Changes in root symbionts during long-term soil and ecosystem development and their ecological role for the maintenance of plant diversity

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Summary

Soil microbiota are increasingly acknowledged as key drivers of plant diversity and community assembly. Indeed, plant communities can be strongly influenced by their associated root microorganisms, such as mutualistic symbionts and soil-borne pathogens. The two main root mutualistic fungal associations are with: i) arbuscular mycorrhizal (AM) fungi, where the fungus enhances the acquisition of inorganic phosphorus (P) and other relatively immobile nutrients; and ii) ectomycorrhizal (ECM) fungi, which enhance plant acquisition of mainly organic sources of nitrogen (N) and P, and provide defence against pathogens. On the other hand, soil-borne pathogens such as oomycetes are parasitic root symbionts that usually cause damping-off in roots; they can have strong detrimental effects on plant health. In this thesis, I studied shifts in root colonisation of AM and ECM fungi associated with two plant species (*Acacia rostellifera* and *Melaleuca systena*), which are both capable of forming multiple symbioses, along a south-western Australian dune chronosequence representing two million years of soil and ecosystem development, and along which there are major changes in soil N and P availability. Furthermore, I evaluated the importance of soil fertility and soil inoculum potential effects on the ECM fungal community composition and richness within individual plant species along the chronosequence. I also studied the role that native species of pathogenic *Phytophthora* and mutualistic ECM fungi may play in plant species coexistence in severely P-impoverished soils in the Southwest Australian Biodiversity Hotspot. To do so, I collected roots and soils from the Jurien Bay chronosequence and grew seedlings of both species in a glasshouse in soils collected from the same chronosequence and measured seedling biomass, mycorrhizal root colonisation, and ECM fungal community composition in roots. I found that AM fungal root colonisation declined with soil age, while ECM root colonisation increased.
This switch in importance between different mutualistic root symbionts is likely related to a decline in total and mineral P, but an increase in the organic fraction of total P. In addition, ECM fungal communities associated with roots from the same two plant species changed in composition during ecosystem development, and this was also related to soil chemical properties, such as pH and different forms of soil P. Finally, I found that native *Phytophthora* species reduced seedling biomass of non-mycorrhizal Proteaceae, while not affecting ECM plant species when both types of plants were competing with each other. Hence, native *Phytophthora* reduced differences in competitive ability between Proteaceae and ECM plant species, likely contributing to the coexistence of these plant species of contrasting strategies. I surmise that this could be related to a trade-off between P-acquisition efficiency and pathogen defence, where Proteaceae are more efficient at acquiring P, but more susceptible to soil-borne pathogens. I conclude that the importance of different symbionts and their communities is strongly associated with edaphic properties, especially P. Furthermore, I show that soil microbiota could play a role in maintaining plant diversity. Soil microbiota are an important part of ecosystems and more studies focusing on them will further our knowledge on plant communities, especially in highly diverse ecosystems.
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Statement of original contribution

The research presented in this thesis is an original contribution to the field of plant and mycorrhizal fungi ecology. The hypotheses and experiments presented and discussed in this thesis are my own original ideas and writing with help of supervisors and co-authors. This thesis has been developed and completed during the course of enrolment in a PhD degree at the University of Western Australia, and has not been used previously for a degree or a diploma at any other institution.

Hans Lambers, Etienne Laliberté and François Teste were the supervisors of this project. They guided me through the process of formulating hypotheses, designing experiments and writing up material for submission. Additionally, Benjamin Turner provided valuable help with soil nutrient analyses and writing advice for chapter 2, and Treena Burgess provided crucial mentoring into experimental design and writing advice for chapter 4. Finally, others who made significant contributions to the research are acknowledged in chapters 2-4.

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Thesis structure

This thesis is in agreement with the Postgraduate and Research Scholarship Regulation 1.3.1.33 (1) of the University of Western Australia, Australia and is presented as a series of three scientific papers. There are five chapters in this thesis; a general introduction, three experimental chapters and a general discussion. The general introduction covers the broad background for the work presented in the thesis in order to justify the research objectives. A more focussed review of literature is presented in the introduction of each experimental chapter. The three experimental chapters are presented in the format of scientific papers that can be read individually or as a part of the whole thesis. All three experimental chapters are currently under review in peer-reviewed scientific journals. Each experimental chapter includes the following sections: summary, introduction, materials and methods, results, discussion, acknowledgements, and references. When appropriate, an extra section is included as supporting information. This “thesis-as-a-series-of-papers” format results in some unavoidable repetition, especially in the materials and methods sections of chapters 2 to 4; I have tried to keep such repetition to a minimum.
Publications arising from this thesis

Primary authored manuscripts: Published or in press.


3. Albornoz, F. E., Teste, F. P., Lambers, H., Bunce, M., Murray, D. C., White, N. E., Laliberté, E. Major changes in ectomycorrhizal fungal community composition and declining diversity within the same host plant species along a 2-million year soil chronosequence. *Molecular Ecology* (accepted).

Primary authored abstracts published in refereed conference proceedings


1. General introduction

This thesis examines factors controlling symbiotic root colonisation and its implications for plant communities in a two-million year chronosequence in the Southwest Australian Biodiversity Hotspot. In this first chapter, I present a review of the overarching concepts and provide context for the questions addressed in the thesis.

1.1. Mutualistic and parasitic root symbiotic associations

Root symbioses have been widely studied across the globe (Tedersoo et al., 2010, 2014; Öpik et al., 2013; Davison et al., 2015). Two main root symbiotic associations are with: i) arbuscular mycorrhizal (AM) fungi, where the fungus enhances the acquisition of inorganic phosphorus (P) and other relatively immobile nutrients, and also can protect their hosts against root pathogens (Wehner et al., 2010); and ii) ectomycorrhizal (ECM) fungi, which help the plant to acquire inorganic P as well as organic sources of nitrogen (N) and P, and also offer pathogen protection (Lambers et al., 2008a; Smith et al., 2015). Furthermore, plants possess other nutrient-acquisition strategies to meet their nutrient requirements besides mycorrhizal associations. These other strategies include forming symbiotic associations with rhizobia, which fix atmospheric N and provide it to their host plants (Lambers et al., 2008a; Houlton et al., 2008). In contrast to mutualistic organisms, soil-borne pathogens, such as oomycetes, are parasitic root symbionts that usually cause damping-off in roots and can have strong detrimental effects on plant health (Brown & Hovmøller, 2002; Gilbert, 2002).

Arbuscular mycorrhizal fungi form associations with more than 80% of all terrestrial plants species (Wang & Qiu, 2006; Brundrett, 2009). Their main role as symbionts is to
increase mineral P acquisition by the plant, as they significantly increase the volume of explored soil (Smith et al., 2015). Recently, growing evidence shows that AM fungi might also provide protection against pathogens to their hosts (Wehner et al., 2010) and can neutralise the negative effects of pathogens for seedling survival and growth (Liang et al., 2015). Ectomycorrhizal fungi associate with far fewer plant species (i.e. 2%) than AM fungi, but they provide the main nutrient-acquisition strategy in many ecosystems (Brundrett, 2009) and are thought to play a major role in nutrient cycling (Read & Perez-Moreno, 2003; Dickie et al., 2014). Ectomycorrhizal fungi can access mineral P and organic forms of N and P, due to their ability to release phosphatases and degrade organic matter (Lambers et al., 2008a; Smith et al., 2015). Furthermore, ECM fungi can play a key role in pathogen defence by either producing antibiotics (Duchesne et al., 1988a,b) or by acting as a physical barrier around root tips (Marx, 1972). Understanding the drivers of plant-mutualist associations will further our knowledge on how plant communities are assembled.

Soil-borne pathogens can have strong negative effects on plant diversity (Cahill et al., 2008) and plant community composition (Anagnostakis, 1987). However, catastrophic diversity losses are associated with invasive pathogens only, rather than with minor pathogens native to the ecosystems (Fisher et al., 2012). Recently, it has been shown how native root pathogens might help to maintain high levels of diversity in tropical rainforests (Bagchi et al., 2014). However, still little is known about the ecological role of native soil-borne pathogens in other species-rich plant communities.
1.2. **Role of root symbionts in plant interactions**

Mycorrhizal fungi can play a key role in plant interactions, especially where soil abiotic conditions hamper seedling performance (Dickie *et al.*, 2002, 2007; Teste & Simard, 2008). Mycorrhizal fungi can either increase their hosts’ fitness, and hence their competitive ability or they can interconnect plants of the same or different species to allow transfer of nutrients, referred to as ‘mycorrhizal networks’ (Simard *et al.*, 2012). Mycorrhizal networks allow colonised plants to acquire nutrients from a larger soil volume, and hence, improve seedlings performance (Teste *et al.*, 2009; Bingham & Simard, 2012). Additionally, plant species associated with N$_2$-fixing rhizobia can produce N-richer litter, which in turn increases N levels in the soil (Facelli & Brock, 2000; Halvorson & Smith, 2009), allowing neighbouring plants to access more N. Therefore, studying the role of mycorrhizal fungi in plant-plant interactions will help to gain a better mechanistic understanding of plant community assembly, but this has been little studied (Bonanomi *et al.*, 2011).

Soil-borne pathogens are thought to play a crucial role in plant interactions (Mills & Bever, 1998; Mordecai, 2011; Laliberté *et al.*, 2015). Indeed, it has been proposed that pathogens can decrease the competitive strength of dominant plant species, promoting the persistence of subordinate ones, and hence increasing biodiversity (Terborgh, 2012; Laliberté *et al.*, 2015). The role of native soil-borne pathogens in maintaining plant diversity has not yet been evaluated in other highly diverse ecosystems such as Mediterranean shrublands.
1.3. **Context-dependency of root symbioses**

Root associations are usually modulated by environmental factors such as soil abiotic properties, vegetation and/or soil biota. Indeed, root colonisation by mycorrhizal fungi and rhizobia can be promoted when soil nutrient availability decreases (Treseder, 2004; Barron *et al.*, 2011). Additionally, different symbionts have particular soil pH range where they can exist (Graham *et al.*, 1994; Aggangan *et al.*, 1996; Coughlan *et al.*, 2000). On the other hand, host identity (Martínez-García *et al.*, 2015) and interactions with other microbes (Founoune *et al.*, 2002; André *et al.*, 2003) can have strong effects on root associations. Understanding what drives root colonisation is paramount to further the knowledge on plant-fungal interactions and community assembly.

Plants respond to changes in nutrient availability by altering their nutrient-acquisition strategies in order to effectively acquire the most limiting nutrient (Lambers *et al.*, 2008b; Lambers & Teste, 2013). In fact, when mineral P availability decreases, AM root colonisation increases compensating for the limited P uptake (Abbott *et al.*, 1984). On the other hand, when soil P is mainly in its organic form, ECM root colonisation tends to increase given their phosphatase release (Antibus *et al.*, 1992). Similarly, root colonisation by rhizobia can be inhibited when soil N levels increase (Barron *et al.*, 2011). This shows that root colonisation is a dynamic process and environmental context needs to be taken into account to better understand host-symbiont interactions. To determine the extent to which soil properties drive root symbioses, it is important to study them within the same host plant species across a range of environmental gradients.

Context-dependency is not only related to soil abiotic properties; other soil and root microorganisms can have strong effects on root symbioses. For example, colonisation
by ECM fungi is thought to be detrimental for AM fungal colonisation. Indeed, several studies have shown negative correlations between AM and ECM fungi, attributing it to a competitive exclusion of AM fungi by ECM fungi (Lapeyrie & Chilvers, 1985; Lodge & Wentworth, 1990; Neville et al., 2002). On the other hand, rhizobia nodulation can be promoted by both AM and ECM fungi (Founoune et al., 2002; André et al., 2003; Lesueur & Duponnois, 2005). By contrast, root infection by soil-borne pathogens can be inhibited if roots are colonised by either AM (Wehner et al., 2010) or ECM fungi (Branzanti et al., 1999). The context dependency of the balance between AM and ECM fungi within-species has rarely been studied (but see Neville et al., 2002; Nilsson et al., 2005).

1.4. Root symbioses during long-term ecosystem development

During long-term soil and ecosystem development (i.e. thousands to millions of years), a series of changes in soils and plant communities occur that strongly influence soil nutrient availability (Walker & Syers, 1976; Wardle et al., 2004). Three phases can be identified during ecosystem development. Phase one is a progressive phase, during which productivity increases Phase two is when productivity reaches a maximum biomass phase. Phase three is the retrogressive phase, during which productivity declines (Odum, 1969; Peltzer et al., 2010). Phase three occurs in the early stages of soil development, where pH is relatively high, N is generally the liming nutrient, since it first has to enter the system mainly through symbiotic N₂ fixation, resulting in low plant productivity (Vitousek & Farrington, 1997; Laliberté et al., 2012). On the other hand, P can be found in relatively high abundance in young soils, mostly in its mineral form (Walker & Syers, 1976). During phase three, pH decreases, N availability increases
through N₂ fixation at faster rates than P diminishes, such that in intermediate-aged soils, both N and P are relatively abundant and co-limit plant productivity (Vitousek & Farrington, 1997; Laliberté et al., 2012, 2013). Phase three ends when ecosystem productivity reaches its maximum (maximal biomass, phase two; Peltzer et al., 2010). Following phase two, ecosystem retrogression occurs, because N and P resources become depleted. However, the decline in P availability is more pronounced than that of N, such that P becomes the limiting nutrient and is found mostly in organic forms (Walker & Syers, 1976; Wardle et al., 2004; Laliberté et al., 2012). Productivity decreases during phase three, whereas plant species richness tends to increase (Wardle et al., 2008; Laliberté et al., 2013; Zemunik et al., 2015).

The relative importance of nutrient-acquisition strategies is expected to change as plants shift from being N- to P-limited during long-term ecosystem development. Indeed, plant species can respond to changes in nutrient availability by altering their nutrient-acquisition strategy in order to most effectively acquire the most limiting nutrient (Lambers et al., 2008b). For example, previous studies have found that AM root colonisation is higher in the mineral soil layer; conversely, ECM fungi predominate in the organic layer (Neville et al., 2002). Furthermore, microbial communities themselves can also be influenced by changes in soil properties during long-term soil and ecosystem development (Clemmensen et al., 2015; Krüger et al., 2015). Yet, effects of soil, microbes, and host identity structuring fungal communities across environmental gradients are hard to disentangle, mainly due to plant species not occurring across the entire gradient. Disentangling these factors will further our knowledge of ecosystem development.

Several plant species can form dual associations with both AM and ECM fungi (Cázares & Smith, 1996; Chen et al., 2000; Adams et al., 2006; Pagano & Scotti, 2008), and in
some cases, a tripartite root symbiosis involving N$_2$-fixing rhizobia occurs, such as in *Acacia holosericea* that forms nodules and AM/ECM associations (Founoune et al., 2002). Plant species that form multiple associations provide opportunities to study shifts in plant resource allocation to different symbiotic associations along ecosystem developmental gradients, while controlling for host identity. Knowledge on multiple symbioses has increased in recent years, but the ecological roles or their drivers still remain poorly understood (but see Chilvers *et al.*, 1987; Jones *et al.*, 1998; Brundrett, 2002; Dickie *et al.*, 2002).

1.5. **Biodiversity hotspot in south-western Australia**

South-western Australia harbours one of the few biodiversity hotspots in the world (Myers *et al.*, 2000). Most south-western Australian soils are old, strongly weathered and nutrient-impoverished (Viscarra Rossel & Bui, 2015), and thus present a major constraint to plant growth (Hopper, 2009). Yet, some of these poor soils host plant communities showing some of the highest levels of local species diversity ever found (Lamont *et al.*, 1977; Cowling *et al.*, 1996; Hopper & Gioia, 2004; Zemunik *et al.*, 2015). Great efforts have been made to understand how such high plant diversity can be maintained in hyperdiverse ecosystems, particularly in tropical rainforests (Wright, 2002; Terborgh, 2012; Laliberté *et al.*, 2015). However, other highly diverse ecosystems, such as Mediterranean shrubland have been poorly studied. A better understanding of the mechanisms through which co-occurring plants interact below ground will provide insights into plant community assembly and biodiversity maintenance in species-rich south-western Australian plant communities.
In the kwongan shrubland, Proteaceae are a well-represented plant family (Zemunik et al., 2015), and this is partly related to their very efficient P-acquisition strategy, cluster roots (Lambers et al., 2012). Cluster roots are short-lived, with a poorly lignified epidermis (Lambers et al., 2008a), thus potentially making them more susceptible to root pathogens (Laliberté et al., 2015). Interestingly, despite their nutrient-acquisition advantage over other species of contrasting strategies, they never dominate in this system (Zemunik et al., 2015). In fact, other strategies, such as ECM associations still remain relatively abundant in cover (Zemunik et al., 2015). It has been proposed that soil-borne pathogens can promote plant diversity by attacking the stronger competitor species with superior P-acquisition strategies, rather than those with less effective strategies to acquire P (Mills & Bever, 1998; Gilbert, 2002; Mordecai, 2011; Laliberté et al., 2015). Recently, Laliberté et al. (2015) proposed that soil-borne pathogens could be promoting plant diversity in P-impoverish soils as a result of a trade-off between P-acquisition efficiency and pathogen defence. Hence, both mutualistic root symbionts and soil-borne pathogens might be a key driver of plant community structure and species diversity in these systems. Nevertheless, the ecological role of native soil-borne pathogens has received little attention in Mediterranean shrublands.

In south-western Australia, there is a two-million year dune chronosequence that follows the Walker & Syers (1976) model of pedogenesis (Laliberté et al., 2012; Hayes et al., 2014; Turner & Laliberté, 2015). Along that chronosequence, there are marked changes in soil abiotic properties (i.e. soil nutrient concentrations and pH) (Laliberté et al., 2012), and vegetation (Zemunik et al., 2015). Indeed, there is an important shift in nutrient-acquisition strategies, partially supporting the Lambers et al. (2008b) model. Soil-associated microbiota in this system has been largely understudied (Krüger et al., 2015; Teste et al., 2016). This chronosequence offers a unique study system for
evaluating changes in root symbiotic associations with fertility gradients during long-term ecosystem development. Additionally, along this chronosequence, two plant species capable of forming multiple symbioses co-occur (*Acacia rostellifera* and *Melaleuca systena*). This provides the opportunity to evaluate how edaphic properties affect mycorrhizal fungi across ecosystem development, while keeping host identity constant.

### 1.6. Outline of this thesis

In this thesis, I studied the effects of long-term soil development on root-associated symbionts such as mycorrhizal fungi (AM and ECM fungi) and N$_2$-fixing rhizobia, and the ecological role of native *Phytophthora* species in the biodiversity hotspot in south-western Australia. To do so, I conducted glasshouse experiments using different native plant species as potential hosts and field-collected soils. Here, I measured plant biomass, percentage of root colonised by mycorrhizal fungi, N$_2$-fixing nodule biomass, ECM fungal community composition, and the effect of *Phytophthora* species on plant competition.

In Chapter 2, I evaluated changes in plant investment in different symbionts during long-term ecosystem development, using two plant species capable of forming multiple symbioses that also co-occur in most of the chronosequence stages: *Acacia rostellifera* (rhizobia, AM and ECM fungi), and *Melaleuca systena* (AM and ECM fungi). This chapter aims to elucidate how plants allocate carbon (C) to different symbionts depending on the most limiting nutrient (N or P).
In Chapter 3, I evaluated changes in ECM fungal communities across the same chronosequence used in the study described in chapter 2. Furthermore, by using soil inoculum manipulations, I aimed to disentangle the effects of edaphic properties from inoculum origin in structuring these communities. This study aims to evaluate what are the main drivers of ECM fungal community assembly during long-term ecosystem development.

Finally, in Chapter 4, I assessed the role of native soil-borne pathogens in plant coexistence in the chronosequence stage that presents the highest plant diversity (i.e. oldest stage). Here, by using plant species of contrasting nutrient-acquisition strategies and different pathogen inoculum treatments, the aim was to provide an experimental test of a recently proposed hypothesis regarding plant coexistence through a potential trade-off between P-acquisition efficiency and defence against pathogens (Laliberté et al., 2015).

1.7. References


2. Shifts in symbiotic associations in plants capable of forming multiple root symbioses across a long-term soil chronosequence

2.1. Preface

The study site is a 2-million years chronosequence in southwestern Australia. Very little is known about the ecology of root symbionts in this system, yet this information is paramount in order to understand how plant communities are assembled and their composition change during long-term ecosystem development. This chapter aims to describe the colonisation pattern that three different symbionts have across this chronosequence and will serve as a baseline for more detailed research. This chapter is currently under review with *Ecology and Evolution*.

2.2. Summary

Changes in soil nutrient availability during long-term ecosystem development influence the relative abundances of plant species with different nutrient-acquisition strategies. These changes in strategies are observed at the community level, but whether they also occur within individual species remains unknown. Plant species forming multiple root symbioses with arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (ECM) fungi, and nitrogen-(N) fixing microorganisms provide valuable model systems to examine edaphic controls on symbioses related to nutrient-acquisition, while simultaneously
controlling for plant host identity. We grew two co-occurring species, Acacia rostellifera (N₂-fixing and dual AM and ECM symbioses) and Melaleuca systena (AM and ECM dual symbioses), in three soils of contrasting ages (c. 0.1, 1 and 120 ka) collected along a long-term dune chronosequence in south-western Australia. The soils differ in the type and strength of nutrient limitation, with primary productivity being limited by N (0.1 ka), co-limited by N and phosphorus (P) (1 ka), and by P (120 ka). We hypothesised that (i) within-species root colonisation shifts from AM to ECM with increasing soil age, and (ii) nodulation declines with increasing soil age, reflecting the shift from N to P limitation along the chronosequence. In both species, we observed a shift from AM to ECM root colonisation with increasing soil age. In addition, nodulation in A. rostellifera declined with increasing soil age, consistent with a shift from N to P limitation. Shifts from AM to ECM root colonisation reflect strengthening P limitation and an increasing proportion of total soil P in organic forms in older soils, since ECM fungi can access organic P via extracellular phosphatases, while AM fungi do not use organic P. Plants shift their resource allocation to different root symbionts depending on nutrient availability during ecosystem development.

2.3. Introduction

Many terrestrial plants form symbiotic associations with soil biota to enhance nutrient acquisition. The most widespread of these associations involves mycorrhizal fungi, which occur in roots of >80% of all plant species (Wang & Qiu, 2006; Brundrett, 2009). The two main types of mycorrhizas are arbuscular mycorrhizas (AM) and ectomycorrhizas (ECM). Arbuscular mycorrhizas enhance the acquisition of inorganic phosphorus (P) and other relatively immobile nutrients, while ectomycorrhizas also
allow plants to access both organic nitrogen (N) and P, as well as sorbed P (Hodge et al., 2001; Leigh et al., 2009; Plassard & Dell, 2010). Some plant species also form root symbiotic associations with N₂-fixing bacteria in nodules, allowing plants to acquire atmospheric N (Gutschick, 1984).

Plants allocate substantial amounts of carbon (C) to sustain symbiotic associations with mycorrhizal fungi or N₂-fixing bacteria (Pate & Herridge, 1978; Smith & Read, 2008). Carbon allocation to AM and ECM fungi can represent >20% of the total C fixed daily in photosynthesis (Bryla & Eissenstat, 2005; Hobbie, 2006). Likewise, C allocation to nodules by N₂-fixing plant species can represent >30% of daily photosynthates (Minchin & Pate, 1973). However, plant investment in symbiotic associations depends strongly on plant nutrient requirements and soil nutrient availability (van der Heijden, 2001; Lambers et al., 2008). The occurrence of AM fungi tends to be more common in neutral soils with low P availability and low organic matter content (Johnson et al., 1991; Coughlan et al., 2000; Smith et al., 2015). By contrast, ECM fungi are more common in acidic soils with lower mineral N concentrations and higher organic matter content (van der Heijden & Kuyper, 2001; Lilleskov et al., 2002). Nitrogen fixation plays a greater role in N acquisition at low soil N availability and is inhibited by N fertilisation (Imsande, 1986; Kanayama et al., 1990). These studies suggest that plants decrease investment in root symbionts when nutrient supply is high, thus allocating C in a manner that increases acquisition of the nutrients that most strongly limit their growth.

Most plant species form associations with only one type of mycorrhizal fungi (e.g., AM or ECM). However, some plant species form dual associations with both AM and ECM fungi (Cázares & Smith, 1996; Chen et al., 2000; Adams et al., 2006; Pagano & Scotti, 2008) and, in some cases, a tripartite root symbiosis involves N₂-fixing microorganisms (e.g., Acacia holosericea; Founoune et al., 2002). Several studies have shown negative
correlations between AM and ECM fungi, and this relationship may reflect competitive exclusion of AM fungi by ECM fungi (Lapeyrie & Chilvers, 1985; Lodge & Wentworth, 1990; Neville et al., 2002). On the other hand, positive relationships between nodulation and both AM and ECM colonisation have been reported (Founoune et al., 2002; André et al., 2003; Lesueur & Duponnois, 2005). The reliance of plants on root symbionts can be better understood by studying within-species shifts in root symbionts with changing soil properties and plant N:P stoichiometry (Jones et al., 1998; Founoune et al., 2002; Neville et al., 2002). Such shifts have rarely been studied (but see Neville et al., 2002; Nilsson et al., 2005; hence, further research needs to identify factors involved in the balance between multiple symbioses.

Long-term soil chronosequences (i.e. gradients of soil age) offer valuable ‘natural experiments’ to study how soil nutrient availability and stoichiometry influence plant-soil interactions (Walker et al., 2010; Turner & Condron, 2013). During tens to hundreds of thousands of years of soil and ecosystem development, changes in soil and plant communities co-occur that strongly alter soil nutrient dynamics (Walker & Syers, 1976; Wardle et al., 2004; Peltzer et al., 2010). In young soils, pH is higher, P is most abundant, and N is generally the key limiting nutrient (Walker & Syers 1976; Turner & Laliberté 2015). As soils develop, pH decreases, soil N accumulates through N\textsubscript{2}-fixation, whilst P availability declines, such that N and P co-limit plant productivity on intermediate-aged soils (Vitousek & Farrington, 1997; Laliberté et al., 2012). Additionally, while soil P decreases with pedogenesis, the organic fraction of it increases, becoming the largest portion in old soils. In strongly-weathered and acidic soils, P can be strongly limiting (Vitousek & Farrington, 1997; Laliberté et al., 2012) and P depletion can be sufficiently severe to cause ecosystem retrogression (Wardle et al., 2004; Peltzer et al., 2010). Soil chronosequences thus provide a unique opportunity
to study changes in plant allocation to different root symbioses with decreasing nutrient availability (Treseder & Vitousek, 2001).

It has been proposed that there is a community-level shift in the relative importance of different nutrient-acquisition strategies (specifically, the type of mycorrhizal association) during ecosystem development (Read, 1991; Lambers et al., 2008). In young soils, ruderal non-mycorrhizal strategies and AM associations should be more common (Lambers et al., 2015), due to their ability to take up mineral P (Lambers et al., 2012; Smith et al., 2015). As soils age, a decrease in AM fungi in favour of ECM fungi and ericoid mycorrhizal associations should occur, because the latter can access sorbed and organic forms of P. Finally, in old severely P-impoverished soils, non-mycorrhizal strategies should become more abundant (Lambers et al., 2008; Zemunik et al., 2015), given their highly effective strategy to acquire sorbed and organic P (Lambers et al., 2012). The validity of this model has been questioned on the basis that vegetation patterns do not follow this model in all chronosequences (Dickie et al., 2013). These models have been evaluated by observing changes in plant species composition across soil age (e.g., Zemunik et al., 2015), rather than evaluating within-species shifts in symbiotic associations. The use of plant species capable of forming multiple symbiotic associations allows for a stronger test of these models by controlling for differences in plant host identity.

We studied changes in root symbiotic associations (AM, ECM, N$_2$-fixing nodules) within two plant species that co-occur across contrasting stages of the Jurien Bay dune chronosequence in south-western Australia (Laliberté et al., 2012, 2014; Hayes et al., 2014). This long-term dune chronosequence shows a marked decrease in soil P and pH (Laliberté et al., 2012; Turner & Laliberté 2015), a shift from N to P limitation with increasing soil age (Laliberté et al., 2012; Hayes et al., 2014), and a high functional
diversity in nutrient-acquisition strategies (Hayes et al., 2014; Zemunik et al., 2015). We grew seedlings of the two focal species in soils of different ages (c. 0.1, 1 and 120 ka) in a glasshouse. We hypothesised that within-species root colonisation shifts from AM to ECM with increasing soil age (Lambers et al. 2008) and that nodulation in A. rostellifera declines with soil age, reflecting the shift from N to P limitation of plant growth along this chronosequence (Laliberté et al., 2012; Hayes et al., 2014).

2.4. Materials and Methods

STUDY AREA

The Jurien Bay dune chronosequence in south-western Australia (30.29° S, 115.04° E), spans two million years of pedogenesis (Laliberté et al., 2012; Laliberté et al., 2014; Turner & Laliberté 2015). We focused on three stages of the chronosequence that are most contrasting in terms of the strength and type of nutrient limitation (i.e. N vs. P limitation; Table 2.1). The youngest dunes (~100 years) are highly calcareous and show little to no soil development (Turner & Laliberté 2015). Soils on these youngest dunes have a relatively high P availability (primarily as mineral P), but low N availability (Turner & Laliberté 2015), and plant growth on these youngest dunes is limited by N (Laliberté et al., 2012; Hayes et al., 2014). Intermediate-aged dunes (~1000 years) are relatively high in both N and P, and plant productivity is highest and co-limited by N, P and possibly other nutrients (Laliberté et al., 2012; Hayes et al., 2014). Old dunes (~120,000 years) are N- and P-depleted and plant productivity is low and strongly limited by P (Laliberté et al., 2012; Hayes et al., 2014). The three dune systems correspond to chronosequence stages 1, 2 and 4 in Hayes et al. (2014) and Laliberté et al. (2014), and form a strong natural nutrient-availability and stoichiometry gradient.
driven by long-term pedogenesis (Turner & Laliberté 2015). These three chronosequence stages are < 10 km apart and are exposed to the same present-day Mediterranean climate, with a mean annual rainfall of 570 mm (Australian Bureau of Meteorology, http://www.bom.gov.au/climate/data/). They are derived from the same parent material (calcareous sand of marine origin; McArthur & Bettenay, 1974; Turner & Laliberté, 2015) and share the same regional species pool, with no barrier to dispersal among the different dune systems (Laliberté et al., 2014).

SPECIES SELECTION

We selected two native plant species that co-occur in the three selected chronosequence stages and form at least two different types of root symbioses: i) *Acacia rostellifera* (Benth.) Pedley (Fabaceae), which forms associations with N₂-fixing rhizobia as well as AM and ECM fungi (based on field sample observations), and ii) *Melaleuca systena* Craven (Myrtaceae), which forms AM and ECM associations (Brundrett, 2009), but does not fix N₂. These species are among the few along the chronosequence that occur at these three distinct stages (Hayes et al., 2014; Laliberté et al., 2014; Zemunik et al., 2015).

SITES SELECTION

Turner & Laliberté (2015) used 10 sites for each chronosequence stage. In this study, we selected a representative plot in each stage (i.e. close to the overall stage mean nutrient concentrations) in which both species co-occur, to ensure compatible soil microbiota (i.e. mycorrhizal and rhizobia inoculum). These plots were Q.Y.17, Q.M.18.
and S.W.35 for young, intermediate-aged and old stages, respectively. For each plot, we collected soil from five nearby sites located at least 200 m apart. Soil property data from all plots were obtained from Turner & Laliberté (2015) (Table 2.1). We only chose one representative plot for each chronosequence stage given the low variability in soil properties within stages compared with that among stages (Laliberté et al., 2012); hence, this is unlikely to confer any biases.
Table 2.1 Main soil properties for the three soil ages used. Estimated soil age, total nitrogen (N), phosphorus (P), organic phosphorus \((P_{\text{org}})\), pH and effective cation-exchange capacity (ECEC) are from Turner and Laliberté (2015). Values are given as means ± standard error \((n = 10)\). * from Hayes et al. (2014).

<table>
<thead>
<tr>
<th>Estimated soil age (ka)</th>
<th>0.1 (Holocene)</th>
<th>1 (Holocene)</th>
<th>120 (Middle Pleistocene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronosequence stage*</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total N (g kg(^{-1}))</td>
<td>0.51 ± 0.01</td>
<td>1.16 ± 0.01</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Total P (mg kg(^{-1}))</td>
<td>351 ± 2.4</td>
<td>432 ± 4.8</td>
<td>20.3 ± 0.5</td>
</tr>
<tr>
<td>P(_{\text{org}}) (% of total P)</td>
<td>0.6 ± 0.2</td>
<td>3.7 ± 0.4</td>
<td>35.6 ± 2.4</td>
</tr>
<tr>
<td>pH (CaCl(_2))</td>
<td>8.2 ± 0.01</td>
<td>7.8 ± 0.01</td>
<td>5.8 ± 0.03</td>
</tr>
<tr>
<td>ECEC (cmol(_c) kg(^{-1}))</td>
<td>24.9 ± 1.5</td>
<td>12.9 ± 0.5</td>
<td>3.8 ± 0.1</td>
</tr>
</tbody>
</table>
GLASSHOUSE EXPERIMENT

Soil collection and potting

Soils were sampled in March 2013 at each of the 15 sites, from the top 45 cm layer. Soil-collecting materials were cleaned and washed with ethanol (70% v/v) between sites to avoid potential contamination. Soils were sieved (2 mm), homogenised and then dried for five days at 35 ºC. This temperature is within the natural range for the region and was selected to ensure that soil biota would persist in the soils (Lucas et al., 1992). Soil from each site was then added to 2.8-l pots.

Six-month old seedlings were grown in a mixture of sterile perlite and sand without fertiliser by Men of the Trees, Hazelmere, Australia. The purchased seedlings were transplanted into pots and watered three times a week for the duration of the experiment (6 months) for a total of 10 replicates per treatment.

Post-harvest analyses

After six months of growth in the glasshouse, seedlings were harvested. Roots were severed and washed over a 1-mm sieve immediately after harvesting to remove soil particles. Shoots and roots were oven-dried for three days at 70 ºC and weighed separately. A subsample of live fine roots (<2 mm diameter) was weighed, cut into 1 to 2 cm segments, and stored in 10 ml tubes for one week at 5 ºC. Dry weight of the subsample was later estimated by calculating root water content. Roots were cleared using potassium hydroxide (10% w/v) for five hours in a water bath at 90 ºC. Following clearing, we used a 5% (v/v) ink in vinegar solution for five minutes in a water bath at 90 ºC to stain roots (Vierheilig et al., 1998). Finally, cleared and stained roots were placed in a 50% (v/v) lactoglycerol mixture for storage.
Root colonisation was determined following the gridline intersect method (Giovannetti & Mosse, 1980) at 200× magnification, counting intersects that had arbuscules or vesicles for AM fungi, a mantle for ECM fungi, or a Hartig net when the mantle was absent. We counted at least 130 intersections for each sample. For A. rostellifera, all nodules were collected, oven-dried at 70 ºC for 48 hours and weighed.

Leaf samples were digested in a mixture of sulfuric and salicylic acid and hydrogen peroxide (i.e. Kjeldahl digest), with N and P detection by automated colourimetry using a Technicon Auto Analyzer II (Technicon Instruments Corp., Tarrytown, New York, USA). Initial dry biomass (B) of planted seedlings was estimated through an allometric regression equation based on seedling height (H) and stem diameter (D) using additional seedlings for each species (A. rostellifera: ln(B) = 0.75 × ln(D² × H) - 2.69, \( R^2 = 0.81, n = 20 \); M. systena: ln(B) = 0.52 × ln(D² x H) – 2.31, \( R^2 = 0.80, n = 20 \)). The relative growth rate (RGR) was calculated (Hunt, 1982). We also recorded initial mycorrhizal colonisation of rehydrated oven-dried roots, using methods described above; initial mycorrhizal root colonisation was either absent or low: for A. rostellifera it was 0.4% and 0% for ECM and AM, respectively; for M. systena it was 1% and 0.4%, respectively. We tested the effects of root rehydration on mycorrhizal colonisation; extraradical hyphae were lost, but percent root length colonisation estimates was not affected (paired t-test; \( P \geq 0.4 \); Table S2.1).

STATISTICAL ANALYSES

We used linear mixed-effect models (Pinheiro & Bates, 2000) to test for differences in mycorrhizal colonisation, nodule biomass, RGR, and leaf N and P concentrations among plant species and chronosequence stages, including the interaction between these two fixed factors. Additionally, we tested for a potential effect of ECM colonisation on
nodulation and AM colonisation using a linear model, with chronosequence stage as a covariate. Site was specified as a random effect, because more than one sample came from each site. In all analyses, residuals were inspected visually to check model assumptions. When models did not meet assumptions (i.e. residuals centred around zero and homoscedasticity), appropriate variance structures were specified in a second model, and both models were compared using the Akaike Information Criterion (AIC) and likelihood ratio tests (Zuur et al., 2009). When a main term was significant, post hoc Tukey tests were performed (Hothorn et al., 2008). All analyses were conducted in R (R Core Team, 2015) using the ‘nlme’ (Pinheiro et al., 2012) and ‘multcomp’ (Hothorn et al., 2008) packages.

2.5. Results

MYCORRHIZAL COLONISATION

Changes in root colonisation by AM fungi differed between species, but these differences depended on soil age (species × stage interaction; \( P \leq 0.05 \); Fig. 2.1a; Table S.2.2). Arbuscular mycorrhizal root colonisation was greater in *M. systena* than in *A. rostellifera* only in the young soils (\( P \leq 0.02 \)), while there were no differences in AM root colonisation between the two species in either intermediate-aged or old soils (\( P \geq 0.4 \)). Arbuscular mycorrhizal root colonisation of both species was greatest on the youngest and intermediate-aged soils, and least on the oldest soils (\( P \leq 0.001 \)).

Both species showed similar patterns of increasing ECM colonisation with increasing soil age, although differences varied between species (species × stage interaction; \( P \leq 0.001 \); Fig. 2.1b; Table S2.2). *Melaleuca systena* generally showed greater ECM
colonisation than did *A. rostellifera* (*P* ≤ 0.001), with the exception of the youngest soil, where the species showed similarly low ECM colonisation (*P* ≥ 0.16). Also, we found no significant relationships between AM and ECM colonisation for both species when soil age was taken into account (*P* ≥ 0.79).

**Figure 2.1** Percentage of root length colonised by (a) arbuscular mycorrhizal fungi (AM; percentage of grid intercepts) and (b) ectomycorrhizal fungi (ECM; percentage of root tips) for *Acacia rostellifera* and *Melaleuca systena* with increasing soil age. Means ± 95% confidence intervals (CI) are shown. Different letters indicate significant (*P* ≤ 0.05) differences among soil ages based on *post hoc* Tukey tests

**NODULE BIOMASS IN ACACIA ROSTELLIFERA**

Total seedling (*P* ≤ 0.001) and nodule biomass in *A. rostellifera* declined with increasing soil age (*P* ≤ 0.001). Therefore, we measured the relative investment in N$_2$-fixing nodules in *A. rostellifera* as the ratio between nodule biomass and total plant biomass. This ratio also declined with increasing soil age (*P* ≤ 0.001; Fig. 2.2; Table 30
We found no correlation between relative nodule production and ECM root colonisation after controlling for differences in soil age ($P \geq 0.98$).

**Figure 2.2** Relative investment in nodules (i.e. ratio between nodule biomass and total plant biomass) of *Acacia rostellifera* seedlings with increasing soil age. Means ± 95% confidence intervals (CI) are shown. Different letters indicate significant ($P \leq 0.05$) differences among chronosequence stages based on post hoc Tukey tests.

LEAF NUTRIENT CONCENTRATIONS AND BIOMASS

Leaf [N] followed a similar pattern for both species across the chronosequence, being highest on intermediate-aged soils ($P \leq 0.003$; Fig. 2.3a; Table S2.2), where soil total [N] was highest (Table 2.1). Leaf [N] was higher in *A. rostellifera* than in *M. systena* across all soil ages ($P \leq 0.001$). Leaf [P] decreased from young to old soils for both species ($P \leq 0.001$; Fig. 2.3b; Table S2.2). Leaf [P] was lower in *A. rostellifera* than in *M. systena* on young and intermediate-aged soils ($P \leq 0.001$), but on old soils both species had similarly low leaf [P] ($P \geq 0.2$). Leaf N:P ratio increased from young to old soils in both species ($P \leq 0.001$; Fig. 2.3c; Table S2.2). On intermediate-aged soils, the N:P ratio of *A. rostellifera* (65 ± 7.9) pointed towards P limitation, while that of *M. systena* (2.1 ± 0.5) pointed toward N limitation.
Figure 2.3 (a) Leaf nitrogen (N) and (b) phosphorus (P) concentrations and (c) N:P ratio of *Acacia rostellifera* and *Melaleuca systena* with increasing soil age. Means ± 95% confidence intervals (CI) are shown. $n = 10$. Different letters indicate significant ($P \leq 0.05$) differences among soil ages based on *post hoc* Tukey tests. Black dashed lines indicate thresholds for N or P limitation, following Güsewell (2004). Grey area indicates thresholds for N or P limitation based on Koerselman and Meuleman (2007).
There were differences in RGR between species, but these depended on soil age (species $\times$ stage interaction; $P \leq 0.01$; Fig. 2.4; Table S2.2). The RGR of $A.\ rostellifera$ was greater on both the youngest and intermediate-aged soils than on the oldest soils ($P \leq 0.02$), while for $M.\ systena$ it was greatest on intermediate-aged and old soils ($P \leq 0.01$), and lowest on the youngest soils ($P \leq 0.01$).

![Figure 2.4](image)

**Figure 2.4** Relative growth rate (RGR) of *Acacia rostellifera* and *Melaleuca systena* seedlings grown on soils of different ages. Means $\pm$ 95% confidence intervals (CI) are shown. Different letters indicate significant ($P \leq 0.05$) differences among soil ages based on post hoc Tukey tests

2.6. Discussion

**SHIFTS IN MYCORRHIZAL COLONISATION**

Consistent with our hypothesis, root colonisation by AM fungi declined with increasing soil age, whereas previous studies have found AM colonisation increasing with declining soil P availability (Abbott *et al.*, 1984; Bentivenga & Hetrick, 1992; Treseder & Vitousek, 2001). However, these studies were conducted at higher soil [P] and across a much smaller soil [P] range (Francis & Read, 1994) than that along the studied chronosequence (Turner & Laliberté 2015). In addition, these studies used species that
only form AM, whereas our study focused on species forming multiple associations simultaneously. Furthermore, soil pH decreased and previous studies have shown that AM fungi tend to dominate on young alkaline-to-neutral soils (Piotrowski et al., 2008; Zangaro et al., 2012), and soil pH < 5 can decrease AM colonisation (Clark, 1997; Coughlan et al., 2000). In our study, pH declined to only 5.8 in the oldest soils, suggesting that pH inhibition did likely not contribute to the effect of soil age on AM colonisation. Our results suggest that AM associations are favoured in younger soils where most P is in mineral forms (Lambers et al. 2008; Turner & Laliberté 2015).

Root colonisation by ECM fungi was about four times greater in the oldest soils than in the youngest soils for both species. Although the oldest soils had a much lower total [P], organic P represented a much larger fraction. Ectomycorrhizal fungi are efficient at accessing organic forms of N and P (Read, 1989; Antibus et al., 1992; Chalot & Brun, 1998). Consequently, ECM colonisation may be related to the organic soil P fraction, consistent with results of Harvey et al. (1976). Old acidic soils might be better suited for ECM fungi than young alkaline soils (Piotrowski et al. 2008; Zangaro et al. 2012), as the optimum conditions for ECM fungi are between pH 4 and 5 (Aggangan et al., 1996; Yamanaka, 2003). Young soils in our study exhibited a pH between 5.8 and 8.2, suggesting that the decline in pH contributed to the increase of ECM fungi with increasing soil age. However, we cannot disentangle potential effects of total P from those due to pH, because total P and pH decline simultaneously during pedogenesis.

Negative relationships between AM and ECM have been interpreted as competitive exclusion of AM fungi by ECM fungi (Chen et al., 2000; Adams et al., 2006). Similarly, colonisation shifts from AM to ECM with soil depth have been found (Neville et al. 2002), with higher ECM colonisation in upper soil layers, where organic matter content is greater. In coniferous forest AM fungi dominate in nutrient-rich soils
with high pH, while ECM fungi dominate in soils with low nutrient availability and lower pH (Nilsson et al. 2005). The lack of a relationship between AM and ECM at any soil age in our study suggests that the observed shift from AM to ECM colonisation was driven by changes in soil properties, rather than reflecting a direct negative effect of ECM fungi on AM fungi.

SHIFTS IN NODULE BIOMASS

Nodulation in A. rostellifera declined with increasing soil age, likely because plant growth on the oldest soils is limited by the availability of P, rather than N. Nodulation might be constrained in old soils by the relatively high P demand of N\textsubscript{2} fixation (Sprent & Raven, 1985; Sprent, 1999; Raven, 2012). Thus, on old soils where both N and P availability are extremely low, legumes might acquire N predominantly via ECM, rather than rhizobia. There was no relationship between nodulation and ECM colonisation in A. rostellifera once differences in soil age were controlled for. These results differ from those obtained by Diagne et al. (2013), who found that ECM fungi promote nodulation under P limitation in A. mangium. However, Diagne et al. (2013) used soils with relatively high P levels (4.8 mg Olsen P kg\textsuperscript{-1}), while resin [P] in our study ranged between 0.6 and 3 mg kg\textsuperscript{-1} (Turner & Laliberté 2015). Furthermore, previous studies have shown that a soil pH < 4.5 can be detrimental for the two main N\textsubscript{2}-fixing rhizobia (Rhizobium and Bradyrhizobium; Graham, 1992; Graham et al., 1994). Since soil pH in the present study ranged from 8.2 to 5.8, the decrease in nodulation is likely related to nutrient limitation, rather than a low soil pH.
SHIFTS IN THE TYPE AND STRENGTH OF NUTRIENT LIMITATION

Both leaf [N] and leaf [P] reflect the low availability of these nutrients in soils (Laliberté et al., 2012, Turner & Laliberté 2015). Furthermore, leaf N:P ratio increased more than 10-fold for A. rostellifera and 20-fold for M. systena from the youngest to the oldest soils, consistent with shifts from N limitation to strong P limitation of plant productivity along the chronosequence (Laliberté et al., 2012, Hayes et al., 2014). Leaf N:P increased markedly in A. rostellifera between the youngest and intermediate-aged soils, while there was no difference between N:P on these two soil ages for M. systena. The change in A. rostellifera was associated with a greater increase in leaf [N], presumably due to its N2-fixation capacity. Foliar N:P in a N2-fixing shrub is also low on young soils along a 120,000 year chronosequence in New Zealand (Richardson et al., 2004), due to high leaf [N] rather than low leaf [P].

The shifts in mycorrhizal colonisation with increasing soil age could be due to changes in inoculum potential, which decreases with increasing soil age for AM fungi, but increases with soil age for ECM fungi (Piotrowski et al., 2008; Zangaro et al., 2012). However, such changes in inoculum potential might be related to longer-term feedback between plants and soil biota that ultimately depend on soil nutrient availability. Future experiments should aim to disentangle the role of such biotic and abiotic effects on the balance of multiple symbioses, to assess the effects of soil abiotic properties and inoculum potential independently.

In conclusion, our results show within-species shifts between different root symbiotic associations during long-term soil and ecosystem development, consistent with those predicted by Read (1991) and Lambers et al., (2008). This might be associated with a shift from N to P limitation of primary plant productivity, soil pH or inoculum potential (Nilsson et al., 2005; Zangaro et al., 2012). Our study supports the hypothesis that the
importance of different mycorrhizal types changes with soil age (Lambers et al., 2008). Our results on intraspecific shifts in nutrient-acquisition strategies complement those of a recent study along the same chronosequence showing that, at the community level, ECM plants become more abundant as soils age (Zemunik et al., 2015). Further work on within-species shifts in symbiotic associations and their functional significance is needed to better understand the role of mycorrhizal fungi during long-term ecosystem development (Dickie et al., 2013).

2.7. Acknowledgements

We thank Hannah Etchells and Jennifer Middleton for assistance in sample collection. Funding was provided by the Australian Research Council (ARC) through a DECRA (DE120100352) to EL and a Discovery Project (DP0985685) to HL and a UWA Research and Development Award granted to FPT. We also acknowledge financial support to FA through the “Becas Chile” scholarship from the Chilean government, the ANZ Holzworth Wildlife Research Endowment, and the University of Western Australia.

2.8. References


2.9. **Supporting Information**

**Table S2.1** Comparison of mycorrhizal root colonisation between fresh and rehydrated roots. Values shown as mean ± SE based on paired t-test

<table>
<thead>
<tr>
<th>Fresh roots</th>
<th>Rehydrated roots</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acacia rostellifera</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECM colonisation (%)</td>
<td>6.5 ± 1.9</td>
<td>6.2 ± 1.1</td>
</tr>
<tr>
<td>AM colonisation (%)</td>
<td>3.1 ± 0.9</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td><strong>Melaleuca systena</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECM colonisation (%)</td>
<td>18.8 ± 1.0</td>
<td>20.3 ± 1.3</td>
</tr>
<tr>
<td>AM colonisation (%)</td>
<td>2.7 ± 0.7</td>
<td>2.4 ± 0.8</td>
</tr>
</tbody>
</table>
Table S2.2 Summary of statistical outputs. Values shown are degrees of freedom (DF), F-test and p-value of individual mixed-effect models of two factors (Stage and Species) and their interaction for each variable.

<table>
<thead>
<tr>
<th></th>
<th>Stage</th>
<th>Species</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>F-test</td>
<td>p-value</td>
</tr>
<tr>
<td>AM colonisation (%)</td>
<td>2</td>
<td>15.74</td>
<td>0.0004</td>
</tr>
<tr>
<td>ECM colonisation (%)</td>
<td>2</td>
<td>36.35</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Nodulation</td>
<td>2</td>
<td>35.21</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>RGR (g g(^{-1}) d(^{-1}))</td>
<td>2</td>
<td>10.16</td>
<td>0.0026</td>
</tr>
<tr>
<td>Leaf N (mg g(^{-1}))</td>
<td>2</td>
<td>14.96</td>
<td>&lt;.0001</td>
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<tr>
<td>P (mg g(^{-1}))</td>
<td>2</td>
<td>53.38</td>
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<tr>
<td>Leaf N:P ratio</td>
<td>2</td>
<td>50.63</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

Nodulation was calculated as total nodule biomass divided by total seedling biomass.
3. Changes in ectomycorrhizal fungal community composition and declining diversity along a 2-million year soil chronosequence

3.1. Preface

After finding such a clear increase in ECM root colonisation with soil age in chapter 1, evaluating potential changes in ECM fungal community during long-term ecosystem development was necessary to fully understand how soil development influenced ECM fungi. This chapter focuses on disentangling potential abiotic from biotic factors structuring ECM fungal communities across the chronosequence. This chapter is currently under review in *Molecular Ecology*.

3.2. Summary

Ectomycorrhizal (ECM) fungal communities co-vary with host plant communities along soil fertility gradients, yet it is unclear whether this reflects changes in host composition, fungal edaphic specialisation, or priority effects during fungal community establishment. We grew two co-occurring ECM plant species (to control for host identity) in soils collected along a 2-million year chronosequence representing a strong soil fertility gradient, and used soil manipulations to disentangle effects of edaphic properties from those due to fungal inoculum. Ectomycorrhizal fungal community composition changed and richness declined with increasing soil age; these changes were linked to pedogenesis-driven shifts in edaphic properties, particularly pH and resin-
exchangeable and organic phosphorus. However, when differences in inoculum potential or soil abiotic properties among soil ages were removed while host identity was held constant, differences in ECM fungal communities and richness among chronosequence stages disappeared. Our results show that ECM fungal communities strongly vary during long-term ecosystem development, even within the same hosts. However, these changes could not be attributed to short-term fungal edaphic specialisation or differences in fungal inoculum (i.e. density and composition) alone. Rather, they must reflect longer-term ecosystem-level feedback between soil, vegetation and ECM fungi during pedogenesis.

3.3. Introduction

Major changes in soil properties occur during long-term soil and ecosystem development, with shifts from nitrogen (N) limitation of plant productivity in young soils, to phosphorus (P) limitation in old, strongly-weathered soils (Walker & Syers 1976; Wardle et al. 2004b; Laliberté et al. 2012). These changes in soil properties are associated with significant changes in plant community composition (Kitayama & Mueller-Dombois 1995; Richardson et al. 2004) and increasing plant diversity (Wardle et al. 2008; Laliberté et al. 2014; Zemunik et al. 2015). In addition, recent studies have highlighted changes in communities of ecologically important root symbionts such as arbuscular mycorrhizal fungi during long-term soil and ecosystem development (Krüger et al. 2015; Martínez-García et al. 2015). However, less is known about changes in ectomycorrhizal (ECM) fungi during long-term ecosystem development (Dickie et al. 2013), despite ECM fungi playing their important role in plant N and P acquisition (Smith & Read 2008; Smith et al. 2015). Understanding how ECM fungal communities change during long-term soil and ecosystem development also has implications for
ecosystem functioning, since ECM fungi act as drivers of soil carbon (C) accumulation (Orwin et al. 2011; Clemmensen et al. 2013; Averill et al. 2014).

Soil abiotic properties such as pH and concentrations of N and P affect ECM fungi (Twieg et al. 2009; Navarro-Ródenas et al. 2012). Fungal communities co-vary with host plant communities along soil fertility gradients (Davey et al. 2015), and host plant communities can control their root-associated fungal communities (Ishida et al. 2007; Gao et al. 2015; Martínez-Garcia et al. 2015). On the other hand, ECM fungal communities may respond in similar, yet independent ways to the same environmental factors driving host plant communities (Toljander et al. 2006; Glassman et al. 2015; Tedersoo et al. 2015). To determine the extent to which soil properties drive ECM fungal communities, it is valuable to study ECM fungal community changes within the same host plant species across a range of soil properties thereby avoiding ‘host identity’ as a confounding factor (Hart et al. 2014; Davey et al. 2015).

Host identity and fungal edaphic specialisation are not the only drivers of ECM fungal communities along soil fertility gradients; the composition of root-associated ECM fungal communities may reflect stochastic colonising events during fungal community assembly (Kennedy & Bruns 2005; Kennedy et al. 2009; Fukami et al. 2010). Such ‘priority effects’ reflect the fact that the order of arrival of colonising ECM fungal species can lead to divergent communities due to competition for limited space (e.g., number of root tips), even under similar soil abiotic conditions (Kennedy & Bruns 2005). Priority effects can persist up to a year after inoculation, as shown for wood-decaying fungi (Dickie et al. 2012); however, whether these effects can persist to drive ECM fungal communities along fertility gradients or long-term ecosystem development remains unknown. Therefore, to better understand what drives host-symbiont selection
during long-term soil and ecosystem development, we need to disentangle the role of inoculum potential from soil abiotic factors driving root colonisation by ECM fungi.

In the present study, we hypothesised that ECM fungal communities vary in composition with soil age, and that changes are driven by soil properties, rather than inoculum potential, specifically soil P availability, which drives plant (Zemunik et al. 2015) and arbuscular mycorrhizal fungal communities (Krüger et al. 2015) along this chronosequence. Additionally, we hypothesised that ECM fungal richness increases with soil age, as a reflection of the increase in total and ECM host plant diversity across the chronosequence (Zemunik et al. 2015). To test these hypotheses, we evaluated soil fertility and soil inoculum effects on the ECM fungal community composition and richness along a 2-million year dune chronosequence in south-western Australia. We conducted a glasshouse experiment with two inoculum treatments to disentangle effects on root-associated ECM fungal communities of edaphic specialisation from those due to inoculum potential (i.e. priority effects). Studying changes in ECM fungal communities along soil fertility gradients within single host plant species helped us better understand environmental drivers structuring ECM fungal communities, independent of host identity. Because these different drivers of ECM fungal communities are difficult to disentangle, studies based on experimental manipulations (i.e. modifying soil properties and inoculum potential) are needed to better understand what drives the composition and structure of ECM fungal communities along soil fertility gradients.
3.4. Materials and Methods

STUDY AREA

The Jurien Bay dune chronosequence, located in south-western Australia (30.29° S, 115.04° E), spans over two million years of pedogenesis over approximately 10 km with no dispersal barriers among stages. The chronosequence has been described in detail elsewhere (Laliberté et al. 2012; Hayes et al. 2014; Turner & Laliberté 2015). This chronosequence provides an exceptionally strong natural nutrient-availability and stoichiometry gradient driven by long-term pedogenesis (Laliberté et al. 2014; Turner & Laliberté 2015; Table 3.1). We used the same classification of five chronosequence stages as in Hayes et al. (2014).

Site selection and soil collection

We used all five chronosequence stages to cover the progressive and retrogressive phases of long-term ecosystem development along this chronosequence (Laliberté et al. 2012; Table 3.1). From each chronosequence stage, we randomly selected five plots (10 × 10 m) from the same set of permanent plots used in earlier studies (Laliberté et al. 2014; Hayes et al. 2014; Turner & Laliberté 2015; Zemunik et al. 2015).

We collected soils in November 2013 and in each plot, we collected 5.3 l of bulk soil from the top 30 cm layer from three randomly-positioned points around the plot (no more than 5 m away from the plot). We also collected 1.6 l of inoculum soil from the top 20 cm layer of soil from seven randomly-positioned points. We used these soil depths to match previous work on soil properties and fungi on this chronosequence (Laliberté et al. 2012; Krüger et al. 2015; Turner & Laliberté 2015; Teste et al. 2016).
The collected soils were then mixed and bulked for each plot, keeping bulk and inoculum soil separate.

In June, July, and August of 2012, using a subsample of stages (i.e. stages 2, 4 and 5), we also collected root and soil samples following the same approach used by Krüger et al. (2015). Description of the methodology used for these samples can be found in Method S3.1 (Supporting Information).

Species selection

We selected two plant species that naturally co-occur across the chronosequence (Zemunik et al. 2016) in order to control for host plant identity, and, therefore, focus primarily on the effects of soil properties and changes in fungal inoculum on root-associated ECM fungal communities. These two plant species were *Acacia rostellifera* (Fabaceae) and *Melaleuca systena* (Myrtaceae). Both species form arbuscular mycorrhizal and ECM associations and *A. rostellifera* also produces N2-fixing nodules (Albornoz et al. 2016).
Table 3.1 Soil properties and vegetation variables among chronosequence stages. Soil properties are from Turner & Laliberté (2015), and vegetation properties are taken from Zemunik et al. (2015).

<table>
<thead>
<tr>
<th>Soil properties</th>
<th>Chronosequence stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>total N (g kg(^{-1}))</td>
<td>0.51 ± 0.01(^{c})</td>
</tr>
<tr>
<td>total P (mg kg(^{-1}))</td>
<td>354 ± 2(^{b})</td>
</tr>
<tr>
<td>resin P (mg kg(^{-1}))</td>
<td>1.4 ± 0.1(^{ab})</td>
</tr>
<tr>
<td>organic P (% of total P)</td>
<td>0.3 ± 0.2(^{a})</td>
</tr>
<tr>
<td>pH</td>
<td>9.26 ± 0.03(^{a})</td>
</tr>
<tr>
<td>exchangeable K (cmol(_{c}) kg(^{-1}))</td>
<td>0.03 ± 0.01(^{b})</td>
</tr>
<tr>
<td>exchangeable Mn (cmol(_{c}) kg(^{-1}))</td>
<td>0 ± 0(^{a})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vegetation properties</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>total plant cover (%)</td>
<td>36 ± 7(^{c})</td>
</tr>
<tr>
<td>ECM plant cover (%)</td>
<td>15.0 ± 6(^{abc})</td>
</tr>
<tr>
<td>ECM plant relative cover (%)</td>
<td>33 ± 10(^{a})</td>
</tr>
<tr>
<td>total plant richness</td>
<td>17 ± 2(^{d})</td>
</tr>
<tr>
<td>ECM plant richness</td>
<td>3.6 ± 0.6(^{b})</td>
</tr>
</tbody>
</table>
GLASSHOUSE EXPERIMENT

Soil treatments

To distinguish between changes in root-associated ECM fungal communities due to soil abiotic properties and those due to changes in ECM fungal inoculum, we used three soil treatments (Table 3.2). Firstly, we used unaltered (i.e. unsterilised) fresh bulk soil from each plot (hereafter referred to as “field soil”) to explore possible differences in ECM fungal community composition and diversity that were due to the combined effects of soil properties and ECM fungal inoculum. Secondly, we triple-pasteurised bulk soil from each plot, followed by inoculation with 7% (v/v) mixed live soil inoculum from all five chronosequence stages (hereafter, referred to as “pooled inoculum”). This second treatment was used to isolate effects of soil abiotic properties, while exposing the host plant to the entire ECM fungal regional species pool across the whole chronosequence. Finally, we used triple-pasteurised, mixed bulk soil from all five chronosequence stages (i.e. mixed in equal proportions, on a volume basis), but with addition of 7% (v/v) live soil plot-specific inoculum from each specific stage (hereafter referred to as “specific inoculum”). This third treatment allowed us to determine if mycorrhizal fungal inoculum (i.e. composition, density) could be an important factor driving responses in ECM fungal community composition, since this treatment maintained abiotic conditions constant. Using small volumes of inoculum soil requires more time for soil biota communities to fully establish (Brinkman et al. 2010), whereas larger volumes provokes confounding local nutrient effects and decreases plant growth (van der Putten et al. 1988). We used 7% as a compromise to minimise the chances of these two undesirable outcomes. The bulk soil from all plots for the pooled and specific inoculum treatments was sterilised via triple-steam pasteurisation at 80°C for two hours.
per day over seven days, following previous studies (Fang et al. 2012; Ryan et al. 2012).

Plant growth

We germinated *A. rostellifera* and *M. systena* seed in triple-steam pasteurised sand at 19° C with a 12 h photoperiod. Twenty days after germination, healthy seedlings were transplanted into 150 soil-filled pots (November 2013). Seedlings were immediately transplanted into pots and watered to 75% field capacity throughout the duration of the experiment. After five (*A. rostellifera*) and seven months (*M. systena*) of growth in the glasshouse, seedlings were harvested by severing shoots from roots. We harvested *A. rostellifera* first because of its higher growth rate than that of *M. systena*. Soil attached to roots was carefully washed away from root systems over a 1-mm sieve and clean roots were immediately freeze-dried. Equipment was thoroughly washed and surface sterilised with 70% (v/v) ethanol between each root system harvested. We subsampled and pooled 20 mg of roots of each species from each soil treatment and plot combination. Finally, we pooled both plant species for each treatment-plot combination in order to maximise the amount of ECM fungal species colonising roots, while still controlling for host identity for a total of 73 samples; two samples did not contain enough root material as plants were too small (five replicates per combination of treatments).

DNA extraction, amplification and high-throughput sequencing

Forty mg of pulverised root material was extracted using Qiagen’s DNeasy Plant Mini Kit with a modified protocol. Several negative controls were used in order to assess possible environmental contamination. DNA extracts were then quantified via real-time quantitative PCR (qPCR) to assess for quality and quantity of genomic DNA (gDNA)
using the primers ITS3-KYO2/ITS4 (Toju et al. 2012). Then, the ITS region was amplified and sequenced (in a single PCR) on an Illumina MiSeq system utilising modified fusion primers with a unique 8bp Multiplex Identifier tag (MID-tag) and custom adaptors for paired-end sequencing on both primers.

The pooled amplicons were purified using Agencourt AMPure XP PCR Purification Kit (Beckman Coulter Genomics, NSW, Australia) following the manufacturer’s protocol. Purified amplicons were electrophoresed on 2% agarose (w/v) gel and pooled in approximately equimolar ratios based on ethidium-stained band intensity to form a MID-tagged DNA sequencing library. Illumina MiSeq sequencing was performed using a MiSeq Reagent Kit v3 (600 cycles) 300 bp paired-end protocol as per manufacturer’s instructions (Illumina, Inc., San Diego, CA, USA). Details on the DNA extraction, amplification and high-throughput sequencing protocol can be found in Methods S3.2 (Supporting information).
Table 3.2 Summary table of inoculum treatments used in the glasshouse experiment.

<table>
<thead>
<tr>
<th>Inoculum treatment</th>
<th>Bulk soil</th>
<th>Bulk soil sterilisation</th>
<th>ECM fungal inoculum</th>
<th>Aim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field soil</td>
<td>each plot separately</td>
<td>none</td>
<td>none</td>
<td>evaluate natural factors</td>
</tr>
<tr>
<td>Pooled inoculum</td>
<td>each plot separately</td>
<td>triple pasteurised</td>
<td>all plots mixed in equal volumes</td>
<td>isolate abiotic effect</td>
</tr>
<tr>
<td>Specific inoculum</td>
<td>all plots mixed in equal volumes</td>
<td>triple pasteurised</td>
<td>each plot separately</td>
<td>isolate inoculum effect</td>
</tr>
</tbody>
</table>
BIOINFORMATICS

Deconvolution of amplicon sequences into sample batches followed the method described in Murray et al. (2015), using Geneious v8.0.2 (Drummond et al. 2011). Sequences were then dereplicated at 100% identity across their full length and clustered at a 97% identity threshold using the UPARSE (Edgar 2013) algorithm implemented in USEARCH (Edgar 2010) to identify operational taxonomic units (OTUs). OTUs were mapped against the original amplicon sequence fasta file from which sequences identified as singletons and/or chimerics were removed. An OTU table was subsequently created using USEARCH identifying the presence or absence of each OTU in each sample. Following OTU identification, representative sequences from each OTU were queried against UNITE, UNITE Envir. or annotated INSD datasets with Mega BLAST (Altschul et al. 1990) using MassBLASTer with default parameters (Abarenkov et al. 2010). Operational taxonomic units with less than 85% similarity to best match and 90% query were removed from the study. Ectomycorrhizal fungal lineages were assigned according to Tedersoo & Smith (2013).

STATISTICAL ANALYSES

Ectomycorrhizal community composition

To visualise differences in composition of ECM fungal communities among chronosequence stages, we calculated the Jaccard dissimilarity among samples and used non-metric multidimensional scaling (NMDS). To test for statistical differences in ECM fungal community composition among stages, we used permutational multivariate analysis of variance (PERMANOVA). When appropriate, pairwise comparisons between each pair of chronosequence stages and adjusted $P$-values for multiple
comparisons. To explore if shifts in plant composition were related to shifts in ECM fungal community composition, we performed a Procrustes test (Lisboa et al. 2014) between vegetation and ECM fungal OTU matrices. Vegetation data from the same plots were obtained from Laliberté et al. (2014).

To evaluate if changes in ECM fungal community composition were related to other factors (i.e. abiotic soil properties and ECM plant diversity and cover), we performed a canonical correspondence analysis (CCA) to test if community composition differences were related to soil properties or vegetation. Relevant explanatory environmental variables were selected by permutation tests with forward selection. Soil property data from all of our sites were obtained from Turner & Laliberté (2015), and plant species richness and cover were obtained from Zemunik et al. (2015).

Ectomycorrhizal fungal richness

To allow meaningful comparisons of fungal OTU richness among samples while standardising for number of sequences, we compared richness estimates using individual-based rarefaction (Gotelli & Colwell 2001) based on the minimum number of sequences per sample found across all samples (447 and 6 sequences for the glasshouse experiment and field-collected samples respectively). Rarefied ECM OTU richness and its relationship with soil properties among chronosequence stages were tested using generalised least squares models. Residuals were inspected visually to check model assumptions. When models did not meet assumptions (i.e. residuals centred around zero and homoscedasticity), appropriate variance structures were specified in a second model, and both models were compared using the Akaike Information Criterion (AIC) and likelihood ratio tests (Zuur et al. 2009). When a main term was significant, post hoc Tukey tests were performed.
Finally, to test the robustness of our models from the glasshouse experiment, we calculated richness for all fungal OTUs found in the study to ensure that our results were not purely driven by an overall trend in fungal OTU richness across the chronosequence (Fig. S3.1a-c). Furthermore, since south-western Australian ECM fungi are poorly studied, we also calculated ECM OTU richness using a more relaxed criterion (i.e. classifying an OTU as ectomycorrhizal if it belonged to an ECM fungal family, or if the reference sequence was isolated from an ectomycorrhiza; Fig. S3.1b-d). Since both models showed the same trend, we only show results using the more conservative 85% similarity with best match. For the field-collected samples, we used 85% similarity with best match. All statistical analyses were conducted in R (R Core Team 2015). Detailed functions and packages used in this study are listed in Methods S3.2 (Supporting information).

3.5. Results

ECTOMYCORRHIZAL OTUs AND LINEAGES

We obtained 532,694 ECM fungal sequences across all root samples from all seedlings in our experiment, representing 128 unique ECM OTUs from 15 ECM fungal lineages. In the field soil treatment, the proportion of OTUs from each lineage varied with chronosequence stage (Fig. 3.1a). Lineages such as /pulvinula, /terfezia-peziza depressa, and /tomentella-telephora became less frequent in older stages of the chronosequence ($P \leq 0.01$; Table S3.1; Supporting Information), while /marcelleina-peziza gerardii increased in frequency ($P \leq 0.01$; Table S3.1; Supporting Information), and /cenococcum was found only in seedlings grown in soils from stages 4 and 5 (Fig. 3.1a). By contrast, /tuber-helvella was found in similar abundance in seedlings grown in soils
from all chronosequence stages (Fig. 3.1a). On the other hand, all chronosequence stages from the pooled and specific inoculum treatments were dominated by the same three lineages ($P \geq 0.2$; Fig. 3.1b-c), with *T. depressa* being the most common one, representing around 76% of all OTUs.

**ECTOMYCORRHIZAL COMMUNITY COMPOSITION**

Ectomycorrhizal fungal community composition strongly co-varied with plant community composition across the chronosequence for seedlings grown in field soil (Procrustes correlation = 0.88; $P \leq 0.0001$), but not for ones in the other soil treatments (Procrustes correlation $\leq 0.3$; $P = 0.09$). In seedlings grown in the field soil treatment, ECM fungal communities differed significantly in composition among all chronosequence stages (Fig. 3.2a; $P \leq 0.05$, after Holm correction for pairwise comparisons), except for stages 2 and 3, which were indistinguishable from each other (Fig. 3.2a). These changes were explained by pedogenesis-driven shifts in abiotic soil properties: pH, organic P, % organic P of total P, and exchangeable manganese (Mn) (Fig. 3.3a; $R^2_{adj} = 0.33$, $P \leq 0.001$). Ectomycorrhizal plant species richness, cover and relative cover were not retained as explanatory variables by our variable selection model for all soil treatments.
Figure 3.1 Proportion of ECM OTUs of all lineages found in roots of *Acacia rostellifera* and *Melaleuca systena* growing in (a) field soil, (b) pooled inoculum, or (c) specific inoculum treatments. Lineages with less than three OTUs per chronosequence stage were pooled in “others”. Each chronosequence stage is represented by 5 sites.
Figure 3.2 Non-metric multidimensional scaling ordination (NMDS) plots showing differences in ECM fungal community composition associated with *Acacia rostellifera* and *Melaleuca systena* growing in soils from all five chronosequence stages for (a) field soil, (b) pooled inoculum treatment, and (c) specific inoculum treatment. Ellipses show the 95% confidence intervals around the mean centroid for each chronosequence stage based on Jaccard’s dissimilarity scores. Black arrow links centroids and shows the chronological sequence of the chronosequence stages from the youngest (stage 1) to the oldest (stage 5) soils. Statistically significant differences in composition were determined by using pairwise comparisons with Holm correction (Holm 1979). For the field soil, composition was different between all pairs of stages ($P \leq 0.001$) except between stages 2 and 3, while there were no differences for the two other treatments.
Figure 3.3 Canonical correspondence analysis (CCA) between ECM OTUs community composition on roots from *Acacia rostellifera* and *Melaleuca systena*, and soil properties among the different chronosequence stages between two inoculum treatments. Relevant explanatory environmental variables ($P \leq 0.01$) were selected by permutation test with forward selection using 9999 number of permutations. Organic phosphorus ($P_o$), percentage of $P_o$ of total P ($\%P_o$), total phosphorus (P), organic carbon (C), exchangeable manganese (Mn) and soil pH were the only variables selected. For the (a) field soil, soil properties explained 33% of total variation ($P \leq 0.001$), while in the (b) pooled inoculum only 13% was explained ($P \leq 0.01$).

In contrast to the field soil treatment, there were no significant differences in ECM fungal community composition for seedlings grown in soils from the pooled inoculum and specific inoculum treatments (Fig. 3.2b-c; $P = 0.13$). However, in the pooled inoculum treatment, differences in ECM fungal community composition could still be partly explained by total P and organic C (Fig. 3.3b; $R^2_{\text{adj}} = 0.13$, $P \leq 0.01$).

**ECTOMYCORRHIZAL RICHNESS**

Rarefied ECM OTU richness in seedlings grown in the field soil treatment was lowest in stage 5 and greatest in stage 1 (Fig. 3.4a; $P \leq 0.04$). Furthermore, ECM OTU richness
was strongly associated with a decline in soil pH and resin P (plant-available P), and with an increase in the percentage of total P as organic P, and in exchangeable Mn (Table S3.5; Supporting Information). On the other hand, there were no differences in rarefied ECM OTU richness among chronosequence stages for seedlings grown in soils from the pooled inoculum treatment (Fig. 3.4b; $P = 0.2$); while in the specific inoculum treatment, rarefied ECM OTU richness was lower in stage 5 compared with stage 2, with no differences among other stages (Fig. 3.4c; $P \leq 0.05$).

Results from the field-collected samples showed the same trends found in the field soil treatment of the glasshouse experiment (Results S3.1). Indeed, relative abundance of ECM lineages changed with chronosequence stage (Fig. S2a), community composition differed among all chronosequence stages ($P \leq 0.01$; Fig. S3.2b), and these changes were explained mainly by soil pH and total P (Fig. S3.2c; $R^2_{adj} = 0.27$, $P \leq 0.001$). Finally, rarefied OTU richness was lower in the oldest stage compared to stage 4 ($P \leq 0.01$; Fig. S3.2d), but only marginally lower than stage 2 ($P = 0.1$; Fig. S3.2d).

**Figure 3.4** Rarefied ECM OTU richness from roots of *Acacia rostellifera* and *Melaleuca systena* grown in soils from five chronosequence stages from (a) field soil, (b) pooled inoculum, and (c) specific inoculum treatments. Values are means (points) with 95% confidence intervals (bars). Different letters indicate significant ($P \leq 0.05$) differences among soil ages based on post hoc Tukey tests.
3.6. Discussion

Our results show major changes in ECM fungal communities and a decline in richness during long-term soil and ecosystem development, in both the field-collected samples and in the glasshouse where plant host identity was constant. Our results indicate a key role of soil pH and P as drivers of ECM fungal community composition during long-term ecosystem development along this chronosequence. Interestingly, our results also show that changes in ECM fungal communities cannot be independently attributed to short-term fungal edaphic specialisation or differences in fungal inoculum, because differences in ECM composition in our glasshouse experiment largely disappeared when seedlings were exposed to the entire fungal species pool or when they were grown under the same abiotic conditions, but exposed to different fungal inoculum. This is likely a result from fungal priority effects when plants have access to a different inoculum pool (Kennedy 2010) or due to an altered competitive balance among ECM fungal species due to soil disturbance (Baar et al. 1999; Teste et al. 2009). Results from our study suggest that long-term rather than short-term feedback between soil, plants and their root-associated ECM fungi plays a key role in structuring ECM fungal communities along this soil chronosequence.

SHIFTS IN ECTOMYCORRHIZAL COMMUNITY COMPOSITION

The importance of soil abiotic properties in driving changes in ECM fungal communities along a long-term retrogressive chronosequence is consistent with our first hypothesis and with other results from tropical rain forests (Peay et al. 2010) and boreal forests (Toljander et al. 2006). Indeed, ECM community composition differed strongly among chronosequence stages in both field-collected samples and in the field soil
treatment of the glasshouse experiment. Other studies have found that ECM fungal communities vary with soil age along other soil chronosequences (Clemmensen et al. 2015; Gao et al. 2015). Similar results were obtained for arbuscular mycorrhizas by Martínez-García et al. (2015) and Krüger et al. (2015). However, Davey et al. (2015) found that ECM fungal OTUs follow the same trajectory as its associated vegetation in two short-term glacier chronosequences in Norway. Similarly, composition of ECM fungal communities on seedlings strongly correlated with the composition of whole plant communities along the entire chronosequence in both the field and glasshouse experiment. Host and symbiont communities are presumably strongly interlinked, but this co-variation probably results from similar responses of plants and fungi to abiotic soil factors, rather than a direct effect of vegetation composition on fungal communities (Tedersoo et al. 2015).

Most ECM fungal lineages showed clear changes among chronosequence stage in the field-collected samples and the field soil treatment. For example, OTUs belonging to the /cenococcum lineage were present solely in the latest stages of the chronosequence, while the /tomentella-thelephora lineage was almost restricted to the youngest stage (absent in stage 4 and only one OTU in stage 5). This is surprising, given that Cenococcum is thought to be a ubiquitous generalist group (Dickie 2007). Nevertheless, in an alpine forest in southern Germany, Baier et al. (2006) found that Cenococcum prefers organic soil layers, while Tomentella prefers mineral soil horizons, and van der Heijden et al. (1999) found in the coastal dunes of the Wadden Isles that the ECM fungal genus Cenococcum does not occur in young calcareous soils. Additionally, Cenococcum is highly stress tolerant (Pigott 2006; Fernandez & Koide 2013), which may allow it to persist in these strongly P-impoverished-soils. On the other hand, previous studies showed that Thelephora species are indicators of young stages of ecosystem development (Jumpponen et al. 2002; Dickie et al. 2013; Davey et al. 2015). These
changes in lineages during ecosystem development may be related to the efficiency of acquiring different forms of N and P of each ECM fungal species, given that the composition of N and P forms vary strongly along the chronosequence (Turner & Laliberté 2015). The few discrepancies on the lineages between field-collected samples and glasshouse experiment might be because in the glasshouse we considered only two host plant species, to the low sequencing depth of the 454 sequencing method (field-collected samples; Supporting Information), as opposed to Illumina (glasshouse root samples).

A significant fraction of the variation in ECM fungal community with soil age was explained by soil abiotic factors, accounting for 27 and 33 % of the total variation in the field-collected samples and field soil treatment, respectively. Soil pH and total P concentration were the strongest drivers for the field-collected samples, while soil pH, organic P concentration and [Mn] were the strongest drivers in field soil treatment. These results support our first hypothesis that ECM fungal communities are partly driven by soil chemical and nutrient availability, and agree with previous studies showing that soil pH (van der Heijden et al. 1999) or P (Roy et al. 2013) can be important drivers of ECM fungal communities at the local scale. Other studies also showed that soil N is a key factor determining ECM fungal communities (Lilleskov et al. 2002; Cox et al. 2010; Sterkenburg et al. 2015). However, those studies have been carried out mostly in systems where plant productivity is limited by N availability, while P becomes the growth-limiting nutrient along the Jurien Bay chronosequence (Laliberté et al. 2012; Hayes et al. 2014).

When plants were exposed to the entire regional ECM fungal species pool, the differences in ECM fungal community composition among chronosequence stages found in the field soil treatment largely disappeared, likely as a result of short-term
colonising events (i.e. priority effects; (Kennedy & Bruns 2005; Dickie et al. 2012). Kennedy et al. (2009) experimentally showed that for four species of the ECM genus *Rhizopogon* colonising *Pinus muricata* the order of arrival (i.e. priority effect) of ECM fungi strongly impacts ECM fungal colonisation dynamics. Conversely, when soil abiotic differences were removed (i.e. specific inoculum treatment), the trend found in the field soil treatment was also not found. This suggests that changes in ECM fungal community composition during ecosystem development cannot be independently attributed to differences in soil abiotic properties or inoculum potential. Rather, we surmise that changes in ECM fungal communities reflect long-term ecosystem-level plant-soil-fungal feedback (Wardle et al. 2004a, 2008). However, we cannot discount the possibility that our experimental soil manipulations might have favoured certain ECM fungal species. For example, our use of 7% of inoculum by volume would have diluted the amount of inoculum which may have favoured fast-growing fungal species. Such potential biases are unavoidable in experimental manipulations to isolate effects of fungal inoculum, because too large of a volume would have confounded abiotic and biotic effects (Brinkman et al. 2010).

DECREASE IN ECTOMYCORRHIZAL FUNGAL DIVERSITY

Our second hypothesis that ECM fungal OTU richness would increase with increasing soil age was rejected despite increases in ECM host plant diversity (Zemunik et al. 2015). In fact, the opposite pattern was found in both field-collected samples and in the glasshouse experiment using unaltered field soils. Other studies have found that ECM fungal diversity increases with soil age (Davey et al. 2015), but these studies were conducted on postglacial soils with more available P, and where soils contain more organic matter. The decline in ECM OTU richness might be accounted for by strong
environmental filtering favouring ECM fungal species that are adapted to the extremely low soil P availability in older soils along the Jurien Bay dune chronosequence (Laliberté et al. 2012; Turner & Laliberté 2015).

CONCLUSIONS

We show how changes in soil properties during long-term pedogenesis influence ECM fungal community composition and richness, even when host identity is constant. Priority effect appears to drive ECM fungal community composition in the short term (Kennedy & Bruns 2005; Kennedy et al. 2009). However, our results suggest that over time, environmental filtering plays a stronger role, and long-term fungal edaphic specialisation becomes an important driver. Hence, we surmise that changes in ECM fungal communities during pedogenesis reflect a synergistic effect of edaphic specialisation and changes in fungal inoculum resulting from long-term feedback between soil, vegetation and ECM fungi. As such, our study advances recent field-based studies of mycorrhizal communities along long-term retrogressive chronosequences (Clemmensen et al. 2015; Martinez-Garcia et al. 2015) and addresses a recent call by Dickie et al. (2013) to use chronosequences as model systems to better understand plant-fungal-soil feedback throughout ecosystem development. Further studies are required to elucidate the functional roles that ECM fungal species play during long-term ecosystem development, particularly with regard to plant P nutrition in severely P-impoverished soils.
3.7. **Acknowledgements**

We thank James Taylor for technical assistance with DNA extraction and amplification. We also thank Dr. Ian Dickie for providing constructive comments on an earlier draft of our manuscript. Funding was provided by the Australian Research Council (ARC) through a DECRA (DE120100352), Discovery Project (DP130100016) and Hermon Slade grant to EL and a UWA Research and Development Award granted to FPT. We also acknowledge financial support to FEA through CONICYT BECASCHILE/DOCTORADO (72130286), the ANZ Holsworth Wildlife Research Endowment, and the University of Western Australia. We also thank the Pawsey centre for computing time on their high-performance computing infrastructure.

3.8. **References**


3.9. Supporting Information

Methods S1: Description of data collection for the field-collected samples

Site selection and sample collection

To first determine how ECM fungal communities varied in the field along the Jurien Bay chronosequence, we selected three contrasting chronosequence stages for this preliminary study that strongly differ in age and nutrient availability (Table S1). These stages correspond to stages 2 (< 7 ka), 4 (~ 120 ka), and 5 (~ 2 Ma) in Hayes et al. (2014). These stages also represent the retrogressive phase of ecosystem development in this system (Laliberté et al. 2012, Krüger et al. 2015). From each chronosequence stage, we selected five plots (10 × 10 m) from the same set of permanent plots used in earlier studies (Laliberté et al. 2014; Hayes et al. 2014; Turner & Laliberté 2015; Zemunik et al. 2015). In June, July, and August of 2012, within each of the 15 plots, we collected root and soil samples following the same approach used by Krüger et al. (2015).

Methods S2: Detailed description of DNA amplification, sequencing, bioinformatics and statistical functions and packages used.

FIELD-COLLECTED SAMPLES

DNA extraction

Extraction of DNA from pooled (June, July, August sampling) root fragments (diameter ≤ 2 mm) was conducted by first pulverising the tissues previously freeze-dried with a custom-built ball mill. After homogenising the bulk tissue mixture, 60 mg was used for DNA extraction by using a plant DNA extraction kit (REDExtract-N-Amp Plant PCR
Kit, Sigma-Aldrich) following the instructions of the manufacturer. For extraction of DNA from the pooled soil samples (June, July, August sampling) we mixed (10 g in total) and extracted with the PowerMax Soil DNA Isolation Kit (Mo-Bio Laboratories Inc., Carlsbad, USA) following the instructions of the manufacturer, with the exception of the fourth step, where samples were vortexed for 10 min, then placed in a water bath at 65 °C, and mixed every 3 min.

**DNA amplification**

Preliminary amplification of the extracted DNA with the polymerase chain reaction (PCR) was optimised with two sets of primer pairs, ITS1-F ITS4 and ITS5-ITS2 (Martin & Rygiewicz 2005). After optimisation, we used 1 μl of the extracted DNA and amplified the ITS region in a 20 μl reaction volume with primers ITS5-ITS2 (White et al. 1999). PCRs were carried out in an Eppendorf Mastercycler® ep (Eppendorf, Hamburg, Germany) under the following conditions: an initial denaturation step at 93 °C for 180 s, followed by 35 cycles with denaturation at 93 °C for 60 s, annealing at 53 °C for 55 s (35 cycles), and extension at 72 for 35 s (+5 s/cycle; 35 cycles); and a final extension step at 72 °C for 10 min. Due to PCR inhibition, DNA extracts were diluted into 1:10. The PCR amplicons were visualised with gel electrophoresis using a 2% (w/v) agarose gel and ethidium bromide for optimisation purposes. Then final PCR’s were conducted with the ITS5 and ITS2 primers with 32 different MID adaptors and also including 5 additional cycles during PCR (40 cycles in total). Amplicons generated from this final PCR, were cleaned twice with the Agencourt AMPure XP beads (Beckman Coulter Genomics, MA, USA). The cleaned PCR amplicons were visualised again on a 2% (w/v) agarose gel for estimation of DNA concentration for the subsequent equimolar pooling in emulsion PCR. The bead:template ratio for 454
sequencing was determined by quantitative PCR against standards of known molarity (Bunce et al. 2012).

454 pyrosequencing

We conducted 454 pyrosequencing with the Roche GS Junior system (Branford, CT, USA) using Lib-A chemistry. Fusion-tagged amplicon generation was conducted in triplicates for each root and soil sample. Pre-processing of the resulting 454 pyrosequence reads was done as in Coghlan et al. (2012) and involved looking for exact MID-tags and primer sequences. Sequencing was performed at the State Agricultural Biotechnology Centre at Murdoch University, Australia.

GLASSHOUSE EXPERIMENT

DNA extraction and amplification

Forty grams of root material was pulverised in 2 ml mini-lyse tubes to disrupt the cell walls immediately prior to DNA extraction. DNA was extracted using Qiagen’s DNeasy Plant Mini Kit with a modified protocol (56 °C overnight digestion). Pulverisation and extraction were conducted under clean conditions and several negative controls were used in order to assess possible environmental contamination.

DNA extracts were quantified via real-time quantitative polymerase chain reaction (qPCR) to assess for quality and quantity of genomic DNA (gDNA), and to assess potential PCR inhibition. Each gDNA extract was diluted into 1/10 using the primers ITS3-KYO2/ITS4 (Toju et al. 2012). From the qPCR results, an optimal DNA concentration (maximising amplifiable input template) was selected for DNA sequencing which was free of inhibition and yielded DNA of sufficient quality.
High-throughput DNA sequencing

The ITS region was amplified and sequenced (in a single PCR) on an Illumina MiSeq system utilising ITS3-KYO2/ITS4 fusion primers (Toju et al. 2012) modified with a unique 8bp Multiplex Identifier tag (MID-tag) and custom adaptors for paired-end sequencing on both primers.

Independent MID-tagged qPCR setup for samples and controls were prepared in a physically separate laboratory and were carried out using each primer set in 25 µl reactions containing 1X PCR Gold Buffer, 2.5 mM MgCl2, 0.4 mg ml-1 BSA, 0.25 mM of each dNTP, 0.4 µM of forward and reverse MID-tag primer, 0.25 µl AmpliTaq Gold, 0.6 µl SYBR Green and 2 µl of gDNA. The cycling conditions for qPCR were as follows: initial heat denaturation at 95°C for 5mins, followed by 40 cycles of 95 °C for 30 s; 50°C for 30 s; 72°C for 45 s followed by final extension at 72°C for 10 min. Multiplex Identifier-tagged PCR amplicons were generated in duplicate for each sample and pooled to minimise the effects of PCR stochasticity. The pooled amplicons were purified using Agencourt AMPure XP PCR Purification Kit (Beckman Coulter Genomics, NSW, Australia) following the manufacturer’s protocol. Purified amplicons were electrophoresed on 2% agarose (w/v) gel and pooled in approximately equimolar ratios based on ethidium-stained band intensity to form a MID-tagged DNA sequencing library. For each MID-tagged qPCR assay, extraction and PCR controls were included, but these did not contained amplifiable DNA, and hence were not incorporated into the pooled MID-tagged DNA sequencing library. Illumina MiSeq sequencing was performed using a MiSeq Reagent Kit v3 (600 cycles) 300 bp paired-end protocol as per manufacturer’s instructions (Illumina, Inc., San Diego, CA, USA) at the TrEnD Laboratory, Curtin University, Australia.
Deconvolution of amplicon sequences into sample batches followed the method described in Murray et al. (2015), using Geneious v8.0.2 (Drummond et al. 2011). Sequences from both field and glasshouse were labelled with the sample from which they came, and all sequences were combined into a single file for processing using USEARCH v6 (Edgar 2010, 2013). Sequences were dereplicated at 100% identity across their full length and singleton sequences were identified. Dereplicated sequences were clustered at a 97% identity threshold using the UPARSE (Edgar 2013) algorithm implemented in USEARCH (Edgar 2010) to identify operational taxonomic units (OTUs). At that stage, chimeric sequences were also identified. OTUs were mapped against the original amplicon sequence fasta file from which sequences identified as singletons and/or chimerics were removed. Additionally, one sample (stage 4 – pooled inoculum treatment; plot S.W.6) was removed from the study as the OTU accumulation curve indicated insufficient sequencing depth for that sample (less than 2000 sequences; Fig. S3.3-5.). An OTU table was subsequently created using USEARCH identifying the presence or absence of each OTU in each sample. Following OTU identification, representative sequences from each OTU were queried against UNITE, UNITE Envir. or annotated INSD datasets using Mega BLAST (Altschul et al. 1990) using MassBLASTer with default parameters (Abarenkov et al. 2010) and visualised in MEta Genome ANalyzer v4 (minScore = 50.0 topPercent = 10.0 minSupport = 1) (Huson et al. 2007). Operational taxonomic units with less than 85% similarity to best match and 90% query were removed from the study. Ectomycorrhizal fungal lineages were assigned according to Tedersoo & Smith (2013).
STATISTICAL ANALYSES

Ectomycorrhizal community composition

To visualise differences in composition of ECM fungal communities among chronosequence stages, we calculated the Jaccard dissimilarity among samples using the function metaMDS() in the “vegan” R package (Oksanen et al. 2015) and used non-metric multidimensional scaling (NMDS). To test for statistical differences in ECM fungal community composition among stages, we used permutational multivariate analysis of variance (PERMANOVA), using 9999 permutations for statistical tests using the function adonis() in the “vegan” R package (Oksanen et al. 2015). When PERMANOVA showed a significant effect of chronosequence stage on ECM fungal community composition, we performed pairwise comparisons between each pair of chronosequence stages and adjusted P-values for multiple comparisons using the function p.adjust() with the “holm” procedure (Holm 1979). To explore if shifts in plant composition were related to shifts in ECM fungal community composition, we performed a Procrustes test between vegetation and ECM fungal OTU matrices with the function protest() in the “vegan” R package (Oksanen et al. 2015).

To evaluate if changes in ECM fungal community composition were related to other factors (i.e. abiotic soil properties and ECM plant diversity and cover), we performed a canonical correspondence analysis (CCA) to test if community composition differences were related to soil properties or vegetation by using the function cca() in the “vegan” R package (Oksanen et al. 2015). Soil property data from all of our sites were obtained from Turner & Laliberté (2015), and plant species richness and cover were obtained from Zemunik et al. (2015). Relevant explanatory environmental variables were selected by permutation tests with forward selection, using 9999 permutations using the function ordistep() in the “vegan” R package (Oksanen et al. 2015). All variables used
in this study are: total organic P, organic fraction of P, pH, Mn, total N, total P, resin P, Cu, Fe, Zn, Mg, K, organic C, total plant richness, total plant cover, *A. rostellifera* cover, *M. systena* cover, and ECM plant richness, cover and relative cover.

**Ectomycorrhizal fungal richness**

Rarefied ECM OTU richness was obtained by calculating the number of ECM OTUs based on the minimum number of sequences obtained across all samples for a given treatment using the rarefy() function in the “vegan” R package (Oksanen et al. 2015). Rarefied ECM OTU richness and its relationship with soil properties among chronosequence stages were tested using generalised least squares models with the function gls() from the “nlme” package (Pinheiro et al. 2012). Residuals were inspected visually to check model assumptions. When models did not meet assumptions (i.e. residuals centred around zero and homoscedasticity), appropriate variance structures were specified in a second model, and both models were compared using the Akaike Information Criterion (AIC) and likelihood ratio tests (Zuur et al. 2009). When a main term was significant, post hoc Tukey tests were performed using the function glht() from the “multcomp” package (Hothorn et al. 2008). In models involving soil properties as explanatory variables, nlme() was used and chronosequence stage was treated as a random effect (Pinheiro et al. 2012).

Finally, to test the robustness of our models, we calculated richness for all fungal OTUs found in the study to ensure that our results were not purely driven by an overall trend in fungal OTU richness across the chronosequence (Fig. S3.1a-c). Furthermore, since south-western Australian ECM fungi are poorly studied, we also calculated ECM-OTU richness using a more relaxed criterion (i.e. classifying an OTU as ectomycorrhizal if it belonged to an ECM fungal family, or if the reference sequence was isolated from an ectomycorrhizal fungus; Fig. S3.1b-d). Since both models showed the same trend, we
only show results using the more conservative 85% similarity with best match. All statistical analyses were conducted in R (R Core Team 2014).

**Figure S3.1** Comparison of OTU richness and rarefied OTU richness among chronosequence stages between all fungal and only ECM OTUs. Comparison of OTU richness (a-b) and rarefied OTU richness (c-d) associated with *Acacia rostellifera* and *Melaleuca systena* among chronosequence stages between all fungal OTUs (a, c) and Only ECM-OTUs (b, d) from plants grown in field soil. Values are means (points) with 95% confidence intervals (bars). Different letters indicate significant ($P \leq 0.05$) differences among soil ages based on *post hoc* Tukey tests.

**Results S1: Field-collected samples**

We found 61 unique ECM OTUs in soils and roots collected from three contrasting chronosequence stages (i.e. stages 2, 4 and 5). These OTUs represented 14 ECM lineages. The proportion of OTUs in the different lineages varied among
chronosequence stages and the number of lineages increased with soil age (Fig. S3.2a). In stage 2, *terfezia-peziza* depressa was the most common lineage, but it became less common in the older stages. Similar patterns were observed for the two lineages *sebacina* and *tomentella-telephora* (Fig. S3.2a), but *boletus* and *inocybe* were found only in the two oldest stages, while *pisolithus-scleroderma* was most frequent in stage 4.

In field-collected soils and roots, ECM fungal community composition strongly co-varied with plant community composition across the chronosequence (Procrustes correlation = 0.79; $P \leq 0.0001$). Furthermore, ECM fungal communities differed in composition among all chronosequence stages (Fig. S3.2b; $P \leq 0.001$, after Holm correction for pairwise comparisons). These changes in composition were explained by pedogenesis-driven shifts in abiotic soil properties: pH and total P concentration (Fig. S3.2c; $R^2_{adj} = 0.27$, $P \leq 0.001$). Neither vegetation nor other soil properties were selected as explanatory variables.

Rarefied ECM OTU richness in the field-collected samples was lower in stage 5 compared with stage 4 (Fig. 3.2d; $P = 0.03$), but the same as stage 2 ($P = 0.15$). Furthermore, rarefied ECM OTU richness was strongly associated with a decline in soil concentration of total P, resin P (plant-available P) and pH, and with an increase of organic P (Table S3.2).
Figure S3.2 Summary of results from field-collected samples. (a) Proportion of ECM OTUs of all lineages found in the field-collected samples. Lineages with less than three OTUs per chronosequence stage were pooled in “others”. Each chronosequence stage is represented by five sites. (b) Non-metric multidimensional scaling ordination (NMDS) plots showing differences in ECM fungal community composition among three contrasting chronosequence stages. Ellipses show the 95% confidence intervals around the mean centroid for each chronosequence stage based on Jaccard’s dissimilarity scores. Black arrow links centroids and shows the chronological sequence of the chronosequence stages. (c) Canonical correspondence analysis (CCA) between ECM OTUs community composition and soil properties among the different chronosequence stages. Relevant explanatory environmental variables ($P \leq 0.05$) were selected by permutation tests with forward selection using 9999 permutations. Total phosphorus (P) and soil pH were the only variables selected and explained 29% of the total variation. (d) Rarefied ECM OTU richness soils from three chronosequence stages. Values are means (points) with 95% confidence intervals (bars). Different letters indicate significant ($P \leq 0.05$) differences among soil ages based on post hoc Tukey tests.
Figure S3.3 Accumulation curve of operational taxonomic units (OTUs) against number of sequences for the field soil samples of the glasshouse experiment. All sites reached a plateau.

Figure S3.4 Accumulation curve of operational taxonomic units (OTUs) against number of sequences for the pooled inocula samples of the glasshouse experiment. All sites reached a plateau.
**Figure S3.5** Accumulation curve of operational taxonomic units (OTUs) against number of sequences for the specific inocula samples of the glasshouse experiment. All sites reached a plateau.

**Table S3.1** Pair-wise comparisons among chronosequence stages for each individual ECM lineage in the field soil treatment. Star symbol shows statistical differences ($P \leq 0.01$).

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Stage pair-wise combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-2</td>
</tr>
<tr>
<td>/cenococcum</td>
<td></td>
</tr>
<tr>
<td>/tuber-helvella</td>
<td></td>
</tr>
<tr>
<td>/marcellina-peziza gerardii</td>
<td></td>
</tr>
<tr>
<td>/pulvinula</td>
<td></td>
</tr>
<tr>
<td>/terfezia-peziza depressa</td>
<td></td>
</tr>
<tr>
<td>/tomentella-thelephora</td>
<td></td>
</tr>
</tbody>
</table>
**Table S3.2** Multivariate relationship between soil properties and ECM OTU richness for the field-collected samples. P-values show significance effect of particular variables.

<table>
<thead>
<tr>
<th>Soil property</th>
<th>DF</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>1</td>
<td>28.2</td>
<td>0.02*</td>
</tr>
<tr>
<td>total P</td>
<td>1</td>
<td>-26.9</td>
<td>0.02*</td>
</tr>
<tr>
<td>resin P</td>
<td>1</td>
<td>-38.3</td>
<td>0.01*</td>
</tr>
<tr>
<td>organic C</td>
<td>1</td>
<td>-7.5</td>
<td>0.08</td>
</tr>
<tr>
<td>organic P</td>
<td>1</td>
<td>14.5</td>
<td>0.04*</td>
</tr>
<tr>
<td>% organic P</td>
<td>1</td>
<td>12.9</td>
<td>0.04*</td>
</tr>
<tr>
<td>Cu</td>
<td>1</td>
<td>-22.9</td>
<td>0.03*</td>
</tr>
<tr>
<td>Fe</td>
<td>1</td>
<td>-6.5</td>
<td>0.09</td>
</tr>
<tr>
<td>Mn</td>
<td>1</td>
<td>-3.9</td>
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</tr>
<tr>
<td>Zn</td>
<td>1</td>
<td>28.8</td>
<td>0.2*</td>
</tr>
<tr>
<td>total N</td>
<td>1</td>
<td>0.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Table S3.3** Multivariate relationship between soil properties and ECM OTU richness for the field soil treatment of the glasshouse experiment. P-values show significance effect of particular variables.

<table>
<thead>
<tr>
<th>Soil property</th>
<th>DF</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8</td>
<td>3.8</td>
<td>0.005*</td>
</tr>
<tr>
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<tr>
<td>resin P</td>
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</tr>
<tr>
<td>organic P</td>
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<td>0.005*</td>
</tr>
<tr>
<td>% organic P</td>
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<td>0.0001*</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>0.0001*</td>
</tr>
<tr>
<td>Zn</td>
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</tr>
<tr>
<td>total N</td>
<td>8</td>
<td>3.4</td>
<td>0.01*</td>
</tr>
</tbody>
</table>
4. Native soil-borne pathogens equalise differences in competitive ability between plants of contrasting nutrient-acquisition strategies

4.1. Preface

In chapters 2 and 3, I found that ECM root colonisation, community composition and richness strongly differed in the oldest stages of the chronosequence compared to the younger ones. Furthermore, a previous study has shown that plant diversity is highest in the oldest stage of the Jurien Bay chronosequence (Zemunik et al. 2015). On the other hand, Laliberte et al. (2015) proposed that a trade-off between P-acquisition efficiency and pathogen defence could promote high plant diversity. Hence, this chapter aim to evaluate the protective role of ECM fungi against native soil-borne pathogens and its implications for plant coexistence. This chapter is currently under review with *Journal of Ecology*.

4.2. Summary

Soil-borne pathogens can contribute to the maintenance of local plant diversity by reducing differences in competitive ability between co-occurring plant species. It has been hypothesised that efficient phosphorus (P) acquisition by plants in P-impoverished ecosystems might trade-off against resistance to root pathogens. This could help explain
high plant diversity in severely nutrient-impoverished ecosystems. However, empirical
evidence of such a trade-off remains scarce. In hyperdiverse shrublands in south-
western Australia, non-mycorrhizal cluster-rooted Proteaceae are very efficient at
acquiring P. However, Proteaceae co-occur with many other plant species using other P-
acquisition strategies, such as ectomycorrhizal (ECM) associations. In a glasshouse
experiment, we grew Proteaceae and ECM plant species from hyperdiverse shrublands
alone and in competition with each other, and in the presence or absence of native soil-
borne pathogens (*Phytophthora* spp.). We hypothesised that native *Phytophthora*
species are more detrimental to Proteaceae than co-occurring ECM plants, due to a
trade-off between highly efficient P acquisition and pathogen defence, and that this
equalises differences in competitive ability between these two plant groups. When
seedlings were grown alone, biomass of non-mycorrhizal plants was reduced in the
presence of *Phytophthora*, while ECM species were unaffected by this pathogen. When
non-mycorrhizal and ECM species were planted together, ECM plants grew better in the
presence of *Phytophthora* than in its absence, because *Phytophthora* reduced the growth
of the non-mycorrhizal competitors. Growth of ECM plants was positively correlated
with percent root colonisation by ECM fungi, but this was only significant when ECM
plants were grown in the presence of *Phytophthora*. Our study shows that native soil-
borne pathogens equalised differences in competitive ability between plants of
contrasting nutrient-acquisition strategies, thus supporting the hypothesis proposing a
trade-off between highly efficient P acquisition and resistance against root pathogens.
We found that non-mycorrhizal cluster-rooted species may be the most efficient at
acquiring the growth-limiting resource, but that co-occurring ECM species are better
defended against root pathogens. Our results suggest that native soil-borne pathogens
and ECM contribute to the maintenance of the plant hyperdiversity in severely P-
impoverished ecosystems.
4.3. Introduction

Most plant pathogens have detrimental impacts on both natural and managed ecosystems, threatening plant biodiversity and productivity in many biomes across the globe (Fisher et al., 2012). In many cases, the pathogens causing a decline in plant diversity have been introduced from other regions (Anagnostakis, 1987; Brown & Hovmøller, 2002). These introduced pathogens can cause significant damage to plants that have not evolved specific defences against those introduced pathogens (Cahill et al., 2008). By contrast, very little is known about the ecological role of native soil-borne pathogens that have co-evolved with plant species in a given region, although a potential role of pathogens to the maintenance of local plant species diversity is receiving increasing attention in recent years (Gilbert, 2002; Bagchi et al., 2010b; Laliberté et al., 2015).

Plant pathogens can contribute to plant species coexistence and thus promote local plant diversity through different mechanisms (Mills & Bever, 1998; Gilbert, 2002; Mordecai, 2011; Laliberté et al., 2015). For example, this can occur through conspecific negative density dependence (Wurst et al., 2015), or by reducing differences in competitive ability between co-occurring plant species (Terborgh, 2012). Negative density dependence (i.e. Janzen-Connell effect) occurs when an increasing density of conspecific individuals leads to the local accumulation of host-specific pathogens, reducing conspecific seedling survival and growth (Janzen, 1970; Connell, 1971; Freckleton & Lewis, 2006). On the other hand, pathogens with low host specificity can still promote plant species diversity by being more detrimental to (or building up larger populations around) plant species showing higher competitive ability, thus enabling less competitive plant species to persist (Bell, Freckleton & Lewis, 2006; Bagchi, Press & Scholes, 2010a). These different effects of pathogens are not mutually exclusive and can
both contribute to the maintenance of local plant diversity (Gilbert, 2002). Determining the ecological role of soil-borne pathogens for plant species coexistence should help us understand how highly-diverse plant communities are maintained (Laliberté et al., 2015).

Highly-diverse plant communities such as tropical rainforests and Mediterranean shrublands often occur on old, strongly weathered, very infertile soils that are particularly low in phosphorus (P) (Huston, 1994; Laliberté et al., 2013). Some of these plant communities exhibit a wide range of nutrient-acquisition strategies (Lambers et al., 2014; Zemunik et al., 2015). Laliberté et al., (2015) surmised that low soil P availability contributes to plant coexistence in these hyperdiverse communities, and that this might be related to a trade-off between P-acquisition efficiency and root defences against pathogens. Indeed, roots that are highly efficient at acquiring P tend to be short-lived, poorly lignified, with a thin epidermis, thus making them more susceptible to root pathogens (Newsham, Fitter & Watkinson, 1995). Evidence of pathogens contributing to plant species coexistence exists for tropical rainforests (Freckleton & Lewis, 2006; Terborgh, 2012), which are renowned for their high plant diversity. For example, Bagchi et al., (2014) experimentally showed that applying fungicides reduced tree seedling diversity in a tropical forest in Belize, pointing to a role of fungal pathogens in maintaining plant diversity. However, to our knowledge, the ecological role of native pathogens on plant interactions and diversity in other highly-diverse ecosystems such as seasonally-dry shrublands has not yet been studied.

Soils in the kwongkan shrublands in south-western Australia are old, strongly-weathered and severely nutrient-impoverished, especially with respect to P (Laliberté et al., 2015; Viscarra Rossel & Bui, 2015). Plant communities in kwongkan are renowned for high plant diversity with contrasting nutrient-acquisition strategies, such as different
mycorrhizal associations and non-mycorrhizal strategies such as cluster roots (Lamont, Hopkins & Hnatiuk, 1984; Zemunik et al., 2015). In particular, non-mycorrhizal, cluster-rooted Proteaceae are particularly successful in these habitats (Lambers et al., 2006; Zemunik et al., 2015), because this nutrient-acquisition strategy is highly effective at acquiring different forms of P (Lambers et al., 2006, 2012). On the other hand, cluster roots are fine short-lived roots (Shane et al., 2004; Lambers et al., 2006), and thought to be highly susceptible to soil-borne pathogens as a trade-off of high efficiency in P-acquisition (Laliberté et al., 2015).

Although cluster roots might be the most efficient strategy at acquiring the growth-limiting soil nutrient, P, in these shrublands (Lambers, Martinoia & Renton, 2015), many other plant species with contrasting P-acquisition strategies coexist with Proteaceae in hyperdiverse, P-impoverished south-western Australian shrublands (Laliberté et al., 2014; Zemunik et al., 2015). In particular, associations with ectomycorrhizal (ECM) fungi are another common nutrient-acquisition strategy, but considered to be less efficient than cluster roots at acquiring different forms of P in P-impoverished soils (Lambers et al., 2008b). However, ectomycorrhizal fungi contribute not only to plant nutrient acquisition (Smith, Anderson & Smith, 2015), but also confer physical and chemical defences against root pathogens (Marx, 1972; Strobel & Sinclair, 1991). Therefore, it is possible that soil-borne pathogens might promote the coexistence of non-mycorrhizal and mycorrhizal plants in P-impoverished soils, because of a trade-off between efficient P acquisition and defence against pathogens; however, to our knowledge this has never been tested experimentally.

Oomycetes are considered to be ecologically important soil-borne pathogens in hyperdiverse south-western Australian shrublands (Laliberté et al., 2015). On one hand, the invasive oomycete Phytophthora cinnamomi Rands has caused devastating damage
to native flora in Australia since it was introduced in the early 1900s (Cahill et al., 2008). For example, in the south-west botanical province approximately 40% of the native flora is susceptible (Shearer, Crane & Cochrane, 2004). On the other hand, south-western Australia also harbours several native species of Phytophthora (Rea et al., 2011; Simamora et al., 2013), whose ecological roles are unknown. In the present study we evaluated how native Phytophthora species could affect the outcome of interactions between ECM Myrtaceae and non-mycorrhizal Proteaceae in hyperdiverse shrublands by reducing differences in competitive ability among these co-occurring species. Specifically, we aimed to test the following hypotheses: i) non-mycorrhizal Proteaceae are more severely negatively affected by the presence of different native Phytophthora strains than ECM species; ii) the presence of native Phytophthora will reduce the competitive superiority of non-mycorrhizal Proteaceae over ECM species; and iii) higher ECM root colonisation will offer greater protection against Phytophthora, and hence increase growth of ECM plants.

4.4. Materials and Methods

Study area and site selection

Our study focused on the oldest chronosequence stage of the Jurien Bay dune chronosequence, located in south-western Australia (30.29° S, 115.04° E), because soils from this oldest chronosequence stage are severely P-impoverished and host the highest plant species and functional diversity (Laliberté et al., 2014; Turner & Laliberté, 2015; Zemunik et al., 2015). The Jurien Bay dune chronosequence spans over two million years of pedogenesis over approximately 10 km and has been described in detail elsewhere (Laliberté et al., 2012, 2013, 2014; Hayes et al. 2014; Turner & Laliberté,
2015; Zemunik et al., 2015). This chronosequence is located in a global biodiversity hotspot (Myers et al., 2000). A detailed description of soil properties along the entire chronosequence can be found in Laliberté et al., (2012) and Turner & Laliberté (2015).

**Soil collection**

Using a network of permanent 10 m × 10 m plots from earlier studies(Laliberté et al., 2014; Hayes et al., 2014; Turner & Laliberté, 2015; Zemunik et al., 2015), we collected soils from five plots in the oldest stage (stage 6 in Turner & Laliberté, 2015) that were at least 1 km apart. We collected ~10 kg bulk soil from each plot. Soils were collected from the top 30-cm layer at three randomly-positioned points within each plot. Soils were air-dried, mixed and sieved through a 2-mm sieve. Then, bulk soil from all plots was sterilised via triple-steam pasteurisation at 80° C for 2 hours per day over seven days, following previous studies (Fang, You & Barbetti, 2012; Ryan et al., 2012).

**Species selection**

We selected six plant species for our experiment: three non-mycorrhizal Proteaceae that form cluster roots (*Banksia attenuate* R. Br., *B. menziesii* R. Br. and *Hakea ruscifolia* Labill.), and three ECM species from the Myrtaceae (*Calothamnus quadrifidus* R. Br., *Eucalyptus todtiana* F.Muell. and *Eremaea asterocarpa* Hnatiuk). Myrtaceae species are solely ECM with the exception of *E. asterocarpa* which can also form arbuscular mycorrhizal association (Zemunik et al., 2015). Seedlings were germinated in triple-steam pasteurised soil and one-month old seedlings were transferred into 1 L pots. At time of planting, small plastic tubes (2 cm diameter, 10 cm long) were inserted next to seedlings to leave space to insert Phytophthora inoculum to allow for infestation of the rhizosphere of growing plants.
Inoculum with ectomycorrhizal fungi

To ensure ECM species would be colonised by ECM fungi, 20 individuals of each ECM species were germinated and grown in non-sterile soils collected from the field for four months. Then, we harvested their roots, cut and mixed them, and used these roots as ECM fungal inoculum. We visually assessed for lesions and damping-off symptoms on roots. Despite the fact that this inoculum containing ECM fungi likely also contained other microorganisms, no traces of damage by pathogens were observed in these seedlings and their roots. We added 50 mg of inoculum with ECM fungi under each seedling (ECM and Proteaceae) during transplantation into triple-pasteurised soils for all Phytophthora inoculation treatments.

Phytophthora inoculum preparation

We selected five strains of Phytophthora representing different native species isolated from kwongkan vegetation (Simamora et al., 2013); Phytophthora arenaria (CBS 127950) and four less common species isolated from kwongkan vegetation during routine surveys, P. taxon cooljarloo (CLJO100), P. taxon kwongan (TCH009), P. aff. rosacearum (HSA2350) and P. rosacearum (HSA1658). Inocula were produced as described in Aghighi et al., (2015). Briefly, a sterile medium made of vermiculite (with 0.1% of millet seed) wetted with V8 juice was inoculated with actively growing mycelium and left for 8–12 weeks at 20ºC for the mycelium to fully colonise the medium.

Experiment 1

Colonised media of strains of Phytophthora, except P. arenaria, were pooled in equal quantities (w/w). After seedlings were transplanted and grown for two weeks in 1 L pots with soils with inoculum with ECM fungi, we added 5 g of (0.4% of total soil weight)
each of the following treatments: i) “– Phytophthora” (double autoclaved inocula), ii) “+ Phytophthora” (mix of P. taxon cooljarloo, P. taxon kwongan, P. aff. rosacearum and P. rosacearum), or iii) “+ P. arenaria”. We used a sample size of 10 seedlings for this experiment. Three days later, pots were watered to field capacity, and then twice weekly to 70% of field capacity. Seedlings were grown in a glasshouse for four months and then harvested to avoid root growth becoming pot-bound.

Experiment 2

Given that P. arenaria did not show to be more detrimental to plant growth than the other Phytophthora strains in Experiment 1, in this experiment all strains were pooled. Furthermore, E. asterocarpa and H. ruscifolia were not used in this experiment, due to poor germination. One individual of either ECM species (C. quadrifidus or E. todtiana) was potted in a 2.7 L pot together with one seedling of B. menziesii and one seedling of B. attenuata for a total of three seedlings per pot. This was done in order to maximise the interaction between Proteaceae and ECM plant species. Each ECM plant was planted with 50 mg of inoculum with ECM fungi as described above. After seedlings were transplanted and grown for two weeks in sterile soils with inoculum with ECM fungi, we inoculated each pot with 5 g of either: i) “– Phytophthora” (double autoclaved inocula), or ii) “+ Phytophthora” (mix of P. arenaria, P. taxon cooljarloo, P. taxon kwongan, P. aff. rosacearum and P. rosacearum). We used a sample size of nine seedlings for this experiment. Three days later, pots were watered to field capacity and then twice weekly to 70% to field capacity. Seedlings were grown for four months in the glasshouse and then harvested as per experiment 1.

Post-harvest analyses

After four months of growth in the glasshouse, seedlings were harvested by severing shoots from roots. Roots were carefully washed over a 1-mm sieve immediately after
harvesting to remove soil particles. Shoots and roots were oven-dried for three days at 60 ºC and weighed separately. Later, roots were re-hydrated in water at 5ºC for 48 hours, and cleared using potassium hydroxide (10%, w/v) for three hours at 90 ºC in a water bath. Following clearing, we used a 5% (v/v) ink-vinegar solution to stain roots (Vierheilig et al., 1998). Finally, roots were placed in a 50% (v/v) lactoglycerol mixture for storage.

Root colonisation by ECM fungi was determined using the gridline intersect method (Giovannetti & Mosse, 1980) at 200 × magnification, counting root tips with an ECM mantle and/or Hartig net when the mantle was not conspicuous. At least 150 total root tips were counted for each sample.

Statistical analyses

All analyses were conducted and figures were drawn in R (R Core Team, 2015). Statistical differences in biomass between species and inoculum treatment were tested using linear mixed-effect models with the function gls() in experiment 1 and lme() in experiment 2 (with “pot” as random effect) from the “nlme” package (Pinheiro et al., 2012). Residuals were inspected visually to check model assumptions. When a given model did not meet model assumptions (i.e. residuals centred on zero and homoscedasticity), a revised model with an appropriate variance structure was used (Supporting Information). The quality of the new model was evaluated using the Akaike Information Criterion (AIC) and likelihood ratio tests (Zuur et al., 2009). When a main term was significant, post hoc Tukey tests were performed using the function glht() from the “multcomp” package (Hothorn, Bretz & Westfall, 2008). Relationship between ECM root colonisation and seedling biomass was calculated by fitting linear regression models using lm().
4.5. Results

Experiment 1

All seedlings survived during this experiment. The two + Phytophthora treatments led to lower seedling biomass in Proteaceae species, compared with those grown in the – Phytophthora treatment; by contrast, the biomass of ECM plant species was unaffected by the presence of Phytophthora (species × Phytophthora treatment interaction; F_{2,10} = 9.99; P ≤ 0.0001; Fig. 4.1). Indeed, biomass for all three Proteaceae species was reduced by 20 to 40 % when plants exposed to Phytophthora were compared with plants grown in the – Phytophthora treatment (P ≤ 0.02; Fig. 4.1). On the other hand, biomass of ECM species was not observed to be affected by either of the + Phytophthora treatments (P ≥ 0.08; Fig. 4.1). No differences were found between + Phytophthora and the + P. arenaria treatments for ECM or Proteaceae species (P ≥ 0.65; Fig. 4.1).

Root to shoot ratio differed significantly between treatments, but this varied among species (species × Phytophthora treatment interaction; F_{2,10} = 3.32; P ≤ 0.0001; Fig. 4.2). For all three Proteaceae, root:shoot ratio was 40 to 50% lower in the two + Phytophthora treatment compared with that in the – Phytophthora treatment (P ≤ 0.01; Fig. 4.2). However, no differences were observed between the two + Phytophthora treatments (P ≥ 0.7; Fig. 4.2). For ECM species, E. asterocarpa showed a lower root:shoot ratio in both + Phytophthora treatments compared with that in the – Phytophthora treatment (P ≤ 0.01; Fig. 4.2); while the root:shoot ratio of C. quadrifidus was only lower when exposed to P. arenaria compared with that in the – Phytophthora treatment (P ≤ 0.05). There was no evidence that the root:shoot ratio of E. todtiana was affected by Phytophthora treatments (Fig. 4.2).
Figure 4.1 Final biomass of non-mycorrhizal cluster-rooted (top row; *Banskia attenuata*, *B. menziesii* and *Hakea ruscifolia*) and ectomycorrhizal plant species (bottom row; *Calothamnus quadrifidus*, *Eremaea asterocarpa* and *Eucalyptus todtiana*) grown under three inoculum treatments: i) – *Phytophthora*, ii) “+ *Phytophthora*” (mix of *P. taxon cooljarloo*, *P. taxon kwongan*, *P. aff. rosacearum* and *P. rosacerarum*), and ii) + *P. arenaria*. Means and 95% confidence intervals (CI) are shown.

Figure 4.2 Root to shoot ratio of non-mycorrhizal cluster-rooted (top row; *Banskia attenuata*, *B. menziesii* and *Hakea ruscifolia*) and ectomycorrhizal plant species (bottom row; *Calothamnus quadrifidus*, *Eremaea asterocarpa* and *Eucalyptus todtiana*) grown under three inoculum treatments: i) – *Phytophthora*, ii) “+ *Phytophthora*” (mix of *P. taxon cooljarloo*, *P. taxon kwongan*, *P. aff. rosacearum* and *P. rosacerarum*), and ii) + *P. arenaria*. Means and 95% confidence intervals (CI) are shown.
We found a positive relationship between total ECM seedling biomass and ECM root colonisation in both + *Phytophthora* treatments, although not for the – *Phytophthora* treatment (Fig. 4.3). Indeed, this relationship was significant for both + *Phytophthora* treatments for *C. quadrifidus* ($P \leq 0.05$; Fig. 4.3), *E. todtiana* ($P \leq 0.01$; Fig. 3) and *E. asterocarpa* ($P \leq 0.01$; Fig. 4.3), while it was not significant in the – *Phytophthora* soil for any of these three species ($P \geq 0.18$; Fig. 4.3).

**Figure 4.3** Relationships between ectomycorrhizal root colonisation and final seedling biomass in three ectomycorrhizal plant species (*Calothamnus quadrifidus*, *Eremaea asterocarpa* and *Eucalyptus todtiana*). Seedlings were grown with three inoculum treatments: i) – *Phytophthora* (red circles and dashed line), ii) “+ *Phytophthora*” (green triangles and solid line; mix of *P. taxon cooljarloo*, *P. taxon kwongan*, *P. aff. rosacerarum* and *P. rosacerarum*), and ii) + *P. arenaria* (blue squares and solid line). Relationships between ECM colonisation and biomass were only significant for + *Phytophthora* and + *P. arenaria* treatments for all ECM plant species. For *C. quadrifidus*, $R^2$ was 0.41 and 0.53, respectively; for *E. todtiana*, $R^2$ was 0.64 and 0.56, respectively; for *E. asterocarpa*, $R^2$ was 0.7 and 0.97, respectively. Solid lines indicate significant relationship ($P \leq 0.05$), while dashed line indicates non-significant relationship ($P \geq 0.05$).
Experiment 2

The effect of *Phytophthora* inoculum treatment on final biomass varied among plant species (species × *Phytophthora* treatment interaction, $F_{1,3} = 17.58; P \leq 0.0001$; Fig. 4.4). Final biomass of both ECM species competing with Proteaceae was greater in the + *Phytophthora* treatment, compared with that in the – *Phytophthora* treatment ($P \leq 0.01$; Fig. 4.4). Conversely, biomass of *B. menziesii* was less in the + *Phytophthora* treatment compared with that in the – *Phytophthora* treatment ($P \leq 0.05$; Fig. 4.4); that of *B. attenuata* was not observed to differ among treatments ($P = 0.2$; Fig. 4.4).

Figure 4.4 Final biomass of non-mycorrhizal cluster-rooted (top row; *Banskia attenuata* and *B. menziesii*) and ectomycorrhizal (ECM) plant species (bottom row; *Calothamnus quadrifidus* and *Eucalyptus todtiana*) when grown together in competition with each other: one ECM plant species planted with both cluster-rooted species under two inoculum treatments: i) – *Phytophthora* or ii) “+ *Phytophthora*” (mix of *P. arenaria*, *P. taxon cooljarloo*, *P. taxon kwongan*, *P. aff. rosacearum* and *P. rosacerarum*). Means and 95% confidence intervals (CI) are shown.
Root to shoot ratio varied among species and *Phytophthora* treatments (species × *Phytophthora* treatment interaction, $F_{1,3} = 18.48; P \leq 0.0001$; Fig. 4.5). Root:shoot ratio of *C. quadrifidus* and *E. todtiana* was almost twice as high in the – *Phytophthora* treatment than in the + *Phytophthora* treatment ($P \leq 0.001$; Fig. 4.5). By contrast, no differences between treatments in root:shoot ratio were found for either of the Proteaceae ($P = 0.1$; Fig. 4.5).

**Figure 4.5** Root to shoot ratio of non-mycorrhizal cluster-rooted (top row; *Banskia attenuata* and *B. menziesii*) and ectomycorrhizal plant species (bottom row; *Calothamnus quadrifidus* and *Eucalyptus todtiana*) grown together in competition with each other: one ectomycorrhizal plant species planted with both cluster-rooted plant species under two inoculum treatments: i) – *Phytophthora*, and ii) “+ *Phytophthora*” (mix of *P. arenaria*, *P. taxon cooljarloo*, *P. taxon kwongan*, *P. aff. rosacearum* and *P. rosacerarum*). Means and 95% confidence intervals (CI) are shown.
Finally, both *C. quadrifidus* and *E. todtiana* showed a significant positive relationship between ECM root colonisation and seedling biomass in the + *Phytophthora* treatment ($P \leq 0.04$; Fig. 4.6), while it was not significant in the – *Phytophthora* treatment ($P \geq 0.64$; Fig. 4.6). There were no statistically significant correlations between ECM root colonisation of Myrtaceae and biomass of competing Proteaceae in the different *Phytophthora* treatments ($P \geq 0.8$).

**Figure 4.6** Relationship between ectomycorrhizal root colonisation and final seedling biomass of two ECM species (*Calothamnus quadrifidus* and *Eucalyptus todtiana*). Seedlings were grown together with competing non-mycorrhizal cluster-rooted species, and exposed to two inoculum treatments: i) – *Phytophthora* (red circles and dashed line), and ii) “+ *Phytophthora*” (green triangles and solid line; mix of *P. arenaria*, *P. taxon cooljarloo*, *P. taxon kwongan*, *P. aff. rosacearum* and *P. rosacerarum*). $R^2$ values were only significant for the + *Phytophthora* treatment for both plant species, and were 0.87 and 0.38 for *C. quadrifidus* and *E. todtiana*, respectively. Solid lines indicate significant relationship ($P \leq 0.05$), while dashed line indicates non-significant relationship ($P \geq 0.05$).
4.6. Discussion

Overall, our results show that non-mycorrhizal Proteaceae were more susceptible to native soil-borne pathogens than ECM plant species, and this translated into a relaxation of competition between species with these two nutrient-acquisition strategies, presumably because non-mycorrhizal Proteaceae species are most effective in acquiring the growth-limiting resource in these soils, P (Lambers, Martinoia & Renton, 2015). In agreement with our hypotheses, we found that biomass gain of Proteaceae was reduced by ~26% in the presence of native *Phytophthora* species, while the growth of ECM species was not affected. This supports the contention that non-mycorrhizal cluster-rooted species are more susceptible to soil-borne pathogens than ECM species (Laliberté *et al.*, 2015). Furthermore, when competing with Proteaceae, ECM species showed higher biomass gain in the presence of native *Phytophthora* species than in their absence, suggesting that the presence of native soil-borne pathogens can modulate competitive interactions between ECM and Proteaceae species. Additionally, this increase in ECM plant biomass in presence of *Phytophthora* was positively correlated with ECM root colonisation, suggesting an important role in pathogen defence by ECM fungi. Our study suggests that soil-borne pathogens may contribute to the maintenance of highly-diverse ecosystems by reducing differences in competitive ability among plant species of contrasting nutrient-acquisition strategies. However, our experimental design does not allow us to quantify how pathogens modulate density-dependent competition among Proteaceae and Myrtaceae (e.g., Gibson *et al.*, 1999; Connolly, Wayne & Bazzaz, 2001). Quantifying how pathogen-mediated negative density dependence varies among co-occurring plant species of contrasting nutrient-acquisition strategies is an important avenue for future research that will help us better understand mechanisms of plant species coexistence in hyperdiverse vegetation (Laliberté *et al.*, 2015).
For the Proteaceae tested here, while not killed by the native Phytophthora species, there was a reduction in overall growth. On the other hand, growth of ECM plants was not negatively affected by the presence of these native Phytophthora species. Branzanti et al., (1999) showed that the inoculation of chestnut seedlings by P. cinamomi or P. cambivora reduces leaf and root size of non-mycorrhizal chestnut seedlings by 43-48%, while not affecting growth of chestnut seedlings previously inoculated with ECM fungi. Our results show how plants with different nutrient-acquisition can have contrasting responses to the same native pathogen. Results support the trade-off between P-acquisition efficiency and pathogen defence proposed by Laliberté et al., (2015). This trade-off could partly explain why Proteaceae do not dominate in severely P-impoverished systems, despite having a more efficient P-acquisition strategy than ectomycorrhizal species (Lambers, Martinoia & Renton, 2015). On the other hand, the fact that we did not find differences in growth among Proteaceae species in the + Phytophthora treatments suggests that coexistence among Proteaceae is not modulated by soil-borne pathogens. In this study, we did not evaluate if non-mycorrhizal Proteaceae promote the local build-up of soil-borne pathogens to a greater extent than ectomycorrhizal plant species, as has been hypothesised (Laliberté et al., 2015). Future studies should evaluate this possibility, as this process could lead to negative plant-soil feedback between Proteaceae and their associated soil biota which might further contribute to plant species coexistence in these ecosystems.

When planted together with Proteaceae, ECM plant biomass gain was greater in the presence of Phytophthora compared with that when grown in the absence of pathogens; conversely, biomass of Proteaceae species was lower. Likewise, several studies have shown how ECM fungi offer protection from pathogens to their host by several mechanisms, such as a physical barrier (Marx, 1972) and the biosynthesis of fungicides (Duchesne, Peterson & Ellis, 1988a; b). Hence, our results suggest that Phytophthora
can affect growth of non-mycorrhizal plant species while not affecting that of co-occurring ECM species, thus conferring an advantage to ECM species in terms of accessing scarce P resources. Pathogen-mediated plant coexistence has been reported in other ecosystems and with herbaceous plants (Burdons & Chilvers, 1974; Mills & Bever, 1998), but to our knowledge this is the first study to show empirical evidence of this for woody plants in a hyperdiverse, seasonally-dry shrubland. Our glasshouse experiment used seedlings rather than mature plants, because the studied species are long-lived, slow-growing woody perennial plants. As such, care must be taken when extrapolating our results to longer-term interactions between mature plants. We believe that our results are relevant for mature plants, because plant competition is mainly for belowground resources in this system, and plant nutrient acquisition primarily takes place in superficial soil layers, where plant nutrients and fine roots of both seedlings and mature plants are concentrated (Dodd et al., 1984).

In both experiments, seedling biomass of ECM plant species was positively correlated with ECM root colonisation, but only in the presence of *Phytophthora*. This suggests an important role of ECM fungi in pathogen defence, as previously shown (Branzanti et al., 1999; Whipps, 2004). A previous study showed detrimental effects of ECM fungi on two *Phytophthora* species when cultured together on agar plates (Branzanti, Rocca & Zambonelli, 1994). On the other hand, no relationship was found between biomass and ECM colonisation in the – *Phytophthora* treatment. In a recent study from the same shrublands studied here, Teste et al., (2016) showed how external hyphal biomass of mycorrhizal fungi was very low in P-impoverished soils compared with that in younger and P-richer soils, despite mycorrhizal root colonisation being high. This, together with our results from the present study, suggests that the main function of ECM fungi in these P-impoverished soils may not be to scavenge nutrients, but to protect ECM plants against root pathogens. This hypothesis deserves further attention as ECM fungi could
still enhance nutrient uptake, which might not be reflected in seedling biomass, but in increased leaf nutrient concentrations (Smith, Anderson & Smith, 2015).

Finally, our results show that native species of Phytophthora were generalist pathogens for both plant families, despite not affecting total biomass gain of ECM plant species. Indeed, root:shoot ratio of not only Proteaceae but also ECM plant species was lower in the presence of Phytophthora compared with that of plants grown in soil without Phytophthora, except for E. todtiana. Oomycetes cause damping-off and root damage (Cohen & Coffey, 1986; Bell et al., 2006), and hence reduce the root:shoot ratio of their hosts. Many invasive Phytophthora species are generalist in Australia (Scott et al., 2009; Scott, Burgess & Hardy, 2013), yet, until now, there was no information about the host-specificity of many native species of Phytophthora. However, Rea et al., (2011) reported that P. arenaria is often associated with non-mycorrhizal, cluster-rooted Banskia species. This observation, taken together with our ECM root colonisation results, provides some evidence that the resistance of ECM plant species is provided by ECM fungi, rather than an intrinsic defence of the ECM plant species themselves. Notwithstanding, we used fresh roots to inoculate both Proteaceae and Myrtaceae species with ECM fungi. This approach likely introduced microorganisms other than ECM fungi. Hence, potential contamination by other endophytes or pathogens cannot be discarded. However, other pathogens would have been introduced equally, irrespective of plant species and treatment. Hence, any potential effects on seedlings would not have obscured our results.

In conclusion, our results show how native soil-borne pathogens can equalise plant competition among seedlings of contrasting nutrient-acquisition strategies. We surmise that root pathogens may play a key role in coexistence of plants with different nutrient-acquisition strategies in these hyperdiverse shrublands. Moreover, we provide further
evidence for the hypothesis that there is a trade-off between P-acquisition efficiency and pathogen defence (Laliberté et al., 2015). We propose that in old, strongly-weathered and severely P-impoverished soils, ECM fungi are important for pathogen defence and potentially the persistence of their hosts. Our results highlight the need for considering soil microbiota in studies on plant interactions as well as plant diversity and ecosystem functioning, since pathogens and mycorrhizal fungi may strongly affect the outcome of plant competition.

4.7. Acknowledgements

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4.8. References


5. General Discussion

This thesis aimed to evaluate the effects of soil properties on root symbioses along a dune chronosequence, which exhibits long-term ecosystem development and pedogenesis, and the role of native soil-borne pathogens in plant coexistence in a biodiversity hotspot. In chapter 2, I showed a shift from arbuscular mycorrhizal (AM) to ectomycorrhizal (ECM) root colonisation as soils age. In chapter 3, I revealed how pedogenesis drives changes in ECM fungal communities and a decline in ECM richness. In chapter 3, I found that native soil-borne pathogens can affect competition between Proteaceae and ECM plant species. Finally, in this chapter I summarise the main findings, highlight the limitations of the studies, and suggest directions for future research.

5.1 Summary of main findings

I found that root symbioses and ECM fungal communities are strongly affected by changes in soil properties during pedogenesis and ecosystem development. Changes in pH, mineral and organic P concentrations induced a shift from AM to ECM root colonisation within the same plant species. Furthermore, pH and organic P concentration were among the most important variables explaining changes in ECM fungal communities during pedogenesis. I also provided evidence for a trade-off between P-acquisition efficiency and pathogen defence in Proteaceae and ECM plant species; this mechanism could be a critical factor in promoting plant coexistence in the south-western Australian biodiversity hotspot.
I found that different forms of P influenced plant resource allocation to different symbionts along a 2-million year chronosequence (chapter 2). These results suggest that AM fungi could be more important for plant P acquisition in young soils where most of the soil P is in mineral form, while ECM fungi are more important as soil P concentrations decrease, but its organic fraction increases with pedogenesis (Walker & Syers, 1976; Laliberté et al., 2012; Turner & Laliberté, 2015). Additionally, declines in soil pH were inversely and positively correlated with abundance of ECM and AM fungi, respectively (chapter 2). These results were also corroborated by the strong effect that pH and P availability had on structuring ECM fungal communities across the chronosequence (chapter 3). Krüger et al. (2015) showed that soil P concentrations drove richness and phylogenetic diversity of AM fungal communities, where sites with the lowest levels of available P harboured a reduced richness and diversity. I conclude that both soil pH and the availability of different forms of P could be important drivers of both AM and ECM fungal communities and success of root symbioses with their hosts. By using the same host species, I attempted to isolate the role of soil properties in driving mycorrhizal root colonisation, community composition and diversity, and furthered our knowledge on long-term plant-fungal-soil feedback.

Chapter 4 showed how native soil-borne pathogens had detrimental effects on plant health, but positive effects on plant species coexistence. Native Phytophthora species strongly reduced growth of non-mycorrhizal Proteaceae species, while not affecting the growth of ECM plant species. It has been suggested that in order to effectively acquire P, Proteaceae species have short-lived, poorly-defended and highly vulnerable roots (Laliberté et al., 2015). On the other hand, previous studies have shown the defensive properties of ECM fungi against root pathogens (Marx, 1972; Branzanti et al., 1994, 1999), and recently the protective role of AM fungi has also been shown (Wehner et al., 2010). These studies taken together with results from chapter 4 suggest that in these old
and severely weathered soils, high plant diversity may be enhanced by a trade-off between P-acquisition strategies and susceptibility to soil-borne pathogens

In chapters 2 and 3, I inferred that the decline in ECM fungal richness and increase in ECM root colonisation was in favour of a few ECM fungal species that were the most efficient at acquiring P. On the other hand, in chapter 4, I showed how in these old soils (i.e. last stage of the chronosequence), one of the ECM fungi’s main roles could be defence against pathogens. Hence, this reduction in ECM fungal richness may not only be driven by their nutrient-acquisition efficiency, but also by their ability to protect their hosts from pathogens. These results show a dual role of nutrient uptake and pathogen defence of ECM fungi. Furthermore, Muler et al. (2014) showed how Proteaceae may facilitate mycorrhizal plant species by releasing nutrients that are otherwise poorly available for plants that do not release substantial amounts of carboxylates in the same ecosystem. Other studies have also shown the facilitative effects that plants can have on species with other nutrient-acquisition strategies (Li et al., 2014; Teste et al., 2014). These studies taken together with results from this thesis suggest that less nutrient-efficient plant species could co-exist with species with superior nutrient-acquisition strategies by possessing better defence against pathogens and tapping into nutrients made available in the rhizosphere of their less well-defended competitors (Fig. 5.1).
Finally, here I show how ECM fungi increased growth of their host only when *Phytophthora* was present, suggesting that an important role of ECM fungi in this ecosystem might be protection against pathogens. Johnson *et al.* (1997) proposed that mycorrhizal associations fall into a mutualism-parasitism continuum based on several studies finding that mycorrhizal root colonisation could have positive to no to negative effects on plant growth. Results in this thesis show how this proposed continuum might be oversimplified by focusing only on nutrient uptake and overlooking other potential benefits that mycorrhizal fungi offer to their hosts, such as pathogen defence. On the other hand, findings by Teste *et al.* (2016) found overall low mycorrhizal external hyphal biomass in the Jurien Bay chronosequence, indicating relatively low nutrient scavenging compared with that in other ecosystems. Based on this study and the results of this thesis, I propose that mycorrhizal fungi might not necessarily act as parasites.
Instead, their main contribution to plants might change depending on environmental context.

5.2 Limitations and implications for future research

This thesis shows a number of limitations in the methodologies and interpretation of results. The use of only two plant species in chapters 2 and 3 and the use of glasshouse trials instead of field experiments are an inevitable simplification of the reality and hence limit ecosystem-level interpretations. Furthermore, I was unable to evaluate the independent contribution of each potential role of mycorrhizal fungi for plant communities (i.e. nutrient uptake and defence against pathogens). In addition, the lack of DNA sequences isolated from south-western Australia prevented me from identifying a greater number of ECM fungal species. Finally, I tested the role of native Phytophthora species on coexistence of only two nutrient-acquisition strategies and community-level interpretations have to be made with caution.

Chapters 2 and 3 focused on only two plant species (i.e. A. rostellifera and M. systena), since they were the only two plant species that co-occurred on most stages of the chronosequence. There are more than 239 mycorrhizal plant species along the Jurien Bay chronosequence with different additional nutrient-acquisition strategies, such as Allocasuarina humilis, which also forms cluster-roots (Zemunik et al., 2015). Co-occurring plant species can have contrasting associated mycorrhizal communities (Martínez-Garcia et al., 2015). Hence, implications to the whole plant community could only be inferred in this thesis. Future studies including a larger number of plant species should unveil whether results from this thesis can be generalised more broadly.
This thesis was based on glasshouse experiments and thus several potential interacting factors were either intentionally or intentionally removed, such as neighbouring plants and organisms from other trophic levels. For example, previous studies have shown how neighbouring plants in a community can have strong effects on root colonisation by symbionts (Dickie et al., 2004; Kennedy et al., 2012; Teste et al., 2014). Furthermore, our soil manipulation experiment in chapter 2 (i.e. soil sieving, mixing, pasteurisation and use of 7% inoculum) could have favoured fungal species that colonise through spores rather than hyphae. Hence, future research should aim to disentangle biotic from abiotic factors in driving mycorrhizal communities by also using field experiments and involving the whole plant community.

Disentangling the relative importance of the complementary roles of mycorrhizal fungi for both nutrient uptake and pathogen defence found in this thesis was not possible. Future studies should aim to quantify the relative contribution of the different benefits ECM fungi can have for plant health by conducting multifactorial experiments using pathogen and mycorrhizal inoculum, and modifying soil nutrient availability. Also, only a few studies have attempted to compare nutrient-acquisition efficiencies among ECM taxa (Courty et al., 2005; Alvarez et al., 2012). This issue needs to be addressed in order to better explain the decline in ECM fungal richness, as well as the changes in composition and diversity of AM fungi found by Krüger et al. (2015).

This thesis shows how poorly understood mycorrhizal fungal diversity is in south-western Australia. Mycorrhizal associations have been consistently studied since their discovery in 1842 (Nägeli, 1842). Yet, in the past 174 years, only a handful of mycorrhizal studies have evaluated fungal diversity in this part of the world (Castellano & Bougher, 1994; Brundrett, 2009) with no studies attempting to isolate and sequence the DNA of fungal specimens. This prevented us from identifying operational
taxonomic units (OTUs) at the species level, and hence making more accurate interpretations of our results. For example, several potential ECM fungal species could have been removed from our analyses given the lack of information of their ecological role. Additionally, ~ 200 OTUs were only identified at family or higher taxonomic level, preventing us from identifying their symbiotic status (e.g., mycorrhizal, saprophytic). There have been studies attempting to identify fungal communities in eastern and southern Australia (Bougher & Malajczuk, 1985; Tedersoo et al., 2009, 2010). Furthermore, most of the best BLAST matches were related to a study conducted in Tasmania, Australia (Tedersoo et al., 2008) which suggest that fungal species in Tasmania are genetically different from those in other parts of the world. Much is still to be discovered, such as endemic mycorrhizal fungal species. These new findings will help to better understand how diverse fungal communities are and their relationship with plant diversity.

Finally, in my thesis, the role of native soil-borne pathogens in plant coexistence was tested for only two nutrient-acquisition strategies (cluster roots and ectomycorrhizas). In the community studied in chapter 3, plant species have different and multiple nutrient-acquisition strategies (Zemunik et al., 2015), and hence, results from this thesis do not explain the persistence of other nutrient-acquisition strategies, such as species with dauciform or sand-binding roots. A trade-off between P-acquisition efficiency and pathogen defence might not be the only mechanism by which hyperdiverse ecosystem can be maintained, and other mechanisms such as facilitation (Muler et al., 2014) could be playing an important or complementary role as well. Future studies should test the generality of the new hypotheses proposed in this thesis by using other nutrient-acquisition strategies and other nutrient-poor highly diverse ecosystems, such as the fynbos in South Africa, tropical rainforests in South America and Asia (Myers et al., 2000) or campos rupestres in Brazil (Oliveira et al., 2015; Silveira et al., 2015). Also,
future research should aim to quantify the relative importance of different factors allowing plant coexistence such as competitive and facilitative interactions through soil microbiota, and its implications for plant diversity.

5.3 Conclusion

This thesis has provided valuable understanding of factors driving root symbioses, and their implications for plant communities. This research will not only contribute to our ability to assess fungal and plant community shifts during ecosystem development, but also support the need to recognise root symbionts as key components in highly diverse ecosystems. Future research could build on this thesis, particularly by further evaluating the mechanisms by which symbionts and pathogens modulate plant interactions (i.e. competition and facilitation) in hyperdiverse ecosystems.

5.4 References


