress and preferentially favoured the growth of *Phytophthora* within the soil. Plants were not droughted or flooded for longer than 24 hours because there was concern that this may have killed or damaged the plants. The dry Mediterranean south-west climate may particularly favour pathogens specialized in tolerating extended dry periods and rapidly exploiting wet periods. The thick-walled oospore resting structures of *P. multivora*, and its ability to sporulate within 24 hours, may allow it to tolerate infrequent rainfall.

In conclusion, *P. multivora* caused significant fine root loss of *E. gomphocephala* under controlled conditions, which may be indicative of its capacity to do the same in natural stands. However, more work is required to confirm pathogenicity on roots *in situ*. Healthy woodlands may have to be artificially infested with *P. multivora* to determine Koch’s postulates, as demonstrated for *P. cinnamomi* (Podger 1972). *In situ* infestation is not an option as areas of woodland free from decline are of high conservation value and the time necessary for disease establishment and expression may be decades. Alternatively, the impact of *P. multivora* as a fine feeder root pathogen may be quantified by measuring the interaction of the pathogen, the root systems and disease progression of affected trees over time, and determining if the significant interactions *in situ* result in a net decrease in plant resources and subsequent decline. Trees infected with *Phytophthora* may also eventually die as a consequence of secondary pathogens and pests and these relationships need to be determined.
Chapter 6. General discussion

6.1 Overview

This research has made a major contribution to our understanding of the causes of *Eucalyptus gomphocephala* decline in Western Australia. The approaches and methods developed, including techniques for measuring the fine root and ectomycorrhizal status of whole trees will be useful in studying declines in other species.

A new species of *Phytophthora*, *P. multivora*, was isolated from the rhizosphere of declining *E. gomphocephala* woodland trees suffering significant fine root loss and Koch’s postulates were satisfied. *Eucalyptus gomphocephala* seedlings grown under controlled conditions infested with *P. multivora*, suffered significant fine feeder root loss and *P. multivora* was reisolated from the rhizosphere and roots of these artificially infested seedlings. *Phytophthora multivora* also caused significant lesions on *E. gomphocephala* when inoculated under-bark. There is strong direct and indirect evidence to support the role *P. multivora* in the *E. gomphocephala* decline syndrome. Further work is required to quantify the contribution of other contributing stress factors on the pathogenicity of *P. multivora* to *E. gomphocephala*.

Direct evidence indicates that *P. multivora* may be involved in the decline of *E. gomphocephala* in the Yalgorup National Park, and is potential involved in the decline of *E. marginata* and *Agonis flexuosa*, including:

- *P. multivora* was associated with declining *E. gomphocephala*, *E. marginata* and *A. flexuosa* trees;
- phosphite injection improved the crown health of declining *E. gomphocephala* trees in infested sites;
- *P. multivora* rhizosphere infestation *in vitro* caused significant fine root loss of
E. gomphocephala and E. marginata seedlings and death of E. marginata seedlings; and

- *P. multivora* caused significant expanding stem lesions when inoculated under the bark of *E. gomphocephala* and *E. marginata* saplings in situ.

Indirect evidence suggests that *P. multivora* may be an inciting factor contributing to *E. gomphocephala* decline, based on:

1. The symptomology of *E. gomphocephala* decline within Yalgorup, which is typical of a *Phytophthora* induced decline, as:
   - declining *E. gomphocephala* trees found on infested sites have significantly reduced fine root and ectomycorrhizal densities;
   - nutrient application improved the crown health of declining *E. gomphocephala* trees in infested sites; and
   - *E. gomphocephala* have suffered at least one period of mass collapse and sudden tree death, followed by extended periods of gradual decline, which is similar to dieback caused by *P. cinnamomi* in native forests.

2. The role of *P. multivora* decline in other woodlands outside the distribution of *E. gomphocephala* including:
   - *P. multivora* is a stem girdling primary pathogen of *E. marginata* in restored bauxite mines, where it has been associated with episodic tree death of 2-6 year old trees (Hardy, personal communication);
   - *P. multivora* is a stem girdling primary pathogen of *Banksia attenuata* in natural ecosystems and has been associated with at least 13 other species in south-west Western Australia (Scott *et al.* 2009, Chapter 4); and
   - *P. multivora* caused significantly larger lesions on under-bark inoculated stems of *E. marginata* and *C. calophylla* than *P. cinnamomi* (Shearer *et al.* 1988).
3. *Phytophthora multivora* has most likely been recently introduced to Western Australia since European settlement, 184 years ago.

*Phytophthora multivora* may either increase decline severity or be the primary driving agent, depending on the site and environmental characteristics. It is also not known whether *P. multivora* only affects the fine roots in *E. gomphocephala*, or if it is able to infect major roots under environmentally favourable conditions. Evidence supporting the role of *P. multivora* in the decline and important questions about its biology and impact follows.

### 6.2 Assessment of the pathogenicity of *P. multivora*

Fulfilment of Koch’s postulates is difficult for fine feeder root pathogens in forest ecosystems, as *in situ* artificial soil infestation of non-infested healthy forest is ecologically irresponsible and decline symptoms might only be expressed a long time after the introduction of the inoculum source (Jönsson *et al.* 2003). The pathogenicity of *P. multivora* was therefore determined using seedlings grown in infested rhizosphere soil *in vitro* and *in situ* under-bark-stem inoculation of *E. gomphocephala* and *E. marginata*. Pathogenicity measured in this way may, however, differ from that evident in natural woodland infections because the host, pathogen and environmental conditions will vary within natural conditions as indicated in Fig. 6.1.
Quantification and replication of stresses affecting naturally growing mature trees is difficult to achieve. For example, the chemical and biological characteristics of natural woodland soil may significantly interact with Phytophthora species affecting their pathogenicity. An understanding of the aetiology of E. gomphocephala decline is required to demonstrate the impact that fine feeder or root and collar disease may have. This may be determined by quantifying the relationship between the inoculum density of P. multivora, fine root condition and tree health under natural conditions. To accurately quantify these, measurements of the whole root system and inoculum dynamics are required, without disturbing the fine root structures. This may be conducted through the use of technologies such as the ground penetrating radar and the
6.3 Direct evidence supporting the role of *P. multivora* within *E. gomphocephala* decline

6.3.1 *Phytophthora multivora* is associated with declining *E. gomphocephala, E. marginata* and *A. flexuosa* trees

*Eucalyptus gomphocephala* has been declining throughout its range, with the most significant declines observed throughout the Yalgorup National Park, south of Mandurah Western Australia (Edwards 2004). *Phytophthora multivora* was isolated from five of 32 sites sampled throughout the *E. gomphocephala* range, from declining *E. gomphocephala, E. marginata* and *A. flexuosa* trees in the Yalgorup woodland (Scott et al. 2009, Chapter 4). Although this initial survey was not extensive enough to accurately measure the relationship between *P. multivora* distribution and decline, the findings justify further work.

6.3.2 Phosphite injection improved the crown health of declining *E. gomphocephala* trees in infested sites

Phosphite is routinely used to control *Phytophthora* diseases (Guest and Grant 1991; Hardy et al. 2001). With our current understanding of the action of phosphite, improvement in the crown condition of phosphite treated trees can only be explained through phosphite either suppressing a *Phytophthora* pathogen or inducing host defence responses, especially as its application has been shown to have no fertilizer effect in the short term (Rickard 2000; Thao and Yamakawa 2009). Phosphite application also significantly reduced lesion extension in *B. prionotes* seedlings caused by isolates which were morphologically identified as *P. citricola* (Bunny 1996), but were probably
*P. multivora* (Scott *et al.* 2009, Chapter 4). Irrespective of confirming Koch’s postulates, phosphite injection significantly improved the crown health of declining *E. gomphocephala*, suggesting the involvement of a *Phytophthora* pathogen in *P. multivora* infested sites. Unless a further, as yet unidentified, *Phytophthora* species is active at these sites, the response of phosphite injection in improving crown health suggests that *P. multivora*, is most likely an inciting factor contributing to the decline syndrome.

### 6.3.3 *Phytophthora multivora* as a fine feeder root pathogen of *E. gomphocephala* and *E. marginata*

*Phytophthora multivora* infestation in vitro caused significant fine root loss on *E. gomphocephala* and *E. marginata* and also death of *E. marginata* (Chapter 5, Experiment 1). On *E. gomphocephala* trees in declining sites there was found to be a significant positive correlation between crown health and the density of fine root and ectomycorrhizae (Chapter 3, Experiment 2), and *in situ* seedling health survival (Chapter 3, Experiments 3). These decline symptoms and significant relationships between seedling survival *in situ* and tree health may be explained by *P. multivora* acting as a fine root pathogen, and potentially a major root pathogen, causing damage to the roots of large trees, a reduction in ectomycorrhizae and subsequently impaired crown health.

As a fine feeder root pathogen, *P. multivora* may not be able to colonize the vascular tissue and girdle the stem, but still cause declines which occur over many years. Fine feeder root pathogens may cause more damage in the presence of other decline pressures and/or exacerbate decline initiated by adverse abiotic factors including drought or flooding. Fine root loss may also impair crucial functions, such as the formation and viability of symbiotic mycorrhizae including ectomycorrhizae, which are
often crucial for micronutrient uptake in extreme environments (Harley 1989; Brundrett 2002), by facilitating drought resistance during dry periods (Bowen and Theodorou 1973; Bolan 1991), and nutrient uptake in nutrient poor and/or limiting soils. Confirmation is needed of how root necrosis caused by \textit{P. multivora} within natural ecosystems impacts on the health of \textit{E. gomphocephala} and \textit{E. marginata} in the presence of other decline pressures.

### 6.3.4 \textit{Phytophthora multivora} is a major/minor root and/or collar/stem pathogen of \textit{E. gomphocephala} and \textit{E. marginata}

\textit{Phytophthora multivora} was shown to be a fine feeder root pathogen \textit{in vitro}. However, under conditions favourable to the pathogen, \textit{P. multivora} may be able to infect major roots and the collar. Compared to fine root necrosis, damage of the minor/major roots and/or the collar/stem would result in more rapid decline through impaired uptake of nutrients and water.

\textit{Phytophthora multivora} has been shown to cause expanding lesions in a range of trees outside of the \textit{E. gomphocephala} woodland and in the cambium of under-bark inoculated stems of \textit{E. gomphocephala} and \textit{E. marginata} saplings \textit{in situ}. Un-assisted infection has yet to be identified (Chapter 3). This study only isolated \textit{P. multivora} from the rhizosphere of declining \textit{E. gomphocephala} and the pathogen was not isolated from minor root or collar lesions on \textit{E. gomphocephala}, despite extensive examination of the roots, collars and stems of declining trees exposed after air spading (Chapter 3, Experiment 3). No \textit{Phytophthora} species was isolated from lesions on minor roots exposed after air spading. As \textit{P. multivora} was subsequently isolated from rhizosphere soil within the same site, the pathogen could have been present in the roots of air spaded trees, but was not isolated because of variations in the population density or due to pathogen dormancy. \textit{Phytophthora multivora} is homothallic and like other homothallic
*Phytophthora* species it may require specific environmental conditions to break oospore dormancy (Jeffers and Aldwinckle 1987; Ferguson 1999). If *P. multivora* is only infecting the fine roots, it is plausible that most of the inoculum would reside in the rhizosphere where seasonal cycles of soil drying may induce its dormancy or mortality. However, if *P. multivora* is able to infect larger roots then it might be buffered to a degree from seasonal soil drying cycles reducing the need for induced dormancy.

When inoculated under-bark, *P. multivora* caused significantly larger lesions on *E. marginata* than on *E. gomphocephala* (Chapter 5, Experiment 2). Therefore, if *P. multivora* is able to infect beyond the fine roots into thicker tissue under natural conditions, it will likely cause more damage on *E. marginata* than *E. gomphocephala*.

A major *E. gomphocephala* dieback event was observed in 1997 near Preston Beach, Yalgorup National Park, with more than 90% mortality in some areas with the decline spreading north and south (Mitchell, D., Department of Conservation and Land Management, personal communication). In the *P. multivora* infested sites, the 1997 mass collapse event may be partially explained by climatic conditions. From 1960 to 2008, Lake Preston in Yalgorup National Park had an average total rainfall of 864 mm with an average September to December rainfall of 177 mm (Station 009679 (BoM 2011a)). For 1996 Lake Preston, received an above average total rainfall of 951 mm and above average spring rainfall in September to December of 285 mm which may have caused mass infection and damage, potentially into thicker tissue beyond the fine roots. The following year in 1997, when decline symptoms were most extreme, rainfall was below average at 698 mm, which could potentially have exacerbated fine root damage through water stress. A climate analysis should be conducted to determine how future declines correspond to climatic variations. To determine if *P. multivora* can cause necrosis of major roots and/or the collar and stem it is important that the major roots and collars of symptomatic stems are examined for the presence of *P. multivora*,

Chapter 6. General discussion
especially in response to incidence of sudden death.

Further work is needed to determine if *P. multivora* is associated with root and collar lesions on rapidly dying *E. gomphocephala* and *E. marginata* trees in Yalgorup, as this would provide strong evidence of its significance in the decline. In the present study, the opportunity to isolate from trees that had collapsed rapidly did not exist, as no such deaths occurred during the study. Low lying areas of declining *E. gomphocephala* throughout Yalgorup, such as areas on the Vasse Soil Complex (McArthur and Bartle 1980), are prone to intermittent flooding, which may facilitate *P. multivora* entry, as demonstrated on *E. marginata* seedlings with *P. cinnamomi* (O'Gara et al. 1996). For *E. gomphocephala* trees, further research is required to determine if *P. multivora* is associated with trees that collapse and die rapidly and if and how *P. multivora* infects plant tissues other than fine roots.

6.4 Indirect evidence supporting the role of *Phytophthora multivora* in *Eucalyptus gomphocephala* decline

6.4.1 Symptomology of *E. gomphocephala* decline around Yalgorup

Some of the symptoms of *E. gomphocephala* decline may be explained by *P. multivora* acting across a continuum as a fine feeder root pathogen associated with chronic decline caused by a pathogen capable of invading and causing damage to thicker roots and/or the collar/stem where it is associated with episodic sudden death. Similar aetiologies have been observed in *E. marginata* decline caused by *P. cinnamomi*, where invasion and damage of roots is variable, involving: significant fine root infection and loss, with little colonization of larger roots including laterals (Somerford *et al.* 1987); invasion and girdling of tap roots with little damage of laterals (Shea *et al.* 1982); and invasion of laterals culminating in the death of trees, when they became girdled at the collar (Shearer *et al.* 1981; Crombie and Tippett 1990).
6.4.2 Fine root and ectomycorrhizal loss and zinc deficiencies

Declining *E. gomphocephala* trees had reduced fine root and ectomycorrhizal density (Chapter 5) with strong evidence of micronutrient deficiencies including zinc, as indicated by foliar nutrient analysis (Eslick 2005) and improvements in crown condition after systemic zinc implants (Chapter 2, Experiment 1). Ectomycorrhizal symbiosis is probably a significant factor necessary for *E. gomphocephala* survival, and could give a competitive advantage over other *Eucalyptus* species by facilitating zinc uptake on calcareous soils. *Eucalyptus gomphocephala* is one of the few *Eucalyptus* species adapted to highly alkaline calcareous soils (Eldridge *et al.* 1994), where it is the dominant species (Semeniuk and Glassford 1989). Zinc is believed to be limited on calcareous soils because the alkaline pH limits the availability of low solubility compounds (Clarke and Graham 1968; Saeed and Fox 1977), and zinc is adsorbed by carbonates (Udo *et al.* 1970). Zinc has been shown to be particularly deficient for *Eucalyptus* plantations grown on calcareous soil and disturbed sites (Dell and Wilson 1985; Haridasan 1985; Wallace *et al.* 1986; Dell and Wilson 1989). Zinc application improved the crown health of *Pinus* plantations grown on calcareous soils within the *E. gomphocephala* forest north of Ludlow (Kessell and Stoate 1936; Kessell and Stoate 1938). A wide variety of ectomycorrhizal fungi has been shown to be associated with *E. gomphocephala* (Legault 2005) and may be involved in zinc uptake. On calcareous soil, vesicular-arbuscular mycorrhizae have been shown to increase zinc uptake (Marschner and Dell 1994) and their presence in *E. gomphocephala* should be investigated. Micronutrient deficiencies associated with *E. gomphocephala* decline, particularly of zinc, may be explained by mycorrhizal and fine root loss which could have been caused by *P. multivora*. Future work is required to test this hypothesis.
6.4.3 *Phytophthora multivora* epidemiology outside of the *E. gomphocephala* woodland

*Phytophthora multivora* is capable of causing aggressive necrosis of secondary phloem, as demonstrated on *E. gomphocephala* and *E. marginata* (Chapter 5, Experiment 2). A *P. multivora* isolate, originally identified as *P. citricola* DEC 236 (Scott *et al.* 2009, Chapter 4), caused significantly larger lesions on under-bark inoculated *E. marginata* and *C. calophylla* stems than *P. cinnamomi* (Shearer *et al.* 1988). Outside the geographic range of *E. gomphocephala*, *P. multivora* has also been shown to infect the collars of *E. marginata* in restored *E. marginata* (jarrah) forest in the Darling Range Western Australia, after bauxite mining (Hardy, personal communication) and of *B. attenuata* in natural ecosystems in Injidup Western Australia, and of at least another 13 species in the south-west of Western Australia (Scott *et al.* 2009, Chapter 4).

Environmental characteristics may cause significant variation in diseases caused by *P. multivora*, especially the invasion of tissue other than the fine roots. *Phytophthora multivora* appears to only infect the collars of *E. marginata* in rehabilitated jarrah forest under conditions of temporary flooding and high temperatures (Hardy, personal communication). Variation in the frequency of isolation throughout south-west of Western Australia of *Phytophthora* isolates, that were previously identified as *P. citricola* (Bunny 1996) but were probably *P. multivora*, are probably due to temporal differences in rainfall and temperature.

6.4.4 *Phytophthora multivora* was probably recently introduced to Western Australia

Recent molecular analysis of *P. multivora* isolates from around the world suggests that *P. multivora* may have been introduced to Western Australia since European settlement (184 years ago), possibly at the beginning of the 20th century. *Phytophthora multivora*
isolates from South African populations were found to be more diverse than isolates from Australia, indicating that *P. multivora* may not be native to Australia but was introduced more recently than in South Africa (Burgess, personal communication). These findings contrast to previous observations from a molecular sequence analysis of the ITS and *cox*I regions of *P. multivora* isolates, which compared the diversity of Western Australia isolates to isolates on GenBank, and suggested greater diversity within Western Australian populations (Scott *et al.* 2009, Chapter 4). Since *P. multivora* has probably been recently introduced to the *E. gomphocephala* woodland, there is a higher chance that it could be a significant pathogen to a wide range of native plants, as these plants are less likely to have evolved effective defence mechanisms. Further work is required to confirm the origins of *P. multivora* and its current distribution within Western Australia, to insure adequate quarantine is enforced to minimize the pathogen spread, and to determine what hosts are affected and how they should best be managed.

### 6.5 Impact of *P. multivora* on other hosts and ecosystems

*Phytophthora multivora* has been associated with numerous host species other than *E. gomphocephala* and *E. marginata* (Burgess *et al.* 2009; Scott *et al.* 2009), and it is likely that many other susceptible species will be identified in the future. At sites measured between 1976 and 2004 in the *E. gomphocephala* forest in Yalgorup National Park, Archibald *et al.* (2005) associated the decline of *E. gomphocephala, Banksia attenuata* and *Allocasuarina fraseriana* to modified fire regimes and competition from *A. flexuosa*. *Phytophthora multivora* may, however, be an inciting factor in the decline of these species, since *P. multivora* has satisfied Koch’s postulates for *E. gomphocephala* (Chapter 5) and been isolated from declining *B. attenuata* (Burgess *et al.* 2009).
We currently do not know what species are susceptible to *P. multivora*. Similarly we do not know what indicator species would be most effective to map the pathogen’s occurrence and distribution. Preferably indicator species would not be susceptible to *P. cinnamomi* but susceptible to *P. multivora*. Some sites that have previously been designated as infected by *P. cinnamomi* may be infested only with *P. multivora* or with both species as both pathogens produce similar symptoms of progressive crown and foliage dieback and wilting. In the future, it will be difficult to differentiate sites infested with *P. cinnamomi* and *P. multivora*. It is critical that the current *P. multivora* distribution be restricted with best available quarantine and the susceptibility of species within infested or potentially infested sites be determined. Effective quarantine, site management and chemical control using phosphite injection may be the most efficient mechanisms to minimize the conservation burden and financial cost.

6.6 Future research

Table 6.1 presents significant outstanding questions of the involvement of *P. multivora* in *E. gomphocephala* decline.
Table 6.1. Future research on the involvement of *Phytophthora multivora* in *Eucalyptus gomphocephala* decline.

<table>
<thead>
<tr>
<th>Disease Characteristic</th>
<th>Outstanding questions</th>
</tr>
</thead>
</table>
| Aetiology              | • To what degree is *P. multivora* responsible for *E. gomphocephala* decline and other potential hosts including *E. marginata* and *A. flexuosa*?  
  • What is the relationship between the distribution of *P. multivora* and *E. gomphocephala* decline? |
| Epidemiology           | • How and where does *P. multivora* survive long dry periods?  
  • What soil, biological and climatic parameters influence *P. multivora* population density?  
  • What parameters influence the isolation success of *P. multivora*? |
| Pathogenicity          | • What biological and physical environmental characteristics influence the pathogenicity of *P. multivora* and the susceptibility of *E. gomphocephala* and other plant species?  
  • Does *P. multivora* only affect the fine roots, or is it capable of infecting thicker tissue including the cambium, and if so under what circumstances?  
  • How does damage caused by *P. multivora* impact plant health?  
  • What is the host range of *P. multivora*? |
| Distribution           | • What is the current distribution of *P. multivora*?  
  • What are the best indicator species to identify and map *P. multivora* distribution? |
| Control                | • What control techniques, can be used to manage the contribution of *P. multivora* to *E. gomphocephala* decline?  
  • How can phosphite injection be used to manage the contribution of *P. multivora* to *E. gomphocephala* decline?  
  • How does the relationship between *P. multivora* and ectomycorrhizal health impact on *E. gomphocephala* decline?  
  • To what extent does zinc deficiency contribute to *E. gomphocephala* decline?  
  • How is *P. multivora* pathogenicity influenced by other decline pressures including: global climate change and site disturbance? |

6.6.1 Testing Koch’s postulates by infecting uninfested healthy vegetation

Artificial infestation of healthy sites may be justified if:

- non-infested sites would unavoidably become infested in the near future without any feasible management or quarantine;
- site infestation does not significantly increase the rate of *P. multivora* spread to other non-infested sites;
- the infestation trial would clearly demonstrate aetiology, and result in improved
decline management, and

- *P. multivora* isolates used were obtained from an adjacent infested area(s).

Currently there is no quarantine of known *P. multivora* infestations within the *E. gomphocephala* distribution, and there are increasing opportunities for pathogen spread associated with the substantial clearing, urban development and other anthropogenic pressures within surrounding areas. Until effective quarantine is enacted, *P. multivora* is likely to continue to be spread into uninfested vegetation, providing ample opportunity to measure disease establishment under natural conditions.

### 6.6.2 Measuring *P. multivora* distribution and disease expression in situ

Measuring how decline symptoms develop as a pathogen spreads temporally and spatially into uninfested vegetation throughout a landscape indicates aetiology and can be a proxy for confirming Koch’s postulates. The distribution of *P. multivora* should be determined as soon as possible, to ensure that the best possible management and quarantine measures can be implemented. Using the isolation techniques applied in this study *P. multivora* distribution should be mapped and the relationship to decline expression determined. However, mapping *P. multivora* distribution in certain sites may be difficult because:

- sites may have been infested for a long time, and have a low population density, resulting in false negatives;
- *P. multivora* may be present at a site, yet dormant due to unfavourable conditions, and current isolation techniques may not always break dormancy of oospores, resulting in false negatives; and
- plants may take many years to exhibit symptoms, making it difficult to quickly identify recent infestations.
Phytophthora multivora disease aetiology can be determined by measuring temporal and spatial inoculum dynamics in relation to decline symptoms within infested sites. Approaches for measuring the influence of inoculum dynamics may be modified from work on the role of P. cinnamomi in dieback throughout the E. marginata forests of the South-West Botanical Province (Shea et al. 1980; Shea and Dell 1981; Blowes et al. 1982; Dell and Wallace 1983; Shea et al. 1983; Shearer and Shea 1987; Kinal et al. 1993) and between the Banksia and E. marginata forests of the Swan Coastal Plain (Shearer et al. 2010). Further examination of E. gomphocephala decline may indicate if P. multivora is a minor/major root and/or collar/stem pathogen and can be determined if P. multivora is found to infect and damage thicker tissue during periods of accelerated decline, as shown for P. cinnamomi in E. marginata dieback (Shearer et al. 1981; Crombie and Tippett 1990).

6.6.3 Improved diagnostics and management of P. multivora disease

To improve diagnostics and management of P. multivora disease, research needs to be done to improve the detection and isolation reliability so that a reliable GIS overview of pathogen distribution can be developed. It is critical to develop specific molecular probes (O’Brien et al. 2009) that can be used to identify P. multivora in soils, irrespective of environmental conditions and dormancy, such as techniques developed for the detection of P. nicotianae in soil (Huang et al. 2010). Interactions of P. multivora with other Phytophthora species needs to be determined. Once the pathogen distribution has accurately been mapped, protectable areas need to be identified and quarantined from future infection, and infected areas need to be managed. The susceptibility of different P. multivora isolates to phosphite and optimum phosphite treatments for reducing damage caused by P. multivora and to reduce pathogen spread
6.7 Concluding statements

Urgent work is required to understand basic aspects of the biology of disease caused by *P. multivora* (Table 6.1) including the aetiology, epidemiology, distribution and control. Until these basic aspects of the disease are understood, we cannot effectively quarantine and manage its spread, let alone manage infected sites.

The role of the *P. multivora* in *E. gomphocephala* decline will vary with time, and location in the landscape, depending upon numerous host, pathogen and environmental characteristics, as described by Manion (1981). An adaptive model is required that shows how different factors influence the *E. gomphocephala* decline syndrome, that can then be refined as further information becomes available. A conceptual model outlining the development of *E. gomphocephala* decline following infestation with *P. multivora* is outlined in Table 6.2. With best available knowledge, the model outlines under what circumstances the current *E. gomphocephala* decline in Yalgorup National Park may be explained by *P. multivora* acting as a fine feeder root and minor/major root and/or collar/stem pathogen and the cascading effects of its pathogenicity. *Phytophthora multivora* is more likely to infect thicker tissue beyond the fine roots during warm wet conditions while damage to root systems is more likely to be expressed during hot extended dry periods of high drought stress (Table 6.2). It is also predicted that *P. multivora* will be most active immediately following warm rainfall events that follow extended dry periods, which would reduce the density of *Phytophthora* antagonistic soil micro-organisms. Therefore, *P. multivora* may be most easy to isolate during the first rains following hot summer dry periods. This model should be updated as more information becomes available, as further work is required to determine what characteristics influence the pathogenicity of *P. multivora* and its range of impact on
individual plants, communities and ecosystems.
Table 6.2. Conceptual analysis of core components and those that cascade from these, for the development of *Eucalyptus gomphocephala* decline attributed to *Phytophthora multivora*. The relationships are tailored to explain the past and current decline syndrome around Yalgorup; however, it is recommended that these relationships be modified as new evidence about the declines becomes available.

<table>
<thead>
<tr>
<th>Phytophthora multivora as a fine feeder root pathogen</th>
<th>Phytophthora multivora as a minor/major root and/or collar/stem pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Predisposing pathogen invasion: Host, Pathogen and Environment</strong></td>
<td><strong>Continuum</strong></td>
</tr>
<tr>
<td>Fine feeder root damage associated with regular seasonal variation and extreme weather cycles of warm wet and dry periods: Predisposing conditions include:</td>
<td>Minor/major root and/or collar/stem necrosis is more likely, in association with cycles of warm wet periods followed by extreme drought. Most extreme on sites prone to inundation and/or modification to fresh groundwater lenses and hydrology. Predisposing conditions include:</td>
</tr>
<tr>
<td>• increased host stress;</td>
<td>• cycles of warmer wet periods followed by drought, including unseasonal cyclonic and El Niño spring, autumn and summer rains;</td>
</tr>
<tr>
<td>• presence of oospores and chlamydospores (resting structures) which can tolerate extreme dry periods;</td>
<td>• warm wet winters; and</td>
</tr>
<tr>
<td>• reduced density of soil organisms which are antagonistic to <em>P. multivora</em>; and</td>
<td>• ponding and anoxic rhizosphere stress.</td>
</tr>
<tr>
<td>• sudden warm wet conditions which would favour rapid <em>P. multivora</em> population increase (life strategy r) and infection before antagonistic soil microbes reduce population density.</td>
<td></td>
</tr>
<tr>
<td><strong>Primary impacts (direct impact on infected plants)</strong></td>
<td><strong>Secondary impacts (outcomes of direct impacts on plants)</strong></td>
</tr>
<tr>
<td>Fine feeder root damage directly impairs nutrient and water cycling and ectomycorrhizae formation. Cascading primary impacts of fine feeder root damage include:</td>
<td>Cascading secondary impacts of fine feeder root damage include:</td>
</tr>
<tr>
<td>• decreased vascular activity;</td>
<td>• decreased critical plant functions including foliar and seed production;</td>
</tr>
<tr>
<td>• decreased mycorrhizal damage and/or decreased mycorrhizal establishment;</td>
<td>• greater dry and dead material which is more flammable; and</td>
</tr>
<tr>
<td>• reduced nutrient uptake, especially on nutrient limiting sites, including zinc limiting calcareous soils;</td>
<td>• death resulting from chronic deterioration.</td>
</tr>
<tr>
<td>• decreased tolerance to hydrological stress;</td>
<td>Cascading secondary impacts of infection of thicker plant material include:</td>
</tr>
<tr>
<td>• increased likelihood of secondary infections; and</td>
<td>• secondary impacts as indicated for fine feeder root damage; and</td>
</tr>
<tr>
<td>• increased opportunity for pathogen population increase.</td>
<td>• death resulting from chronic deterioration and girdling.</td>
</tr>
<tr>
<td><strong>Secondary impacts (outcomes of direct impacts on plants)</strong></td>
<td><strong>Tertiary impacts (ecosystem impacts)</strong></td>
</tr>
<tr>
<td>Cascading secondary impacts of fine feeder root damage include:</td>
<td>Cascading tertiary impacts of infection of thicker plant material include:</td>
</tr>
<tr>
<td>• decreased critical plant functions including foliar and seed production;</td>
<td>• tertiary impacts as indicated for fine feeder root damage, occurring more frequently during periods of sudden tree death.</td>
</tr>
<tr>
<td>• greater dry and dead material which is more flammable; and</td>
<td></td>
</tr>
<tr>
<td>• death resulting from chronic deterioration.</td>
<td></td>
</tr>
<tr>
<td><strong>Tertiary impacts (ecosystem impacts)</strong></td>
<td></td>
</tr>
<tr>
<td>Cascading tertiary impacts of fine feeder root damage include:</td>
<td></td>
</tr>
<tr>
<td>• impaired site hydrology, nutrient cycling, recruitment, community plant population dynamics, fauna population dynamics; and</td>
<td></td>
</tr>
<tr>
<td>• increased fire frequency until sites become degraded, then decreased fire frequency and more shrub fires.</td>
<td></td>
</tr>
</tbody>
</table>
Appendix

Appendix 1. Study sites for research chapters 2, 3, and 5, and sites from which *Phytophthora multivora* isolates WAC 13200, 13201, 13202, 13203 and 13204, where isolated as described in Chapter 4 and Scott *et al.* (2009). *Phytophthora multivora* isolates stored at Department of Agriculture and Food Western Australia Plant Pathogen Collection, Perth, Australia.
## Appendix 2

(a) Schematic representation of a theoretical fine root system (< 2 mm in diameter) attached to the largest main lateral root. The dashed line delineates the area inside the perimeter of the outer root tips defined as the ‘total reach’. Each square represents 1m². (b) Worked example showing how the fine root total density score (TDS) was determined. The method for determining the fine root TDS is given in Chapter 3, section 3.2.2.

<table>
<thead>
<tr>
<th>Grid</th>
<th>Percentage cover</th>
<th>Average fine root density (D)</th>
<th>Fine root (DSG)</th>
<th>Area inside each grid within the total reach (m²) (Ag)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>20</td>
<td>20</td>
<td>0.05</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>35</td>
<td>22.75</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>25</td>
<td>25</td>
<td>0.35</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>35</td>
<td>35</td>
<td>0.08</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>40</td>
<td>36</td>
<td>0.95</td>
</tr>
<tr>
<td>6</td>
<td>95</td>
<td>40</td>
<td>38</td>
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Area within total reach (Atr) (m²) = 10.46

Total density score (fine roots) = ((0.05/10.46)*20)+((0.5/10.46)*22.75)... = 21.10
Appendix 3. (a) Schematic representation of a theoretical ectomycorrhizal system attached to the largest main lateral root. The dashed line delineates the area inside the perimeter of the outer root tips defined as the ‘total reach’. Each square represents 1m$^2$. (b) Worked example showing how the ectomycorrhizal total density score (TDS) was determined. The method for determining the ectomycorrhizal TDS is given in Chapter 3, section 3.2.2.

<table>
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<th>Ectomycorrhizal (DSG)</th>
<th>Area inside each grid within the total reach (m$^2$) (Ag)</th>
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Area within total reach (Atr) (m$^2$)=10.58

Total density score (fine roots) = (((0.35/10.58)*15)+((0.08/10.58)*30))... = 18.78
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